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Continued Inside Cover

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Bioscience Biotechnology Research Communications, BBRC is a broad based internationally indexed official publication of Society for Science & Nature (SSN) since 2008. The international journal publishes peer reviewed original research papers, exciting reviews and short communications in basic and applied areas of life sciences and the upcoming state of the art technologies, including Biology and Medicine on a fast track. The young editorial team of BBRC tries hard to provide a high quality flawless format of scientific communication for the popularization and advancement of science, worldwide. During these 10 years more than 1000 peer reviewed research papers of very high quality have been published in BBRC and authors like Kiran Shaw Majumdar of Biocon, Bangalore have contributed to BBRC helping it achieve high readership in a short span of time. Reviewing the published research articles, it becomes evident that on an average, about 7 papers out of 10 are subjected to healthy revisions in BBRC making quality reading. We owe this achievement to our reverend reviewers! We hope the standards set by BBRC will improve further making this international journal unique and easily accessible to the scientific fraternity across the globe. In its tenth year of successful existence as a scholarly publication, BBRC has now become an open access Thomson Reuters ISI ESC/Clarivate Analytics USA Indexed journal also approved by University Grants Commission (UGC) Ministry of Human Resource Development, Government of India, New Delhi and has a NAAS-2017, Government of India, Indian Council of Agricultural Research (ICAR) New Delhi rating of 4.38 and SJIF (2017) 4.196.

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Description of intestinal parasites found in some snakes of Al-Diwaniyah, Iraq

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ABSTRACT

A survey of parasites was conducted in 130 snakes collected from five sites of Al-Diwaniyah Province (City Center, Afak, East Hamzah, Al-Badair and Nafer districts of Iraq. The snakes belong to 8 species of two families Colubridae and Biodae, which contain *Platyceps ventromaculatus, P. rogers, Malpolon monspesslana, Sapalerosophis clifordi, Dolichophis mesopotamicus, Rhynchocalamus melanocephalu, Eryx jaculus and Natrix tessellate*. Eighty-three samples of snakes 63.84% were infected with five species of internal parasites which include two protozoans, *Isospora sp. and Cryptosporidium sp*, two species of nematodes *Kalicephalus sp*. and *Strongyloides sp*. and one species of cestode *Oochoristica tandani*. Through the site of intestinal parasites in the digestive tract was mostly small intestine, higher percentage of them were detected in other parts of digestive tract. Results also showed that single parasitic infections were most common in comparison to other infections. This is the first survey of intestinal parasites from snakes in Iraq where detailed description of the intestinal parasites are reported for the first time from snakes of Iraq.

KEY WORDS: SNAKES, REPTILE, INTESTINAL PARASITES, IRAQ

INTRODUCTION

Al-Diwaniyah province, (180 KM south of Baghdad) is one of the southern provinces and its territory is part of the plain sedimentary Iraqi, which is characterized by the simple decline from north-west to the south and south-east also show minor differences and other local in the surface of the province because of several factors, most important process of wind sedimentation and can be explained nature by dividing the province. The first part consists of the flood plain, which includes most areas of the province and the area of the shallow and semi-shallow depressions, which the second part represents, the third part which is located in the sand dunes area. Such as the districts of Afak and AL-Badir, and the fourth part, which is represented by the sandy area and

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covers the southwestern part of the province in the area between the west of the Euphrates River and the Western administrative boundaries of the province, (Al-Janabi & Ghaleb, 1992).

There are about 2,900 species of snakes spread in all countries except the North and South Poles, the Hawaiian Islands, Ireland and New Zealand. The anatomical and physiological behavior of the scale diaphragms in the body is the easiest way to distinguish between these species, (Vitt & Caldwell, 2013 McAllister *et al.* (2015).

Some of these species are venomous and some other nonvenomous. The poison is mainly used to kill prey or defend itself. Snakes are not at all harmful and many of them are highly beneficial to human beings as they feed on rats and mice and their skin is of great value as it enters in the illegal manufacture of bags and shoes. In addition, the venom of some snakes is used in preparing antidotes for poisoning, (Pasi, 1992; Caswell *et al.*, 2014, Yimming et al. (2016).

Reptiles, including snakes, are exposed to a variety of pathogens, which may be bacterial, fungal, viral, parasitic agents. There are many studies have indicated that snakes are the intermediary or definitive hosts for many of the internal parasites, such as round worms such as *Angusticaecum sp*, *Porocephalus crotali*, *capillaria sp*. as well as intestinal protozoa such as *Eimeria sp*., *Isospora sp*., *Caryospora sp*., *Tyzzeria sp*, (Klingenberg, 2000 Parc 2008, Rataj et al., 2011).

Cryptosporidium sp. is an intermediate host of *Toxoplasma sp*. (Duszynski & Upton, 2009), as well as it is a blood parasite similar to *Plasmodium, Haemoproteus mesnili* and *Haemoproteus balli* (Telford, 2009; Jacobson, 2007). It is also affected by external parasites, most notably ticks such as *Amblyomma sp*. and mites like *Ophionyssus natricis* (Rataj *et al*., 2011; Pietzsch *et al*., 2006 McAllister *et al*. (2015).

According to our literature review, despite of wide distribution of these snakes in Iraq, there is not much comprehensive and adequate published data about intestinal parasites of these snakes. Therefore the current study was conducted to prepare list of intestinal parasites of these snakes in Iraq.

MATERIAL AND METHODS

EXAMINATION OF COLLECTED SNAKES AND THEIR PARASITES

The dead snakes were kept in the refrigerator at 7° C and dissected within seven days while living species were anesthetized with ether after the removal of the fangs (Fontenot & Font, 1996). Then process of snakes dissection was carried out according to the method of Jacobson, (1978), where the snakes were placed on a large piece of cork designed for this purpose. Then they were opened from abdominal side, starting from annual slit towards the fore front. The digestive tract and its components were then removed by sharp scissors in a sterile Petri dish to look for intestinal worms. It was also examined near the yellow sac around the pancreas adjacent to the stomach as well as the liver and lungs. In case of isolation of intestinal worms, it was washed with water and kept in containers of %70 ethyl alcohol, then added drops of glycerin. For the purpose of clarification and confirmation, use of acetocarmine dye was carried out to pigment tapeworms, trematoda, and acanthocephalus. Nematodes were placed in lactophenol solution and then observed on a clean glass slide using Canada's balsam (Chaiyabutr & Chanhome, 2002).

For the study of intestinal protozoa a sample of the stool was examined in direct smears with the use of some illustrative pigments such as iodine and Zell-Nelson. All intestinal parasites were examined under low and high magnifications (10x,40x) and necessary measurements were taken using ocular and stage micrometer. Identification of parasites was carried out using characters described by Rataj *et al*., (2011).

FIGURE 1. Some species of snakes.

STATISTICAL ANALYSIS

The results were analysed by Completely Randomized Design (CRD) which was adopted as a one-way and two-way laboratory experimental design, as well as a comparison of the averages using a Least Significant Difference (LSD) under probability level of $P \leq 0.05$

RESULTS

Of the 130 snakes samples examined,83 snakes, 63.84% were found to be infected with different species of parasites (Table 1) and five species of intestinal parasites were recorded which included two species of protozoans one species of cestoda and two species of nematode. The results show that the distribution of intestinal parasites in digestive tract of snakes, tapeworms was found only in the large and small intestine being 80 and 50 % respec-

Table 1. Number & percentage of snakes infected with internal parasites.

Species of parasites	Species of snakes (n=130)															
	P.ventromaculatus (n=42)		*P.roger s* (n=1)		*M.monspessulana* (n=13)		*E.jaculus* (28)		*S.clifordi* (n=11)		*N.tessellata* (n=19)		*D.mesopotamicus* (n=15)		*R.melanocephalus* (n=1)	
	N(%)	S.I*	N(%)	S.I*	N(%)	S.I*	N(%)	S.I*	N(%)	S.I*	N(%)	S.I*	N(%)	S.I*	N(%)	S.I*
Oochoristica tandani	5(11.90)	2.8	0(0)	0	0(0)	0	2(7.14)	1	0(0)	0	11(57.89)	2	2(13.33)	1	0(0)	0
Kalicephalus sp.	10(23.81)	1.20	0(0)	0	1(7.69)	1	21(75)	1.66	3(27.27)	1	7(36.84)	1.42	2(13.33)	1	0(0)	0
Strongyloides sp.	1(2.38)	1	0(0)	0	0(0)	0	2(7.14)	1	0(0)	0	0(0)	0	0(0)	0	(0)0	0
Isospora sp.	8(19.05)	3.30	0(0)	0	2(15.38)	3	2(7.14)	10	4(36.36)	1.75	2(10.53)	4	3(20)	3.30	0(0)	0
Cryptosporidium spp.	5(11.90)	2.40	0(0)	0	4(30.77)	1.25	3(10.7)	2	5(45.45)	1.20	7(36.84)	2	4(26.67)	2.50	0(0)	0
LSD(P≤0.05)	To compare the infection rate between parasites species=14.40 To compare the infection rate between snakes species=N.S								To compare the severity infection between parasites species=1.26 To compare the severity infection between snakes species=1.26							
	F.calculated:4.12 F.table:2.32 F.calculated:2.15 F.table:2.23								F.calculated:7.03 F.table:2.32 F.calculated:3.32 F.table:2.32							

*S.I: Severity of infection

tively, while nematodes, *Kalicephalus sp*. were found in three different places of the gastrointestinal tract, stomach, small and large intestine being 31.82, 52.27 and 20.45% respectively. *Strongyloides sp*, was found only in the small intestine at a rate of 100%. *Isospora sp*. was recorded in stomach, small and large intestine being 42.86, 85.71 and 9.52% respectively. Similarly, Cryptosporidium *spp*. was recorded only in the small and large intestine being 28.57 and 75%, respectively.

The statistical analysis showed no significant differences in infection rates according to species parasites, while significant differences in infection rates were found according to location of infection, (Table 2). It is clear from (Fig. 12) that the highest incidence of intestinal parasites was in single infections with one species of parasite with 65.62 % dominance , followed by binary infections with 36.14 %, while triple infections (three or more parasites) ranked 7.22 %. Significant differences were found in the types infection at the probability level ($P \leq 0.05$).

1. *Isospora sp.*(Henyon,1923)

Species of intestinal protozoa were recorded in smears prepared from all snakes except *P. roger's* and *Rhynchocalamus melanocephalus*. In the isolates from stomach, small and large intestinal ,the immature cysts of *Isospora sp*. were detected, being oval with one end was having rounded boundary, and the other was thin and had a wall consisting of two thin layers and was with a micropyle at the high end, which was about 19-20 33 x 20 micrometers. The life cycle of this parasite was of direct nature and the host was perhaps infected by mature cysts along with food or water contamination, (Fig.2)

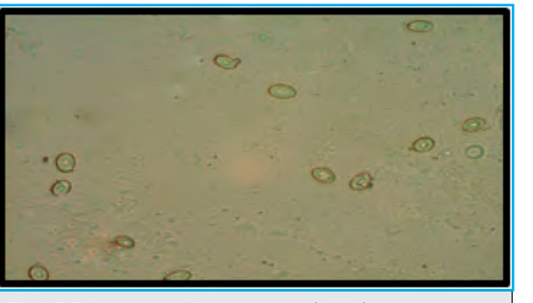

FIGURE 2. Cysts of Isospora sp. (100x)

2. *Cryptosporidium spp.* (Tyzzer, 1907)

The cyst of *Cryptosporidium spp* isolated from small and large intestinal of all types of snakes except the two species *P. roger*'s and *R. melanocephalus showed* cysts which were spherical or oval-shaped, containing eight spores with double-walled being about 5.1 x 4.5

Table 2. Number &infection rate of intestinal parasites in digestive tract in snakes.

Species of parasites	Number of parasites	Site of infection					
		stomach		Small intestine		Large intestine	
		n	%	n	%	n	%
Oochoristica tandani	20	0	0	16	80	10	50
Kalicephalus sp.	44	9	20.45	23	52.27	14	31.82
Strongyloides sp.	3	0	0	3	10	0	0
Isospora sp.	21	2	9.52	18	85.71	9	42.86
Cryptosporidium spp.	28	0	0	21	75	8	28.57
LSD(P≤0.05)	To compare the infection rate according to species of parasites =N.S To compare the infection rate according to site of infection =33.98						
	F.calculated:0.31 F.table:3.83 (to species parasites) F.calculated:20.91 F.table:4.45 (to site infection)						

micrometers and were characterized by having a direct life cycle, (Fig. 3).

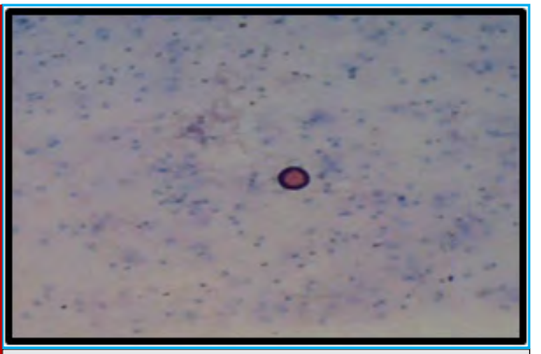

FIGURE 3. Cysts of *Cryptosporidium spp.*(100x)

3. *Oochoristica tandani* (Luhe, 1898)

This species of cestoda was found in both the large and small intestines of *P. ventromaculatus, E.jaculus, N. tessellata* and *D. mesopotamicus* which were tall and thin worms with a length of about 90 mm and a width of 0.77 mm. The head was equipped with four suckers without spines followed by the neck area consisting of 40 immature proglottids followed by mature proglottids, after about 10 pieces of the gravid proglottids, were 18 proglottids and all types of proglottids had length greater than the width by about 4 times. The ovary consisted of 5-4 lobes while number of testes had 37-45 test, both ventral longitudinal excretory ducts and narrow dorsal ducts showed small anastomosing branches (fig. 4, 5 & 6).

4. *Kalicephalus sp.* (Rudolphi, 1819)

This nematode worm had the highest percentage of isolated intestinal worms, it was found in all species of snakes except *Platyceps rogers* and *Rhynchocalamus melanocephalus*, and was found in both the large

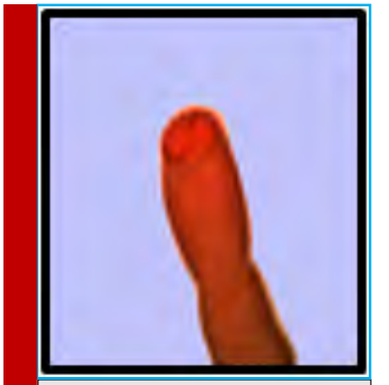

FIGURE 4. *Oochoristica tandani*

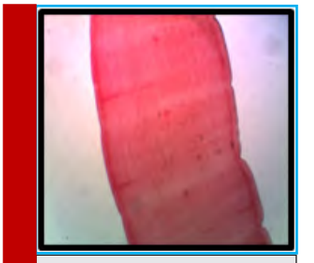

FIGURE 5. Immature proglottids

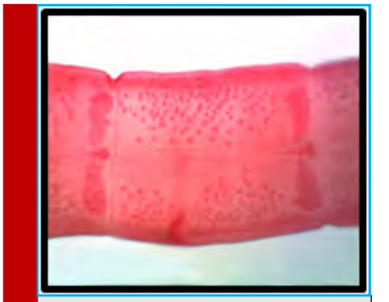

FIGURE 6. Mature proglottids Scolex(100x)

and small intestines as well as stomach, this nematoda female had short, strong worms with a white color that did not exceed 6 mm in length. The mouth was equipped with four pyramid cuticular structures, it had esophagus funnel shaped containing three small teeth and with strong kaitinine lining, excretory pore was near to posterior end, uterine branches were opposed or parallel and were full of eggs with posterior end being conical, (fig. 7,8 & 9).

FIGURE 7. Anterior end of *Kalicephalus sp.* female(100x)

FIGURE 8. Eggs of *Kalicephalus sp.* female(100x)

FIGURE 9. Posterior end of *Kalicephalus sp.* female(100x)

5. *Strongyloides sp.* (Grassi, 1879)

This nematode worm was found in the small intestines of only two species of snakes*: P. ventromacu- latus* and *Eryx jaculus*. The isolated female length was about 3.5 mm and white color and was covered with a soft layer of cuticle. The oral cavity was small and followed by a cylindrical shaped esophagus. The uterus had a thin wall and was filled with eggs that appeared in the shape of two tubes full of eggs (Fig.10,11 & 12).

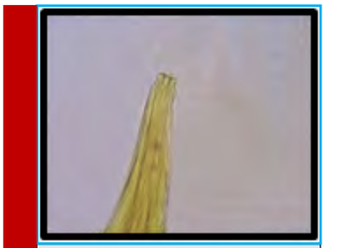

FIGURE 10. Anterior end of *Strongyloides sp.* female(100x)

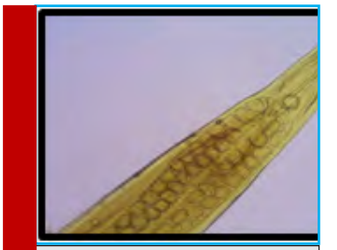

FIGURE 11. Eggs of two tubes (100x)

FIGURE 12. Posterior end of *Strongyloides sp.* female(100x)

DISCUSSION

The results of the present study indicate that the total infection rates of snakes were 63.84 %, which is lower than that recorded by Chaiyabutr & Chanhome (2002) in Thaland, Santora *et al.*, (2013) in southern Italy and Nasiri *et al.*, (2014) being 75,95 and 73.56 % respectively, and is higher than that recorded by Dusen *et al.*, (2010) in the north-west of Turkey and Rataj *et al.*, (2011) in Scandinavia, which amounted to 27.27 and 47 % respectively. The different recorded ratios (above and below) may be due to the variation of the number of

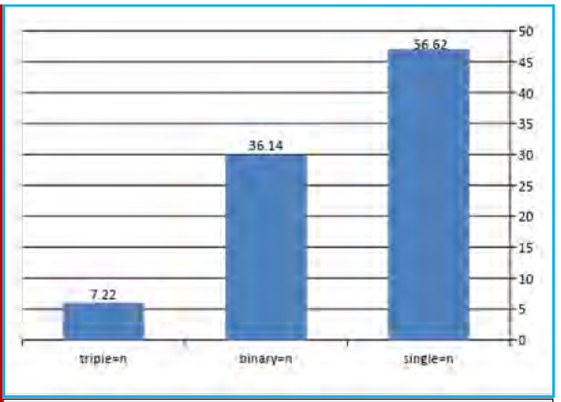

FIGURE 13. Types of infection & percentage of infected snakes with intestinal parasites.

specimens examined, their species, sizes and sources of food, as well as their location and the nature of their livelihood, as well as some of the research was conducted on the captive snake and some on wild species, some of them on aquatic species.

In this study, there were five species of intestinal parasites, the intestinal protozoa was the most widespread, the reason for this was that most of the snakes were subjected to pressure conditions of cages. In the case of infection a of single snake it was put to the cysts of spores with waste, which led to the pollution of cages and water sources, as well as the fact that these parasites had a direct life cycle. A high incidence of nematode infection was observed compared to tapeworms, this is consistent with Chaiyabutr & Chanhome's study of (2002) in Thailand, Santos *et al*., (2006) in Iberian Peninsula and Dusen *et al*., (2010) in the north-west of Turkey and Ribas *et al*., (2010) in the north-east of Spain and of Fontenot & Font's (1996) in south-eastern Louisiana in aquatic vipers, which pointed to a higher incidence of tapeworms compared to nematodes.

This is due to the availability of intermediate hosts which play a major role in the incidence of tapeworms. Also observed was that the number of intestinal parasites located in the intestines is much higher than the numbers present in the stomach and large intestine may be due to the fact that the intestinal environment suitable for the parasites because of the integration of the physiological characteristics of the food in addition to its presence in a soluble and ready for absorption and this is consistent with Santora *et al*., (2013) and Dusen (2010).

The results of the current study showed that the incidence of one species of parasite was the highest percentage compared with binary and triple infections. The reason for this may be due to the living and environmental competition between the parasites of the host.

The results of the study indicate that snakes of medium size are most susceptible to parasitic infections, reaching the highest level within the group which ranged between 50 - 100 cm. This result is consistent with the reports of Santora *et al*., (2013) and Capizzi *et al*., (2008). This may be due to the fact that medium-sized snakes are more active than small and large species and therefore have a wide food diversity, Small snakes are always inactive and poorly nourished. While few infection in large snakes due to having evolution immune system (Santora *et al*., 2013; Capizzi *et al*., 2008; Poulin, 2007). Parasitic infection occurs under the influence of a biological agents which include host, parasite and carrier, such as host species, genus and feeding nature (Osgood & Schall, 2004).

This study indicated only one species of tapeworm was found, which was found in the small and large intestine. It was recorded in only four species of snakes: *Platyceps ventromaculatus, Eryx jaculus, Natrix tessellata and D. mesopotamicus*, being 11.9, 7.14, 57.89 and %13.3, respectively. It is worth mentioning this species is not only in snakes, as it was previously recorded in Iraq by Al-Hashimi (2006) in Al-Anbar in his study of Iraqi reptiles. It has been isolated by King & Babero (1974) in Australian Kangaroo *Dipodomys ssp*. in Nevada by Bursey *et al*., (1996) in the Australian lizards called *Moloch horridus* and Rataj *et al*., (2011) in Scandinavia from lizards and Bursey *et al*. (1996) pointed to the presence of more than 74 species, which is concerned with the infection of different species of reptiles, Their life cycle is indirect and requires beetles and some insects as an intermediate host to complete their life cycle. While *Kalicephalus sp*. was one of the most common nematodes recorded during the current study, it was found in the stomach, small and large intestine and has been isolated from most species of snakes and the highest percentage was in the *Eryx jaculus*, which was 75% and the lowest in *Malpolon monspesslana*, which amounted to 7.69%, while *Strongyloides sp*. was found only in two species of snakes, *Platyceps ventromaculatus and Eryx jaculus* with a percentage of 2.38 and 7.14 respectively. This species was universally recorded in snakes by Fontenot & Font (1996) in South-East Louisiana in group of aquatic snakes at a rate of 4 % and Rataj *et al*., (2011) in Scandinavia snakes and turtles at a rate 3.7 %.

Protozoa included two species such as *Isospora sp. and Cryptosporidium spp Isospora spp* which were recorded in most species of snakes. The highest percentage was the *S. clifordi*, which was 36.36 % and the infection was obtained by swallowing the cysts containing spores with contaminated water and food, which is globally registered in snakes by Asmundsson *et al*., (2001) in Ecuador and Chaiyabutr & Chanhome (2002) in Thailand and McAllister *et al*. (2015) in Oklahoma.

Cryptosporidium spp. was recorded in six species of snakes and highest percentage was registered in *S. clifordi*, which was % 45.45. This finding agrees with that of Brower & Cranfield (2001) who have shown similar data in snakes of Animal Farm Zoo in Baltimore, Maryland USA. Our present study of the histological changes caused by the parasite considerably agrees with that of Kuroki *et al*., (2008) in Japan and Rataj *et al.,* (2011) in Scandinavia and Yimming *et al*., (2016) in Thailand. This protozoa was recorded in 57 different species of reptiles, including 40 species of snakes, 15 species of lizards, and 2 turtles (Xiao *et al*.,2004). The incidence of species without apparent disease in mammals and birds (Ramirez *et al*., 2004) with the exception of *C. serpentis*, which leads to clear pathological symptoms such as abdominal bloating, low weight and lethargy of infected animals,This species is concerned with reptiles only (Fayer *et al*., 1997). The infection with *Cryptosporidium* is obtained by swallowing cysts containing spores with water and food or through contaminated soils, (Rosenthal,1997).

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In-silico identification of phytohormone pathway genes in *Camellia sinensis* and expression analysis under combined water and herbivore stress

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ABSTRACT

Tea (*Camellia sinensis*) is a popular beverage worldwide. Abiotic and biotic stresses due to recent climate change have significant effect on yield of tea. Plant hormones such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) plays an important role in regulating plant defense responses to different kind of stresses. In this study homologous phytohormone genes of ABA, JA, SA and ET pathway in tea plant were identified from the public domain transcriptomic database and the expression of the rate-limiting genes of phytohormone pathway were analyzed in tea plants subjected to combined water and herbivore stress to understand the interaction among the stress-induced phytohormone pathways genes. Vegetatively propagated TV1 clones of tea plant were subjected to three level of water stress treatments: 1) well watered control 2) mild water stress 3) severe water stress for three months and then infested with *Hyposidra talaca* (looper caterpillar). The constitutive expression (without infestation) of the rate-limiting genes of ABA and ET pathway were positively regulated by water stress whereas JA and SA pathway genes were negatively regulated. On looper caterpillar infestation (induced expression) water stressed plants showed significant decrease in expression of the rate-limiting phytohormone genes except *ACC* synthase. Our study showed that on herbivore infestation well watered plants have higher capacity to induce the phytohormone genes and water

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stress played a major role in regulation of gene expression than herbivore infestation. The presence of an initial water stress not only affected the tea plant constitutive defense but also significantly altered the phytohormone defense gene expression towards subsequent herbivore stress. The water stressed tea plant with weak induced expression of defense associated phytohormone genes may be at a higher risk for incidence of pest and pathogen attack compared to well watered plants.

KEY WORDS: *CAMELLIA SINENSIS*, EXPRESSION ANALYSIS, HERBIVORE INFESTATION, *HYPOSIDRA TALACA*, *IN SILICO* IDENTIFICATION; PHYTOHORMONE PATHWAY

INTRODUCTION

Tea, *Camellia sinensis* is a major economic crop worldwide and its young leaves are used for preparing beverage. Huge losses in tea leaf yield is incurred due to the present climate change scenario. As the climatic change event is expected to increase the incidence of water shortage and outburst of insect population, the monoculture cultivation of tea may face severe crop loss in recent future. Drought is one of the major abiotic stress that influences the quality and productivity of crops by growth inhibition, increase in organic solutes concentration and changes in the endogenous phytohormones content (Wijeratne et al., 2007; Bhagat et al., 2010, Aimar et al. 2011, Chen and Chen, 2012 and Anderegg et al. 2015).

Biotic stressors such as insects and pathogens, also contribute significantly towards the enormous damage to the crops (Hammond-Kosack and Jones, 2000). Plants have both inherent and adopted mechanisms to cope with the environmental stresses by producing certain proteins and secondary metabolites that are toxic or have repellant effect on the biotic agents (Rani and Jyothsna, 2010; War et al., 2011a; War et al., 2011b; War et al., 2012). Abiotic and biotic stresses in plants trigger the activation of a number of phytohormone pathway genes which simultaneously activate other interconnected defense network to help plants sustain the stress period (Fraire-Velázquez et al., 2011). The primary phytohormones abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), ethylene (ET) are involved as messengers triggering the specific defense pathways against environmental stress and may act individually or in combinations depending upon the stress perceived, (Atkinson and Urwin, 2012, Verma et al., 2016; Wani et al., 2016).

ABA is produced in response to water-deficit stress (Osakabe et al., 2013). Enhanced accumulation of ABA in *Arabidopsis thaliana* seedlings has been reported under drought conditions (Huang et al., 2008). Exogenous application of ABA delay wilting and is reported to induce drought tolerance in plants (Lu et al., 2009). ABA functions both synergistically and antagonistically with JA, SA and ET signaling pathways which play a dominant role during biotic stress (Chen and Yu, 2014). Under the combination of abiotic and biotic stresses, ABA mostly acts as an antagonist to JA/SA/ET making the plant susceptible to disease and pathogen attack (Rejeb et al., 2014). However, a positive interaction has also been observed, whereby an increase in ABA level under abiotic stress results in stomatal closure which prevents the entry of biotic agents and protects the plants from both biotic and abiotic stresses (Melotto et al., 2006). SA, an endogenous growth regulator, induces systemic acquired resistance (SAR) in plants against different pathogens, particularly microbes and serves as a signal molecule by producing pathogenesis related (PR) proteins (Gao et al., 2015; Verma et al., 2016).

SA is also involved in plant response to different abiotic stresses such as drought, temperature variations, heavy metals and osmotic stress (Rivas-San Vicente and Plasencia, 2011). JA, a key regulator of plant response to pathogens and insects, is involved in both direct and indirect defenses of plants to herbivory (Creelman and Mullet, 1995). JA also participates in plant's response to drought and salinity (Riemann et al., 2015). ET, the gaseous phytohormone for defense, helps in both direct and indirect response of plants to abiotic and biotic stresses. The effects of ET can be transitory or long lived as its biosynthesis shows a diurnal rhythm and controls its own biosynthesis (Eyidogan et al., 2012; Gamalero and Glick, 2012; Verma et al., 2016).

Depending on the type of stress perceived by the plant, different signaling pathways are activated which synergistically or antagonistically influence the type of response generated. The interactions among the different signal transduction pathways are considered as crosstalk between the pathways which helps the plants to sustain the stress period (Rejeb et al., 2014). In the event of climatic change where both the abiotic and biotic stress will co-occur, it is largely unknown how the phytohormone based plant defense network would behave as majority of the studies so far considered single stress factor either abiotic or biotic at a time. It also remain unpredictable how the presence of an initial stress affects the plant defense network on perception of a subsequent stress. As the frequency and extent of drought as well as insect infestation are projected to increase due climate change it is essential to understand the response of plants to combined stress conditions. Knowledge of the molecular mechanisms underlying these effects is very limited. The

molecular study of the stress-related hormonal genes and their interactions would help to understand the synchronization of plant constitutive and induced defense responses to insect infestation in plants under abiotic stress. Tea plant faces water stress event round the year and also plethora of insect infestation. Looper caterpillar infestation stand out to be the most destructive insect infestation in terms of crop loss. The water stress may change the overall metabolism of the tea plant and alter its defense interaction with the biotic agents. It will be vital to know the interaction of the phytohormone defense gene network in perception of combined water and herbivore stress.

In this study, a comparative genomics approach was undertaken to mine the phytohormone pathway genes of *C.sinensis* and the expression pattern of the rate limiting genes of the four phytohormone pathway (ABA, JA, SA, ET) was analyzed in a clone (TV1) of tea plant which was subjected to different regime of water stress (abiotic stress) treatment with subsequent insect infestation (looper caterpillar). This study will help to better understand the stress-induced phytohormones defense interaction at transcriptional level in tea plant under combined water and herbivore stress.

MATERIALS AND METHODS

IN SILICO IDENTIFICATION OF PHYTOHORMONE PATHWAY GENES IN *C. SINENSIS*

The genes or transcripts involved with ABA, JA, SA and ET phytohormone pathway in *C. sinensis* were mined from different databases using *Arabidopsis* sequences as reference. The *Arabidopsis* full-length coding sequences (CDS) were collected from the TAIR database (https://www.arabidopsis.org/) and subsequently the sequences were subjected to BLASTN with *C. sinensis* Expressed Sequence Tags (EST), Transcriptome Shotgun Assembly (TSA) and Non-Redundant (NR) nucleotide databases of NCBI. The sequences showing significant similarity with an E-value ≤ $1e^{-15}$ were selected and assembled using CAP3 program to remove redundancy and get consensus sequences. Each of those sequences were screened for the presence of open reading frame (ORF) using NCBI ORF Finder (Wheeler et al., 2003) and sequences with the longest ORF having both start and a stop codon were sorted out. The sequences were further subjected to BLASTX with NCBI NR (Non-Redundant) protein database to confirm their annotation. Based on the BLAST annotation and alignment results, the sequences found similar to *Arabidopsis* reference sequences and nearby plant species sequences were retained and others were filtered out. The best representative sequences were subjected to blast and functional classification following the Gene Ontology (GO) scheme using BLAST2GO suite (Conesa and Götz, 2008). The transcripts were classified into the major GO categories, namely, cellular component, molecular function and biological process. Further the rate-limiting gene sequences of the phytohormone pathways were used in expression study.

MOTIF AND DOMAIN IDENTIFICATION

MEME and MAST programs (Bailey and Elkan, 1994; Bailey and Gribskov, 1998) were used for the identification of the motif cluster present in the phytohormone pathway gene sequences. MEME program performs motif discovery on DNA, RNA or protein datasets. Whereas, MAST program searches sequences for matches to a set of motifs and sorts the sequences by the best combined match to all motifs. The sequences from *C. sinensis* and *Arabidopsis* were analyzed together for the easy identification of common motifs between them. The motif discovery mode was set to normal. The maximum number of motif was set to 20. Domain search was performed using CD-search tool available at the conserved domain database (CDD) of National Center (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

SELECTION OF GENE FOR qRT PCR AND PRIMER DESIGN

The rate limiting gene/enzyme of each phytohormone pathway was searched from literature references and the expression of the corresponding genes was analyzed across different treatments. For the ABA pathway, the reaction catalyzed by 9-cis-epoxycarotenoid dioxygenase (*NCED*) i.e- the oxidative cleavage of neoxanthin is considered as the rate-limiting step (Tan et al., 1997; Qin and Zeevaart, 1999) and chosen for our study. In the ET biosynthesis pathway, the conversion of S-AdoMet to 1-aminoacyclopropane 1-carboxylate (*ACC*) by *ACC* synthase is taken as the rate-limiting step (Wang et al., 2002). Allene oxide synthase (*AOS*), the first enzyme in the branch pathway leading to the formation of JA act as rate-limiting step in JA biosynthesis (Harms et al., 1995; Sivasankar et al., 2000). In the SA pathway, isochorismate synthase (*ICS*) acts as the rate-limiting enzyme (Serino et al., 1995; Gaille et al., 2003).Thus *NCED* gene for ABA, *ACC* synthase gene for ET, *AOS* gene for JA and *ICS* gene for SA pathway was chosen for gene expression analysis. The primers for the corresponding genes were designed using primer3 (http://frodo.wi.mit.edu/primer3/) software. The primers were designed as such that the product length was within 100-200 base pair. List of the primers used in the study is provided in Supplementary Table 1.

EXPERIMENTAL SET UP AND SAMPLE COLLECTION

Two years old vegetatively propagated TV1 clones of tea plant were collected from the nursery of Tocklai Tea Research Institute, TRA, Jorhat. The plants from nursery were replanted in black poly-sleeves (18 cm diameter and 23cm height) with field soil (sandy loam, pH 4.8-5.1, bulk density 1.3-1.4 Mg m^{-3}, single super phophate 0.5 kg. m^{-3} of soil) and allowed to acclimatize for 30 days in natural environmental condition with sufficient irrigation. After the acclimatization period the plants were transferred to a polyhouse and were allowed to acclimatize within the polyhouse for 10 days before starting the stress experiments. The plants were covered with nets for protecting it from external pest infestation. Thereafter the plants were subjected to three level of water stress treatment: 1) well-watered control; 2) mild water stress; 3) severe water stress. The plants in each drought stress level received same amount of water and watered simultaneously. The well-watered control plants received water every 3rd day such that the soil remained constantly moistened. Mild drought stressed plants were watered once the soil water content drops to 7-8% and received 40-50 % of water supplied to the well-watered control plants. The severe water stressed plants were watered when the soil water content reaches 4-5 % and received around 15–20% the amount of water supplied to well-watered control plants. At the end of the three months the plants were rehydrated with small amount of water overnight in all the treatment and then subjected to *Hyposidra talaca* (looper caterpillar) infestation.

Rehydration of plants was done to ensure that the gene expression of plants reflect the effect of pulsed water stressed treatment which is often faced by tea plant in natural environment rather than continuous drought. The leaves were collected before insect infestation i.e - at 0 hours (0 TPI), and after insect infestation i.e - 24, 48 hours (24 TPI, 48 TPI) in all the treatments. The leaves collected at 0 TPI were taken as undamaged control. As only the non-damaged control tissue was measured at the initial time point (time = 0 hrs), the main effect of time represents time post herbivory infestation or time post infestation and is designated at TPI. For each treatment and for each time point there were three biological replicates. Once the plants were used to collect sample they were discarded and not used further in the experiment. For all the treatment the third leaf of tea plant from top was collected and immediately stored at -80^{0}C to prevent any enzymatic activity.

RNA ISOLATION AND cDNA PREPARATION

Total RNA was extracted from 100 mg of each sample according to the protocol of Zaman et al., 2016. RNA integrity was determined using a 1% agarose gel and concentration was quantified using an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany). After verifying the integrity of the RNA, equal concentration of RNA from each sample was used for first strand cDNA preparation using QuantiTect Rev. Transcription Kit (Cat No./ID: 205311, QIAGEN, Germany).

QUANTITATIVE REAL-TIME PCR ANALYSIS

Four important genes known to be involved in the rate-limiting step of the phytohormone biosynthesis pathway (ABA, JA, ET, SA) in tea were selected based on the comparative *in-silico* identification of homologous genes. The expression of the selected four genes was studied in tea plant subjected to three level of water stress treatments with subsequent insect infestation. Quantitative Real-Time PCR was performed in a Roche Light Cycler 480 real time machine (Roche, Germany) using QuantiTect SYBR Green PCR Kit (Cat No./ID: 204145, QIAGEN, Germany). The reverse transcribed first strand cDNA of each sample was used as template in the assay and amplified by gene-specific primers. The PCR was performed in 10 µl reaction volume and prepared according to the protocol mentioned in the kit manual. In short, 3.5 µl of supplied PCR grade water was mixed with 0.5 µl of forward and reverse primer and 5 µl of 'QuantiTect SYBR Green I Master Mix' to get a final volume of 9.5 µl. Finally, 0.5 µl of template was added. The relative expression levels of all the genes were calculated using the ddCt method. The raw Ct values were normalized against Ribulose-1, 5-bisphosphate carboxylase/oxygenase housekeeping gene. The log 2 fold change values for all the samples was calculated relative to well-watered control sample at 0 TPI.

STATISTICAL ANALYSIS

The transcript relative expression values were log 2 fold transformed and analyzed with a 3 × 3 (T x TPI) mixed model analysis of variance (ANOVA) followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing. "T" stands for water stress treatment and "TPI" stands for time post infestation. All the expression data are expressed as mean ± standard deviation (SD). Each expression value is the mean of three biological replicates. Data were analyzed using IBM SPSS Statistics, Version 20.0.

RESULTS AND DISCUSSION

IDENTIFICATION AND ANALYSIS OF PHYTOHORMONE PATHWAY GENES

Gene mining of the four phytohormone pathways (ABA, JA, SA and ET) resulted in a large number of *C. sinen-*

sis homologues for each gene (Table1). CAP3 clustering removed the redundancy of the sequences, still a significant number of homologues were retained after clustering in many instances. In the ABA pathway few genes like ABA 8'-hydroxylase, 9-cis-epoxycarotenoid dioxigenase (*NCED*) etc. were represented by more than one homologue of *C. sinensis* having significant similarity and they possessed most of the common motifs present in corresponding *Arabidopsis* reference sequences (Table 1, Fig.1 (A, B)). Homologue gene mining of JA pathway resulted in the identification of nine genes and each gene best representative homologue is listed in Table1. For the SA pathway a total of four genes were mined but only three gene homologues were retained. The *C. sinensis* homologues obtained for salicylic acid carboxyl methyltransferase gene had an E-value >1e^{-15} and hence it was not considered for further study. Two full-length sequences of ET pathway genes namely 1-aminocyclopropane-1-carboxylate synthase (*ACCS*) and 1-aminocyclopropane-1-carboxylate oxidase (*ACCO*) were obtained based on keyword search in the NCBI NR nucleotide database.

Further the result of Blast2GO program with the details of sequence similarity, GO classification, enzyme list, InterPro scan domain etc. for the four pathway genes are provided in Supplementary Table 2. MEME/MAST search, for the putative functional and common motif occurrence showed that most of the motifs are conserved among the *C. sinensis* homologues and its *Arabidopsis* counterpart except in few cases, where it was seen that few motifs were missing in *C. sinensis* homologues. This may be due to the presence of partial transcript sequences of *C. sinensis*. Few representative genes displaying the occurrence of common motifs between *Arabidopsis* and *C. sinensis* homologues are shown in Fig.1 (A, B), Supplementary Fig. 1 (A-D) & Supplementary Fig. 2 (A-D). The result of the functional domain identification using CD-search tool showed the presence of conserved domains between *Arabidopsis* and *Camellia sinensis* homologues (Supplementary Table 3). The presence of conserved common motif and domain in sequential pattern between the phytohormone pathway genes of *C. sinensis* and *Arabidopsis* clearly confirms their identity and support the results of our comparative genomics approach.

Gene expression of the rate-limiting genes 9-cis-epoxycarotenoid dioxygenase (*NCED*), Allene oxide synthase (*AOS*), 1-aminocyclopropane-1-carboxylate synthase (*ACC* synthase) and Isochorismate synthase (*ICS*) of ABA, JA, ET and SA biosynthesis pathway respectively was studied at different time point in the three water stress treatment with subsequent insect infestation. The relative gene expression values discussed here are expressed in terms of log 2 Fold Change and the well watered treatment at 0 TPI is taken as control for calculation of relative gene expression fold change for other time points and treatments.

Considering the expression of rate limiting genes at 0 TPI (without insect infestation) the expression of *NCED* gene of ABA pathway in case of mild and severe water stress treatment was higher than well watered plants. Mild water stress plant showed a log 2 FC value of 1.31 while severe stress plant showed a value of 1.62 (Fig.2 (A)). In mild water stress plants the *AOS* gene of JA pathway had almost similar expression value with control and the difference was not statistically significant. Whereas in severe water stress plants there was significant down regulation of *AOS* expression with a log 2 FC value of -2.49 compared to control plants ($P< 0.05$, Fig.2 (B)). *ICS* gene of SA biosynthesis pathway showed higher transcript accumulation in mild water stress plant with a value of 2.84 whereas in severe stressed plants it was down-regulated with a value of -4.04 compared to control ($P< 0.05$, Fig.2 (C)). In case of ET pathway, with the increase in water stress intensity the expression of *ACC* synthase gene also increased proportionally with a value of 1.88 and 2.59 in mild and severe water stress treatment respectively (Fig.2 (D)). The transcript expression at 0 TPI represent the constitutive expression of tea plant and it mainly reflect the effect of the water stress treatment on the expression of the phytohormone pathway genes. The expression at 24 TPI and 48 TPI mainly represent the induced expression of the tea plant after insect infestation under different levels of water stress treatment.

At 24 TPI, the expression of *NCED* gene in well-watered plants increased significantly (log 2 FC: 2.13) compared to control at 0 TPI. However the expression at 48 TPI was somewhat less (log 2 FC: 1.42) compared to 24 TPI. In the mild stress plants there was up-regulation at 24 TPI followed by down-regulation at 48 TPI with a value of -0.04. The severe stress plant showed a decline in expression pattern with the increase of time (1.62 at 0 TPI, 1.38 at 24 TPI and 0.04 at 48 TPI). *AOS* gene of JA pathway at 24 TPI showed significant induction in expression (log 2 FC 3.05, $P<0.05$) of well-watered plants. As the time elapsed the gene expression declined at 48 TPI (Fig.2 (B)). However the expression was still significantly higher compared to 0 TPI. Mild and severe stressed plant followed the same trend with increase of transcript accumulation at 24 TPI and then a decline at 48 TPI. The expression of *ICS* gene in case of control and severe stressed plants increased at 24 TPI and decreased at 48 TPI whereas in mild stressed plant the expression declined both at 24 TPI and 48 TPI (Fig.2 (C)). Expression of *ACC* Synthase of ET pathway increased at 24 TPI and then declined at 48 TPI in all the water stress conditions (Fig.2 (D)). Two way ANOVA showed that for the

Table 1. Results of in-silico mining of *Camellia sinensis* phytohormone genes..

Arabidopsis reference sequence accession numbers	Sequence Name	Associated Pathway	Total No. of Homologs (EST + NR + TSA) before CAP3	Total sequences after CAP3 assembly (Contig + singleton)	Camellia sinensis homologous sequences retained after processing	CAP3 (Contig, singleton) Number	Accession No.s of Contigs & singletons	Blast Annotation	BlastX similarity percentage and best hit organism
AT4G19230, AT2G29090, AT5G45340, AT3G19270	ABA 8'-hydroxylase (ABA8ox)	Abscisic acid pathway	22	5	ABA 8'-hydroxylase (ABA8ox) [Singleton1, Singleton2]	Singleton1	HP733304.1	ABA 8'-hydroxylase	86% Citrus sinensis
						Singleton2	HP764411.1	PREDICTED: abscisic acid 8'-hydroxylase 4	83% Vitis vinifera
AT1G52400	ABA glucosidase		12	6	ABA glucosidase [Contig1]	Contig1	KA279844.1, KA279587.1, HP733896.1, GH710784.1, GH710770.1, FE942881.1	beta-glucosidase-like protein	99% Camellia sinensis
AT2G27150	abscisic aldehyde oxidase (ABAO)		6	1	abscisic aldehyde oxidase (ABAO) [Contig1]	Contig1	KA288987.1, HP767578.1, KA286688.1, KA282538.1, HP727479.1, HP770168.1	PREDICTED: aldehyde oxidase 4-like	87% Vitis vinifera
AT1G16540	molybdenum cofactor sulfurase		3	2	molybdenum cofactor sulfurase [Contig1]	Contig1	HP742704.1, JK475554.1	PREDICTED: molybdenum cofactor sulfurase-like	70% Vitis vinifera
AT4G18350, AT3G14440, AT1G30100, AT3G24220, AT1G78390	9-cis-epoxycarotenoid dioxigenase (NCED)		18	4	NCED [Singleton1, Contig2]	Singleton1	HP727751.1	9-cis-epoxycarotenoid dioxygenase 1	88% Diospyros kaki
						Contig2	HP765237.1, BJ999395.1	putative 9-cis epoxycarotenoid dioxygenase	88% Daucus carota subsp. sativus
AT1G67080	neoxanthin synthase (NSY)		2	1	NSY[Contig1]	Contig1	KA283257.1, HP702818.1	neoxanthin synthase	73% Citrus sinensis
AT1G52340	xanthoxin dehydrogenase (XD)		7	6	XD[Contig1]	Contig1	HP701207.1, JK341963.1	short chain alcohol dehydrogenase	79% Citrus sinensis
AT5G67030	zeaxanthin epoxidase (ZEP)		4	1	ZEP[Contig1]	Contig1	HP760589.1, HP739212.1, HP727722.1	zeaxanthin epoxidase 1	82% Vitis vinifera

AT3G45140	13-lipoxygenase/LOX2	Jasmonic acid pathway	20	5	LOX2[Contig1]	Contig1	KA280187.1, DY523322.1, HP756288.1, FE942952.1	lipoxygenase	99% Camellia sinensis
AT4G16760, AT5G65110, AT1G06290, AT3G51840, AT2G35690	acyl-CoA oxidase		38	13	ACX[Contig4, Contig5]	Contig4	FE943071.1, KA288172.1, KA280456.1, HP709220.1, HP724420.1 , HP740917.1, HS399721.1, KA280456.1	Peroxisomal acyl-coenzyme A oxidase 1-like	70% Nicotiana tomentosiformis
						Contig5	HP754805.1, KA298085.1, KA300729.1	acyl-coenzyme A oxidase 2	85% Brassica oleracea var. oleracea
AT3G25760, AT3G25770, AT3G25780	allene oxide cyclase		46	5	AOC[Singleton1, Singleton2]	Singleton1	HP769131.1	allene oxide cyclase	74% Camellia sinensis
						Singleton2	KA282377.1	allene oxide cyclase	99% Camellia sinensis
AT5G42650	allene oxide synthase (CYP74A1)		4	3	AOS[Contig1]	Contig1	KA283779.1, HP717071.1	cytochrome P450 allene oxide synthase	77% Populus trichocarpa
AT5G07010	hydroxyjasmonic acid sulfotransferase		5	4	hydroxyjasmonic acid sulfotransferase (AtST2a)[Contig1]	Contig1	KA297677.1, HP755751.1	PREDICTED: flavonol sulfotransferase-like	74% Vitis vinifera
AT1G19640	jasmonic acid carboxyl methyltransferase		1	NA	NA	KA286401.1	KA286401.1	Jasmonate O-methyltransferase, putative	67% Ricinus communis
AT2G46370	jasmonic acid-amino acid synthase		6	4	JAR[Contig1]	Contig1	HP705426.1, KA286035.1	JAR1-like protein	80% Nicotiana attenuata
Keyword Search	OPC-8:0-CoA ligase		NA	NA	NA	NA	FS957551.1	OPC-8:0-CoA ligase	NA
AT1G20510	OPC-8:0-CoA ligase		6	4	OPCL1[Contig1]	Contig1	HP750023.1 ,KA293135.1, KA286488.1	4-coumarate--CoA ligase-like 5	76% Sesamum indicum
AT2G06050	OPDA reductase		9	2	OPR3[Contig1]	Contig1	HP743074.1, KA280376.1, HS399901.1, HP725651.1, HP746582.1, HS398253.1, HS398153.1, KA302045.1	PREDICTED: 12-oxophytodienoate reductase 3	82% Vitis vinifera

AT1G18870, AT1G74710	isochorismate synthase	Salicylic acid pathway	5	2	ICS[Contig1]	Contig1	HP734410.1, KA297692.1,	isochorismate synthase, putative	69% Ricinus communis
AT2G23620, AT2G23600, AT2G23560, AT4G37150	methyl salicylate esterase		13	6	Methyl_salicylate_ esterase [Singleton1]	Singleton1	KA281093.1	PREDICTED: polyneuridine-aldehyde esterase	63% Vitis vinifera
AT2G43840, AT2G43820	salicylic acid glucosyltransferase		14	8	salicylic acid glucosyltransferase [Singleton1, Contig2]	Singleton1	KA286158.1	PREDICTED: UDP-glycosyltransferase 74F2	71% Vitis vinifera
						Contig2	HP768085.1, KA296966.1	PREDICTED: UDP-glycosyltransferase 74E1-like	80% Vitis vinifera
Keyword Search	1-aminocyclopropane-1-carboxylate synthase	Ethylene pathway	NA	NA	NA	NA	EF205149.1	1-aminocyclopropane-1-carboxylate synthase	NA
Keyword Search	1-aminocyclopropane-1-carboxylate oxidase		NA	NA	NA	NA	DQ904328.1	ACC oxidase	NA

NCED gene expression there was significant main effect for time post infestation and interaction effect between water stress treatment and time post infestation whereas for *AOS*, *ICS* and *ACC* synthase gene expression both the main effect (water stress treatment, time post infestation) and there interaction effect was significant (Fig 2 A- D). The homogeneity and specificity of the single PCR product was determined by the melting curve and melting peak of the four analyzed genes and are provide in Supplementary Fig. 3 (A-D), Supplementary Fig. 4 (A-D) For supplementary data please see:https://drive.google.com/drive/folders/1qVqzAqW1IEgkKnh6RA75zkdVIUxx0xH?usp=sharing.

It can be seen that as the water stress (0 TPI) increased the transcript abundance of *NCED* gene also increased (Fig.2 (A)). Water stress is known to increase the expression of *NCED* gene followed by accumulation of ABA (Shinozaki and Yamaguchi-Shinozaki, 2007; Wang et al., 2009). The function of ABA in the control of stomata closure and the responses to abiotic stress is well-established (Mittler and Blumwald, 2015). ABA integrates various stress signals and is known to controls stress responses during water deficit stress (Raghavendra et al., 2010; Ye et al., 2012). After insect infestation the expression of *NCED* gene in well watered plant showed higher induction than mild and severe stressed plants (24 TPI) and then there was a drop in transcript level at 48 TPI for all treatment with well watered plant retaining the highest transcript abundance.

When the expression folds were analyzed for statistical significance main effect of water stress treatment (T) on *NCED* gene expression was not statistically significant ($P > 0.05$). However the main effect of time post infestation (TPI) and interaction effect (TxTPI) was significant ($P<0.05$). Herbivore infestation and the interaction of water stress treatment and time post infestation significantly regulated the expression differences. The strong induction of *NCED* expression in well-watered and mild stressed plants along with the increase in *AOS* gene expression of JA pathway at 24 TPI (Fig. 2(A) , Fig.2(B)) strongly suggest the synergistic role of *ABA* and JA signaling pathway genes in plant herbivore defense. On the other hand severe stressed plant produced a smaller induction for both *NCED* and AOS gene. It is very likely that water stress severity negatively affected the induced response of the *ABA* and JA biosynthesis pathway genes on herbivore attack. *ABA* have been reported to interact with JA signaling and enables *N. attenuata* plants to mount a full defense response against chewing herbivores (Dinh and Baldwin, 2013).

The expression of *AOS*, JA pathway rate-limiting gene, was significantly down-regulated in severe water stressed plants with a value of -2.49 compared to well watered control plants (0 TPI, $P<0.05$, Fig. 2 (B)). Irrespective of water treatment on herbivore infestation *AOS* gene expression increased at 24 TPI for all

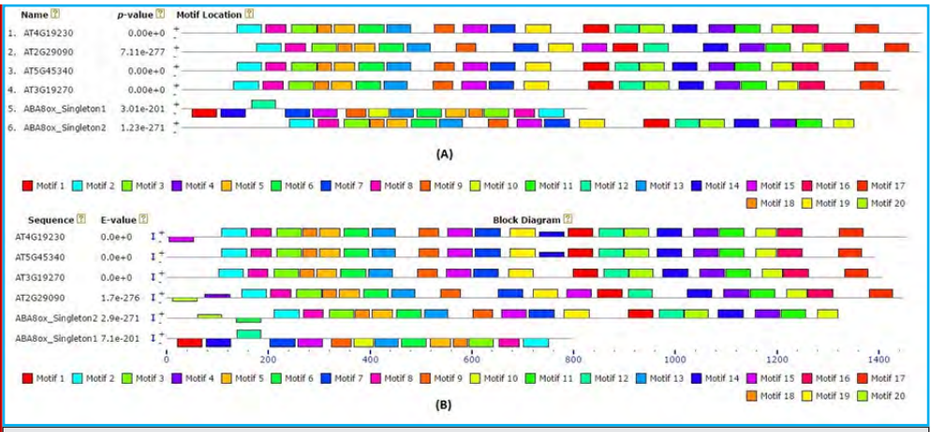

FIGURE 1. (A) Schematic representation of occurrence and distribution of motifs in the ABA 8'-hydroxylase sequences of *Arabidopsis* and *Camellia sinensis* - MEME program output (B) Schematic representation of motifs and sorting of *Arabidopsis* and *Camellia sinensis* ABA 8'-hydroxylase sequences by the best combined match to all motifs - MAST program output.

the plants with control showing maximum transcript abundance. It is noteworthy to mention that severe stressed plant had maximum induction value when the induced expression fold change of individual treatment at 24 TPI is calculated relative to its own expression at 0 TPI. Wound induced elevated level of *AOS* gene expression was found to correlate with the increase in endogenous JA content (Wilmowicz et al., 2016). JA is mainly involved in biotic stress response and its role biotic defense is well established. The detailed molecular mechanisms of the role of jasmonates for drought stress signaling are still unclear (Riemann et al., 2015). Plant

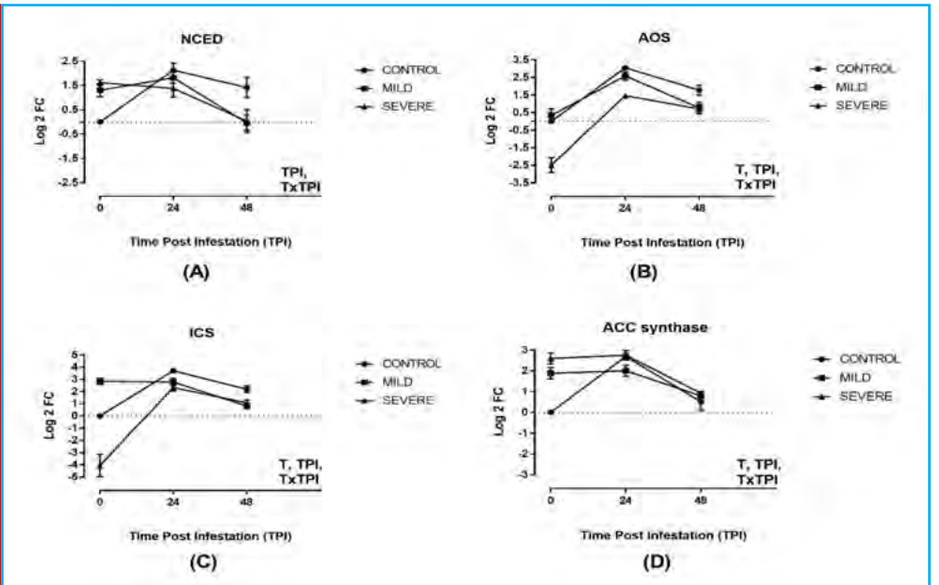

FIGURE 2. Expression of 9-cis-epoxycarotenoid dioxygenase (*NCED*) gene of ABA pathway (B) Expression of Allene oxide synthase (*AOS*) gene of JA pathway (C) Expression of Isochorismate synthase (*ICS*) gene of SA pathway (D) Expression of 1-aminocyclopropane-1-carboxylate synthase (*ACC synthase*) gene of ET pathway. Significant main effect of water stress treatment and time post infestation are indicated by T and TPI respectively, while significant interaction are indicated by TxTPI ($P<0.05$). All the expression data are calculated relative to well watered control sample at 0 TPI and are expressed as mean ± standard deviation (SD). Each expression value is the mean of three biological replicates.

responses to combined abiotic stresses and biotic stress are largely controlled by different signaling pathways that may interact and inhibit one another (Suzuki et al., 2014). In our study the expression of *AOS* gene of JA biosynthesis pathway is clearly suppressed in severe water stressed plants (0 TPI, 24 TPI). High transcript accumulation of *AOS* gene in controls and mild water stressed plants compared to severe stressed plants indicates that water availability play an important role in stronger constitutive and induced JA pathway defense. Plants under increasing drought stress may be at danger of herbivore infestation with lower activation of JA pathway defense. In some plants, it has been reported that a specific abiotic stress enhanced the resistance of plants to biotic stress (Rouhier and Jacquot, 2008). However, in most cases, prolonged exposure of plants to abiotic stresses, such as drought resulted in the weakening of plant defenses (Mittler and Blumwald, 2010). Plants under combinations of abiotic and biotic stresses may prioritize responses to address the potentially more damaging abiotic stress (Atkinson et al., 2013).

Increase in *ICS* gene expression at 0 TPI in mild water stress points towards the role of SA pathway in moderate water stress condition. SA significance has been increasingly recognized in enhanced plant abiotic stress-tolerance via SA-mediated control of major plant-metabolic processes (Khan et al., 2015). Studies extensively found and reviewed the role of SA pathway in the improvement of plant abiotic stresses tolerance such as drought (Horváth et al., 2007; Pal et al., 2013; Fayez and Bazaid, 2014; Miura and Tada, 2014). In induced response (24 TPI) well watered plant maintained higher *ICS* gene expression followed by mild and severe water stressed plant. It is known that upon insect attack usually two signaling pathways Salicylic acid (SA) and Jasmonic acid (JA), mediate plant responses. Severe water stress negatively affected the *ICS* gene expression at constitutive and induced level. The lower expression of SA biosynthesis pathway gene in water stress tea plant may increase their susceptibility to herbivores and necrotrophic pathogens. A significant amount of literature mentioned that the induction of the SA signaling pathway suppresses JA signaling (Niki et al., 1998; Preston et al., 1999; Koornneef et al., 2008a, 2008b).

However, in our study, we have seen a completely different picture where SA and JA associated gene expression increased co-currently in control and mild stressed plants. Whereas in case of severe stressed plant both the genes were significantly down-regulated. Thus JA and SA pathway associated gene expression may not always act antagonistically and may act in synchrony according to the type and severity of stress it is undergoing. In tea cultivation, water stress severity is an important factor which needs to be taken care to avoid disastrous pest and pathogen attack due to weakening of SA and JA associated plant defense.

ACC synthase expression showed maximum peak in severe stressed plants at 0 TPI. The increased constitutive expression in water stressed plants may be associated with ET signaling role in osmotic stress adjustment. ET signaling is known to act as an important controller of the hormone-regulated defense pathways in biotic stress (Broekgaarden et al., 2015) as well as helping plants to adjust to drought stress (abiotic stress) by increasing the compatible solutes accumulation (Cui et al., 2015). Pairwise comparison showed that induced expression value was not significant between control and severe stressed plant ($P > 0.05$, Fig.2 (D)). However it was significant between control and mild stressed plant ($P < 0.05$, Fig.2 (D)). When the fold change at 24 TPI in all treatment is calculated relative to its own expression at 0 TPI the increase in well-watered fold induction in maximum. Thus the increase of *ACC* synthase expression in response to herbivore infestation points towards ET signaling role in biotic stress signaling and well-watered plant have higher capacity of transcript induction on insect infestation. Ethylene transcripts mediated role in plant stress and pathogen responses have been already reported in literature (Abeles et al., 1992; O'Donnell et al., 1996). In rice plant, *ACC* oxidase and *ACC* synthase transcript up regulation in response to feeding by the brown planthopper, *Nilaparvata lugens* (Stal) have been documented (Zhang et al., 2004).

Overall from our study it has been seen that water stress positively affected ABA and ET pathway genes with increase in constitutive expression. However JA and SA pathway genes known to be involved with herbivore and pathogen defense signaling were negatively affected in severe stressed plants. In case of induced expression (24 TPI), severe water stressed plants showed significant decrease in expression of the rate-limiting phytohormone genes compared to well water plants except *ACC* synthase gene. The crosstalk among the phytohormone pathway transcriptional defense in combination with other intrinsic defense mechanism will ultimately govern the of tea plant response.

The presence of abundant antagonistic or synergistic interactions among the pathways provide the plant with an extensive regulatory potential for the activation of specific defenses (Vos et al., 2013). When combination of water and herbivory stress was applied, it seems to be most likely that plant transcriptional defense machinery was largely controlled by water stress severity and herbivory stress regulation of phytohormone genes was overpowered by water stress gene regulation. Thus our results clearly shows that well-watered plants on insect infestation will have higher capacity to induce the expression of phytohormone genes than water stressed plants.

CONCLUSION

It can be concluded that the presence of an initial water stress not only affected the tea plant constitutive defense but also significantly altered the phytohormone defense gene expression towards subsequent herbivore stress. On prolonged drought events tea plant with weak induced defense may face higher incidence of pest and pathogen attack.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Comparative analysis of plant growth regulators on bud break in *Prosopsis* and *Tecomella* for sustainable agriculture in arid and semi-arid regions of India

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ABSTRACT

Prosopsis cineraria commonly known as Khejri and *Tecomella undulata* known as Rohida are Golden Trees of Thar Desert in India belonging to the family *Fabaceae and Begnoniaceae* respectively. Both the plants are multipurpose tree whose almost all parts are used in pharmaceutical industry for preparing medicines. The medicinal uses of this plant has necessitated large scale production and as raw material to medicinal industry, leading to its over-exploitation. Both the trees are important component of desert Ecosystem of India as biomass producer and enrich desert soil, fix atmospheric nitrogen and provide a green coverage. Both contribute to ecological stability of the region and providing extensive support to human beings, livestock and the nutrient deficient soils. The plant tissue culture techniques can play an important role in propagation and qualitative improvement of plants of medicinal aspects. Axilary nodes, and shoot tips were aseptically cultured on MS basal medium fortified with different concentrations of cytokinins (BAP, Kinetin) and auxins (NAA, IAA,IBA) along with sucrose as energy source. The plant growth regulators act in synergistic way to proliferate shoots of almost 1-5cm lengths which were rooted on rooting media to regenerate the whole plant. In vitro developed complete seedlings were acclimatized and propagated in vitro for mass cultivation.

KEY WORDS: BUD BREAK; BAP; MICRO PROPAGATION; *TECOMELLA, PROSOPSIS*

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INTRODUCTION

Woody trees are vital to arid environments because of their ecofriendly and multipurpose nature, and the fact that they are well able to tolerate drought situations. *Prosopis* and *Tecomella* are the principal genera in these regions, and both have great biological diversity and ecological plasticity. These are used worldwide in arid regions to improve the local economy. These tree species are biologically diverse and are well adapted to stress as a result of multiple interbreeding species. Leguminous tree products are economically important sources of food, fodder, firewood, and timber. Improvement in quality attributes through selection, modification and mass production of germplasm is desirable. Propagation through seeds is the most common practice for raising quality trait seedlings for new plantations in arid areas. such as cuttings, suckers, air layering and tissue cultures are available but more efforts towards their refinement are still required, particularly with regards nursery and laboratory techniques, before commercial cultivation (Baksha *et al.,* 2007; Biswas *et al.,* 2009; Roy, 2008). Plant cell tissue culture has offered a very novel technique to mass multiply, true to type and providing disease resistant plants in controlled conditions.

Prosopis cineraria commonly known as khejri belongs to pea family, *Fabaceae* and is a multipurpose tree of desert in Western Rajasthan. It is also called kalptaru, 'wonder tree' and the 'king of desert. (Singh *et al.* 2013, Tarachand *et. al.* 2012). It is native to arid portions of Western and the Indian subcontinent, including Afghanistan, Iran, India, Oman, Pakistan, Saudi Arabia, the United Arab Emirates, and Yemen. In India it is found in the various parts of Rajasthan, Gujarat, Haryana, Uttar Pradesh and Tamil Nadu (Rathore *et al.,* 1991). It is regarded as a backbone of rural economy being a good biomass producer and fixes atmospheric nitrogen and provides a green coverage and in turn helps in the enrichment of desert soil. It contributes to ecological stability of the region and providing extensive support to human beings, livestock and the nutrient deficient soils (Chaudhry 2011, Panwar *et al.,* 2014 and Hua et al. (2015).

The tree is well adapted to arid and semi-arid conditions of the Indian desert, due to their well-developed and expansive tap root system which reach up to a length of 20 m, often reaching out the ground water resources (Gehlot *et al.,* 2008). Pods of this plant locally called "Sangri" are considered as dry fruits of desert. Pods contain various phytoconstituents like tannins (gallic acid), steroids (stigma sterol, campestral, sitosterol, etc.), Flavones derivatives (prosogerin A, B, C, D, and E), alkaloids (spicigerine, prosophylline), etc. have been isolated from the sangri pods (Gehlot *et al.,* 2008). The ashes of bark are rubbed over the skin to remove hair. Fresh Leaves juice mixed with lemon juice is used for dyspepsia; extract of crushed pods is used for earache, toothache, pain relief from fractured bones (Garg and Mittal 2013).

The whole plant is used in the Indigenous System of Medicine as a folk remedy for various ailments like leprosy, dysentery, bronchitis, asthma, leucoderma, piles, muscular tremor and wandering of the mind. It is also known to possess anthelmintic, antibacterial, antifungal, antiviral and anticancer activities. *Tecomella undulata* belongs to family *Begnoniaceae* is an important medicinal plant. Wide range of therapeutic activities has been attributed to this plant. The plant is excellent blood purifier hence rewarding in hepatitis. Bark forms a major constituents of various herbal formulations like Livoplus, Liv-52, Livosan,Herboliv, Amylcure for curing inflammatory hepatic disease. The leaves have oleanolic acid, ursolic acid and betulinic acid which are strong prohibitors of HIV (Nandwani *et al.,* 1995; 1996). It has been reported to be used in phyto remediation of soil contaminated with crude petroleum oil its common agro forestry tree in arid and semi arid regions used for research, including bio fertilizer aspect as well as afforestation programmes (Rathore *et al.,* 1991 and Shekhawat *et al.,* 1993).

The plant is propagated mainly using seeds and the tree is slow growing species. Till date the woody plants lack suitable methods for vegetative propagation on mass scale. Seeds are on prime importance for extensive plantation. The seeds are available from month of April to June. Freshly harvested seeds have more potential for germination as compared to unripe fruits or ripe fruits collected in June. So present investigation was undertaken with an objective to devise one suitable protocol for bud initiation and plant propagation using nodal tissues in both *Tecomella* and *Prosopsis* (Bhansali, 1993).

MATERIAL AND METHODS

The seedlings of *Prosopsis* and *Tecomella* were used in the present study for bud break and shoot proliferation, which were procured from Tau Devi Lal Herbal Park, Near Khizrabaad Highway, Yamunanagar District, Haryana, India. Axillary nodes were selected as explants to avoid genetic alterations and somaclonal variations observed using indirect regeneration. The disease free axillary buds were collected from 4 weeks old healthy plant. The explants were excised and the contaminants were washed under running tap water for 4-5 minutes, followed by washing in the liquid detergent Tween -20 (few drops / 100 ml solution) and then rinsed with running tap water. The cleaned explants were surface steri-

lized with variable concentration of mercuric chloride (0.1%-0.5%) under laminar air flow. The explants were subjected to different time intervals to optimize the sterilization procedure and then rinsed 4-5 times with sterilized distilled water. After trimming the cut ends, equal sized, surface sterilized explants were cultured on the culture medium defined by Murashige and Skoog, (1962). Among all experiments, a treatment of (0.1% and 0.3%) $HgCl_2$ for 3 minutes proved to be the best for sterilization in *Prososopsis* and *Tecomella*, a large percentage (85%) of explants. The surface sterilization was optimized and this helped in preventing blackening of tissue on exposure to mercuric chloride and establishment of clean cultures.

Axillary nodes were excised from the young seedlings grown *in vivo* conditions and were cultured on different shoot proliferation media (Table 1,2) consisting of different concentration and combination of cytokinin and auxin as plant growth regulator. Axillary node explants were implanted vertically. Test tubes and flask containing the explants were sealed with sterilized cotton plugs and incubated for 4 weeks at 25±2°C under a 16 hour photoperiod. Radiation source was supplied by soft white fluorescence tubes. For each treatment, a minimum of 3 replicates were carried out. Percent shoot proliferation and multiple shoot formation was recorded after four weeks of culture.

RESULTS AND DISCUSSION

The bud break, shoot proliferation and exploring the potential for multiple shoots regeneration in *Prosopsis and Tecomella is* an utmost requirement for propagation of this plant.Sterilization of explants is a crucial step in plant tissue culture and to achieve 100 percent sterilization explants were subjected to various concentration of mercuric chloride (0.1%-0.5%) along with antifungal supplement and a prior ethanol treatment. Maximum sterilization (80%) was observed using a concentration of 0.3% of mercuric chloride for 3 min. A higher concentration of 0.5% of mercuric chloride burn the tissue and cause blackening at the edges resulting in 23 % sterilization while a lower concentration of 0.1% of mercuric chloride resulted in only 40% sterilization. It was found that mercuric chloride alone at a concentration of 0.3% for 3 minutes is sufficient to sterilize the explants without a prior treatment of ethanol and antifungal supplements.

The axillary nodes were cut in equal size and surface sterilized using standard procedure. A concentration of 0.1% $HgCl_2$ for 3 minutes resulted in 85% of sterilization of explants while as compared a lower concentration of $HgCl_2$ was less effective in sterilization process. Even a concentration of 0.05 for 5 minutes resulted in 51% decontamination of explant. However a pretreatment of ethanol (rinse) and Bavistin (45min) resulted in higher percentage of healthy sterilized tissue.

The axillary nodes excised from *in vivo* grown young seedlings were sterilized using standard mercuric chloride solution at 0.1% concentration for 3 min which was sufficient to obtain 80% sterilization. The proliferation of shoots from cultured explant was remarkably influenced by the type of concentration of the growth regulator used. Axillary node cultured on MS medium supplemented with different concentration of BAP alone showed the best results.

TECOMELLA UNDULATA

Complete plant development through tissue culture strongly relies on synergistic effects of growth hormone or say auxin and cytokinin which play a significant role in cell differentiation and whole plant growth in *Tecomella*. Different concentrations of cytokinin and auxins were used alone or in combination for initiation of shoot proliferation. BAP was taken alone without any auxin in combination at different concentrations (0.1,0.5,1.0 mg/L) resulted in bud break and shoot proliferation of up to 2cm from sterilized axillary nodes cultured . Further increase in BAP concentration from 1.5 to 2.0 mg/L resulted in shoot proliferation 1-2 cm in length with a regeneration frequency of 50% in approximately 10 days. Similar results were obtained using axillary nodes in *Prosopsis* by Pareek et al., 2012 and Kumar and Singh (2009) who supported the effectiveness of BAP and KIN in regeneration of multiple shoots in *Prosopsis*. Similar concentration of BAP alone at 0.2 mg/l found to be effective in axillary bud break in *Baccopa monnieri*, (Sharma et al., 2010).

While on further increase in BAP concentration from 2 to 3 and 3.5mg/l found to be inhibitory. No bud break or initiation in shoot proliferation was noticed on higher concentration which clearly demonstrates that there is decrease in plant cell differentiation with much higher concentration of hormones. With an increase in concentration of growth regulators the days required to bud break also increased which is further supported by our present studies. The results totally contradict the finding of Kumar and Singh (2009) who observed one to multiple shoots on medium containing higher concentration of BAP (5.0mg/l) along with IAA at (1.0mg/l). Indole acetic acid was used as auxin in combination with BAP as cytokinin. The concentration of BAP was kept constant (1.0mg/l) and IAA as auxin was used in combination at four different concentrations (0.01,0.02,0.05,0.1mg/l) resulted in bud break and shoot proliferation of 1-2cm in length with an increase in regeneration frequency

from 50%to 65% and bud break initiated in lesser time period of 8 days. This combination reveals the synergistic action of cytokinin to act and promote cell differentiation along with auxin. After getting positive response of BAP along with IAA, the concentration of BAP was raised to 1.5mg/l and was kept constant while the concentration of IAA was altered (0.01,0.02,0.05,0.1mg/l).The shoot length increased in size to 3 cm as compared to earlier with bud break and shoot proliferation in 7 days.

The regeneration frequency also get increased to 95%.Our results well corroborate with the findings of Lal and Singh(2010) in *Celastrus paniculatus* using different cytokinin BAP and Kinetin(0.5,1.0,2.0mg/L) along with auxins (IAA, NAA, and 2,4-D) using nodal explants from mature tree of this species and observed less number of shoots per explants and 100% bud break on MS medium supplemented with BAP.(1.0mg/L). Yadav et al in 2011 reported that season of collection of explants showed direct influence on bud break in *Celastrus paniculatus* using shoot tip explants obtaining highest percentage (90%) bud break and multiple shoot formation(4/explant)on MS medium containing 1.0 mg/l BAP.

On further increase in BAP concentration from 1.5 to 2.0mg/l with IAA in combination at four different concentrations (0.01, 0.02, 0.05, 0.1mg/l) no significant increase was observed. Although the bud break and shoot proliferation was observed which was almost same resulting in 1-2 shoots / explants of 1-2 cm in length with a regeneration frequency of 60% only .The results clearly depicts the synergy between auxin and cytokinin to act in combination for bud break and shoot proliferation but a higher concentration of BAP was found to be inhibitory whether used alone or in combination with IAA. Present results strongly contradict to that observed by Warrier et al in 2010 in *Aegle marmelos* where higher concentration of BAP (2.5mg/l) was beneficial for inducing multiple shoots. (Table-1 and figure 1)

PROSOPSIS CINERARIA

Different cytokinins and auxins were employed alone or in combination for bud break and shoot proliferation in *Prosopsis*. Eighteen different media combinations were used for *Prosopsis* micropropagation which was recalcitrant to grow taking different concentrations of growth hormones. Six different medium were used having BAP (0.5.1.0,1.5, 2.0mg/l) along with NAA as auxin at two different concentrations of (0.5 and 1.0 mg/l). Four medium consisted of BAP along with IBA where IBA was kept constant at a concentration of (1.0mg/l) while the BAP concentration was raised from (1.0 mg /l to 4.0 mg/l). Further six medium consisted of kinetin in combination with two different auxins ie. NAA and IBA. NAA was kept constant while kinetin was used at two different concentrations of (2.0 and 3.0 mg/l),while with IBA the concentration was kept constant at 1.0 mg/l and kinetin was used at very high concentration of(5.0,7.0,9.0,10.0mg/l)to search out the possibility of multiple shoots at much higher concentration.

Six more combinations of auxin and cytokinin includes Zeatin in combination with IBA,where IBA concentration was kept constant at 1.0 mg/l but Zeatin concentration was altered (1.0,1.5,2.0,2.5,3.0,4.0). Although a higher concentration of Zeatin was also used similar to kinetin (5.0,7.0,9.0,10.0mg/l) but the higher concentration as observed earlier in case of *Tecomella* were found to inhibitory and no bud break or shoot proliferation was observed in *Prosopsis*.

On increasing the concentration of BAP step by step from 0.5to2.0mg/l and keeping the NAA concentration constant at 0.5mg/l. At lower concentrations bud break was observed in 15 to 20 days at 0.5 and 1.0mg/l of BAP while on further increase in concentration (1.5and 2.0mg/L)1-2 shoots per explants were observed of 1-1.5cm in length with higher regeneration frequency as compared to lower concentrations used within same time frame (Figure 2). When NAA concentration was raised from 0.5 to 1.0mg/l in combination with BAP at two different concentrations of 0.5and 1.0mg/l no significant change was observed, moreover the medium resulted in bud break only.

Similar findings were reported by Singh et al. (2014) in *Shorea robusta* a woody valuable tree species using nodal explants. They reported that BAP at 1.0mg/l along with NAA at a concentratons of 0.5mg/l found to be the best medium for shoot initiation and proliferation. Similar findings observed by Tyagi et al (2010) in *Capparis deciduas* using axillary shoots observed shoots of 2cm in length when cultured on MS medium supplemented with 2mg/l BAP along with 0.5mg/l NAA.in 2010.

Girijashanker (2011) in *Acacia auriculiformis* a multipurpose tree of medicinal forestry observed higest percentage of shoots induction on BAP (2mg/l)along with NAA at (0.1mg/L). In combination of Zeatin with IBA, IBA was kept constant at 1.0mg/l while the zeatin concentration at different concentration resulted in 1-2 shoots per explants of 1-5cm in length in 45 days with a regeneration frequency of 55%. Similar combinations have been reported by Hua et al. (2015) to be effective in *Pitaya* for shoot proliferation, they used 3.0uM Zeatin in combination with IBA at 0.5mg/l resulting more vigorous multiplication of shoots. With an increase in Zeatin concentration has positive effects on bud break and shoot length elongation as a maximum of 5cm length shoots were observed on 4.0mg/l concentration, while an further increase in concentration of Zeatin has inhibitory effects on cell differentiation same as kinetin.

Table 1. Composition of MS medium supplemented with different growth regulators and result obtained after incubation of *Tecomella undulata* nodal culture.

S.No.	Media Code	Growth Hormones (mg/L)				No. of shoots	Shoot length	% of regeneration	Days of initiation
		BAP	IAA	ABA	NAA				
1	MS - 1	---	---	---	---	Control			
2	MS - 2	0.1	---	---	---	Bud break	---	16	9
3	MS - 3	0.5	---	---	---	Bud break	---	30	10
4	MS - 4	1.0	---	---	---	Bud break	---	25	9
5	MS - 5	1.5	---	---	---	1	1.5	50	13
6	MS - 6	2.0	---	---	---	1	1.0	45	12
7	MS - 7	2.5	---	---	---	Bud break	---	35	11
8	MS - 8	3.5	---	---	---	---	---	---	11
9	MS - 9	1.0	0.01	---	---	---	---	---	8
10	MS - 10	1.0	0.02	---	---	1	1.5	60	7
11	MS - 11	1.0	0.05	---	---	1	1.0	55	8
12	MS - 12	1.0	0.1	---	---	2	1.0	65	7
13	MS - 13	1.5	0.01	---	---	2	1.5	80	7
14	MS - 14	1.5	0.02	---	---	3	2.5	95	7
15	MS - 15	1.5	0.05	---	---	1	1.5	80	9
16	MS - 16	1.5	0.1	---	---	Bud break	---	50	11
17	MS - 17	2.0	0.01	---	---	Bud break	---	54	7
18	MS - 18	2.0	0.02	---	---	Bud break	---	70	8
19	MS - 19	2.0	0.05	---	---	Bud break	---	65	7
20	MS - 20	2.0	0.1	---	---	1	1.0	60	9
21	MS - 21	2.5	0.01	---	---	1	1.5	65	10
22	MS -22	2.5	0.02	---	---	2	1.0	55	9
23	MS - 23	2.5	0.05	---	---	2	1.0	60	11
24	MS -24	2.5	0.1	---	---	2	1.0	55	10
25	MS -27	1.5	---	---	0.01	Bud break	---	70	8
26	MS -28	1.5	---	---	0.1	Bud break	---	65	9
27	MS -29	2.0	---	---	0.01	1	1.5	55	7
28	MS - 30	2.0	---	---	0.1	---	---	---	8

The results depicts that higher concentrations of hormone should not be taken as alternative to mass propagate plants in shorter period of time. BAP was used at different concentrations (1.0,2. 0,3.0,4.0), while IBA concentration was kept constant at 1.0mg/l resulted in 1-2 shoots per explants of 2-3cm in length in 35-40 days approximately. NAA was kept constant at a concentration of (1.5mg/l) and using kinetin at two different concentration (2.0 and 3.0mg/l) the shoot length increased in size from 2 to 4cm almost double while the initiation process of shoot proliferation also shorten by 10 days from 40 to 30 days. The results well corroborate with that of Dhabhai et al. (2010) in *Acacia nilotica* a nitrogen fixing tree through direct regeneration using nodal segments cultured on MS medium supplemented with Kinetin (1.0mg/l along with NAA at 0.6mg/l they observed highest no of shoots of 2-3 cm in 15-20 days of culture. Multiple shoots were not observed on any of the media used only one or two shoots were observed otherwise the shoots just proliferated to 2-4 cm in 15 days. Similar findings were observed in *Vitex negundo,* where MS medium supplemented with BAP individually enhanced the induction of multiple shoots within an average time of 8 to 12 days, while BAP (2.0mg/L) in combination

FIGURE 1. Bud break on MS medium supplemented with different growth regulators in *Tecomella undulate*. A) BAP (0.1mg/L),B) (0.5mg/L)andC) (1.0mg/l).

with NAA (0.5 mg/L) resulted in a higher percentage (93%) of multiple shoot formation. The results were further supported in *Gloriosa superba*, *Rauvolfia serpentine* and *Boerrhavia diffusa* (Baksha et al., 2007; Hassan and Roy, 2005 Biswas et al., 2009). MS media without growth regulators failed to induce bud break from nodal segments of *Tecomella* which is probably due to insufficient level of endogenous growth regulators in explants to induce bud break and it required an exogenous supply.

Our present investigation focused on initial bud break. Supplementation of cytokinins like BAP at dif-

Table 2. Composition of MS medium supplemented with different growth regulators and result obtained after incubation of *Prosopis cineraria* nodal culture.

S. No	Media code	Growth hormones (mg/L)				No. of shoots	Shoot length (cm)	% of Regeneration	Day of initiation
		Zeatin	IBA	BAP	NAA				
1	MS - 1	1.0	1.0	---	---	---	---	30	30
2	MS - 2	1.5	1.0	---	---	2	1.5	30	30
3	MS - 3	2.0	1.0	---	---	1	1.5	50	30
4	MS - 4	2.5	1.0	---	---	1	5	50	30
5	MS - 5	3.0	1.0	---	---	1	5	60	40
6	MS - 6	4.0	1.0	---	---	1	4.5	60	40
7	MS - 7	---	1.0	1.0	---	2	2.5	30	40
8	MS - 8	---	1.0	2.0	---	1	2.5	40	40
9	MS - 9	---	1.0	3.0	---	1	3.0	25 30	
10	MS - 10	---	1.0	4.0	---	1	2.5	35 30	
11	MS - 11	---	---	0.5	0.5	Bud break	---	15	15
12	MS - 12	---	---	1.0	0.5	Bud break	---	20	20
13	MS - 13	---	---	1.5	0.5	2	1.5	20	20
14	MS - 14	---	---	2.0	0.5	1	1.0	20	20
15	MS - 15	---	---	0.5	1.0	Bud break	---	20	10
16	MS - 16	---	---	1.0	1.0	Bud break	---	10	15
17	MS - 17	---	---	---	1.5	1	2	10	30
18	MS - 18	---	---	---	1.5	1	4	15	40

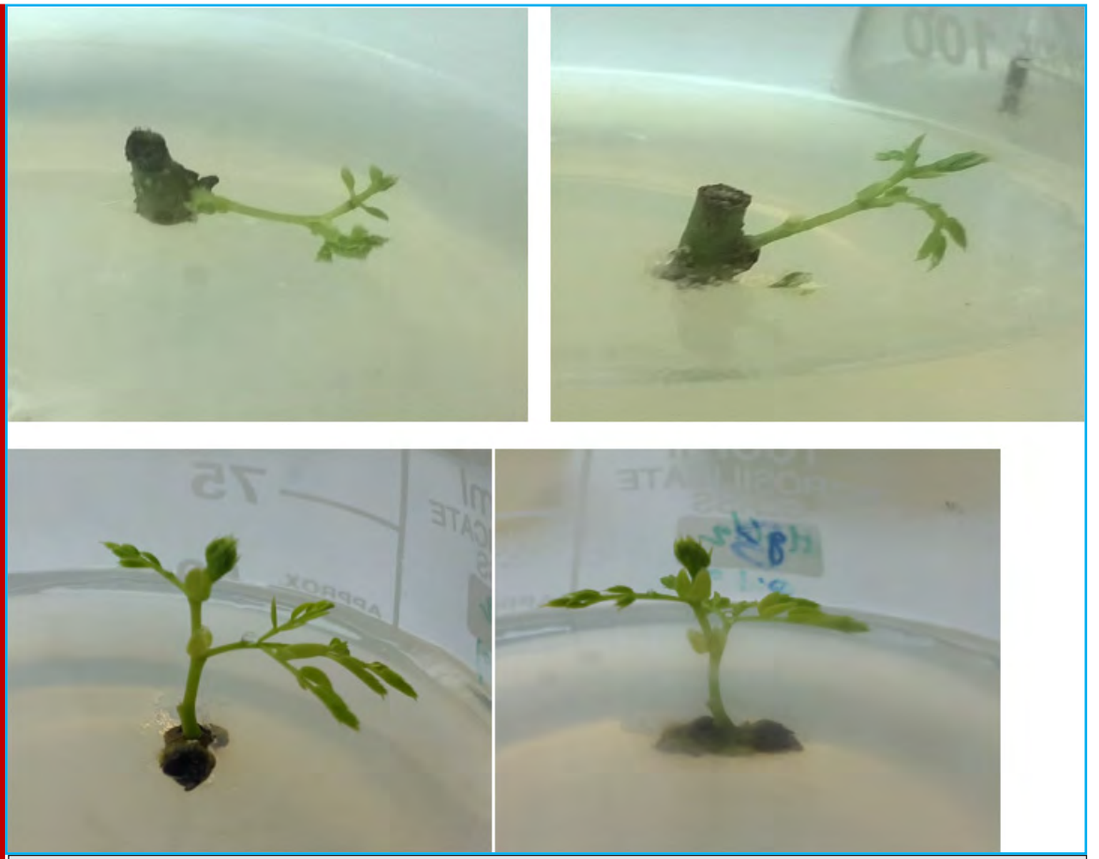

FIGURE 2. Shoot propagation on MS medium supplemented with growth regulators (BAP 1.5mg/l + NAA 0.5mg/l)

ferent concentration induces bud break as well as initiation of shoot. Cytokinin supplemented media nullifies the effect of apical dominance and promote axillary bud prolifiration into shoots. This is due to exogenous applications of cytokinins disturbs the internal polarity and change the genetically physiology of explants, resulting in organogenesis. Similar effectiveness of cytokinins in promoting in vitro auxillary bud break have been reported in many forest plants. Auxins and cytokinins have synergistic action in plant cells proliferation and differentiation. The ratio of auxin to cytokinin has major impact on growth and bud break. The release or absorption of one growth regulator is regulated by the presence of other.

CONCLUSION

Multiplication of germplasm through plant tissue culture methods is one of the applications of biotechnology via which elite trees can be mass produced rapidly. MS medium without growth regulators failed to induce bud break from nodal segments of *Tecomella* and *Prosopsis* probably due to insufficient level of endogenous growth regulators in explants to induce bud break and it requires an exogenous supply of growth hormones. Our present investigation was merely focused on initial bud break and shoot proliferation with axillary buds in *Tecomella* and *Prosopsis*. Both the plants have importance as medicinal value as reported in ancient literature. Both can withstand the adverse conditions prevalent in semi-arid and arid regions. With temperature 500°F in winters and 110-114°F in summers. The tree provides grazing material to livestock and plays an important role in livelihood and ecosystem preservance. Both are excellent soil binders and important constituents of arid vegetation system. The National Research Centre Institute for Arid Horticulture, Bikaner (CIAH), Central Arid Zone Research Institute (CAZRI), Jodhpur and Arid Zone Forestry Research Institute (AFRI), Jodhpur are working to collect germplasm, and to improve and propagate fruit and forest trees found in hot arid zones. Still there is a need to cope up with overgrazing and exploitation of both trees as important structure for livelihood of local people residing in deserts areas. New protocols are indeed a basic requirement to propagate such trees on

large scale multiplication through tissue culture technology .Conclusively this study has established the bud break and shoot initiation from axillary node of both woody taxa. Further research in order to develop plantlets and their acclimatization to field conditions will be next stepping stone .Meanwhile this protocol offers an efficient method to initiate shoot proliferation and also paved the path to improve vegetation coverage in arid and semi-arid regions of India.

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Relationship among combining ability, heterosis and genetic distance in maize (*Zea maize* L.) inbred lines under water-deficit conditions using line × tester and molecular analysis

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ABSTRACT

This study was conducted to investigate the relationships of combining ability, hybrid performance and genetic distance using Sequence Related Amplified Polymorphism (SRAP) data and other data sets obtained from analysis of agronomic performance of the CIMMYT maize inbred lines. For this purpose, 13 lines and four testers were crossed through controlled pollination in a line × tester design scheme to develop 52 hybrids. These hybrids were evaluated together with two standard checks (KSC704 and KSC705) for grain yield under two soil moisture environments for two years (2014-2015). Pair-wise genetic distance (GD) was estimated based on Jaccard (J) and simple matching (SM) coefficients. The variance components of specific combining ability (SCA) were higher than general combining ability (GCA), hence non-additive gene effects contributed to hybrid performance. There was no coincidence between the SRAP data and morphological assessments in this study. Significant and positive association of general combining ability with mid parent heterosis (MPH) under drought stress conditions is an indicator that GCA can be useful to predict MPH during selections under water stress conditions. However, correlations of genetic distances with heterosis under both conditions were too low to be predictive of hybrid vigor.

KEY WORDS: WATER STRESS, GENETIC DISTANCE, HYBRID PERFORMANCE, LINE × TESTER, SEQUENCE RELATED AMPLIFIED POLYMORPHISM

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INTRODUCTION

Maize is one of the most important crops worldwide, which serves as food, animal feed and raw materials of bioenergy. It is stated that, maize is queen of cereal crops due to high yielding potential and genetic diversity. The global production of this crop has increased during last years. However, its yield is reduced due to water deficit, which is one of the most important environmental factors affecting agricultural productivity worldwide, (Prasanna, 2012, Aminu et al., 2014 Li et al., 2017).

Heterosis, a powerful phenomenon in the evolution of plants, has been used extensively in crop production. However, identification and selection of appropriate parental combinations which produce superior F1 hybrids, is one of the most important stages in heterosis utilization (Mohammed et al., 2014). Hybrid breeders have always been concerned to the selection of appropriate parental lines without making all of the possible crossing among the available lines. Selection of parents with various genetic backgrounds is hardly substantial in the development of hybrids having optimal expression of heterosis (Hallauer et al., 2010 Pheirim et al., 2017).

During the past decades, several procedures have been utilized for prediction of heterosis, including performance of parental lines, combining ability, genetic diversity which determined using multivariate analysis of morphological and agronomic traits and molecular markers (Mohammadi et al., 2008). Selection based on phenotypic traits is extremely influenced by environmental factors, and the presence of genotype × environment interactions can hide the actual genetic value. Moreover, because of strong dominance effects of genes controlling maize yield, hybrid performance may not be predicted from the performance of parental lines, reliably. Furthermore, in breeding programs with a large numbers of inbred lines, making and evaluation of all of the possible crosses is not only expensive and boring, but also practically difficult and time consuming (Mohammadi et al., 2008).

In maize, several methods have been expanded for the prediction of hybrid performance by means of genetic markers (Frisch et al., 2010; Maenhout et al., 2010). Considering the cost and time which is required for field evaluation of hybrids, the utilization of genetic markers for identification of best heterotic combination of parental lines can be a suitable alternative (Mohammed et al., 2014). Molecular markers have been widely utilized in breeding programs, as a tool for the selection of the best parental lines of crosses; and as potential tools for the prediction of the heterosis from a certain cross.

Parental genetic distance has been considered as a feasible indicator for hybrid performance (Melchinger, 1999). Breeders are strongly interested to the prediction of hybrid performance from parental genetic distance. Because the preferable crosses could be identified by means of genetic distance before field evaluation of all hybrid combinations. This can increase the efficiency of hybrid breeding programs, (Mohammed et al., 2014). Estimated genetic distances can be related with hybrid performance from field experiments, and the extension of molecular marker systems such as sequence related amplified polymorphisms (SRAP) have considerably amended the power of the genetic distance estimation between genotypes.

Several researchers have used genetic distance to predict hybrid performance (Dhliwayo et al., 2009; Devi and Singh, 2011; George et al., 2011); however, their results were inconsistent with each other. Some researchers reported a positive correlation between marker based genetic distance and hybrid performance (Amorim et al., 2006; Srdic et al., 2007; George et al., 2011), while other researchers have reported no correlation between these two phenomenon (Balestre et al., 2008; Dhliwayo et al., 2009; Devi and Singh, 2011). Hence, the potential utilization of molecular markers in predicting the amount of hybrid performance in maize needs more research. Though significant associations were found between hybrid performance and genetic diversity in several investigations, the level of association varied widely from one study to another. Moreover, the reliability of molecular markers in estimating genetic distance depends on several factors such as the number of markers, their mode of inheritance and uniform distribution across the genome (Hahn et al., 1995; Mohammadi et al., 2008).

To the best of our knowledge, there is a little information about the association of genetic divergence and hybrid performance in CIMMYT maize inbred lines, which asks for studies to determine genetic distance as suitable predictor of hybrid performance in this germplasm. It is also needed to examine combining ability of parents as predictor of heterosis and F1 performance comparing with genetic distance measured by molecular markers. Therefore, this study was conducted to 1) investigate the possibility of predicting the hybrid performance using SRAP data and other data sets acquired from analysis of agronomic performance of the CIMMYT maize inbred lines; 2) determine associations among genetic distance of molecular markers in parents, heterosis, F1 performance, general combining ability (GCA) and specific combining ability (SCA) effects of parents and crosses, and compare the strategies to determine hybrid performance based on parental genetic distance (GD), GCA and SCA for heterosis; and 3) evaluation of coincidence between the SRAP data and morphological data.

MATERIALS AND METHODS

PLANT MATERIALS AND EXPERIMENTAL SITE

The experiment was conducted during two years (2014-2015) at the Research Farm of Agriculture and Natural Resources Research Center, Kermanshah, Iran (longitude of 47° 26' E, latitude of 34° 8' N and altitude of 1346 m) on a silty clay loam soil. The mean annual precipitation and temperature are 538 mm and 12.2 °C for the region, respectively. In this study, a set of 13 inbred lines were selected and crossed through controlled pollination with four temperate maize testers using a line × tester matting design to produce 52 hybrid combinations. The origin and pedigree of the lines and testers are given in Table 1.

FIELD EXPERIMENT

In this experiment, 52 hybrids derived from line × tester matting scheme along with two standard checks were planted in the field according to a randomized complete block design (RCBD) with three replications, at two moisture environments (normal and water stress). Each plot was included 2 rows of 4 m long with an inter-row spacing of 0.75 m and in-row plant spacing of 18 cm. Under the normal and stress moisture environments, plants were irrigated when 50% and 65% of the total available soil water was depleted from the root zone, respectively. Soil moisture was measured based on standard gravimetric methods (Clarke Topp et al., 2008). The irrigation was applied by using a basin irrigation system. The amount of water for each irrigation treatment was measured using a volumetric counter. Grain yield per plot was recorded on five randomly selected plants per replication.

SRAP ANALYSIS OF INBRED LINES

Genetic characterization of all of the inbred lines and testers was done using a set of 30 SRAP primer pairs. Genomic DNA was extracted from fresh leaves of each line or tester according to the method of Murray and Thompson (1980). PCR reactions were performed in a 10µl reaction mix and amplified products were resolved by using 6% polyacrylamide gel followed by silver staining.

STATISTICAL ANALYSES

Analysis of variance (ANOVA) for each moisture environment was conducted using the PROC GLM of SAS (SAS Institute 2008). Genotypes were considered as fixed effects while years, moisture environments and replications were considered as random. The SAS program was used for the line × tester analysis to compute the SCA effects (Singh and Chaudhary, 1977). The GCA effects of lines and testers, the SCA effect of crosses, and their interactions with the year were estimated based on the factorial mating design as follows:

$$Y_{ijk} = \mu + g_i + g_j + s_{ij} + e_k + ge_{ik} + ge_{jk} + se_{ijk}$$

Table 1. Information on maize lines and testers used in the study

Parents	Name of lines and testers/pedigree	Origin
Line 1	4-CHTSEY,2002/1389/9=1390/13=1391/10	CIMMYT germplasm
Line 2	4-CHTSEY,2002/1389/19=1390/21=1991/70	CIMMYT germplasm
Line 3	7-CHTSEY,2002/1389/33=1390/33=1391/61	CIMMYT germplasm
Line 4	7-CHTSEY,2002/1389/35=1390/37=1391/64	CIMMYT germplasm
Line 5	K18 × 2-CHTHIY, 2002/1389/59=1390/73=1391/43	derived from cross k18 × CIMMYT originated line
Line 6	K18 × 2-CHTHIY, 2002/1389/61=1390/77=1391/46	derived from k18 × CIMMYT originated line
Line 7	XT03	Derived from unknown China -source
Line 8	4-CHTSEY, 2002/1390/5=1391/6	CIMMYT germplasm
Line 9	4-CHTSEY, 2002/1390/9=1391/8	CIMMYT germplasm
Line 10	7-CHTSEY, 2002/1390/41=1391/22	CIMMYT germplasm
Line 11	20-CHTSEY,2002/1390/45=1391/25	CIMMYT germplasm
Line 12	20-CHTSEY,2002/1390/53=1391/31	derived from CIMMYT germplasm
Line 13	MO17 × 6-CHTHEY, 2002/1390/69=1391/40	derived from cross MO17 × CIMMYT originated line
Tester 1	MO17	CL. 187-2 × C103
Tester 2	0K18	derived from MO17 changes
Tester 3	A679	A B73 back-cross derived line [(A662 × B73)(3)]
Tester 4	K166B	derived from CIMMYT germplasm

Where Y_{ijk}; is performance of the hybrid when ith line is crossed to jth tester, in the kth year, μ is the overall mean, g_i is the effect of the ith line, g_j is the effect of the jth tester, s_{ij} is the interaction of the ith line with the jth tester, e_k is the effect of the kth year, $(ge)_{ik}$ is the interaction of the g_i and e_k, $(ge)_{jk}$ is the interaction of the g_j and e_k, $(se)_{ijk}$ is the interaction of s_{ij} and e_k.

For each cross combination (P1 × P2) mid parent heterosis (MPH) was calculated according to Falconer and Mackay (1996) as follows:

$$\text{Mid parent heterosis (MPH)} = [(F1 - MP) / MP] \times 100$$

where F1 is the mean of the F1 hybrid performance and MP is mean performance of two parental inbred lines.

Better parent heterosis (BPH) was calculated as:

$$BPH = [(F1 - BP) / BP] \times 100$$

where BP = mean of the better parent.

Genetic distance (GD) between each pair of parents was estimated from the binary matrix, using Jaccard and simple matching coefficients through the NTSYSpc version 2.0. Cluster analysis was done based on the UPGMA method. For evaluation of the correlation between two similarity matrices (molecular and phenotypic data), Mantel test in NTSYS software was applied. Mean of the trait in each moisture environment was used to calculate correlation coefficients between genetic distance, grain yield, MPH, BPH, GCA and SCA.

RESULTS AND DISCUSSION

Presence of an appropriate value of heterosis for grain yield and predicting hybrid performance is important in hybrid breeding programs. The degree of heterosis may influence by genetic diversity of the germplasm being used. The magnitude of heterosis which was observed in this study indicates that there is an opportunity to use this germplasm for extending hybrid varieties appropriate for stress and non-stress conditions.

Analysis of variance of grain yield for each of the two moisture environments showed significant genotype effects, indicating the existence of genetic variation among the genotypes. However genotype × year interaction was non-significant in both moisture environments, indicating that the genotypes were consistent over the years (Table 2). Nevertheless, significant differences ($p < 0.01$) among parents and F1 hybrids in both moisture environments were found and indicated that the data was suitable for genetic analysis of line × tester design. The mean squares for lines and testers which determine the GCA effects were also significant and showed the predominance of additive gene action in controlling grain yield. However, the mean squares of testers were higher than that of the lines in normal conditions and it was vice versa for water stress conditions.

Table 2. Analysis of variance (ANOVA) for combining ability of total grain yield based on line × tester matting design under normal and drought stress conditions.

Source of variation	df	Mean squares (MS)	
		NS	S
Year (Y)	1	18.11	17.41
Block (R)	4	37.18**	1.20
Genotype (G)	53	14.99**	19.33**
F1 vs. Check	1	2.65	39.26
Check	1	35.02**	6.02*
F1	51	14.84*	19.20**
Lines (L)	12	21.31**	36.36**
Testers (T)	3	46.94**	35.45**
L×T	36	10.00**	11.89**
G×Y	53	2.56	0.85
(F1 vs. Check) × Y	1	0.65	5.38
Check × Y	1	1.84	0.04
F1 × Y	51	2.61	0.77
L × Y	12	2.85	0.38
T × Y	3	0.71	0.86
L × T × Y	36	2.69	0.95
Error	212	2.32	1.12
σ^2A	-	1.96	1.91
σ^2D	-	4.88	7.29

*,**Significant at 0.05 and 0.01 probability levels, respectively. NS: Non-stress, S: Stress

The significance of line × tester interaction revealed that SCA was also important in the control of grain yield and indicated that non-additive gene effects also play an important role in the controlling of this trait (Table 2).

Average grain yield for parents (Table 3) and hybrid combinations (Table 4) showed a remarkable reduction under water stress and it ranged from 8.68 ton/ha for L8 × T1 to 15.16 ton/ha for L9 × T4 under normal conditions. However, under water stress conditions, this ranged from 4.95 ton/ha for L10 × T1 to 11.99 ton/ha for L5 × T3. The GCA effect was positive and significant for two parents of T3 and T4 under both normal and water stress conditions (Table 3). However, under normal conditions four parents and under water stress conditions five parents showed significant and positive values of GCA for grain yield (Table 3). Under normal conditions five hybrids and under water stress conditions seven hybrids expressed significant and positive values of SCA for grain yield (Table 4). Moreover, under normal conditions five hybrids and under water stress conditions eight hybrids showed significant and negative SCA for this trait.

Table 3. Grain yield (GY) means and general combining ability (GCA) values for parental lines used in this study under normal (NS) and water stress (S) conditions.

Parents	Grain yield (GY) (Ton/ha)		General combining ability (GCA) (Ton/ha)
	NS	S	NS
L1	5.85	2.77	-1.12
L2	4.14	3.07	0.41
L3	5.49	2.40	0.57
L4	6.39	3.85	0.98**
L5	5.30	2.72	0.37
L6	6.33	3.22	0.54
L7	3.77	2.73	0.07
L8	4.91	2.82	-1.90**
L9	4.17	2.62	0.65
L10	7.15	3.05	-0.83*
L11	6.18	3.21	-0.98**
L12	5.74	2.57	1.33**
L13	5.70	2.88	-0.09
T1	4.56	2.34	-0.71**
T2	4.80	2.71	-0.63**
T3	5.27	3.06	0.75**
T4	5.53	3.04	0.59**

*, ** Significant at 0.05 and 0.01 probability levels, respectively.
NS: Non-stress, S: Stress

Mid parent heterosis (MPH) and better parent heterosis (BPH) values were significant and positive for all hybrids under both normal and water stress conditions. MPH ranged from 70% for L10 × T1 to 230% for L7 × T3 under normal conditions and from 0.63% for L10 × T3 to 3.15% for L5 × T3 under water stress conditions. The range of BPH was from 39% for L10 × T1 to 183% for L7 × T3 under normal conditions, and from 0.62% for L10 × T3 to 2.92% for L5 × T3 under water stress conditions (Table 4).

SRAP data showed low genetic distances among parental lines. Distances ranged from 0.181 for L12 × T4 to 0.423 for L11 × T2 based on Jaccard coefficient, and from 0.142 for L12 × T4 to 0.358 for L9 × T1 based on simple matching coefficient (Table 4). The average genetic distance among inbred lines of this study based on Jaccard and simple matching coefficients were 0.33 and 0.25, respectively; indicating low levels of polymorphism among them. Ndhlela et al. (2015) stated that low genetic distances can be attributed to the mixing of germplasm by CIMMYT for population improvement at the expense of hybrid breeding.

In field experiments, the most expensive and time consuming step of hybrid breeding programs is the identification of inbred lines expressing higher heterosis (Mohammadi et al., 2008). Plant breeders often have used SCA of hybrids in identifying better parental lines for extension of hybrid combinations. However, when a large numbers of inbred lines are available in a breeding program, more useful tools are needed. In maize, genetic distance determined by molecular markers is the main strategy which has been followed for determination of hybrid performance and its potential for this purpose has been evaluated in several researches. In these researches, the extent of correlation differed greatly from one trait to another and also varied extensively with the germplasm used in different studies.

The correlation coefficients of GD calculated based on Jaccard and simple matching coefficients were negligible and non-significantly different from zero for each of TGY, MPH and BPH (Table 5). Therefore, prediction of hybrid performance for grain yield based on genetic distance estimated by SRAP markers cannot be a practical approach and this was in agreement with the results of Dhliwayo et al. (2009), Devi and Singh (2011) and Ndhlela et al. (2015). However, some studies have reported powerful correlation between hybrid performance and parental genetic distance (Melchinger, 1999; Singh and Singh, 2004).

Mohammadi et al. (2008) suggested that insufficient genome coverage, sample size of the parental lines and progenies and different levels of dominance effects on traits are some important reasons for the low correlation between genetic distance and hybrid performance. Other possible reason for this issue is utilization of unlinked markers to the trait in estimation of genetic distance. For solving this problem, Bernardo (1992) suggested identifying of specific marker loci with close linkage to chromosomal segments controlling target traits. Although genetic distance was not a reliable predictor of hybrid performance, some promising approaches such as BLUP (Best Linear Unbiased Prediction) along with molecular marker data have been extended for predicting hybrid performance using genetic distances. However, in this study significant and positive associations were observed between TGY, BPH and MPH with SCA effects of crosses. Moreover, a significant and positive correlation was found between GCA and MPH under water stress conditions (Table 5). This correlation is an indicator that GCA can be useful to predict MPH during selections under water stress conditions.

In this study, correlations based on genetic distance estimates using simple matching coefficient were relatively higher than correlations based on GD estimated using Jaccard coefficient. This shows that the extent of correlation coefficient was not only impressed by the germplasm under study, but also by the genetic distance measures.

Cluster analysis grouped the 17 lines and testers into three major groups (Fig. 1). However, the cluster analy-

Table 4. Grain yield (GY) means, specific combining ability (SCA), mid parent heterosis (MPH) and better parent heterosis (BPH) estimates for 52 F_1 hybrids of line × tester under normal (NS) and water stress (S) conditions, and genetic distance (GD) between respective parental lines using simple matching (SM) and Jaccard's (J) coefficients based on SRAP markers.

Hybrids	GY (Ton/ha)		SCA (Ton/ha)		MPH (%)		BPH (%)		GD J	GD SM
	NS	S	NS	S	NS	S	NS	S		
L1×T1	10.55	7.35	-0.01	-0.43	103**	188**	80**	166**	0.327	0.245
L1×T2	10.73	7.56	0.08	1.04*	101**	176**	83**	173**	0.359	0.250
L1×T3	12.59	8.24	0.56	-0.81	126**	183**	115**	170**	0.308	0.221
L1×T4	11.23	9.00	-0.63	0.20	97**	210**	92**	196**	0.350	0.275
L2×T1	12.32	6.87	0.23	-0.34	183**	154**	170**	124**	0.353	0.270
L2×T2	12.57	7.79	0.38	0.45	181**	170**	162**	154**	0.292	0.196
L2×T3	11.62	6.79	-1.94**	-1.17*	147**	122**	121**	121**	0.290	0.206
L2×T4	14.72	10.92	1.32	1.06*	204**	257**	166**	255**	0.312	0.240
L3×T1	14.21	7.28	1.96**	-0.28	183**	207**	159**	203**	0.276	0.211
L3×T2	12.21	7.63	-0.13	0.37	137**	199**	122**	182**	0.349	0.255
L3×T3	11.61	7.71	-2.11**	-0.80	116**	183**	112**	152**	0.301	0.225
L3×T4	13.84	7.75	0.28	0.71	151**	185**	150**	155**	0.280	0.221
L4×T1	12.20	6.66	-0.46	-0.26	123**	115**	91**	73**	0.395	0.324
L4×T2	11.42	8.41	-1.33	-0.75	104**	157**	79**	119**	0.388	0.289
L4×T3	14.73	8.93	0.60	-0.39	153**	159**	130**	132**	0.350	0.270
L4×T4	15.15	10.00	1.19	1.40**	154**	191**	137**	160**	0.376	0.314
L5×T1	14.59	6.76	2.54**	-0.11	196**	167**	175**	148**	0.388	0.324
L5×T2	10.82	8.36	-1.33	-1.56**	114**	208**	104**	207**	0.370	0.279
L5×T3	14.51	11.99	0.98	0.80	175**	315**	174**	292**	0.333	0.260
L5×T4	11.16	9.57	-2.20**	0.87	106**	232**	102**	215**	0.398	0.343
L6×T1	11.97	7.22	-0.26	-0.48	120**	160**	89**	124**	0.291	0.216
L6×T2	12.21	6.77	-0.10	-0.87	119**	129**	93**	111**	0.297	0.201
L6×T3	12.79	10.58	-0.90	-0.24	121**	237**	102**	229**	0.283	0.201
L6×T4	14.79	10.86	1.26	1.59**	149**	247**	134**	238**	0.306	0.235
L7×T1	11.78	6.47	0.03	0.92*	183**	155**	158**	137**	0.240	0.181
L7×T2	11.70	9.90	-0.14	-0.35	173**	264**	144**	262**	0.347	0.255
L7×T3	14.92	9.98	1.70*	0.40	230**	245**	183**	227**	0.331	0.255
L7×T4	11.46	10.74	-1.59*	-0.96*	146**	272**	107**	254**	0.256	0.201
L8×T1	8.68	7.62	-1.10	0.43	83**	195**	77**	170**	0.232	0.176
L8×T2	10.11	5.09	0.24	-0.20	108**	84**	106**	80**	0.327	0.240
L8×T3	11.21	5.21	-0.04	0.91*	120**	77**	113**	71**	0.290	0.221
L8×T4	11.98	5.71	0.90	-1.14*	129**	95**	117**	88**	0.259	0.206
L9×T1	12.38	8.84	0.05	0.71	184**	256**	172**	237**	0.422	0.358
L9×T2	11.61	5.79	-0.81	-1.07*	159**	117**	142**	114**	0.408	0.314
L9×T3	13.02	7.16	-0.78	0.32	176**	152**	147**	134**	0.390	0.314
L9×T4	15.16	8.76	1.53*	0.04	212**	210**	174**	188**	0.412	0.358
L10×T1	9.92	4.95	-0.93	0.00	70**	84**	39**	62**	0.342	0.265
L10×T2	11.92	6.79	0.98	0.02	100**	136**	67**	123**	0.340	0.240
L10×T3	12.75	4.96	0.43	0.89	105**	63**	78**	62**	0.336	0.250
L10×T4	11.67	6.23	-0.48	-0.90*	84**	105**	63**	105**	0.354	0.284
L11×T1	11.07	5.42	0.38	-1.18**	106**	95**	79**	69**	0.373	0.324
L11×T2	11.59	10.48	0.80	1.82**	111**	254**	87**	227**	0.423	0.348
L11×T3	11.60	6.67	-0.56	0.81	103**	113**	88**	108**	0.368	0.309
L11×T4	11.39	5.82	-0.61	-1.45**	94**	86**	84**	81**	0.356	0.314

L12×T1	12.78	7.57	-0.24	0.34	148**	208**	123**	194**	0.220	0.172
L12×T2	13.20	7.82	0.09	0.40	150**	196**	130**	189**	0.385	0.304
L12×T3	14.88	8.88	0.39	0.06	170**	216**	159**	191**	0.298	0.235
L12×T4	14.07	8.62	-0.25	-0.80	150**	207**	145**	184**	0.181	0.142
L13×T1	9.41	5.26	-2.18**	0.69	83**	102**	65**	83**	0.329	0.240
L13×T2	12.93	6.58	1.25	0.72	146**	136**	127**	129**	0.234	0.147
L13×T3	14.72	6.06	1.66*	-0.80	168**	104**	158**	98**	0.310	0.216
L13×T4	12.16	5.20	-0.73	-0.62	117**	76**	113**	71**	0.353	0.270

*, **Significant at 0.05 and 0.01 probability levels, respectively.
NS: Non-stress, S: Stress

Table 5. Correlation coefficients of grain yield (GY), Mid parent heterosis (MPH) and Better parent heterosis (BPH) with each of SRAP-based genetic distance estimates using Jaccard (GDJ) and simple matching (GDSM) coefficients, general and specific combining ability (GCA and SCA, respectively) under normal (NS)and water stress (S) conditions.

Environments	GY (Ton/ha)		MPH (%)		BPH (%)	
	NS	S	NS	S	NS	S
GDJ	-0.023	0.028	-0.028	-0.03	-0.063	-0.045
GDSM	0.027	0.062	0.006	0.012	-0.02	-0.015
GCA	0.012	-0.243	0.049	0.661**	0.202	-0.073
SCA	0.689**	0.323**	0.565**	0.318**	0.528**	0.309**

*, **Significant at 0.05 and 0.01 probability levels, respectively.
NS: Non-stress, S: Stress
GY, grain yield; MPH, Mid parent heterosis; BPH, Better parent heterosis; GDJ, Jaccard genetic distance; GDSM, Simple matching genetic distance; GCA, General combining ability; SCA, Specific combining ability.

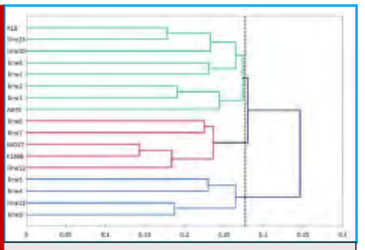

FIGURE 1. Dendrogram depicting genetic relationships among parental lines involved in line × tester analysis, based on SRAP data using UPGMA method and Jaccard's coefficient.

sis based on phenotypic traits and SRAP markers could not separate parents based on geographical or ecological data. Moreover, in this study there was no coincidence between the SRAP data and morphological estimations, which indicated poor association and agreement of molecular marker diversity with that of phenotypic one (r= 0.15 under normal and r= 0.10 under water stress conditions). Several reasons are given for the discordance between these two sets of data. Accumulation of some characteristics having adaptive value in specific habitats subjected to similar ecologic conditions (Steiner and Los Santos, 2001), differences between the evolutionary rates of phenotypic traits with adaptive value and those originating from selectively neutral DNA (Linhart and Grant, 1996), selection pressure for homogenization of different traits in parental germplasm and the different genomic regions evaluated with both markers (Amini et al., 2011), are some of these probable reasons.

In conclusion, prediction of heterosis is critical and valuable in hybrid breeding programs. In this regard, a potentially powerful approach is the application of genetic distance specified by molecular markers. In this study, associations between genetic distance estimates (GDJ and GDSM) with heterosis effects were negligible and non-significant. Thus, prediction of heterosis based on genetic distances estimated by SRAP markers cannot be a practical approach. Use of unlinked markers to the target traits, insufficient genome coverage, sample size of the parental lines and progenies and different levels of dominance effects on target traits are some of probable reasons for the low correlations between genetic distance and hybrid performance. On the other hand, identifying of specific marker loci with close linkage to chromosomal segments controlling target traits and application of statistical methods such as BLUP along with molecular marker data are some of the solutions for this problem. A significant and positive association

among GCA and MPH under drought stress conditions is an indicator that GCA can be useful to predict MPH during selections under water stress conditions.

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Screening in healthy individuals for risk of falls by employing various tools: A clinical study

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ABSTRACT

Body posture involves the relative position and control of the musculature with respect to each other. Muscles play a pivotal role in maintaining the balance which controls the posture. Present study involves the use of TETRAX® related tools to determine the risk of falls in different young adults group. In this approach we employ modified Romberg's Test, Static Postural Alignment Examination and TETRAX® Posturographic method in order to validate our hypothesis. We have chosen subjects of different age group such as 20-25 years, 26-30 years, 31-35 years and 36-40 years designated as Group A, B,C and D respectively. Present approach segregated all the subjects on the basis of demographic analysis. Comparison was done in different age groups for risk of fall. In descriptive analysis, it was concluded that there is high risk of fall is observed in case of group A as compared to other groups. Statistical analysis did not prove the same due to the limitation in number of subjects.

KEY WORDS: SCREENING; HEALTHY INDIVIDUALS; POSTURE; BALANCE; RISK OF FALL

INTRODUCTION

Body Posture involves the relative position and control of the musculature with respect to each other (Andre et al., 2015). Muscle recruitment is necessary for maintaining the postural control; therefore muscle should be active for maintaining the balance. Balance is a condition in which all forces acting on the body are being at equilibrium such that the centre of gravity lies within the base of support (Quitschal et al., 2014). Balance and posture is being defined by the System Model of Balance, its components are visual, somato-sensory and vestibular. The impairments in balance can be a consequence of changes in motor, sensory and integrative aspects of motor control (Rey et al., 2017). The central nervous system integrates all the cues coming from the environmental organiza-

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tion and the sensory system orients it. Incidence of high risk of fall and increased postural sway is being seen in older adults and due to these falls there are higher incidence of fractures other than any injuries (Villiers and Kalula, 2015), whereas some literatures report that young adults are also affected with poor posture control and balance and therefore, more cases of upper extremity fractures have been reported in young adults due to falls (Quitschal et al., 2014, Akdeniz et al., 2016 and Aydın et al., 2017).

Various tools or measures were employed such as plumb line or weighted line for normal postural alignment, visual inspection for postural alignment and interactive posturography system for postural stability (Paillard et al., 2015). Researchers also employed measures of the initial stance position, centre of alignment, mean sway path, total excursion and the zone of stability and considered as reliable measures of postural control (Anna et al., 2015 and Hung et al., 2016). Amalgamation of other tools were also being employed such as TETRAX® biofeedback video games for balance training is a feasible adjunctive program that may augment conventional therapy in persons with chronic hemiplegic stroke (Hung et al., 2016 and Yun & Yoo, 2016). The study concluded that there is relation risk of fall, balance and neuromuscular function in 60 years women (Akdeniz et al., 2016).

Aforementioned studies employed the TETRAX® tool in combination with other interventions but there is limited study on the age group 20-40 years. Therefore, there is need to determine that whether the risk of fall is the problem in geriatric population only or is it an alarming problem within young adults of age group (Garcia & Navarro, 2016 and Rey et al., 2017).

As there is higher risk of fall and stability index in individuals with low physical activity. The aim of this study is screening of balance and postural control in individuals. The objective of this study is to determine the risk of fall in the healthy young population by employing measures such as Modified Romberg's Test, TETRAX® Posturographic method and Static Postural Alignment Examination. These tests have intrinsic characteristic features such as accuracy, non-invasive and weight distribution etc. (Lee et al., 2016) which make them reliable tools for the determination of risk of fall among different age group and also on gender bias.

MATERIAL AND METHODS

Young healthy adults between age 20-40 individual were included in the study and were with 10 subjects each in four respective Groups A (20-25) years, B (26-30) years, C (31-35) years and D (36-40) years as per their age. The study was performed at RLJT Hospital & Research Centre, Jhunjhunu. All subjects were included after performing Romberg's test. Exclusion criteria history of neurological (Guler et al., 2012) or musculoskeletal disorder, laxity in joints pregnancy (Aydın et al., 2017), ankle sprain, marked cognitive impairment (Avni et al., 2006), low vision, insufficient English and Hindi language skills lack of interest to participate The experiment was conducted on the basis of Demographic data and Anthropometric characteristics of the subjects was recorded including Name, Age, Gender, Height and Weight, therefore BMI was also recorded prior to the study. Manual Screening for Postural Control was done by Modified Romberg's test and Plumb line Posturography method.

Tools employed for the screening of healthy individuals for risk of falls: Modified Romberg's Test was employed in four different conditions and each condition must be fulfilled in order to move to the next condition. All the conditions were performed standing with feet together and arms crossed. Condition 1: Each subject would be supposed to stand on the floor (firm surface) for 15 seconds eyes open. Condition 2: Each subject would be supposed to stand on the floor (firm surface) for 15 seconds eyes closed.

Condition 3: Each subject would be supposed to stand to on the memory foam with eyes open for 30 seconds. Condition 4: Each subject would be supposed to stand on the memory foam with eyes closed for 30 seconds. Documentation- It would be done by PASS/FAIL condition.

Failure of the test was defined under three parameters i.e 1) The subjects open their eyes in the condition 2 and 4 2) The subjects move their feet or arms to maintain stability 3) Beginning to fall or requiring intervention of the examiner to maintain the balance within 30 seconds threshold time. Static Postural Alignment Examination: Normal postural alignment in standing can be determined by using a plumb line. In standing position, the centre of mass lies at the second sacral vertebrae. Static posture is examined by using the plumb line method in standing position with the feet apart, normal stance width. When viewed from the Sagittal plane the vertical line of gravity (LOG) is expected to fall close to the external auditory meatus, anterior to the shoulder joint, anterior to the thorax, posterior to the hip joint, and slightly anterior to the knee and ankle joint.

TETRAX® Posturographic method: Tool used is TETRAX® Computerized Static Posturographic tool. This system helps in the screening of the Static Postural balance in healthy individuals. The subjects was asked to stand in the middle of the four force plates which would be placed at the right rear, right front, left rear and left front of the patient's feet. The sensor plates would then record the weight bearing based on vertical pressure

being applied to the platform via the heels and the toes. Documentation was done in eight different conditions i.e.:

1. Normal open position (NO) that is standing straight with eyes open
2. Normal closed position (NC) that is standing straight with eyes closed
3. Pillow open (PO) that is standing straight on pillows with eyes open
4. Pillow Closed (PC) that is standing straight on pillows with eyes closed
5. Head right (HR) that is standing straight on sensor plates with eyes closed, head turned to the right
6. Head left (HL) that is standing straight on sensor plates with eyes closed, head turned to the left.
7. Head back (HB) that is standing straight on sensor plates with neck in extension
8. Head Forward (HF) that is standing straight on sensor plates with neck fully flexed.

The study was performed at same time of the day. Each position is maintained for 30 seconds. Tetrax® Software computed the following parameters. Risk of falls with Numeric value of (0-100)

- Low fall risk: 0-35
- Moderate fall risk- 36-57
- High fall risk- 58-100

Data Analysis: The data analysis was done using Statistical Package for Social Sciences software Version 17 applying the descriptive data included mean standard deviation were also calculated Anova test was applied to calculate the difference in between four groups using F distribution . The data was analysed using the F- value and the F crit value. If the F value is greater than the F crit value then the null hypothesis is rejected but if F value is less than the F crit value then the research hypothesis is rejected.

Table 1. Demographic and anthropometric data of the subjects

Characteristics	Mean ± Standard Deviation
Age	30.77 ±5.78
Height (cm)	164.25 ±7.70
Weight (kg)	64.43 ± 13.83
Body Mass Index (kg/m)	23.98 ± 4.35
Gender (Male/Female)	24/16

RESULTS

Segregation of healthy individuals according to demographic analysis:

Subjects (40) were included in the study with mean age of 30.77 ± 5.78 years. The mean height was 164.25 ± 7.70 cm and mean weight was 64.43 ± 13.83 kg. The mean BMI was also calculated as 23.98 ± 4.35 and mean risk of fall was calculated as 34.25 ± 20.32. The ratio of male and female in the group under investigation was 3:2 (Table 1).

Comparison within groups for risk of fall: Comparison was done between the risk of fall 34.25 ± 20.32 years of 4 different age groups 30.77 ± 5.78 years and it was found that there was no significant difference seen in the risk of fall between different age group of healthy population. (F value> F crit value), (p value > 0.05) (Table 2.)

For Intra- group comparison, descriptive analysis was done in which each group subjects were classified under low risk of fall, moderate risk of fall and high risk of fall

It was found that in Group A equal number of people fall under the category of moderate and high risk of fall. In Group B 50% of the population was under low risk of fall and 20% of population is under high risk of fall .As

Table 2. Comparison of risk of fall and Age

Age (Mean ±SD)	Risk of fall (Mean ±SD)	F value	p value	F crit value
30.77±5.78	34.25±20.32	2.575758	0.068935	2.866266
Significant at F value Significant at ≤0.05 level or> F critical value				

Table 3. Result analysis

ANOVA						
Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	2847.5	3	949.1667	2.575758	0.068935	2.866266
Within Groups	13266	36	368.5			
Total	16113.5	39				
Significant at ≤0.05 level or Significant at F value > F critical value						

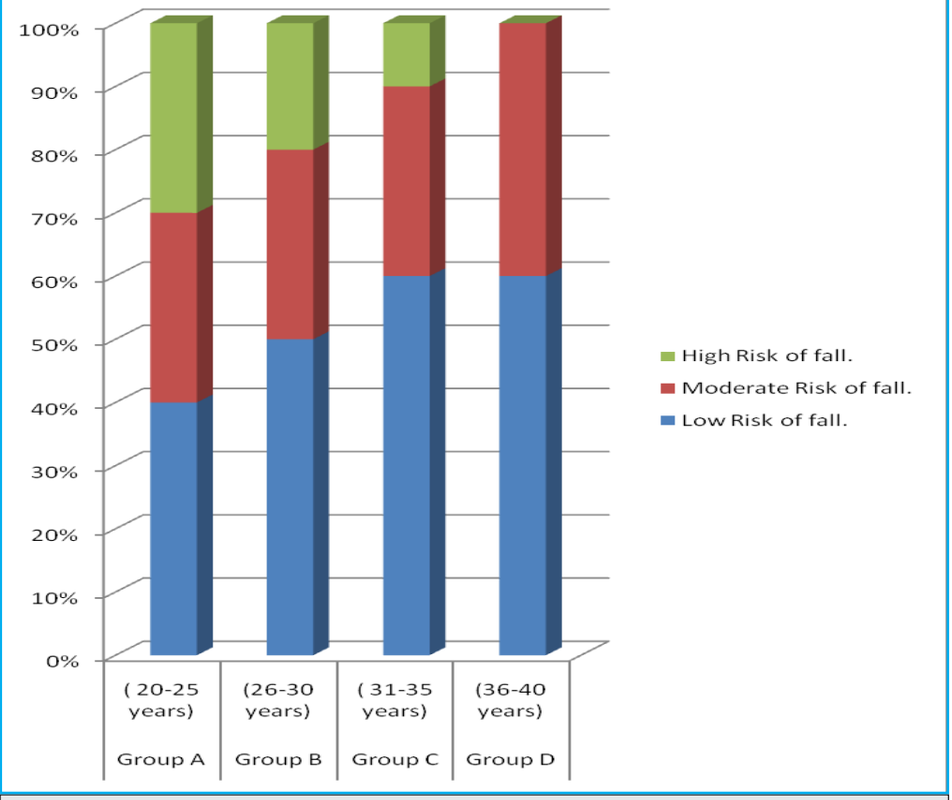

FIGURE 1. Illustrate the intragroup risk of fall in percentage

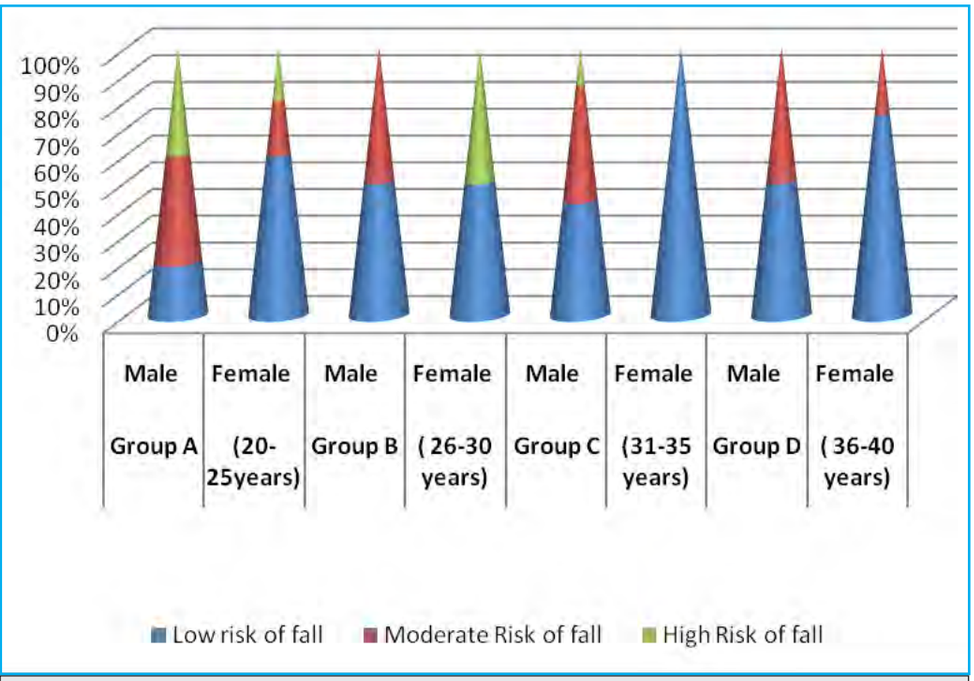

FIGURE 2. Representation of percentage Risk of fall on the basis of gender

Table 4. Intragroup relation between Demographic details and Risk of fall

Characteristics	Group A (20-25 years)	Group B (26-30 years)	Group C (31-35 years)	Group D (36-40 years)
Gender (Male: Female)	5:5	6:4	7:3	6:4
Age (Mean ± SD)	23±1	28±1.55	33±1.44	34±1.46
Height (In cm) (Mean ± SD)	167±8.89	163.44±7.59	164±6.93	162±7.41
Weight(In kg) (Mean ± SD)	62±14.50	60.5±8.54	69±9.31	67±19.9
BMI (Mean ± SD)	22±4.58	22.73±3.49	26±2.57	26±5.31
Risk of fall (Mean ± SD)	43±21.70	37.6±19.20	28±18.48	25±17.10

Table 5. Intragroup categorization of risk of fall

Risk of fall	Group A (20-25 years)	Group B (26-30 years)	Group C (31-35 years)	Group D (36-40 years)
Low Risk of fall.	4	5	6	6
Moderate Risk of fall.	3	3	3	4
High Risk of fall.	3	2	1	0

we move towards the higher Age groups C and D There was large decrement in the risk of fall, In Group D 0% Population is under high risk of fall (Figure .1).

It was also found that in higher age group i.e. Group D neither Males nor Females fall under the category of high risk of fall and in Group B and C females were not seen in the category of moderate risk of fall (Table 5 Table 6 & Figure 2).

Statistically, Anova test was used and it was seen that F value is less than F-crit value therefore the Research hypothesis was rejected. But through descriptive analysis there is significant difference seen in risk of fall within the Age groups

DISCUSSION

In previous studies TETRAX® tool was being used to assess balance and postural control in individuals with some disorders, may it be neurological or musculoskeletal (Dunsky et.al., 2017 and Claeys et.al., 2016). One study had revealed the reliability of TETRAX® tool in young adults also with low physical activity According to this study, TETRAX® measured low fall of risk with lower body endurance with increment in vigorous activity and total activity score (Akkaya et al 2015). Researchers employed these tools for the risk of fall in geriatric population (Garcia, 2016 & Rey et al., 2017) but none of the studies has screened only the young individuals. Therefore we were aimed to introduce TETRAX® TOOL for measuring risk of fall among various young individual groups.

The most important finding of this study is that with increase in age, within the group descriptive analysis showed that 0 % of the subjects were under high risk of fall. Previous studies also indicated that there is no correlation between age and risk of fall. Through statistical analysis, F value is higher than F crit value. Therefore,

Table 6. Classification of Risk of fall on the Basis of gender

		Low risk of fall	Moderate Risk of fall	Moderate Risk of fall
Group A (20-25years)	Male	1	2	2
	Female	3	1	1
Group B (26-30 years)	Male	3	3	0
	Female	2	0	2
Group C (31-35 years)	Male	3	3	1
	Female	3	0	0
Group D (36-40 years)	Male	3	3	0
	Female	3	1	0

statistically no significant difference is being seen in the risk of fall between the various age groups. It can be ascribed due to the fact that strict exclusion criteria and small population size can be major setback in this finding. Furthermore the subjects were also screened only for one time. However descriptive analysis concluded that there is significant difference seen in risk of fall within the Age groups. In futuristic approach larger population may allow evaluation of correlation between risk of fall and age statistically also. In descriptive analysis, young age group (20 - 25 years) showed high risk of fall as compared to older age group (36 - 40 years) which can be due to the equal weight distribution on both the lower limbs of older age group but low sample size is the limitation of this discussion.

CONCLUSION

Present study involves the employment of TETRAX® tool in combination with the Modified Romberg's Test and Static Postural Alignment Examination to determine the risk of fall within young adults of different age group. Earlier reported literatures employ various tools in geriatric population for risk of fall. Limited study was performed in case of young adults. Therefore we were directed to employ the tools in young adults for risk of fall. Our results concluded that in descriptive analysis, there is significant difference in the percentage of risk of fall in different age group. Risk of fall is greater in younger adults as compared to the subjects having more age according to descriptive analysis. But statistically our study did not proof the same due to the limited number of subjects. The results of this study demonstrates difference is being observed in risk of fall within the group of healthy population but after statistical analysis there was no significant difference was being seen between the different age groups of healthy population, therefore the research hypothesis is being rejected.

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A dynamic effect of infectious disease on prey predator system and harvesting policy

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ABSTRACT

The paper deals with a model that describes a prey predator system with disease in the prey population where we have investigated the effect of harvest on the disease when vaccination strategies fail to recover the infected prey population. Many infectious diseases like varicella, which is a highly transferable infection caused by the varicella zoster virus and causes even death if untreated. When the disease affected the prey species, prey species is divided into two categories: susceptible prey and infected prey. From infected prey, the disease is transmitted to the susceptible prey species. It is assumed that infection effect both prey and predator species, but the disease is debilitating and ultimately causing death for predators. Once a predator is infected, it can be considered to be dead and infected prey does not recover due to failure of vaccination strategies. The infected prey species are subjected to harvesting at low and high harvesting rates. It is shown that effective harvesting of infected prey can control the spread of disease and prevent predator species from extinction. Equilibrium points are obtained by linearization and Jacobian matrix. The local and global stability of the various equilibrium points of the system was investigated. It is observed that coexistence of both the prey and predator species is possible through non-periodic solution due to the Bendixson-Dulac criterion. With the help of Routh-Hurwitz criterion and Liapunov function, local and global stability of the non-periodic orbits are determined. Some numerical simulations have been carried out to justify the results obtained.

KEY WORDS: PREY-PREDATOR MODEL, EQUILIBRIUM POINTS, STABILITY ANALYSIS, HARVESTING ACTIVITY

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INTRODUCTION

Mathematical models have become important tools in analyzing the dynamical relationship between predator and their prey. The predator prey system is one of the well-known models which have been studied and discussed a lot. The Lotka-Volterra predator prey system has been proposed to describe the population dynamics of two interacting species of a predator and its prey (Lotka, 1925, Volterra, 1931, Arb Von et al., 2013), Lotka–Volterra equation are of form

$$\begin{cases} \frac{dx}{dt} = ax - bxy \\ \frac{dy}{dt} = cxy - dy \end{cases} \quad (1.1)$$

Where x and y are the prey and predator respectively; a is the growth rate of the prey (species) in the absence of interaction with the predator (species), b is the effect of the predation of species to species, c is the growth rate of species in perfect conditions: abundant prey and no negative environmental impact and d is the death rate of the species in perfect conditions: abundant prey and no negative environmental impact from natural cause. One of the unrealistic assumptions in the Lotka-Volterra model is that the growth of the prey populations is unbounded in the absence of the predator. Murray (Murray, 1989) modified the Lokta-Volterra model and the model were based on assumptions that the prey population exhibits logistic growth in the absence of predators, then the model obtained:

$$\begin{cases} \frac{dx}{dt} = ax\left[1 - \frac{x}{k_1} - b\frac{y}{k_2}\right] \\ \frac{dy}{dt} = cy\left[1 - \frac{y}{k_2} - d\frac{x}{k_1}\right] \end{cases} \quad (1.2)$$

where a, b, c, d, k_1, k_2 are all positive constants. This model was investigated and the conditions for stability obtained.Ecological populations suffer from various types of diseases. These diseases often play significant roles in balancing the population sizes. Most important models for the transmission of infectious diseases descend from the classical SIR model (Kermack and McKendrick,1927). In the past decades, several epidemic models with disease in prey have been extensively studied in various forms and contexts, for example, by Hethcote, (2000), Hethcote et al., (2004), Johri et al., (2012), Nandi et al., (2015), Sujatha et al., (2016), Mbava, (2017) and Yang, (2018).

In particular, a predator-prey model with disease in the prey and analyzed a model of a three species eco-epidemiological system, namely, susceptible prey, infected prey and predator (Chattopadhyay and Arino,1999). Another prey-predator model with harvesting activity of prey which has been observed is that when the harvesting activity of prey is taken into consideration, then the population size of predator decreases and the naturally stable equilibrium of the model becomes unstable (Singh and Bhatti, 2012). A mathematical model to study the response of a predator-prey model to a disease in both the populations and harvesting of each species (Das, 2014), the model with two-stage infection in prey, the early stage of infected prey is more vulnerable to predation by the predator and the later stage of infected pests is not eaten by the predator (Nandi el al., 2015), harvested prey – predator model with SIS epidemic disease in the prey population (Sujatha et al., 2016). The predator–prey model with disease in super-predator are investigated and obtained the results that in the absence of additional mortality on predator by a super - predator, the predator species survives extinction (Mbava, 2017). A diffusive predator-prey model with herd behavior has been developed and the local and global stability of the unique homogeneous positive steady state is obtained (Yang, 2018).

A compartmental mathematical model based on the dynamics of the infection and apply vaccination strategies with herd immunity to reduce the intensity of disease spread in the prey-predator ecosystem (Bakare et al., 2012). We considered the work proposed by E.A. Bakare, because sometimes vaccination strategies become ineffective, in that case dynamic changes developed in the system, which we were investigated in the present work. We are trying to demonstrate the effect of vaccination when it failed to recover from the disease One of the purposes of this article is to explore the complex effect of the prey predator model in epidemiological system due to failure of vaccination strategies. The proposed model is characterized by a pair of first order nonlinear differential equations and the existence of the possible equilibrium points along with their stability is discussed. And finally, some numerical examples are discussed.

MATERRIAL AND METHODS

We shall consider the following prey predator system for analyzing it mathematically,

$$\begin{cases} \frac{dx}{dt} = -ax + bxy - cxz(1-\phi) \\ \frac{dy}{dt} = hy - exy - fyz(1-\phi) \\ \frac{dz}{dt} = hz - exz + fyz(1-\phi) - gz \end{cases} \quad (2.1)$$

Where x, y and z stand for the density of susceptible predator, susceptible prey andinfected prey populations, respectively.And the parameters 'a' is the natural death of the healthy susceptible predator, 'b' is the number of contact between susceptible prey and healthy susceptible predator, 'c' is the number of contact between healthy susceptible predator and infected prey, 'e' is the number of contact between healthy susceptible predator with infected prey and susceptible prey, 'f' is the number of contact between healthy susceptible prey and infected prey, 'g' is the harvesting rate of infected prey , h is the per capita birth rate of susceptible prey (per time) and infected prey and ϕ is the proportion of those successively vaccinated at birth.

The model consists of basic assumptions that we have made in formulating the model are: The relative birth rate for infected prey and that of susceptible prey remains the same.The disease is severely weakened and ultimately causing death for the predators. Once a predator is infected, it can be assumed to be dead. We will therefore consider only susceptible predator andinfectious disease spreads among the prey population by contact, and the rate of infection is proportional to the infected and the susceptible prey.The predator makes no difference between susceptible and infected members of the prey population. The predator becomes infected by consuming the infected prey. The rate of predator infection is proportional to the product of infected prey and susceptible predators.The infected prey does not recover.

To begin with, let us find the equilibrium points of the system (2.1)

The system (2.1) has the following equilibrium points:

$$E_0(0,0,0),\ E_1\left(\frac{h-g}{e},0,\frac{-a}{c(1-\phi)}\right),\ E_2\left(0,\frac{g-h}{f(1-\phi)},\frac{h}{f(1-\phi)}\right),\ E_3\left(\frac{h}{e},\frac{a}{b},0\right),\ E_4(x^*,y^*,z^*)$$

Where x^*, y^*, z^* are given by $x^*=\frac{h+fy^*-g}{e}$, $y^*=\frac{fa+cg}{fb+cf(1-\phi)}$, $z^*=\left(\frac{-a+by^*}{c(1-\phi)}\right)$

In the next section, let us discuss the stability of the five equilibrium points in the next which are obtained above.

RESULTS AND DISCUSSION

Stability Analysis: In this section, we analyzed the local behavior of the system (2.1) around each equilibrium point. The Jacobian matrix of the system of state variables is as follows:

$$J(x,y,z)=\begin{bmatrix} -a+by-cz(1-\phi) & bx & -c(1-\phi)x \\ -ey & h-ex-fz(1-\phi) & -fy(1-\phi) \\ -ez & f(1-\phi)z & h-ex+fy(1-\phi)-g \end{bmatrix}$$

To determine the stability of the equilibrium points, we look at the most useful techniques for analyzing nonlinear system is the linearized stability technique by theorem1.

Theorem 1:

Let $v(\lambda)=\lambda^3+A_2\lambda^2+A_1\lambda+A_0$ There are at most three roots of the equation $v(\lambda)=c$. Then the following statements are true:

a) If every root of the equation has absolute value less than one, then the equilibrium point of the system is locally asymptotically stable and equilibrium point is called a sink.

b) If at-least one of the roots of the equation has an absolute value greater than one, then the equilibrium point of the system is unstable and equilibrium point is called a saddle.

c) If every root of the equation has an absolute value greater than one, then the system is sourced.

d) The equilibrium point of the system is called hyperbolic if no root of the equation has absolute value equal to one. If there exists a root of the equation with absolute value equal to one, then the equilibrium point is called non-hyperbolic (i.e. one eigenvalue has a vanishing real part).

Let us preparefour propositions in order to discuss the local stability around each equilibrium point.

Preposition 1: For system (2.1),

The equilibrium point E_0 is locally asymptotically stable if h<1 and h<g.

Proof: The Jacobian matrix at E_0 (0,0,0) is given by

$$J(E_0)=\begin{bmatrix} -a & 0 & 0 \\ 0 & h & 0 \\ 0 & 0 & h-g \end{bmatrix}$$

The Eigenvalue corresponding to the equilibrium point E_0 (0,0,0) are –a, h, h-g. Only one Eigen value is negative and other two depends upon the value of h i.e. Birth rate of susceptible and infected prey. Then by theorem 1, we obtain $E_0$is locally asymptotically stable if h<1 and h<g.

Preposition 2: For system (2.1), The equilibrium point E1is locally asymptotically stable if $(1-\phi)\sqrt{ah-ag}<1$ and af+cg<1 and af+cg<1.

Proof: The Jacobian matrix at $E_1\left(\frac{h-g}{e},0,\frac{-a}{c(1-\phi)}\right)$ is given by

$$J(E_1)=\begin{bmatrix} 0 & \frac{b(h-g)}{e} & \frac{-c(1-\phi)(h-g)}{e} \\ 0 & g+\frac{fa}{c} & 0 \\ \frac{-ae}{c(1-\phi)} & \frac{-fa}{c} & 0 \end{bmatrix}$$

If the corresponding Eigenvalues are λ_1, λ_2, λ_3 then

$$\lambda_1 = -(1-\phi)\sqrt{ah-ag}$$
$$\lambda_2 = (1-\phi)\sqrt{ah-ag}$$
$$\lambda_3 = \frac{af+cg}{c}$$

Thenby theorem 1, we obtain $E_1\left(\frac{h-g}{e},0,\frac{-a}{c(1-\phi)}\right)$ is locally asymptoticallystable if $(1-\phi)\sqrt{ah-ag}<1$ and af+cg<1.

Preposition 3:For system (2.1),
The equilibrium point E_2 is locally asymptotically stable if $\sqrt{h^2-gh}<1$ and $bg+bh\phi<af+bg\phi+bh+ch$.

Proof: The Jacobian matrix at $E_2\left(0,\frac{g-h}{f(1-\phi)},\frac{h}{f(1-\phi)}\right)$ is given by

$$J(E_2)=\begin{bmatrix} -a+\frac{b(g-h)}{f(1-\phi)}-\frac{ch}{f} & 0 & 0 \\ \frac{e(h-g)}{f(1-\phi)} & 0 & -g+h \\ \frac{-eh}{f(1-\phi)} & h & 0 \end{bmatrix}$$

If the corresponding Eigenvalues are λ_1, λ_2, λ_3 then

$$\lambda_1 = -\sqrt{h^2-gh}$$
$$\lambda_2 = \sqrt{h^2-gh}$$
$$\lambda_3 = \frac{-af+bg-bh-ch-bg\phi+bh\phi}{f}$$

Then by theorem 1, we obtain $E_2\left(0,\frac{g-h}{f(1-\phi)},\frac{h}{f(1-\phi)}\right)$ is locally asymptotically stable if $\sqrt{h^2-gh}<1$ and $bg+bh\phi<af+bg\phi+bh+ch$

Preposition 4:For system (2.1),
The equilibrium point E_3 is neutral if eigenvalue is imaginary.

Proof: The Jacobian matrix at $E_3\left(\frac{h}{e},\frac{a}{b},0\right)$ is given by

$$J(E_3)=\begin{bmatrix} 0 & \frac{bh}{e} & \frac{c(\phi-1)h}{e} \\ \frac{-ae}{b} & 0 & \frac{af(\phi-1)}{b} \\ 0 & 0 & \frac{af(1-\phi)-bg}{b} \end{bmatrix}$$

If the corresponding Eigenvalues are λ_1, λ_2, λ_3 then

$$\lambda_1 = -\frac{(bg-af+af\phi)}{b}$$
$$\lambda_{2,3} = \pm i\sqrt{ah}$$

One Eigen value l1 is negative if af<bg+afϕ and the remaining two Eigen values l2 and l3 are imaginary. The Eigenvalues are purely imaginary, its real parts are exactly 0. The equilibrium point $E_3\left(\frac{h}{e},\frac{a}{b},0\right)$ is neutral.Then by theorem 1(d), we obtain this preposition.

Let us discuss the stability of the E_4 by Routh-Hurwitzcriterian.Local stability of the system (2.1) around the non-zero equilibrium point E_4.

The Jacobian matrix at $E_4(x^*,y^*,z^*)$ is given by

$$J(E_4)=\begin{bmatrix} -a+by^*-cz^*(1-\phi) & bx^* & -c(1-\phi)x^* \\ -ey^* & h-ex^*-fz^*(1-\phi) & -fy^*(1-\phi) \\ -ez^* & f(1-\phi)z^* & h-ex^*+fy^*(1-\phi)-g \end{bmatrix}$$

Where x*, y*, z* are given by

$$x^*=\frac{h+fy^*-g}{e},\quad y^*=\frac{fa+cg}{fb+cf(1-\phi)},\quad z^*=\left(\frac{-a+by^*}{c(1-\phi)}\right)$$

The characteristic polynomial for the Jacobian matrix J (E_4) is given by

$$\lambda^3+A_1\lambda^2+A_2\lambda+A_3=0$$

Where

$$A_1 = a-by^*+cz^*(1-\phi)-2h+2ex^*+fz^*(1-\phi)+g-fy^*(1-\phi)$$
$$A_2 = (-a+by^*-cz^*(1-\phi))(2h-2ex^*-fz^*(1-\phi)+fy*(1-\phi)-g)+$$
$$(h-ex^*-fz^*(1-\phi))(h-ex^*+fy^*(1-\phi)-g)+bex^*y^*-c(1-\phi)x^*ez^*$$
$$A_3 = (h-ex^*-fz^*(1-\phi))((a-by^*+cz^*(1-\phi))(h-ex^*+fy^*(1-\phi)-g)+c(1-\phi)x^*ez^*\}$$
$$-bex^*y^*(h-ex^*+fy^*(1-\phi)-g)-befx^*y^*z^*(1-\phi)-cf(1-\phi)^2x^*y^*ez^*$$

According to Routh-Hurwitzcriterian, $E_4(x^*,y^*,z^*)$ is asymptotically stable if and only if $A_1>0$, $A_3>0$ and $A_1A_2-A_3>0$.

Theorem 2. (E_0) is globally stable.

Proof. Let a Liapunov function be,

$$V(x,y,z)=x+y+z.$$
$$\frac{dV}{dt}=-ax-cxz(1-\phi)-exy-exz-gz+bxy+h(y+z)<0,\ if\ b,h<0.$$

The theorem above, then implies that (E_0) is globally asymptotically stable.

Now, let us find the global stability of the system (2.1) around all the equilibrium points for different 2-D planes by using Bendixson-Dulac criterion.

Theorem 3. E_2 is globally asymptotically stable in y-z plane.

Proof. Let,

$$H(y,z)=1$$

It is obvious that $H(y,z)>0$ if $y>0$ and $z>0$ if and.

Now, we denote

$$F_1(y,z)=hy-fyz(1-\phi),$$
$$F_2(y,z)=hz+fyz(1-\phi)-gz,$$
$$\Delta(y,z)=\frac{\partial}{\partial y}[F_1H]+\frac{\partial}{\partial z}[F_2H],$$

Then,

$$\Delta(y,z) = 2h - fz(1-\phi) + fy(1-\phi) - g.$$

Thus, $\Delta(y,z) < 0$ for all $y > 0$ and $z > 0$ if $h < 0$ and $f(1-\phi) < 0$ Therefore, by using Bendixson-Dulac criterion, there will be no periodic orbit in the y-z plane.

In the similar manner, we can show in the x-z plane for E1 with the condition $\Delta(x,z) < 0$ for all $x > 0$ and $z > 0$ if $h > 0$, in the x-y plane for E_3 with the condition $\Delta(x,z) < 0$ for all $x > 0$ and $y > 0$ if $h,b < 0$ and in the same way E_4 can be globally asymptotically stable in x-y,y-z and x-z planes.

We have performed some numerical simulation to study the role of harvesting on the prey predator system and we illustrate the dynamical and complex features of the model using MATLAB. In the starting, we fixed all parameters to ensure that the three classes of populations survive. Numerical simulations explain the effect of the parameters on the complex behavior of a given system (2.1).

(i) Let us consider following set of parameters,

a = 1.0; b = 1.5; c = 0.1; h = 0.5; e = 1.5;f=0.1;g=0.7,ϕ=0.91,

With initial condition x (0) =0. 8, y (0) =1. 70, z (0) =0. 75. For this set of parameter, we get the following variation of the population of the healthy predator, susceptible prey and infected prey with respect to time, which isillustrated below in figure 4 (a) and figure 4 (b).

(ii) Let us consider following set of parameters,

a = 1.0; b = 1.5; c = 0.1; h = 0.5; e = 1.5;f=0.1;g=0.1;ϕ=0.91;

With initial condition x (0) =0. 8, y (0) =1. 70, z (0) =0. 75. For this set of parameter, we get the following variation of the population of the healthy predator, susceptible prey and infected prey with respect to time, which is illustrated below in figure 4(c) and figure 4(d).

It is observed that effective harvesting of diseased prey, increase the growth rate of the susceptible predator population. If the value of harvesting rate g≥0. 7 then the infected prey population decreases more rapidly, but if the value of g<0.7 then infected prey population decreases slowly that shown in fig. 4(a), 4(b), 4(c) and 4(d) respectively. In this analysis, we have also observed that the whole population of the susceptible predators may be wiped out due to increase in the number of the susceptible and infected preys. This result shows that the system is biologically well behaved. In another case when the diseased prey can be washed out, a rational use of the stability criterion of non-zero equilibrium point may be useful for ecological balance. In this case, the parameters of the system should be regulated in such a way that stability criterion of non-zero equilibrium is satisfied but infected prey remains low enough. Some-

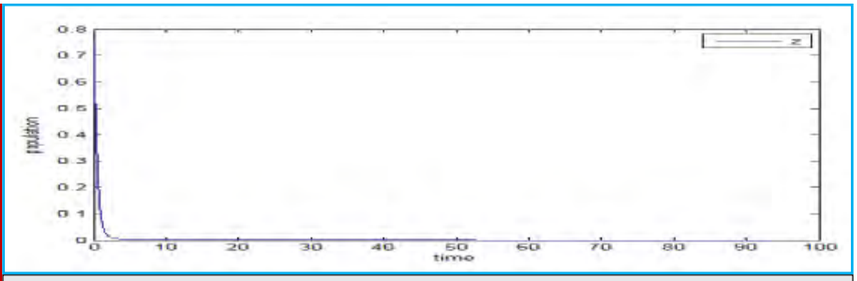

FIGURE 4a. Represents the effect of high harvesting on the population of the infected prey as time goes on.

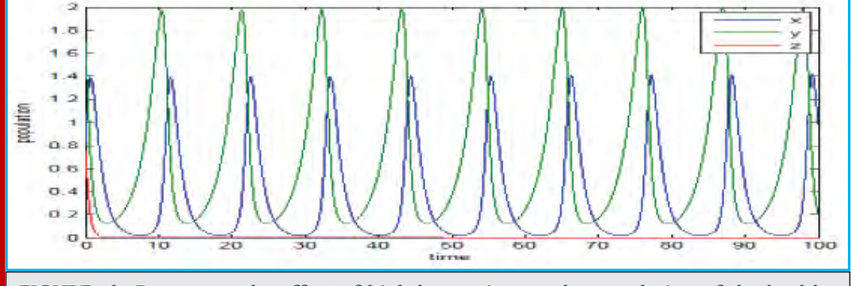

FIGURE 4b. Represents the effect of high harvesting on the population of the healthy predator, susceptible prey and infected prey as time goes on.

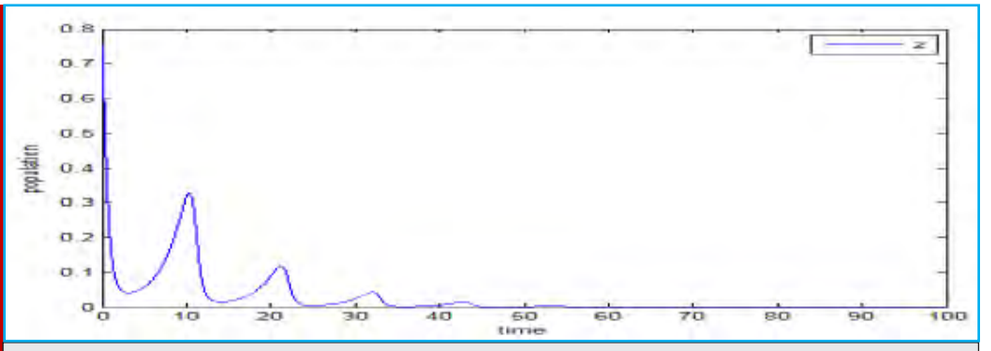

FIGURE 4c. Represents the effect of low harvesting on the population of the infected prey as time goes on.

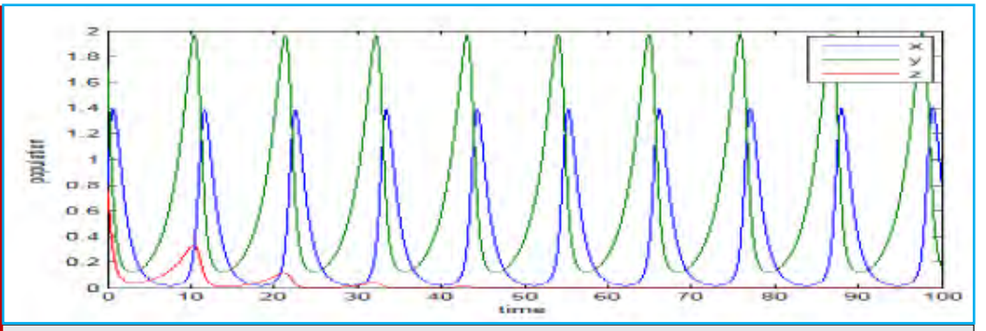

FIGURE 4d. Represents the effect of low harvesting on thepopulation of the healthy predator, susceptible prey and infected prey as time goes on.

times, harvesting became a suitable option for prevention of the population rather than the vaccination strategies. Therefore, effective harvesting became essential for the survival of the population.

CONCLUSION

A non-linear system based on the epidemic SIR model has been studied and discussed. Conditions for local and global stability at various equilibrium points were obtained. We have illustrated the effective harvesting of diseased prey in the whole system and reveal that the increases of predator population when the harvesting rate of infected prey population increases. We may conclude that effective harvesting of diseased prey may be used as a biological control for the spread of disease. And maintain balance in these species populations by preventing in the predator population to extinction. Finally, some numerical simulations illustrate and supplement our theoretical analysis by considering different parameter values. Low harvesting and high harvesting rates play an important role in this analysis. Global stability of equilibrium E_0 shows that disease free equilibrium always exists. In future other effecting condition can be used to save the predator population by introducing alternative food for predator rather than diseased prey.

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Effects of probiotic in expression of RUNX-2, ALP, OCN and CASP-3 genes in Wistar albino rat receiving 2Gy gamma radiation

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ABSTRACT

Today, radiotherapy is responsible for most of the therapies in a variety of cancers. However it could causes harm effects like bone deficiency. Bone reinforces human body. On the other side probiotics are living microorganisms that have been used in many studies to reduce the incidence of certain cancers or treat them. Therefore we decided to study if it is possible to use probiotics against bone lost. Male albino Wistar rats for four weeks were put under a diet of two types of probiotic *Lactobacillus casei* and *Lactobacillus acidophilus*. After the diet, the groups were subjected to radiation with a cobalt 60 apparatus. Then the bone marrow immediately was extracted and examined the expression of the osteogenic genes (RUNX-2, OCN, ALP) and CASPASE-3 (which is effective in apoptosis) by the Real Time PCR machine for the first time .In this study, we found that osteogenesis was much higher in groups with the probiotic diet than those without the probiotic diet. *Lactobacillus acidophilus* was also found to be more effective than *Lactobacillus casei*. Also, the use of these probiotics increases the expression of the CASPASE-3 gene in the common pathway of apoptosis, which means that probiotics increase apoptosis. This study showed probiotics could repair the harmful effects of ionizing radiation on bones.

KEY WORDS: BONE MARROW, RADIATION, PROBIOTICS, *LACTOBACILLUS ACIDOPHILUS, LACTOBACILLUS CASEI*

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INTRODUCTION

All living things, including humans, are constantly exposed to natural and human sources of ionizing radiation. Effective dose of the background is about 2.4 mSv per year. The major man-made origin of human exposure is radio diagnosis and Radiation therapy for cancer patients, (Wang, et al., 2016). Radiotherapy uses high-energy rays to kill cancer cells. This may be done alone or in mixture with other treatments such as surgery or chemotherapy, (Spyropoulos, et al., 2011). Radiation therapy is an important part of the treatment regimen in various human malignancies, and for many non-treatable pain management. It is estimated that 50-70% of all oncology patients are treated with radiation therapy, or a combination of chemotherapy and radiation therapy programs, (Michelin et al., 2004). To eliminate most of the malignant tumors, ionizing radiation requires approximate dose regimens near tolerance to adjacent tissues. On the other hand tissues that contain renewal cells, such as bone marrow and gastric mucosal mucosa, require fast cell proliferation and there are more susceptible to toxic effects of ionizing, (Michelin, et al., 2004). Bone damage in due to radiotherapy has been confirmed in epidemiological and animal studies. Bone is one of the most common natural tissues, and would have harmful effects a like fractures and loss of bone marrow function after radiotherapy, (Mego et al., 2013) osteogenesis associated genes are, RUNX-2, ALP, OCN and adipogenesis associated genes are PPAR-γ and C/EBPα, (Mansouri-Tehrani et al., 2015).

It should be noted that apoptosis is a cell death mechanism with various physical and biological causes. It plays a major role in many natural and physiological processes, as well as in the pathogenesis of various diseases, (Liu et al., 2013). Apoptosis can be activated through the extrinsic and mitochondrial dependent pathway. All paths eventually lead to caspase activation. (Jilka, et al., 1998) on the other hands probiotics are living microorganisms that help to preserve the beneficial microbial balance in humans or other hosts as drugs or dietary supplements. Most probiotics belong to a group of lactic acid producing bacteria (Lactobacilli, Streptococci, and Bifidobacterium). Some of the inhumane strains are used in the fermentation of dairy products, while others are human intestinal biochemistry, (Weiss et al., 2011). Lactobacilli and Bifidobacterium are generally known as probiotics due to their beneficial effects on health and include various effects, such as deprivation and inhibition of pathogens in the intestine, increased integration of intestinal epithelium and modulation of the host immune system both locally and systemically, (Dobrzyńska et al., 2015). So, based on the above, we decided to use probiotics as a diet to reduce the effects of radiation on the bone marrow and reduce apoptosis.

MATERIAL AND METHODS

36 male Wistar rats with weighting of 220 ± 220 g were purchased from the Tehran University of Medical Sciences Pharmaceutical Research Center in 6 groups at the animal house for four weeks prior to exposure to radiation. The groups were kept in special cages under constant ambient conditions at 22 ± 2 ° C and the light was adjusted for 12 hours of light and 12 hours of darkness. Water and special food were provided to animals without restrictions, except during tests. All experiments were conducted on the basis of ethical standards for animal behavior.

THERE WERE SIX RATS IN EACH GROUP:

1. Non-radiation and non-probiotic group (control group)
2. Non-radiation group with Lactobacillus casei probiotics
3. Without radiation and with probiotic consumption of Lactobacillus acidophilus
4. Group with irradiation and no probiotic consumption
5. Radiation and probiotic *Lactobacillus casei* group
6. Radiation and probiotic group *Lactobacillus acidophilus*

GAVAGE

0.1 g of each bacterium is equivalent to 10^{10} CFU / g, the amount of each serving was set for each group. Then for each rat, the calculated amount of each drug was dissolved in one ml of PBS buffer (pH 7.2) and Daily, once for *Lactobacillus acidophilus* and three times for *Lactobacillus casei,* the calculated data was fed to a stomach rat with a gavage needle. Also, control groups received 1 ml of PBS buffer per day.

IRRADIATION

The mice were anesthetized with ethical standards. At the center of radiotherapy at Imam Khomeini Hospital in Tehran, 60 cobalt irradiation devices were exposed to 2Gy and a dose rate of 100 cGy /min in a field with a size of 34.8 cm in 34.8 cm and an SSD of 80 cm were placed. Mice were sacrificed by displacement of the neck. The animal skeletal was washed in 70% ethanol. We discrete the muscle and cut the two ends of the thigh bone. 10 ml syringe by a 27-degree needle was injected from one end of the thigh bone and was poured from the other end of thigh bone into the test tube. Using centrifuge at 1000 rpm for seven minutes, the solvent phase was discarded. These test

tubes were placed in a freezer at a temperature of minus 80 (-80) degrees.

TOTAL RNA EXTRACTION

We extract a 5mm^3 piece of bone marrow tissue and added 1 ml of RNA extraction solution and then homogenized the mixture with the homogenizer. 200 µl of cold chloroform was added to the solution and the tubes were shaken vigorously for 15 seconds. Then the tubes were incubated for 5 minutes in ice. The tubes were centrifuged for 15 min at 4 ° C and 12,000 rpm. After centrifugation, three layers were formed in each vial from up to down including the aqueous phase RNA, the protein phase in the middle with white color, and the green phenolic phase at the bottom. The upper phase was slowly detached and transferred to a new 1.5-ml sterile tube. Equilibrium solution was added to cold isopropanol and incubated after mixing for 10 min at -20° C, then centrifuged for 15 min at 4 ° C and 12,000 rpm. To remove any impurities, the superfluous solution was discarded. The RNA precipitate was rinsed gently with a milliteratanol; after adding ethanol 80%, the tubes were slowly gutted several times and then centrifuged for 10 min at 4 °C and 12,000 rpm. The supernatant was removed slowly and the sediment was placed for 10-15min at the laboratory temperature to dry. RNA deposition added to twenty microliters of treated water with DEPC. The solution was placed on a hot plate at 50-55 ° C for 5 minutes to solve the RNA, then the tubes were kept in - 80 ° C freezer.

EVALUATE RNA QUALITY

To evaluate RNA quality, electrophoresis gel was used. Before electrophoresis, all devices were treated with DEPC water. Electrophoresis was performed in 1% agarose gel. The gels were stained with ethidium bromide.

DETERMINE THE CONCENTRATION OF EXTRACTED RNA

To determine the concentration of RNA, the BioTek Nano Drop device was used. The OD= 260/280 expresses the purity of the extracted RNA and has an inverse relationship with RNA contamination with the protein. The aforementioned ratio close to 2 represents the absence of contamination with the protein.

The ratio 260/230 was also used to check out the amount of RNA contaminated with the materials used for extraction. It is desirable that this number is also close to 2.

SYNTHESIS OF CDNA

The master mix was made and added to each tube (all the work was done on ice). One microgram of the extracted RNA was used to synthesize cDNA. As a result, the amount of the required RNA was calculated based on its concentration in the sample and added to the tube. The tubes were transmitted to the thermocycler, and the cDNA synthesis reaction was performed according to the following procedure.

Finally, the cDNA was maintained at -20 ° C.

Table 1. Materials for synthesis of cDNA

Quantity	ingredients
µl2	RT Buffer (x5)
µl0.5	primer(50µM) oligo dT
µl5/0	Primer:Random Hexamer(100µM)
µl0.5	Reverse TranscriptaseEnzyme
The final volume is 10 µL	Sterilized water treated with DEPC

Table 2. Thermosilocera device program for reverse transcription reaction

The type of reaction	Temperature (°c)	time
Synthesis of single-stranded cDNA	37	15 min
Enzyme inactivation	85	5 s

PRIMER

Primers used in these experiments were designed and tested using NCBI and Gene Runner software. By the company Sinoclon with OD about 2 was made as freeze-dried. The table 3 shows the characteristics of the primers used in this thesis.

REAL-TIME PCR STEPS

All ingredients were removed from the freezer and let to melt gently. Master Mix was kept in aluminum foil to protect it against light. According to the table 4, a mixture of the desired materials was prepared to do real time PCR with final volume of 20 microliter. All reactions were repeated twice. Special microcircuits were put in a cold box with pins and the ingredients were added to each of them according to the instructions below. All the microtubules completely were sterilized to avoid error in results. To control the contamination of the reaction during the test, one sample without cDNA was considered for each gene, as a template called the NTC.

INGREDIENTS FOR REAL TIME PCR

The results of Real Time PCR were obtained from the Corbett-6000 device.

Data analysis:

the results of the Real Time were analyzed through a Fafel test. After analysis, one-way analysis of variance

Table 3. Characteristics of primers

primer	Sequences	Tm	length	OD
OCN	F:CAGACCTAGCAGACACCATGAG	59.2	22	2
	R:GGACATGAAGGCTTTGTCAGAC	58.5	22	2
ALP	F:CGTTTTCACGTTTGGTGGCT	59	20	2
	R:ACCGTCCACCACCTTGTAAC	58.9	20	2
RUNX-2	F:GGCCACTTACCACAGAGCTA	58.1	20	2
	R:AGGCGGTCAGAGAACAAACT	58.3	20	2
CASPASE-3	F:AGCTGGACTGCGGTATTGAG	59.35	20	2
	R:ATGGCGCAAAGTGACTGGAT	57.3	20	2

Table 4. Materials for Real Time PCR

Final concentration	Volume	Materials
X1	µl10	Master Mix 2X
ng/reaction100≥	µl2	cDNA
µM4/0	µl 8/0	µM10forward primer
µM4/0	µl 8/0	µM 10 reverse primer
-	µl4/6	water RNase- Free

(ANOVA) was performed. Because there was a meaningful difference between the different groups, we set up the Tukey HSD Post-hoc test and compared the different groups.

RESULTS AND DISCUSSION

Data analysis results for RUNX-2 gene between different research groups are shown in figure 1. It can be seen that all the groups had a higher expression than the control group (first group), while only the second group had no significant difference compared to the control group. In groups three, four, five and six, there is a significant increase compared to the second group. The fourth group is also the only group that has a significant difference compared to the third group, the difference is also significant. The last significant difference was observed in this chart is for groups five and four, which expresses the decline of group five compared to group four. Data

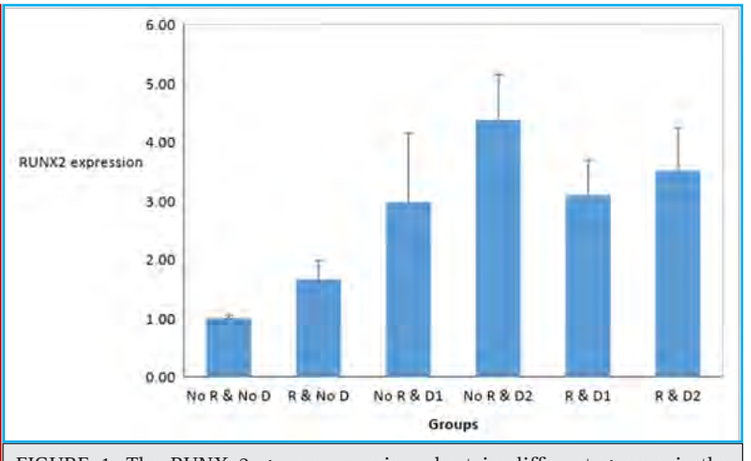

FIGURE 1. The RUNX-2 gene expression chart in different groups is the vertical axis of the gene expression and the horizontal axis of the tested groups. R means radiation, D1 is probiotic *Lactobacillus casei* and D2 probiotic *Lactobacillus acidophilus.*

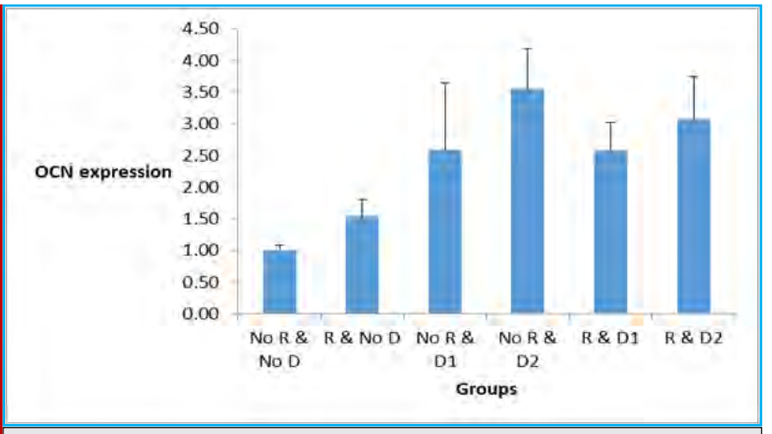

FIGURE 2. The OCN gene expression chart in different groups is the vertical axis of the gene expression and the horizontal axis of the tested groups. R means radiation, D1 is probiotic *Lactobacillus casei* and D2 probiotic *Lactobacillus acidophilus.*

analysis results for OCN gene between different research groups are revealed in figure 2 .

Figure 2 shows a significant increase in the expression of the three, four, five, and six groups relative to group one. And all groups except group 1 have a significant increase compared to the second group.

Figure 3 shows data analysis for ALP gene between different research groups.

The four, five, and six groups showed a significant increase compared to the control group in Figure 3. It can also be seen that groups four and five have a significant increase compared to the second group. The last result indicated in this chart is the increase in ALP gene expression in groups four, five and six compared to the third group.

Figure 4 reveals data analysis for CASPASE-3 gene between different research groups. Increasing the expression of groups 3, 4 , 5 and 6 was significantly different from that of the control group. Fourth and sixth groups also had a significant increase compared to the second group. The fourth group is the only group that has significantly increased relative to the third group. The fifth and sixth groups also had a significant decrease compared to the fourth group, which is visible on the chart.

In order to better understand the conclusions and conclusions about the results of the study, the mechanisms of the effect of probiotics should be considered first. The mechanism of action of these probiotics includes the production of inhibitor compounds, competition for binding sites, competition for food, elimination of tox Enhances

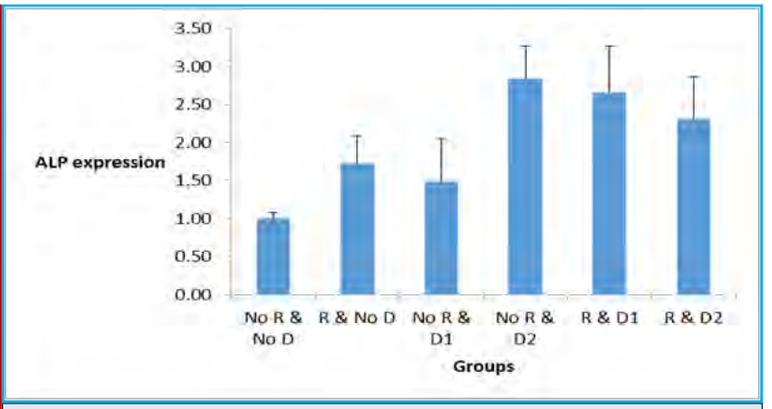

FIGURE 3. The ALP gene expression chart in different groups is the vertical axis of the gene expression and the horizontal axis of the tested groups. R means radiation, D1 is probiotic *Lactobacillus casei* and D2 probiotic *Lactobacillus acidophilus.*

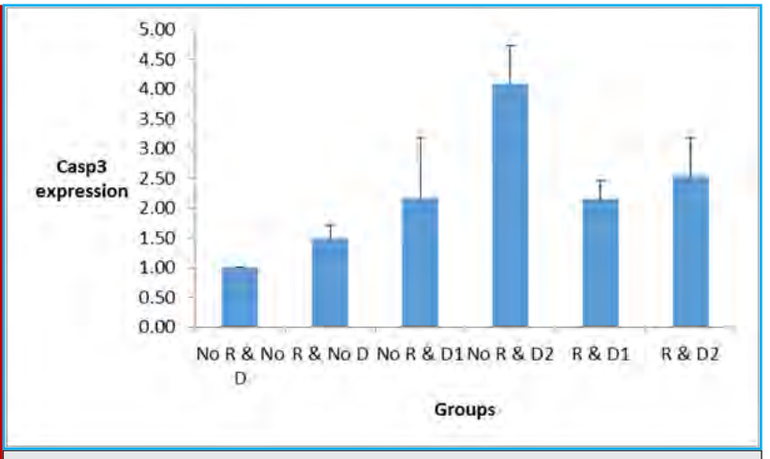

FIGURE 4. The CASPASE-3 gene expression chart in different groups is the vertical axis of the gene expression and the horizontal axis of the tested groups. R means radiation, D1 is probiotic *Lactobacillus casei* and D2 probiotic *Lactobacillus acidophilus*.

the immune system by increasing the level of cytokines, immunoglobulins activating macrophages and mononucleosis, and increasing the activity of natural killer cells, self-immune modulation and the production of TNFα and interleukin 6.in receptors and ultimately boosting the immune system. The tumor necrosis factor effect has three pathways: one way reaches to caspase-3, and the second can be terminated into three different functions, namely pre-apoptosis, proliferation and cellular differentiation, and the third pathway is the anti-inflammatory route .

Caspase-3:

All groups under the probiotic diet were more likely to increase than the control group because of the mechanism of probiotic effects on TNF alpha, which increases the expression of CASPASE-3. Groups under the Lactobacillus acidophilus probiotic diet, in the presence, and in the absence of radiation, gave rise to the expression of the CASPASE-3 gene in comparison to the only radiation group, since probiotics express the expression of CASPASE-3 in accordance with the above mentioned mechanism. *Lactobacillus acidophilus* group has increased expression in lactose-bacillus casei in the presence and absence of radiation, which probably indicates a greater effect of *Lactobacillus acidophilus* than *Lactobacillus casei*. The *Lactobacillus acidophilus* group has a greater expression than the *Lactobacillus acidophilus* group with radiation, which indicates radiation and *Lactobacillus acidophilus* neutralize each other. Perhaps radiation in the bone marrow may produce an immunological status that is more favorable than the pathway of differentiation and proliferation cells arrive.

RUNX-2:

All groups under the probiotic diet were more likely to be exposed than the control group and the radiation group, as probiotics increase the cellular differentiation and multiply their pathways. The *Lactobacillus acidophilus* group has been shown to have a higher expression than *Lactobacillus casei* (both in the presence and in the absence of radiation), indicating a greater effect of *Lactobacillus acidophilus*.

ALP:

Groups that have taken *Lactobacillus acidophilus* (both in the presence of radiation and in the absence of radiation) have a greater expression than the control group, which is probably due to the effect of *Lactobacillus acidophilus*. *Lactobacillus casei* group has a significant increase in expression in comparison with the control group, probably due to the synergistic effect of the radiation and probiotics. Because according to the radiation signal path and the osteoblastic signal path, radiation can ultimately cause cellular sensitivity so that the cell shows the counter-effects of radiation. This issue of the effects of signals can be investigated in the future. Groups that have taken *Lactobacillus acidophilus* (both in the presence of radiation and in the absence of it) have a higher expression than the *Lactobacillus casei* group; it indicates the greater effect of *Lactobacillus acidophilus*. The *Lactobacillus casei* group (in the presence of radiation) has a greater expression than *Lactobacillus casei* which is due to synergistic effect.

OCN:

All groups under the probiotic diet have increased expression in both the control group and the radiation

group because probiotic activates the second alpha-TNF pathway, resulting in differentiation and proliferation.

CONCLUSION

This study shows, the use of probiotics increase the expression of osteogenic genes and apoptosis, except in cases where this increase was not significantly expressed. According to the results and analysis, probiotic *Lactobacillus acidophilus* has a greater effect on *Lactobacillus casei*. That is, this probiotic increased the expression of the two groups of radiation and control. Although it also expands the gene for apoptosis. In general, the use of probiotics in this study increased the expression of osteogenic genes and apoptosis, except in cases where this increase was not significantly expressed. In some studies, we observed that radiation combined with two probiotics has different effects of synergistic and inhibitory effects for two probiotics, which is probably due to the effect of various probiotics in their signal paths, which is not exactly clear and can be tracked and researched.

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Design of a probe type *in situ* electronic grain moisture measurement system

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ABSTRACT

With the increased mechanization of harvesting operation, crop is harvested at high moisture and cannot be stored for longer period unless it is dried. Thus there is a need of equipment for determining moisture of grain. The same equipment may be used for determining moisture at the time of harvest also. The importance of grain-moisture and the use of electrical moisture meters for measurement are discussed briefly. References to earlier work with such moisture meters are cited (Young 1983, Nelson 2000), and a brief description of variation of electrical or dielectric properties with grain moisture are discussed. The information available in the literature on the dielectric properties of grain is reviewed from the viewpoint of its usefulness in grain-moisture measurement. Factors other than moisture that influence the dielectric properties of grain are elaborated. The authors have designed an *in situ* grain moisture meter for major crops like wheat, paddy, soybean. Most of the moisture meters available are desk type and are designed for *in situ* moisture measurement (Rai et al 2005). At present moisture meter and samplers are not integrated. In this design sensor and grain sampler are integrated in one unit. It will be low cost, portable and easy to use.

KEY WORDS: MOISTURE CONTENT, CAPACITANCE, DIELECTRIC PROPERTIES, SENSOR, DESICCANT, DISTILLATION. GRAVIMETRIC, PROBE

INTRODUCTION

Moisture is an important criterion for pricing and procurement (at minimum support price) of agriculture produce. Farmers with high moisture produce is penalized directly by a lower price for excess moisture and farmer with low moisture produce is penalized indirectly for supplying dry matter instead of water to the purchases.

Inexpensive method with ease and sufficient accuracy for determining moisture content of grain is not available for farmers. In most of the cases farmer relies upon his experience to estimate the moisture content. Farmers normally used observation by biting or feel-

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ing. Regardless of the method used for determining the moisture content, there are possibilities of error in making the determination. The major problem is that of securing a sample which is representative of the entire lot of material. To reduce the possibilities of error several samples should be obtained from different location in the bin, container or bag. Usually a large sample is obtained which is taken to the laboratory for determining the moisture content. Moisture content determination within one percent accuracy is sufficient to establish storage period for grains, as considerable money is lost each year because of over drying (Rai et al 2005).

The authors have theoretically designed a hand held grain moisture meter for exclusive use at farmers level, Krishi Upaj Mandi, ware houses and Food Corporation of India during procuring and storage. The aim is to develop a device for measuring grain moisture, based on the principle of capacitance measurement. At present moisture meter and sampler are not integrated. It is proposed that a digital hand held battery operated portable moisture meter with integrated grain sampling unit and display unit will be designed. This unit will be tested rigorously at mandi with paddy, soybean and wheat at large scale. The sensor would be of capacitance type either in the cylindrical shape or miniature parallel plates to be integrated in the sampling device. The principle of operation will be based on dielectric method i.e. Capacitance (Dass et al 2010).

The sensor and measurement device will be internally connected on a single module. Attempts will be made to make the system compact and rigid.

Usually farmers bring their produce to Mandi for its sell. The keeping quality of grain largely depends on the moisture content. Therefore, moisture measurement at Mandi will be helpful in finalizing drying process for safe storage of food grains.

MOISTURE MEASUREMENT TECHNIQUES

Various grain moisture measurement techniques are available i.e. direct as well as indirect methods. The direct method includes oven method, drying with desiccant and distillation method (Rai et al 2005, Hall 1970, Zeleny 1960). Direct methods involve either gravimetric or volumetric procedures. Such methods are cumbersome and time consuming. Other methods such as infrared and microwave (Nelson 2004) are very costly and needs elaborate calibration from variety to variety. It is thought to use capacitance (dielectric) measurement method as it is easy, simple and low cost (Nelson 1987).

PROPOSED DEVICE

The proposed device will be fabricated along with capacitive probe type sensor. The device will be developed consisting following components:

- The size and shape of grain sampler will be optimized.
- The sampled grain will be directly taken into sensor for better accuracy and instant measurement.
- A sampling probe for insertion into grain heaps/ bags will be developed and standardized.
- A LCD based digital display integrated with the sampler and sensor for quick measurement of moisture.
- The Geometry of probe will be optimized / redesigned.
- The sample holder may be in the form of Auger (parkhi). Various sizes of parkhi's will be fabricated and the capacitance response in the moisture range 8-25% will be studied.
- Sizes, shape and capacity of sensor will be optimized for best result in above range.
- If needed some modular arrangement will be made to incorporate grain collector of miniature size to increase the sample volume.
- The circuit will be designed using Op Amps, Analog and Digital ICs having LCD Display (Theraja 1980).
- Additional feature of above system will be equipped with graphical display system of the moisture indicators.
- Initially the meter will be calibrated for wheat, paddy and soybean crop. Thereafter, depending on the requirement and accuracy obtained other commodities may be taken for calibration (Rai et al 2005).

OPERATING PRINCIPLE

A coaxial type sensor (fig. 1) will be developed using dielectric properties of grain moisture.

Capacitance of Coaxial type sensor is given by

$$C = 2\pi H\varepsilon/\{\log(R-r)\}$$

(where R and r is radius of outer and inner cylinder and H = height of the cylinder)

$$= K_2\varepsilon,$$

Where ε = dielectric constant, K_2 = constant for fixed geometry (R,r,H)

Different sizes of coaxial type sensor will be fabricated for the purpose. Since a capacitor is physical device, a change in its capacitance is due to dielectric constant K_2. The ratio of dielectric constants of water, dry grain and air is 80:5:1 respectively (Nelson 1982, Nelson 1991). Calibration curve will be obtained (Gough 1983). Various grain samples with different moisture levels will be collected for each crop. Measurement will be performed at length with different shape/size of sensors and will be excited in the range of radio frequency i.e. 1-50 MHz and frequency optimization will be carried out. Signal Conditioning and amplification will be carried out as per requirement. Initially it will be calibrated for paddy, soybean and wheat. A conceptual sampling probe is shown in fig 2.

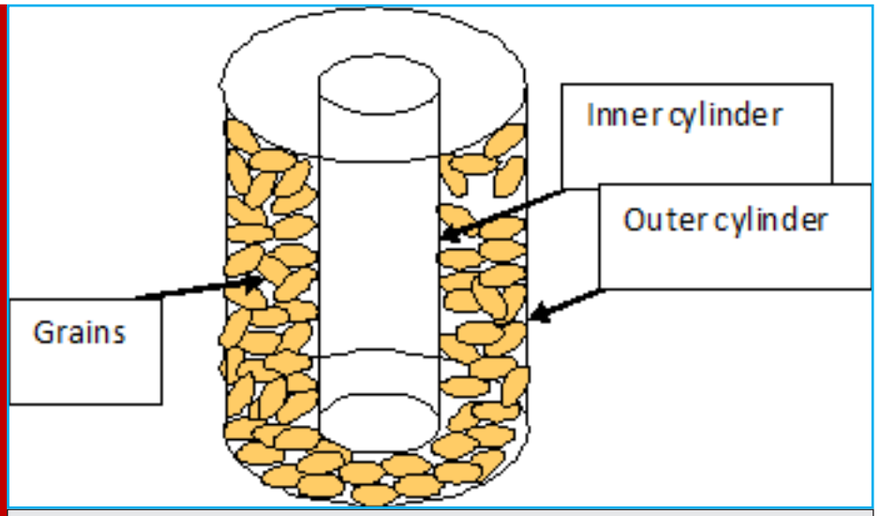

FIGURE 1. Coaxial Type Sensor

FIGURE 2. Parakhi (Grain sampling device)

PROCESS SCENARIO

The circuitry will consist of RF Oscillator, Signal conditioning circuit, Amplification and Display units etc (Mathur 2002, Hall 1986, Gaonkar 1989). The process diagram is shown in fig 3 and excitation circuit in fig 4 (Kant 2010).

MATERIALS AND METHODS

The following methodology is proposed:

- The present status of moisture measurement will be visualized by visiting FCI, SWC, farmer's field, Krishi Upaj Mandi etc.

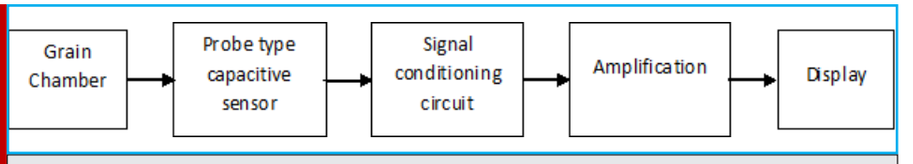

FIGURE 3. Block diagram of the System

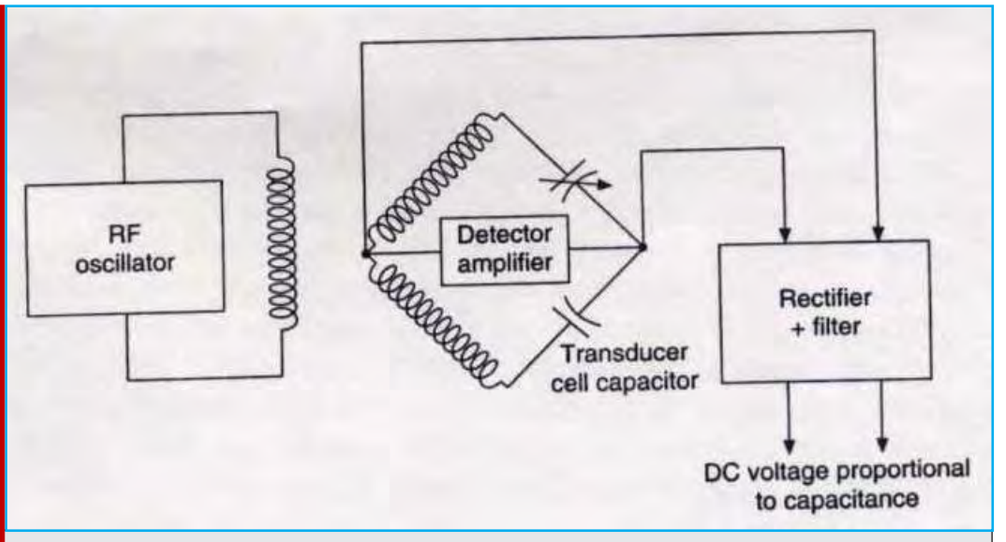

FIGURE 4. Bridge circuit for measurement of grain

- The standard procedure laid down by Association of Analytical Chemist (AOAC) method will followed during calibration and testing of unit at different moisture levels (8-25%).
- Once relationship of the sensor output and correlation factor for other variable is established, the design of the sensor in respect of size, shape etc will be freeze.
- The design work of electronic circuit consisting of oscillator, signal conditioning, A to D conversion etc (Mottershead, 1985) will be taken into account.
- The integration of sensor and other measuring unit will be done.

CALIBRATION AND TESTING OF THE DEVICE

The relationship between the electrical properties and grain moisture content vary with grain type, variety, temperature, packing density etc. For calibrating the device, samples of known moisture content will be prepared (Gough 1983).

- Long duration performance of the system will be tried in Mandi / Warehouses.
- Moisture range will be 8-25% with accuracy of +/-1 % and will be calibrated for Wheat, Paddy and Soybean in the first phase.
- Testing and field trial will be conducted (Gough 1983).

RESULT AND DISCUSSION

This device will facilitate easy, simple and accurate insitu measurement of grain moisture of different crop. This will be useful for monitoring moisture migration of grain stored at Food Corporation of India, central and state warehouses, farmer's field, Krishi Upaj Mandi, etc.

Before harvest during each Rabi and Kharif crop season, the Government of India announces the minimum support prices (MSP), to facilitate procurement of food grains. Government establish a large number of purchase centres at various mandis and key points. For paddy maximum limit of moisture content is notified as 17 percent on wet basis (GoI 2017).

For Wheat procurement more than 18,000 procurement centers are operating in Rabi Market Season (RMS) 2018-19 & for Rice procurement more than 35,000 procurement centres are operating in Kharif Market Season (KMS) 2017-18.

Its utility will be extended to these centers for ensuring procurement as per schedule of specification notified by Government of India (GoI 2017) i..e. .moisture content 17 % (wet basis). Thus there is need and demand of thousands of moisture meters to decide the condition of crop falling under uniform specification, to avoid unnecessary rejection of lot due to over moisture.

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Inventorization of electronic waste management, engaging with consumption: A Survey in Bhopal

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ABSTRACT

The existing practices of electronic waste in India suffer manyproblems like the difficulty in inventorization, legislation, poor awareness of electronic waste management and unhealthy conditions of informal recycling. The study was focused on inventories of e-waste in Bhopal city which is in the capital of the state and generates a database for the future plan and provide statistical data affecting the generation of E-Waste in Bhopal as well as prepare an inventory of electronic waste. This can help to prepare an action plan for Waste Electrical and Electronic Equipment which can be formulated and give a quantitative and qualitative analysis of WEEE generation in Bhopal City. The main objective of the study inventorization of seven electronic sub-sectors like refrigerators, mobile phones, television computers, air conditioners, washing machines and waste batteries in Bhopal city of the state. The present practices of electronic and electrical waste of management having many drawbacks like unhealthy conditions of informal recycling, the difficulty in inventorization, poor awareness inadequate legislation, these are the critical issues, during the site visit interviews total 120 questionnaires were collected, 50 questionnaires business entities and 70 from household and collect 432MT e-waste during the survey.

KEY WORDS: E-WASTE, RECYCLE, INVENTORIZATION, REUSE, DISPOSAL

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INTRODUCTION

Electronic waste management becomes burning issue all over the world, because growth in demand and consumption of electronic goods had led huge amount of waste and this waste become a new type of waste called electronic waste. So there is dire need to adopt sustainable practices so that we can handle a waste, (Schwarzer 2005). Electronic Waste, as the name represent, comprises of electronic and electrical equipment such as computers, cell phones and other electrical devices which are destined for recycling or disposal, (Robinson 2009). The amount of electronic dump generated globally has been estimated to reach about 72 billion tons annually by 2017, (Bisschop 2014).

E-waste informal processing sitescan be located near agricultural fields and other cropland where heavymetals and other contaminate can penetrate into the soil where food isgrown, (Song and Li 2014). Human exposure to halogenated flame retardants (HFR) overdermal adsorption by skin wipe. Dermal absorption would be animportant exposure route for HFR. Liu et al. (2017).

Developing countries leading the pack in accepting electronic waste since thedeveloped world today include China, India, Pakistan, and Nigeria, (Garlapati 2016).

The electrical and electronic equipment's are largely classified into three parts as:- comprising of household usages like air conditioners, refrigerators and dishwashers, washing machines are 'white goods,' computers, printers, fax machines, scanners, etc are'; 'grey goods,' comprising of TVs, camcorders, cameras are 'brown goods, Sinha (2007). PCBs, dioxins, and heavy metals in their essential forms, Lead, Nickel, Cadmium and Lithium are found in used batteries, abundant the ones being massproducedin electric vehicles. Organophosphate flame retardants and plasticizers in urine example of the people living in an e-wastedismantling site (Lin et al., (2017).

Despitethe risk of many developing countries do not have proper regulationsand policies in place to protect the local people and environment. Example, in Nigeria exquisite metals are removed from circuit boardsby using acid, and then dumping them onto the ground or into streams, (Kiddee et al., 2013).

The University of the Negev researchers used cathoderay tube exhibit the financial incentive system. Theproper recycling facilities would offer a higher price for the CRTs thatcould be earned thru the informal recyclers manually dismantling them.This would provide motivation for the informal recyclers totake the collected CRTs to the formal sector to go through recycling.These material incentives encourage a relationship between theinformal and formal sectors, (Davis and Garb, 2015).

'Meeting the needs of the present generations without compromising the capacity of upcoming generations to meet their own needs by Hester et al., (2012). Faster obsolescence and subsequent up-gradation of electronics product, are forcing consumers to discard old products, which in turn accumulate huge e-waste to the solid waste stream, (Bhat and Patil, 2014).

Informal recycling practices: preferably, all electronic waste should be recycledinformal recycling facilities. However, because of the formal electronic wastefacilities are costly to operate construct as well as, especially in lessdeveloped countries, informal recycling sites are prevalent. Theinformal e-waste sector consists of sites that excerpt the valuable parts of the electronics and electrical equipment using crude recycling and disposal methods usuallywithout any kind of shelter equipment such as goggles or gloves or theassistance of technology, (An et al. 2015).

Technological innovations and intense marketing engender a rapid replacement process, the Basel Convention, which is reduction of Tran's boundary movements of hazardous and other wastes including the minimization and prevention of their generation, the environmentally sound management of such wastes and transfer as well as use of technologies. Sthiannopkaoand Wong (2013).

A Draft Strategic Plan has been proposed for the implementation of the Basel Convention, The Draft Strategic Plan takes into account existing regional plans, strategies or programs, the decisions of the Conference of the Parties and its subsidiary bodies, ongoing project activities and process of international environmental governance and sustainable development and also calls the management and effective involvement by all concerned stakeholders essential for the aims of the Basel Declaration within the approach. Of interrelated and equally support strategies are proposed to support the concrete implementation of the activities as indicated1989 in the website(**http://www.basel.int/**).

MATERIAL AND METHODS

SITE SELECTION

The study of electronic waste was conducted in Bhopal cities which is one of the biggest city, located in Madhya Pradesh. The electronic waste generators and business entities, household, institutions are the consumers of the electronic and electrical equipment's they were targeted in this study the survey location is based on the socioeconomic status of the area as well as population density of Bhopal.The population of Bhopal metropolitan area that extends beyond Bhopal city was 1,886,100. The total effective literacy rate was 85.24%, with male and female literacy respectively at 89.2% and 80.1%according to Census in 2011 next will be held on 2021.

FIGURE 1. Map of Bhopal city

STUDIES AND DATA COLLECTION

Field data were collected through the questionnaire-based survey; paper studies of their official sites give a better result than mail survey. The study was targeted following categories of electronic and electrical equipment such as televisions, mobile phones, personal computers air conditioners, refrigerators, washing machines for quantifications and estimations of e-waste produced, we collecteddata from the government office, household private sectors second-hand shops.

EMAILING QUESTIONNAIRE

E-mail is the most useful method for collecting data a standardized e-mail was attached to the questionnaire so that it would attract the recipient to read the email and participate in answering the questionnaire.

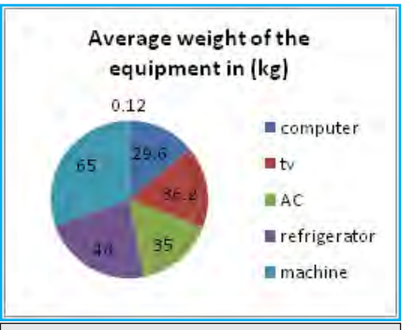

FIGURE 2. Average Weight Of the Equipment

RESULTS AND DISCUSSION

COMPUTERS

A Computer is an electronic device which contains many toxic components such as mercury PCBs, CRT chromium, and cadmium. A Monitor having 6% lead which is dangerous for our health as well as affects our environment generally, electrical and electronic equipment is sent to

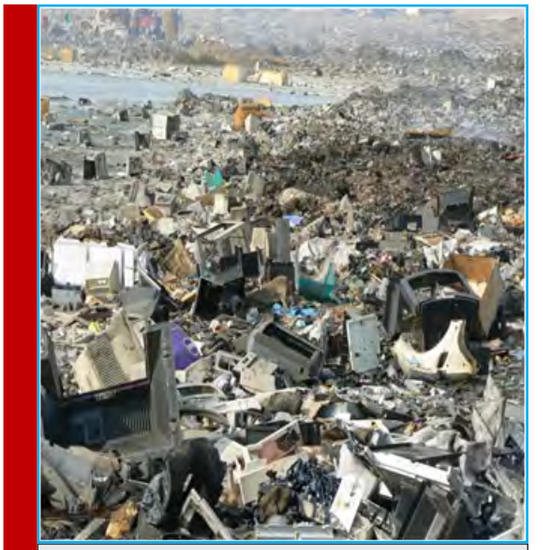

FIGURE 3. Discarded E-Waste

the landfilling, which produces toxic element, like cadmium lead mercury into the atmosphere ground and soil releases negative impact.

TELEVISION SETS

The average weight of the television is 36.2 kg, during the survey, find that total around 8952 televisions were used, since last 5 years the generation of electronic waste is approximate 2173 kg so the yearly generation 1644 kg/year.

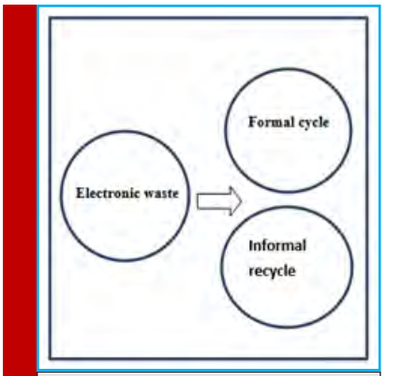

FIGURE 4. Electronic waste handing

REFRIGERATORS

Total of 9374 refrigerators are in use, and according to respondents most of the refrigerators have an average lifespan of 10-12 years. Maximum discarded refrigerators are used for exchange purposes to buy new refrigerator so the yearly generation is 13256.77 kg/year.

AIR CONDITIONERS

On an average it is found that in every two household, one number of AC is being possessed by the respondent, brand new air conditioners were found to be used for a long time. Maximum respondents have discarded their air conditioner by exchange offer only and few respondents have given to scraping dealer, total 1123 air conditioners are used so the e-waste yearly generation is 14.23kg/year.

WASHING MACHINES

Total 7543 washing machines are used on an averageand it is found that each household respondent possessed one number of washing machines. Brand new washing machinewas found to be used for a long time. Many respondents have discarded their washing machine either by exchange offers or by giving to scrap dealer so the e -waste yearly generation is 5389kg/year.

Home	Hospital	Government sectors	Private sectors
Pc	pc	Fax machine	Boiler
Fan	ECG device	Xerox machine	Mixture
Fridge	Microscope	Scanner	Incubator
Washing machine	Incubator	Fan	Fan
Refrigerator	fan	Tube lights	etc.
CD player	etc.	Air conditioner	

FIGURE 5. Source of electronic waste

COMPUTATION OF ELECTRONIC WASTE GENERATION IN LAST YEAR FROM DATA COLLECTED

Reduction of volume

Method which subtract the hazardous parts of waste materials from non-hazardous parts, includes in volume reduction these methodsare basically use for capacity reduction also price of deposing of e-waste also reduce waste steam capacity and divided into two parts:-

- Waste concentration
- Source segregation

The technique for waste reduction can be simple and economical by segregation of wastes. Different types of metal are present in a waste material can be treated simply which can improve the metal value of sludge. Waste can be recycled by the technique of vacuum filtration also gravity or inverse osmosis or ultra-filtration etc.

Table 1. Total generation of batteries

S. No	Total Generation of Batteries	Quantity
1	Average Weight	30 kg
2	Total Usage of Batteries in no.	3845
3	E-Waste of Batteries in Last 5 years in no.	4784
4	Yearly Batteries Generation in KG	8942kg/year
5	Batteries generation in MT	8.94 mt/year

Table 2. Total Generation of Air-Conditioners

S. No.	Total Generation of AC	Quantity
1	Average Weight in 1 AC	35 kg
2	Total usage of AC in no	1123
3	E-Waste of AC in Last 5 years in no.	1565
4	Yearly AC Generation in KG	14.23 kg
5	AC generation in MT	14.22 mt/year

Table 3. Total Generation of Refrigerator

S. No	Total Generation of Refrigerator	Quantity
1	Average Weight in 1 Fridge	48 kg
2	Total Usage of Refrigerator in no	9374
3	E-Waste of Fridge in Last 5 years in no	2045
4	Yearly Fridge Generation in KG	13256.77 kg/year
5	Refrigerator generation in MT	13.25 mt/year

Reuse and recycle

In this method, we can reduce the price of waste removal and raw materials also deliver profitable waste income. Repair facility of waste materials can be provided onsite as well as off-site. Reverse osmosis, condensation, electrolytic repair, filtration are the physical and chemical method which is used to recover and reuse the waste material

Table 4. Total Generation of television

S. No.	Total Generation of TV	Quantity
1	Average Weight in 1 T.V.	36.2 kg
2	Total usage of TV in no.	8952
3	E-Waste of T.V. in Last years in no	2173
4	Yearly T.V. Generation in KG	1644.25 kg/year
5	Yearly T.V. Generation in MT	16.44 mt/year

Table 5. Total Generation of Washing Machine

S. No	Total Generation of Washing Machine	Quantity
1	Average Weight	65 kg
2	Total Usage of Washing Machine in no.	7543
3	E-Waste of WM in Last 5 years in no	3687
4	Yearly WM Generation in KG	5389 kg/year
5	Washing Machine generation in MT	53.89 mt/year

Sustainable product design

- **Redesign the product**:- use less amount of hazardous materials, to design the product example redesign the new computer which has flatter lighter and integrated materials
- **Use of renewable materials and energy**: - plant-based chemical are used to make bio-based plastics.
- **Use non-toxic and non-renewable materials**:- material which are non-renewable use such that they can recycle and reuse. Example some parts of processor product as dell and Gateway lease.

CONCLUSION

Electronic waste has suffered major concern in maximum countries in the world, mainly those countries where electronics and electrical waste is imported as well as unregulated function processed and generating significant opposing environmental effect the evaluation of the study indicates that in 2016, electronic and equipment waste is calculated and the total collection of e waste is estimated in around 432 MT in Bhopal city which is in the Madhya Pradesh and increases year by years. According to the report, 16 registered recycle they collect the electronic and electrical waste and process the electronic and electrical equipment waste in India. Reuse and recycle system were implemented in India it is very important issue for sustainability, globally and domestically both.

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An efficient protocol for *in-vitro* regeneration of *Vitex negundo* an important medicinal plant

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ABSTRACT

An efficient in vitro protocol has been established for propagation of elite plant of *Vitex negundo* L. (Verbenaceae) commonly known as Nirgundi. It is a large woody aromatic and multipurpose medicinal shrub. It is used medicinally throughout the greater part of India. This species is widely used in Chinese herbal medicine and is the second most important for treatment of chronic bronchitis. Leaf extract of this plant possess antibacterial and antitumor activity. In the present study, nodal segments of *Vitex negundo* were taken as source of explants and grown on MS media with 3% Sucrose and 0.8% agar-agar, supplemented with different concentrations of BAP, KIN (0.5 – 3.5 mgl^{-1}) and TDZ (0.5-2.0), with various auxins (NAA, IBA, TIBA), incubated under a photoperiod of 16h illumination of light and 8h dark at 25±2°C. MS + 1 mgl^{-1} BAP was found to be the best concentration for shoot regeneration (90%). The regenerated shoots were sub-cultured for rooting, using different concentrations of IBA and NAA. Present optimized micropropagation protocol offers the possibility of germplasm conservation and mass cultivation of this important medicinal plant.

KEY WORDS: *VITEX NEGUNDO, REGENERATION, NODAL EXPLANT, CALLOGENESIS*

INTRODUCTION

Medicinal plants have been the subject of curiosity since times immemorial (Constable, 1990). Almost every civilization has a history of medicinal plant uses. About 80% of the people living in developing countries depend on indigenous medicines to meet their primary health care needs. About 85% of these traditional medicines involve the consumption of plant extracts. Out of 250 species of the genus *Vitex*, near about 14 species have been found to occur in India. *Vitex negundo* L. (Verbenaceae) is a perennial aromatic, large woody shrub, tri or

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penta-foliate leaves with purple color flower in branched tomentose cymes. It is commonly called as Chaste tree, Nirgundi (Hindi) and Monk's pepper. It is an important agro-forestry tree (200–300 cm high) found throughout the greater part of India and has been included in the list of valuable plant species due to its wide use in the Indian system of medicine (Kapur et al., 1994). It posseses various medicinal properties (Muthuswamy et al., 2012; Basri et al., 2014; Bano et al, 2015; Lad et al., 2016).

The plant possesses anti-arthritic, hepatoprotective, anti-inflammatory, anti-allergic, insecticidal, antioxidant, antibacterial, immunomodulatory, antifungal as well as mosquito repellant activities (Islam et al., 2013; Zheng et al., 2014; Singh et al., 2015; Lad et al., 2015; Lad et al., 2016). Leaves are aromatic, used as an antifertility drug (Bhargava, 1986) and possess snake neutralizing activities, (Minu et al., 2012) (Muthuswamy et al., 2012; Durairaj et al., 2014) Dharmadasa et al., (2016) also reported the anti-snake venom properties. Leaves are antiparasitic and used as alternative vermifuge and anodyne. They are also very effective to reduce inflammatory swellings of joints in rheumatism and relieve catarrh and headache. Root is used as tonic, diuretic and expectorant. It regulates hormones, enhances breast milk production and possesses progesterogenic properties as well (Au et al., 2008; Arora et al., 2011; Basri et al., 2014; Haider et al; 2017).

Betulinic acid, ursolic acid and β-sitosterol are some of its active constituents, isolated from its leaves which have been found to possess anti-cancer, anti-HIV and angiogenic properties, respectively (Basri et al., 2014). In nature the species propagates through stem cutting and seeds. Based on our preliminary investigations propagation with vegetative cuttings is very slow and the survival rate is very limited. Propagation through seeds is hindered due to poor germination. Thus conventional propagation through seeds and vegetative cutting is not an adequate solution to meet the demand for this rare medicinal plant. Hence this study was carried out to develop an efficient protocol for its mass cultivation.

MATERIAL AND METHODS

Nodal explants were excised from elite plants of *Vitex negundo* growing in medicinal plants garden, School of Studies in Botany, Jiwaji University, Gwalior (M.P). The excised nodal explants of *V. negundo* were washed for 10 min under continuous stream of running tap water. Surface sterilization was done by treating the explants with 4% (v/v) Tween-20 (detergent; SRL, Pvt. Ltd, Mumbai, India) and rinsed with distilled water. These explants were then treated with 2% (w/v) bavistin solution (Systemic fungicide; BASP India Ltd., Mumbai India) for 5 min and followed by treatment with freshly prepared 0.1% $HgCl_2$ (SRL, Mumbai, India) for 3 min with continuous shaking under a laminar flow cabinet. These explants were finally washed 2-3 times by sterile distilled water prior to implantation in semisolid media.

The MS (Murashige and Skoog, 1962) basal medium was supplemented with 6-Benzylaminopurine (BAP), 6-Furfuryl-aminopurine (KIN), Thidiazuron (TDZ), Indole-3-butyric acid (IBA), 2,3,5-triiodobenzoic acid (TIBA), α-naphthalene acetic acid (NAA), at various concentrations and in various combinations for rhizogenesis. Full and half strength MS basal medium with IBA and NAA at different concentration was employed. All the plant growth regulators were procured from SRL and Himedia-Qualigens, SRL, Glaxo, CDH, Titan biotech and Himedia. 3% (w/v) sucrose (SRL, Mumbai, India) was used as Carbon source, solidified with 0.8% agar-agar and pH was adjusted to 5.75 using 0.1 N NaOH or 0.1 N HCl. 20 ml media (aprox.) was dispended in each 150×25 cm test tube (Borosil, India), tightly covered with air tight plastic test tube caps and sterilized by autoclaving at 1.06 $kgcm^{-2}$ at 121°C for 15 min. The explants were cultured in vertical orientation in test tubes containing semisolid medium. Cultures were maintained at 25±2 °C temperature with a relative humidity of 55±5 % under regular cycle of light (450-460 µW cm^{-2}) by cool day light emitted from fluorescent incandescent tubes (40 W, Philips &tFinolex, India) of 16 hr light followed by 8 hr dark period.

After root formation, healthy plantlets with well developed root system were removed from medium and washed under running tap water to remove the medium. These are then transferred to plastic pots (5 cm diameter) containing autoclaved mixture of soil, sand and vermicompost (1:1:1). Subsequently acclimatization was achieved by covering the plastic pots with polythene bags to maintain humidity. Plants were irrigated with $1/10^{th}$ of major salts of MS media. After 1 week, 3-5 holes are made in the poly bags. Plants were irrigated after every 5 days. The potted plants were maintained in the culture room. After 30 days the plantlets were potted in earthen pots with garden soil.

The shoot response of explants was evaluated after 35 days of culture in terms of percentage of explants producing shoots, average number of shoots per explant and average shoot length per explant. For root response, percentage of shoot producing roots, average number of roots per explant and average root length was recorded. All the values have been reported as mean value along with standard error (Mean ± SE).

RESULTS AND DISCUSSION

An ever increasing demand of uniform medicinal plants based medicines warrants their mass propagation through plant tissue culture strategy. Tissue culture technology is

Table 1. Effect of growth regulators on shooting during in-vitro culture of *Vitex negundo* L. on MS media.

Cytokinins (mgl1)		% of shoot induction	Average number of shoots per explant (Mean ± SE)	Shoot Length (cm) (Mean ± SE)
Control	0	10	0.12 ± 0.01	0.72 ± 0.01
BAP	0.5	80	3.48 ± 0.34	2.64 ± 0.59
	1.0	90	4.29 ± 0.07	3.28 ± 0.31
	1.5	80	2.59 ± 0.37	2.01 ± 0.27
	2.0	70	3.11 ± 0.82	1. 81 ± 0.14
	2.5	50	2.40 ± 0.30	1.78 ± 0.07
	3.0	40	2.10 ± 0.15	1. 62 ± 0.14
	3.5	30	2.00 ± 0.03	1.50 ± 0.18
KIN	0.5	70	2.43 ± 0.03	1.86 ± 0.18
	1.0	60	2.87 ± 0.24	3.02 ± 0.24
	1.5	80	3.47 ± 0.14	3.33 ±0.08
	2.0	70	2.8 ± 0.2	2.17 ± 0.20
	2.5	60	2.62 ± 0.18	1.81 ± 0.34
	3.0	50	1.92 ± 0.20	1.61 ± 0.16
	3.5	40	1.64 ± 0.07	1.63 ± 0.16
TDZ	0.5	60	2.16 ± 0.16	1.63 ± 0.19
	1.0	50	2.4 ±0.28	1.46 ± 0.06
	1.5	30	1.75 ± 0.25	1.7 ± 0.09
	2.0	20	2.33 ± 0.33	1.43 ± 0.12

potent and has opened extensive areas of research for biodiversity conservation. Tissue culture protocols have been developed for a wide range of medicinal plants, which includes endangered, rare and threatened plant species. (Sharma *et al.*, 2010). Conventional propagation methods are unable to meet the demand of the pharmaceutical industries and drug research. Therefore, it is necessary to develop a non-conventional method for propagation to fulfill the demands of the drug market (Rathore et al. 2008). *In vitro* propagation methods offer a powerful tool for conservation of germplasm and mass-multiplication of threatened plant species (Murch et al. 2000). It helps in micropropagation of large number of plant in shorter time period, irrespective of season and serves as an alternative source of plant propagation (Yadav and Singh, 2012; Yadav et al., 2013; Groach et al., 2014). This method can be employed in multiplying important endangered plant species which are difficult to propagate by conventional means and saves the plant from the extinction.

In order to establish an efficient in vitro micropropagation protocol for commercial exploitation of this plant, nodal explants of *V. negundo* were inoculated on MS medium supplemented with varied concentration (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 mgl^{-1}) of cytokinins (BAP, KIN) and TDZ (0.5-2.0 mgl^{-1}). The nodal segments cultured on growth regulator free MS medium showed minimum signs of bud break even after 15 days of inoculation. The average number of shoots induced on MS basal medium was 10% with an average shoot length of 0.72 ± 0.01 cm after 35 days of culture (Table 1).

However, addition of cytokinin was essential for differentiation of multiple shoots from the nodal explants. Of the three cytokinins tried, BAP was most effective over the other two for induction of multiple shoots. Similar effect have already been reported in various taxa like *Cassia angustifolia* (Agrawal et al., 2002), *Spilanthes acmella* (Pandey and Agrawal 2009), *Aegle marmelos* (Yadav and Singh 2011), *Tylophora indica* (Faisal et al., 2007), *Achyranthes aspera* (Ishwarya et al., 2018), *Vitex trifolia* (Ahmad and Anis, 2014).The nodal segments responded by initial enlargement of dormant axilary buds followed by bud break within a week and multiple shoot induction and proliferation within 15 days of culture on BAP containing media. 1 mgl^{-1}BAP was optimum in inducing 90% morphogenic culture with an average of 4.29±0.07 shoots per explant having an average shoot length of 3.28±0.31 cm after 35 days of culture (Table 1, Fig.1A). BAP at 3.5 mgl^{-1} displayed poor morphogenic response both in terms of average number of shoots and average

FIGURE 1. In vitro plant regeneration through nodal segment and establishment of *Vitex negundo* L. (A) Cultures showing shoots on MS medium with BAP (1.0 mgl^{-1}), (B & C) Multiple shoot development from nodal explants on MS with BAP + IBA (1+0.50 mgl^{-1}) (D) Formation of light creamy callus on TDZ (E) In vitro rooted shootlet with half MS medium + 0.75 ml^{-1} IBA (F) Acclimatized plantlets, 35 days old (G) Plants in pots.

Table 2. Effect of BAP 1 mgl1 and 1.5 mgl1 KIN in combination with different concentrations of auxins on shoot bud regeneration from nodal explants of *Vitex negundo*

Concentration of growth regulators in mgl1	IBA	TIBA	Percentage of explants producing shoots (%)	Number of shoots per explant (Mean ± SE)	Shoot Length (cm) (Mean ± SE)
BAP 1	0.25		60	2.66 ± 0.33	3.23 ± 0.09
	0.50		90	6.12 ± 0.63	3.85 ± 0.21
	0.75		70	3.14 ± 0.40	3.78 ± 0.39
		0.25	50	2.8 ± 0.37	3.02 ± 0.17
		0.50	70	3.5 ± 0.42	3.53 ± 0.08
		0.75	60	2.5 ± 0.34	3.28 ± 0.10
KIN 1.5	0.25		70	4.37 ± 0.41	2.83 ± 0.26
	0.50		80	5.88 ± 0.38	4.13 ± 0.43
	0.75		60	4.16 ± 0.60	3.18 ± 0.17
		0.25	60	4.33 ± 0.42	3.21 ± 0.20
		0.50	80	5.2 ± 0.53	3.71 ± 0.15
		0.75	70	4.38 ± 0.47	3.32 ± 0.12

Results were recorded after 35 days and are presented as Mean ± Std. Error.

Table 3. Effect of growth regulators on rooting pattern of *Vitex negundo* L. during *in-vitro* culture (full and ½ strength of MS media)

Growth regulator (mgl1)		Percentage of explants producing roots (%)	Average number of roots per explants	Average root length (cm)
MS Full + IBA	0.50	70	5.71 ± 0.71	1.88 ± 1.20
	0.75	80	6.75 ± 0.52	2.33 ± 2.21
	1.0	60	4.33 ± 0.42	1.86 ± 2.10
MS half + IBA	0.50	80	7.57 ± 0.92	3.25 ± 3.76
	0.75	90	12.12 ±0.83	4.98 ± 2.50
	1.0	70	8.9 ± 0.89	4.53 ±3.92
MS full + NAA	0.50	70	4.85 ± 0.76	1.32 ± 0.77
	0.75	80	6.25 ± 0.59	2.23 ± 2.62
	1.0	60	4.33 ± 0.42	1.71 ± 3.37
MS half + NAA	0.50	70	6.8 ± 0.86	1.74 ± 0.07
	0.75	90	8.42 ± 0.89	2.66 ± 0.30
	1.0	60	7.33 ± 0.66	1.95 ± 0.13

shoot length (Table 1). On increasing the concentration of BAP, induction of multiple shoots was comparatively low and average shoot length too decreased (Table 1). Except BAP, all the tried concentrations of KIN and TDZ showed poor morphogenic response in term of average number of induced shoots and shoot length (Table 1). Considerable callusing at the basal cut end of nodal segment along with formation of multiple shoots was also reported in the present study which agrees with the study on *Azadirachta indica* (Arora et al., 2010) which showed similar results (Fig.1 D). The formation of callusing at the basal cut ends of nodal segment due to the action of accumulated auxins at the basal cut proliferation, especially in the presence of cytokinins (Marks and Simpson, 1994). The present study also revealed the synergistic effect of BAP in combination of auxin for effect shoot regeneration which has also been reported in studies of *Celastrus paniculatus* (Lal et al., 2010).

The highest number of shoots (6.12±0.63) developed was observed in MS with BAP 1 + IBA 0.50 mgl^{-1} (Table 2 Fig.1 B & C). The highest proliferation rate (90%) was also found at the same combination of plant growth regulators in the medium.

The best results were observed on a medium containing BAP and IBA which is supported by earlier studies in *Chonemorpha grandiflora* (Nishitha et al., 2006), *Vitex negundo* (Ahmad and Anis, 2011), *Launaea cornuta* (Ambajo and Matheka, 2016). *Mimosa pudica* (Bianchetti et al., 2017) *Tylophora indica* (Najar et al., 2018), *Ceropegia juncea* (Binish, 2018), In these studies also synergitic effects were observed when Cytokinin was used in combination with auxin. Among the two different types of auxins employed for root induction on in vitro excised shoots of *V. negundo*, IBA was found to be most effective. A maximum of 90% shoots induced an average of 12.12±0.83 roots with an average root length of 4.98±2.50cm after 3 weeks on half strength MS medium augmented with 0.75 mgl^{-1} IBA (Table 3). The roots were induced directly from the shoot base without callus formation at this concentration. (Table 3, Fig.1 E). Similar responses have been already reported in *Spilanthes acmella* (Pandey and Agrawal 2009, Yadav and Singh 2010), (Reddy et al., 2014), *Ceropegia juncea* (Binish, 2018),.However, at higher concentration of IBA, the number of roots and root length showed decline. Compared to IBA, poor rooting response was observed at the concentration of IBA + full MS and NAA + full and half MS. The tissue culture derived plantlets (Fig.1 F & G) were acclimatized in the field condition with 90% survival. Such micropropagated plants were found to be morphologically similar to the mother plant.

An efficient protocol has been developed for regeneration of *Vitex negundo* which offers a great potential to cater the needs of different pharmaceutical industries. In the present study, enhanced *in vitro* regeneration of plants with combination of plant growth regulators such as BAP, KIN, TDZ, IBA and TIBA was observed. This will be helpful in understanding the callogenesis and organogenesis through the nodal explants and to facilitate the mass propagation of *Vitex negundo*.

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Nutritional assessment of different date fruits (*Phoenix dactylifera L.*) varieties cultivated in Hail province, Saudi Arabia

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ABSTRACT

Date fruits are an imperative crop, especially cultivated in the hot-arid regions of the world having extraordinary nutritional and therapeutic value. In this study, we performed nutritional profiling and mineral analysis of different varieties of date fruits cultivated in north-western region of Saudi Arabia. Among the sample tested, we found that moisture contents was highest in Helwah Hail (23.83 ± 0.49%) and Berhi (23.20 ± 0.10%). Moreover, ash and protein content was found to be more in Ajwah (2.50 ± 0.53%) and Hamra (4.34 ± 0.06%) respectively. Similarly, total fibre percentage of the tested sample varied from 4.35 ± 0.05% to 5.13 ± 0.12% and monosaccharaides was found highest in Helwah Hail and Deglet Shewaish. However, mineral analysis showed that Ajwah date fruits, Asilah, Nabtat Saif and Barni had high amount of calcium, magnesium, sodium and potassium respectively. The present finding helps in understanding the nutritional status and significance of different date varieties cultivated in north-western region of Saudi Arabia (Hail Region). However, lesser known varieties can be improved through better horticulture practices as a valuable product. Further, this study reveals that, the consumption of these date fruits would have several nutritional health effects.

KEY WORDS: NUTRIENT ANALYSIS, PROXIMATE ANALYSIS, DATE FRUITS, HAIL PROVINCE, MINERALS

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INTRODUCTION

The date palm (*Phoenix dactylifera* L., family Arecaceae) is one of the oldest fruit trees on the earth and is closely associated with the life of the human beings in the Middle East countries including the Kingdom of Saudi Arabia (Al-Abdoulhadi et al., 2011). Saudi Arabia is considered as the mother country of date palm trees and is second largest producer of date fruits in the world, with more than 300 types of dates, each with its own taste and texture, but only around 50–60 cultivars are used commercially. In 2013, date production in Saudi Arabia reached 1,065, 032 tons, from 3·7 million trees (Assirey 2015; Allbed et al., 2017). However, few studies have also showed that the Kingdom occupies the first rank in the world in terms of average per capita consumption of dates per year, which reached 34.8 kg/year in 2003 (Al Shreed et al., 2012). Date fruits have great importance in human nutrition owing to their rich content of essential nutrients which include carbohydrates sugar ranging from 65% to 80% on dry weight basis mostly of inverted form (glucose and fructose). Fresh varieties have a higher content of inverted sugars, the semi dried varieties contain equal amount of inverted sugars and sucrose, while dried varieties contain higher sucrose, (Aldjain et al., 2011; Hamad et al., 2015).

The nutritional value of dates is due to their high sugar content as well as other important micro and macro nutrients such as potassium (2.5 times more than bananas), calcium, magnesium and iron. Other important components are proteins, fat, vitamins, dietary fiber, fatty acids, polyphenols, antioxidant and amino acids, (Chandrasekaran et al., 2013). In addition, date fruit has been recommended in folk remedies for the treatment of various diseases like diabetes, obesity, cancer and heart diseases. Recently, it has been found that date fruit might be of benefit in glycemic and lipid control of diabetic patients and have also been identified as having antioxidant and anti-mutagenic properties due to their high levels of poly-phenolic compounds and vitamins (Vayalil, 2012; Parvin et.al., 2015; Khalid et al., 2016). In appreciation of its fruits, the date tree is referred to as the sacred tree, the tree of life, and the bread of the desert (Ghnimi et al., 2017).

With the increase in obesity and overweight among Saudi nationals, especially young males and females due to the life style and food habits, healthier balanced food may be one of the solutions to this problem (Al-Hazzaa et al., 2012). Date fruits are a perfect food that can provide the necessary minerals. Moreover dates can be given to children instead of chocolates that contain various fats and additives that may subject them to health problems. Dates have longer shelf life and can be stored safely even at the high temperature of the Arabian Peninsula. Dates don't require cooking or processing. All of these advantages make dates one of the best food stuff to be consumed (Taha et al., 2015). Considering the nutritional facts and importance of date fruits studying their nutritional quality is increasingly being recognized as a worthy and important task. Our objective was to evaluate the nutritional status and mineral composition of various varieties of Dates fruit cultivated in Hail Province, Kingdom of Saudi Arabia.

MATERIAL AND METHODS

Sample collection and preparation: Thirty two varieties (Nabtat Saif, Khlas, Hamra, Ajwah, Shaishi, Barni, Sabbakah, Seghae, Roshodyyah, Nabtat Ali, Umm-Hamam, Meskany, Rezazy, Asailah, Gasbah, Shaqraa, Meneifi, Sultanah, Wannanah, Umm Kebar, Dhahesyyah, Helwah, Helwah Hail, Helwah Baqqa, Shebeby, Umm-Khashab, Fankha, Berhi, Maktoomy, Sukkari, Deglet Shewaish and Majhoolah) of date palm fruits were collected from local markets and date fruits farms of Hail Province, Kingdom of Saudi Arabia. Subsequently, samples were washed with distilled water and the seeds were removed. Later on, samples were grinded into uniform mixture and stored in air tight containers until further analysis.

Determination of moisture and ash content: Two grams sample were placed into the petri-dish and dried in an oven at 105°C for three hours. The dried sample was cooled in a desiccator for 30 min and weighed to a constant weight. The percentage loss in weight was expressed as percentage moisture content on dry weight basis. However, determination of ash contents were performed in triplicates and percentage residual weight was expressed as ash content (Bashir et al., 2015).

Determination of total protein and fat percentage: 2g samples taken into thimble and placed into Soxhlet apparatus for the determination of fat content using petroleum ether (60 to 80°C) for 5 hours. Moreover, determination of total proteins was performed by using Kjeldahl method (AOAC, 2006).

Determination of total fiber: From the pounded sample, 2.00 g were used in triplicates for estimating the crude fibre by acid and alkaline digestion methods using 20% $H_2SO_4$and 20% NaOH solutions (AOAC, 2006).

Carbohydrate determination: Carbohydrate content was calculated using the following formula: Available carbohydrate (%) = 100 – [protein (%) + Moisture (%) + Ash (%) + Fibre (%) + Crude Fat (%)](Bashir et al., 2015).

Determination of mineral contents: One gram of dried sample and 50 ml of 20% Nitric acid (HNO3) were added to Erlenmeyer flask. The mixture was heated to 70–85 ^{0}C

for 48 h. During heating period the volume of the flask was maintained at the same level by intermittently adding 20% nitric acid. After the completion of digestion the content of Erlenmeyer flask was filtered using Nalgene filter (Thermo scientific) unit. The filtrate was collected in 100 ml volumetric flask and allowed to cool. After cooling the volume was made up to 100 ml using deionized water (Milli Q) and analyzed with ICP-MS. For the sample preparation all the glassware was washed with deionized water and rinsed three times with 20% nitric acid (Ahmad et al., 2017).

Statistical analysis: All the experiments were carried out in triplicates. The data were analyzed statistically with SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Mean was statistically compared by Duncan's multiple range test at P <0.05% level.

RESULTS AND DISCUSSION

Date fruits have huge scope and potential for use as food or as healthy food products because of an important source of nutrition as well as economic significance. Proximate analysis of date fruits are considered important in grading, preservation, storage and processing of dates. The average proximate composition and mineral analysis of date fruits are presented in Tables 1,2 & 3.

Table 1. Proximate composition of date fruits

Sample Name	Moisture (%)	Ash (%)	Fat (%)	Protein (%)	Total Fibre (%)
Nabtat Saif	18.03g ± 0.25	1.95 abcdef ± 0.19	0.43 abcd ± 0.025	2.70 def ± 0.10	4.52 bc ± 0.08
Khlas	18.73 gh ± 0.21	1.31 ab ± 0.09	0.45 abcdef ± 0.050	2.90 ghi ± 0.10	4.40 ab ± 0.10
Hamra	10.36 a ± 0.33	1.84 abcdef ± 0.55	0.55 ghij ± 0.500	4.34 q ± 0.06	4.35 a ± 0.05
Ajwah	14.56 d ± 0.59	2.50 ef ± 0.53	0.42 abc ± 0.015	3.15 kl ± 0.05	4.62 cd ± 0.08
Shaishi	15.97 ef ± 0.45	1.76 abcdef ± 0.05	0.52 efghij ± 0.085	3.29 lm ± 0.01	4.66 cde ± 0.04
Barni	11.23 ab ± 0.21	2.27 cdef ± 0.72	0.49bcdefghi ± 0.030	2.95 hij ± 0.05	4.39 ab ± 0.01
Sabbakah	14.87 de ± 0.35	2.51 f ± 0.34	0.42 abcd ± 0.040	3.25 klm± 0.05	4.65 cde ± 0.05
Seghae	12.43 c ± 0.11	2.14 bcdef ± 0.51	0.50 cdefghij ± 0.020	2.64 cde± 0.06	4.87 fghij ± 0.03
Roshodyyah	18.70 gh ± 0.30	2.15 bcdef ± 0.47	0.46 abcdef ± 0.060	2.29 a ± 0.04	4.95 hijk ± 0.05
Nabtat Ali	15.63 def ± 0.15	2.04 abcdef ± 0.46	0.48bcdefgh ± 0.020	2.60 cd ± 0.10	4.85 fghi ± 0.05
Umm-Hamam	18.50 gh ± 0.10	1.24 a ± 0.15	0.39 a ± 0.135	2.50 bc ± 0.10	4.95 hijk ± 0.05
Meskany	19.50 hi ± 0.40	1.54 abcd ± 0.41	0.58 j ± 0.080	3.39 mn ± 0.01	4.80 efgh ± 0.20
Rezazy	11.97 bc ± 0.86	2.47 ef ± 0.57	0.49 bcdefghi ± 0.010	2.88 fgh ± 0.01	4.88 ghij ± 0.02
Asailah	16.20 f ± 0.61	1.67 abcde ± 0.15	0.45 abcdef ± 0.050	2.34 ab ± 0.05	4.95 hijk ± 0.05
Gasbah	22.06 lmn ± 0.05	1.54 abcd ± 0.30	0.43 abcde ± 0.030	2.72 defg ± 0.11	4.69 cdef ± 0.21
Shaqraa	22.13 lmn ± 0.49	1.68 abcdef ± 0.27	0.47 abcdefg ± 0.030	2.75 defg ± 0.05	4.86 fghi ± 0.04
Meneifi	22.57 mn ± 0.21	1.48 abcd ± 0.57	0.49 bcdefghi ± 0.035	3.75 p ± 0.05	4.85 fghi ± 0.05
Sultanah	21.53 klm ± 0.95	1.43 abc ± 0.06	0.50 cdefghij ± 0.060	3.08 ijk ± 0.07	4.90 ghij ± 0.10
Wannanah	20.47 ijk ± 1.13	1.40 ab ± 0.10	0.47 abcdefg ± 0.030	3.53 no ± 0.12	4.90 ghij ± 0.10
Umm Kebar	22.83 no ± 1.98	1.56 abcd ± 0.37	0.41 ab ± 0.020	3.68 op ± 0.17	4.90 ghij ± 0.20
Dhahesyyah	18.89 gh ± 0.11	1.67 abcde ± 0.46	0.46 abcdef ± 0.010	3.58 op ± 0.18	4.80 efgh ± 0.00
Helwah	18.57 gh ± 0.32	2.31 def ± 0.40	0.49 bcdefghi ± 0.010	3.30 lm ± 0.10	4.72 defg ± 0.08
Helwah Hail	23.83 o ± 0.49	1.87 abcdef ± 0.65	0.56 hij ± 0.060	2.95 hij ± 0.05	4.80 efgh ± 0.20
Helwah Baqqa	21.07 jkl ± 1.53	1.69 abcdef ± 0.35	0.51 defghij ± 0.015	2.80 efgh ± 0.20	4.85 fghi ± 0.05
Shebeby	19.47 hi ± 0.15	1.59 abcd ± 0.42	0.46 abcdef ± 0.015	3.20 kl± 0.20	5.13 k ± 0.12
Umm-Khashab	21.53 klm ± 0.31	1.80 abcdef ± 0.26	0.57 ij ± 0.050	2.70 def ± 0.10	4.78 defgh ± 0..07
Fankha	18.10 g ± 0.71	1.83 abcdef ± 0.48	0.52 fghij ± 0.000	2.70 def ± 0.10	5.00 ijk ± 0.10
Berhi	23.20 no ± 0.10	1.50 abcd ± 0.00	0.45 abcdef ± 0.010	2.96 hij ± 0.06	4.93 hij ± 0.07
Maktoomy	15.17 def ± 0.55	1.59 abcd ± 0.17	0.39 a ± 0.010	3.15 kl ± 0.15	5.03 jik ± 0.02
Sukkari	20.13 ij ± 1.70	1.85 abcdef ± 0.83	0.42 abcd ± 0.010	2.75 defg ± 0.15	4.95 hijk ± 0.10
Deglet Shewaish	14.40 d ± 0.10	1.55 abcd ± 0.13	0.49 bcdefghi ± 0.010	2.65 cde± 0.05	5.05 ik ± 0.05
Majhoolah	15.03 def ± 0.40	1.58 abcd ± 0.58	0.46 abcdef ± 0.006	3.10 jk ± 0.10	4.95 hijk ± 0.05

Means bearing different superscript letters are significantly different at p < 0.05.

Table 2. Carbohydrate and monosaccharide sugar analysis of date fruits

Sample Name	Monosaccharide (%)	Carbohydrate (%)
Nabtat Saif	36.13 ef ± 0.66	72.23 i ± 0.35
Khlas	42.25 h ± 0.77	72.05 hi ± 0.05
Hamra	45.28 ij ± 0.82	78.69 m ± 0.34
Ajwah	45.29 ij ± 0.82	74.23 j ± 0.65
Shaishi	36.17 ef ± 0.66	73.83 j ± 0.37
Barni	36.13 ef ± 0.66	77.37 l ± 1.07
Sabbakah	48.39 k ± 0.88	74.23 j ± 0.09
Seghae	43.92 i ± 0.80	77.39 l ± 0.39
Roshodyyah	37.53 f ± 0.68	71.71 ghi ± 0.26
Nabtat Ali	43.92 i ± 0.80	74.12 j ± 0.42
Umm-Hamam	37.53 f ± 0.69	72.36 i ± 0.01
Meskany	37.53 f ± 0.69	70.12 ef ± 0.31
Rezazy	40.51 g ± 0.73	77.56 l ± 0.86
Asailah	43.92 i ± 0.80	74.11 j ± 0.51
Gasbah	39.97 g ± 0.73	68.24 cd ± 0.17
Shaqraa	34.89 de ± 0.63	68.07 bcd ± 0.61
Meneifi	56.68 n ± 1.03	66.94 ab ± .010
Sultanah	46.18 j ± 0.83	68.58 d ± 1.06
Wannanah	50.77 l ± 0.92	69.10 de ± 0.90
Umm Kebar	50.77 l ± 0.92	67.12 abc ± 2.25
Dhahesyyah	39.44 g ± 0.71	70.88 fgh ± 0.28
Helwah	27.36 a ± 0.49	70.54 fg ± 0.52
Helwah Hail	56.62 n ± 1.02	66.39 a ± 0.01
Helwah Baqqa	40.51 g ± 0.73	68.75 d ± 1.66
Shebeby	53.62 m ± 0.97	70.42 f ± 0.11
Umm-Khashab	32.65 c ± 0.59	68.72 d ± 0.41
Fankha	36.13 ef ± 0.66	72.19 i ± 0.11
Berhi	48.39 k ± 0.88	66.92 ab ± 0.06
Maktoomy	34.38 d ± 0.62	74.59 jk ± 0.51
Sukkari	43.92 i ± 0.81	69.94 ef ± 0.71
Deglet Shewaish	56.67 n ± 1.03	75.76 k ± 0.04
Majhoolah	30.66 b ± 0.56	75.09 jk ± 0.25

Moisture and ash contents: Our results showed that, the moisture content in all the evaluated sample varies from (10.36 ^{a} ± 0.33 - 23.20 ^{no} ± 0.10). Hamra date varieties had lowest moisture percentage among the selected varieties. Which indicates that, hamra date have low water content and could be good for long term storage compared to other cultivars. The low moisture content would not be more inclined to decay, since nourishments with high dampness substance are more inclined to perishability. It might be profitable in perspective of the specimen timeframe of realistic usability (Shaba et al., 2015). However, Berhi had highest moisture content among the evaluated verities. Similarly, previous studies have been reported moisture content 10%- 25%. This indicates that, our results were in accordance with the previous studies (Rehman et al., 2012; Al-Harrassi et al., 2014). The ash content of the selected varieties was found to be in the range of 1.31% ± 0.09 - 2.50% ±0.53. Ghnimi S et al., 2017 reported ash content of date fruits in the range of 1.4 % - 2.3%. However, earlier studies reported ash content of various date fruits varieties ranging from 0.9 % - 2.0 % (Al-Harrasi et al., 2014). This results was in agreement with our obtained quantification.

Total protein and fat content: Total protein content was determined and it was found that, among the tested sample Hamra date had highest amount of protein 4.34$^{\%}$ ± 0.06. However, Roshodyyah had lowest amount of protein 2.29%^{a} ± 0.04. Statistically it was found that, all the samples were significantly different at $p < 0.05$. High content of protein in hamra varieties suggest that, it could be of good potential for nutritional benefits. Moreover, earlier studies reported average protein content of fresh and dried dates is 1.50 - 2.14%, respectively (Kazi et al., 2015). On the other hand our result showed that, tested samples had results ranging from 2.29-4.34. Our results were in accordance with previous studies (Al-Harrasi et al., 2014). Fat content was found to be significantly different at $p < 0.05$. The fat content in several date fruits varieties ranged from 0.3% -0.6%. Similarly, previous studies reported percentage of fat in accordance with our results (Assirey 2015; Khalid et al., 2016).

Total fibre content: Table 1 showed that, the percentage of total fibre was adequate and ranged from 4.39% - 5.13 %. Total fibre content for all the varieties of date fruits found to be significantly different at $p < 0.05$. However, Shebeby variety had significantly higher ($p < 0.05$) than the other varieties. Moreover, Barni was significantly lower ($p < 0.05$) than the rest of the selected varieties. Al-Harrasi 2014 reported average total fibre content in date fruits was 2.5%, this was lower than our reported values. This could be due to environmental as well as duration of fruits collection. On the other hand, few studies suggested that, the total average fiber could be from 5%- 8% (Nasir et al., 2015).

Total carbohydrate content and monosaccharide content: Our result showed that, all the samples were significantly different at $p < 0.05$ as presented in table 2. Moreover, highest carbohydrate contents were found in Hamra dates and were significantly higher ($p < 0.05$) than the other varieties. In addition to that, Helwah dates had low amount of carbohydrates than rest of the varieties. On the other hand monosaccharide sugar was found to be highest in Helwah Hail followed by Deglet

Table 3. Mineral analysis of date fruits

Sample	Calcium (%)	Magnesium (%)	Sodium (%)	Potassium (%)
Nabtat Saif	0.0122i ± 0.00021	0.0051e ± 0.00012	0.0538 s ± 0.00100	0.72 n ± 0.013
Khlas	0.0102g ± 0.00021	0.0061f ± 0.00012	0.0355 m ± 0.00062	0.43 d ± 0.008
Hamra	0.0091 f ± 0.00015	0.0122 k± 0.00021	0.0517 r ± 0.00095	0.76 o ± 0.014
Ajwah	0.0182n ± 0.00032	0.0040 d ± 0.00006	0.0203 f ± 0.00038	0.53 g ± 0.009
Shaishi	0.0162m ± 0.00032	0.0020 b ± 0.00006	0.0172 d ± 0.00032	0.56 ij ± 0.010
Barni	0.0132j ± 0.00026	0.0030 c ± 0.00006	0.0385 o ± 0.00068	0.96 u ± 0.017
Sabbakah	0.0152l ± 0.00026	0.0030 c ± 0.00006	0.0172 d ± 0.00032	0.78 p ± 0.014
Seghae	0.0142k ± 0.00026	0.0061 f ± 0.00012	0.0182 e ± 0.00032	0.78 p ± 0.014
Roshodyyah	0.0122i ± 0.00021	0.0040 d ± 0.00006	0.0223 h ± 0.00042	0.53 gh ± 0.009
Nabtat Ali	0.0112 h± 0.00021	0.0071 g ± 0.00010	0.0436 u ± 0.00079	0.81q ± 0.014
Umm-Hamam	0.0091f ± 0.00015	0.0051 e ± 0.00012	0.0203 f ± 0.00038	0.40 c ± 0.007
Meskany	0.0091 f ± 0.00015	0.0020 b ± 0.00006	0.0213 g ± 0.00036	0.47 e ± 0.008
Rezazy	0.0142 k ± 0.00026	0.0020 b ± 0.00006	0.0406 p ± 0.00074	0.67 m ± 0.012
Asailah	0.0091f ± 0.00015	0.0152 l ± 0.00026	0.0294 k ± 0.00053	0.46 e ± 0.008
Gasbah	0.0061c ± 0.00012	0.0020 b ± 0.00006	0.0122 b ± 0.00021	0.19 a ± 0.003
Shaqraa	0.0071d ± 0.00010	0.0051 e ± 0.00012	0.0152 c ± 0.00026	0.55 i ± 0.010
Meneifi	0.0102g ± 0.00021	0.0030 c ± 0.00006	0.0152 c ± 0.00026	0.58 j ± 0.010
Sultanah	0.0081e ± 0.00015	0.0102 i ± 0.00021	0.0172 d ± 0.00032	0.52 g ± 0.009
Wannanah	0.0040b ± 0.00006	0.0061f ± 0.00012	0.0213 g ± 0.00036	0.48 e ± 0.009
Umm Kebar	0.0071d ± 0.00010	0.0061 f ± 0.00012	0.0203 f ± 0.00038	0.49 f ± 0.009
Dhahesyyah	0.0112h ± 0.00021	0.0010 a ± 0.00000	0.0385 o ± 0.00068	0.60 k ± 0.010
Helwah	0.0102g ± 0.00021	0.0030 c ± 0.00006	0.0284 j ± 0.00053	0.52 g ± 0.009
Helwah Hail	0.0152l ± 0.00026	0.0020 b ± 0.00006	0.0406 p ± 0.00074	0.70 n ± 0.013
Helwah Baqqa	0.0102g ± 0.00021	0.0020 b ± 0.00006	0.0254 i ± 0.00047	0.53 gh ± 0.009
Shebeby	0.0081e ± 0.00015	0.0071g ± 0.00010	0.0182 e ± 0.00032	0.55 hi ± 0.009
Umm-Khashab	0.0102g ± 0.00021	0.0102i ± 0.00021	0.0426 q ± 0.00079	0.41 c ± 0.007
Fankha	0.0030 a ± 0.00006	0.0081 h ± 0.00015	0.0294 k ± 0.00053	0.63 l ± 0.011
Berhi	0.0071d ± 0.00010	0.0122 k ± 0.00021	0.0375 n ± 0.00068	0.64 l ± 0.011
Maktoomy	0.0122 I ± 0.00021	0.0030 c ± 0.00006	0.0324 l ± 0.00059	0.53 g ± 0.009
Sukkari	0.0081e ± 0.00015	0.0081 h ± 0.00015	0.0294 k ± 0.00053	0.25 b ± 0.004
Deglet Shewaish	0.0091f ± 0.00015	0.0071 g ± 0.00010	0.0294 k ± 0.00053	0.47 e ± 0.008
Majhoolah	0.0102 g± 0.00021	0.0051e ± 0.00012	0.0112 a ± 0.00021	0.55 i ± 0.010

Shewaish dates 56.67% and 56.62%, respectively. High amount of monosaccharide sugar could be due to freshness of the sample. However, Majhoolah had 30.66% monosaccharide sugar, this was significantly lower ($p < 0.05$) than the other varieties. Similarly, it was observed that, earlier reports suggested total carbohydrate content as well as monosaccharide sugar ranged from 50-70%, this was in accordance with our results (Al-Harrasi et al., 2014; Assirey, 2015; Khalid et al., 2016).

Mineral Analysis: Results of mineral analysis (calcium, magnesium, sodium and potassium) showed that, all the date varieties are rich source of minerals. Moreover, our result showed that, all the date varieties were significantly different at p $p < 0.05$ as presented in table 3. In addition to that, we found that, calcium was highest in Ajwa dates , when compared with other selected varieties. However, Fankha dates had lowest calcium concentration 0.0030%. In case of magnesium, Asailah was found to have 0.0152% followed by lowest concentration of magnesium in Shaishi and some of ther varieties of dates. Sodium was quantified highest in Nabtat Saif 0.0538% followed by 0.0122% in Gasbah dates. In addition to that, potassium was found be highest in Barni dates 0.96%. Similarly, all the quantified minerals reported were in accordance with earlier studies (Nasir et al., 2015; Parvin et al., 2015; Shaba et al., 2015).

CONCLUSION

Dates fruits are an extremely famous and oldest food known to human beings and it has been proven to contain high levels of carbohydrate, proteins, vitamins, crude fibers and essential minerals. Therefore, dates not only delicious with sweet taste and a fleshy mouth feel but also considered as an almost ideal food that provides a wide range of essential nutrients with many potential health benefits. Our study revealed baseline information on different date varieties grown in Hail region of Saudi Arabia. The results showed that, ash and protein content was highest in Ajwah (2.50 ± 0.53) and Hamra (4.34 ± 0.06) dates, respectively. Similarly, monosaccharaides sugar content was found highest in Helwah Hail and Deglet Shewaish. Mineral analysis showed that Ajwah date fruits, Asilah, Nabtat Saif and Barni had high amount of calcium, magnesium, sodium and potassium respectively. However, lesser known varieties grown in this region can be improved through better horticulture practices as a valuable product and results obtained from the investigation in this study may help in expanding the utilization of these date palm varieties for commercial gain.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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On the use of brushless digital DC motor and fuzzy logic controller for hearts left ventricular assist device (VAD)

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ABSTRACT

The developments of technology and increase in clinical requirements have caused creation of a novel type of blood pump. The present study has been conducted using controlled brushless DC motor and fuzzy logic for hearts left ventricular assist device. We have used a fuzzy logic system based on a mathematical one that analyzes analog input values in terms of logic variables which can take on continuous values between 0 and 1 in contrast to classical or digital logic. This system operates on discrete values of either 1 or 0. The clinical system includes a blood pump, a centrifugal pump, a brushless DC motor, a solenoid, power supply and control. In order to control the ventricular blood pressure and the blood volume (cardiac output), a fuzzy logic controller has been used. The method applied in this research provides conditions for the designer to describe the status in a few words clearly to make control decision based on linguistics structures. The structures and rules could be developed or changed by the physician for personal use. Permanent magnet brushless direct current motor (BLDC) has been the main element in majority of ventricular assist devices (VAD) development. To this end, an Implantable Centrifugal Blood Pump has been developed at the Institute Dante Pazzanese of Cardiology (IDPC) to assist patients with cardiovascular diseases. In order to make a high quality controller, it is important to use reliable virtual BLDC model. Permanent Magnet Synchronous Machine (PMSM) has implemented the differential equations for this motor using a state-space model. This could be the main contribution of this study. The results obtained from this study could be applied by further studies in order to enhance the motor model. Therefore, the results could lead to reliable simulations for the proposed controller.

KEY WORDS: LEFT VENTRICULAR ASSIST DEVICE, BRUSHLESS DC MOTOR, FUZZY LOGIC, CARDIOVASCULAR DISEASE

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INTRODUCTION

The brushless motors (BLDC) have been the main component of propulsion in the creation of majority of the Ventricular Assist Devices (VAD). Among the features used in implantable pumps, no brush is existed and this could avoid the wear observed in other electrical motors and implantable systems. The operation at high rotational speeds and small size are also factors that support this use (Fonseca 2003, Bock et al., 2008, Leao et al., 2012, Viswajith et al., 2017).

The three-phase brushless electric motor has a synchronous permanent magnet on the rotor and coils on the stator located, usually star connected with inverter control for bridge-type H (Bock et al., 2008; Fonseca 2003; Hsieh & Liao 2010). The operation of a BLDC is accomplished through strategic switching of the coils, as well as in a stepper motor. The switching is performed by a circuit that supplies current to the motor coils as a function of rotor position. The phase current of a BLDC, usually rectangular, is synchronized with the Back Electromotive Force (BEMF) to produce maximum torque and constant speed, with the trapezoidal BEMF as the main feature of control (Shao 2003; Shao et al., 2003 Leao et al., 2012).

The MATLAB/Simulink software was employed as virtual environment for simulation, since it allows data and results integration with other software including Comsol Multiphisycs to model ICBP. The dynamic model is required to study transients of the motor drive system and steady state. The immediate currents are crucial for power computation and electromagnetic torque is underlying for drive system performance evaluation. These features become a significant factor in uses such as VAD and are different from industrial appliances that may not be underlying (Krishnan, 2010).

This study has been divided into two main parts: the first part consist of mathematical modeling that Simulink block uses to represent BLDC motor and the second part describes the virtual implementation with help of Matlab / Simulink blocks diagram to represent the electromechanical actuator. Moreover, the main objective of this study is to apply the block Permanent Magnet Synchronous Machine (PMSM) to present the actuator from ICBP to begin researches current and speed control (Paraspour & Hanitsch 1994; Guyton C, 1986).

The dynamics of the aortic valve plays a critical role in the understanding of heart failure and its treatment using the continuous flow left ventricular assist device (LVAD). Maintaining proper and active dynamics of the aortic valve is important when the LVAD is used as a bridge-to-recovery treatment. This treatment requires that the LVAD pump control must be adjusted so that a proper balance between the volume of blood contributed through the aortic valve and that contributed though the pump must be maintained. That is, the pump control must be adjusted so that the pump does not take over the entire pumping function in the circulatory system but instead must share with the left ventricle in ejecting the total amount of blood needed by the circulatory system (Viswajith et al., 2017).

VENTRICULAR ASSIST DEVICE (VAD)

A ventricular assist device (VAD) is mechanical pump supporting heart function and blood flow in people with heart insufficiency. The devices could support the function of the left, right, or both heart ventricles. Ventricles are the lower chambers of human heart. The VAD includes tubes to carry blood out of heart and send it to blood vessels, a power source, and a control unit to

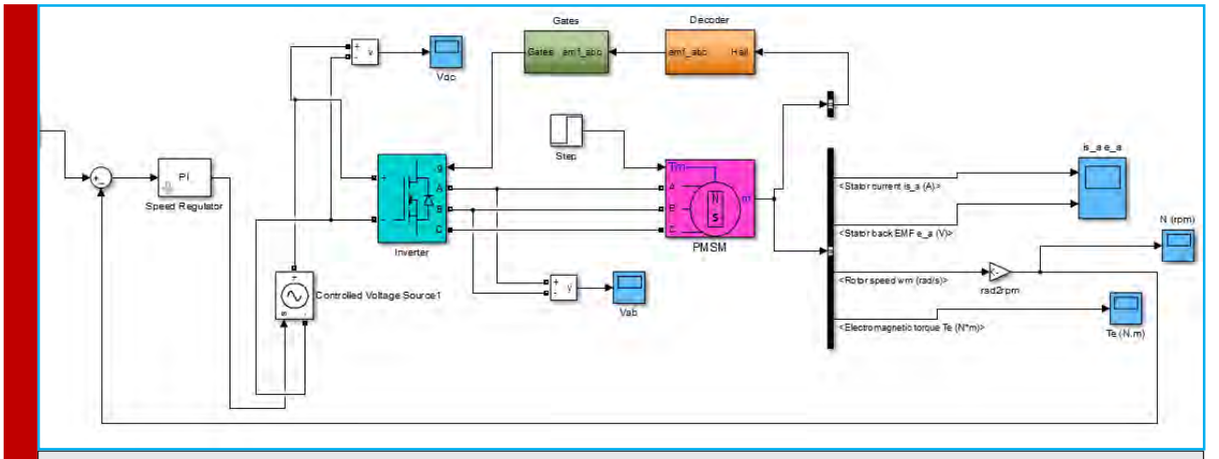

FIGURE 1. The block diagram BLDC

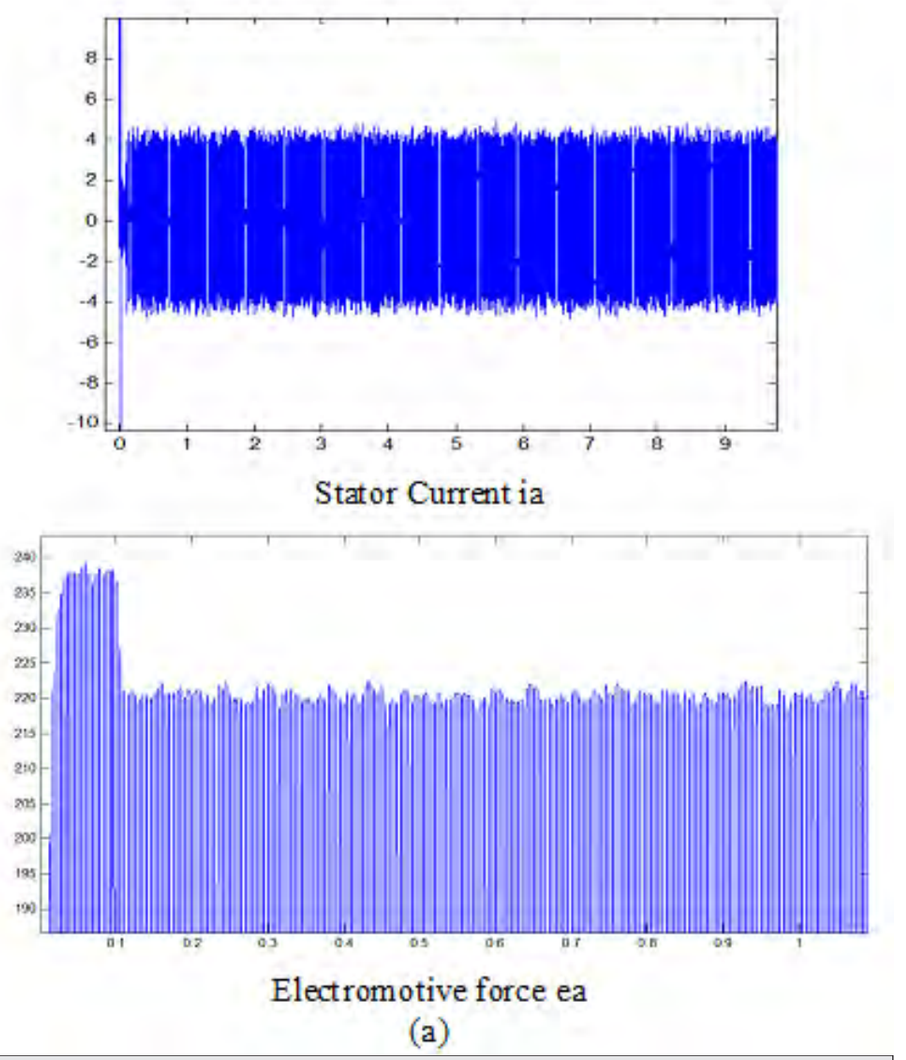

FIGURE 2. Outputs of waveform; (a) Stator Current & Electromotive force; (b) DC bus Voltage; (c) Rotor speed; (d) Voltage

monitor device function. The device may be used to support the heart until it recovers, to support the heart while waiting for a heart transplant, or to help heart work better in case of being eligible for a heart transplant (Parasspour & Hanitsch 1994, Goldowsky 2004,Patel 2005, Gill et al 2006).

Surgery is required to connect the VAD to heart. The surgery will be performed in a hospital. Patients will have general anesthesia and will not be awake or feel pain during the surgery. The patients will receive anticlotting medicine through an intravenous (IV) line in arm. A breathing tube connected to a ventilator will help patients' breath. A surgeon will open the chest and connect heart's arteries and veins to a heart-lung bypass machine. The surgeon will place the pump in the upper part of belly wall and connect the pump to heart using a tube. Another tube will connect the pump to one of the major arteries. The VAD will be connected to the control unit and power source outside the body. When the heart-lung machine is switched off, the VAD will support blood flow and take over heart's pumping function, (Libre etal.,2011; Nicolaescu 2012).

BRUSHLESS DC MOTOR (MOTOR DESIGN)

The brushless motors (BLDC) could be the main component of propulsion in the development of majority of the Ventricular Assist Devices (VAD). Among the features

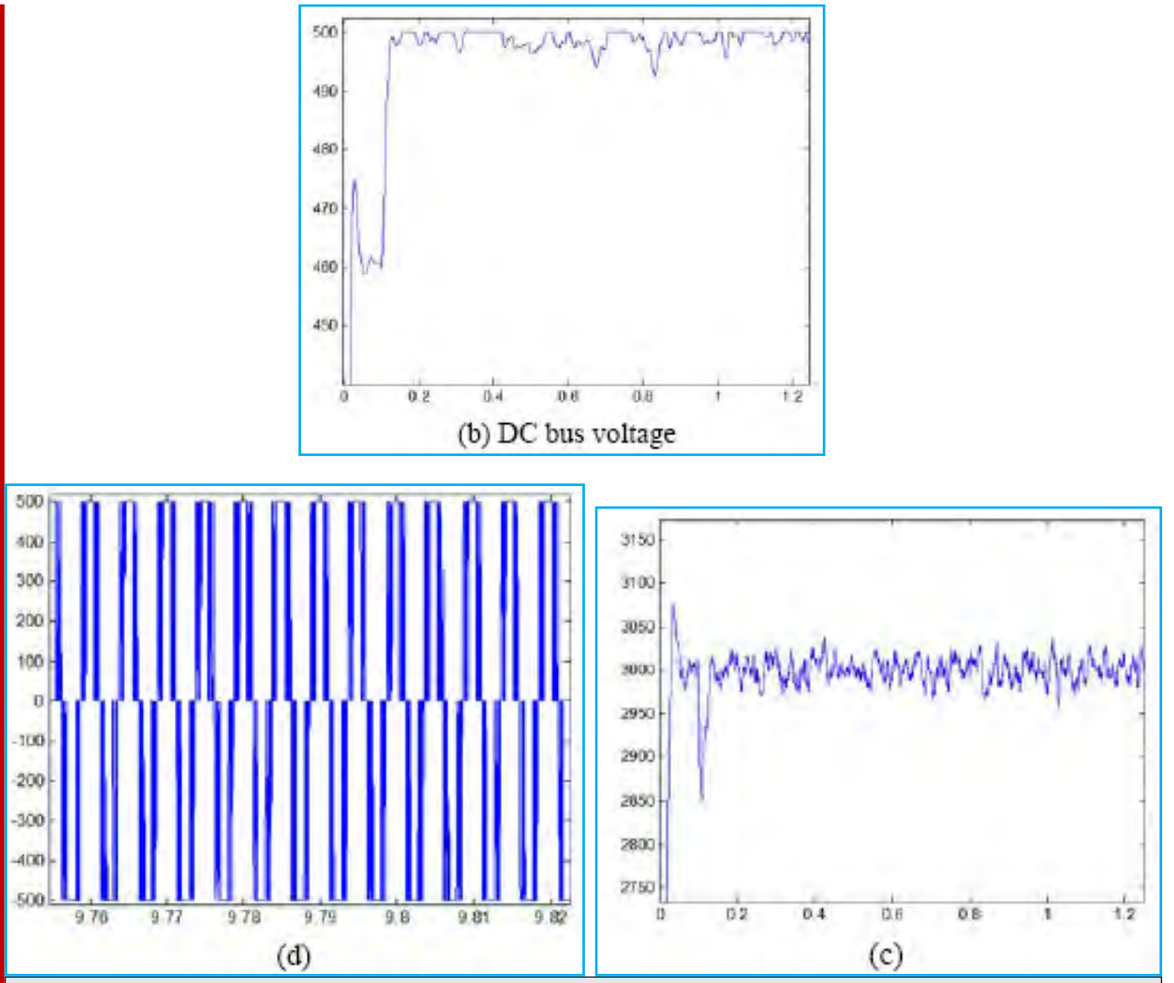

FIGURE 2. (*Continued*)

used in implantable pumps, no brush is existed and this could avoid the wear observed in other electrical motors and implantable systems. The operation at high rotational speeds and small size are also factors that support this use .The three-phase brushless electric motor has a synchronous permanent magnet on the rotor and coils embedded on the stator, usually star connected with inverter control for bridge-type H.

The BLDC operation is accomplished through strategic switching of the coils, as well as in a stepper motor. The switching is performed by a circuit that supplies current to the motor coils as a function of rotor position. The phase current of a BLDC, usually rectangular, is synchronized with the Back Electromotive Force (BEMF) to produce maximum torque and constant speed using the trapezoidal BEMF as the main feature of control . The sensor less control was used as redundant position rotor control in absence of other position sensors as source of failures and should be avoided in implantable devices. Lack of using others sensors could reduce the number of wires for motor control and this is the fact that is associated with the surgical practice and could reduce post-operative complications, (Andrade et al., 2008 Andrade 2010).

ANALYSIS OF THE SIMULINK MODEL

The model performed in MATLAB/SIMULINK used blocks of the Sim Power Systems toolbox. The BLDC was simulated using a block of Permanent Magnet Synchronous Machine (PMSM) with a trapezoidal back electromotive force (BEMF) signal (Leão et al., 2009). The BLDC is connected to an inverter and is supplied by a variable source of Direct Current (DC). The source is adjusted by a Proportional Integral (PI) control with feedback of the

motor speed. The rotor position information is toggled between hall signal and sensor less estimator in 0.5sec of the simulation time. Hence, the behavior of redundant system could be simulated, if the information of rotor position is given by the hall sensor to stop operation.

Figure 1 has illustrated block diagram to study the dynamic of the actuator electromechanical of ICBP. The LPF was adjusted to 500Hz of cut-off frequency for the speed of 2000rpm and 300Hz of cut-off frequency for the speed of 1500rpm.

Figure 2 has presented the output waveform of the simulated model.

ANALYSIS OF FUZZY CONTROLLER

A heart assist device coupled with the natural heart forms a complicated system. In fact, there are two control strategies. The heart assist system pumps either synchrony to the heart rhythm or asynchrony. In both cases, the systolic pressure and the blood volume flow (cardiac output) are considered as controlling parameters. The controlling parameters are the motor speed and the switching frequency of the solenoid. In the diastolic phase, the motor active length will be reduced due to the axial rotor movement. The speed and the motor current are increased and the losses are decreased. An additional speed control during this time improves the motor efficiency. The synchrony pumping is relatively simple to control, but the heart assistance is not fully effective and the mobility of the patient is restricted (Paraspour & Hanitsch 1994; Guyton 1986, Zadeh 1987, Zadeh L. A., 1978, Zadeh 1987, Pedrycz 1989).

The fuzzy controller has two input variables including blood pressure and cardiac output. The output variables are set speed of the actuator. For purpose of fuzzification of variables in each case, five linguistic terms (very low, low, medium, high and very high) are defined in Figure 3. The membership function of the heart parameters is shown in Figure 4.

Figure 5 illustrates the block diagram to analyze the dynamics of Block diagram BLDC when using fuzzy block.

CONCLUSION

The present study has presented and analyzed an electromechanical left ventricular heart assist device driven by a brushless D.C motor and controlled by the fuzzy set theory. For an implantable device, various restrictions should be considered such as fixed low voltage, constant magnetic flux, and upper limit of current density to avoid significant temperature increase and low volume requirements. According to the equations of the electromechanical system and the above mentioned restrictions, an optimization method has been developed in this study.

The optimization method has been used to design and make prototype drive and its electronic control. In combination with the blood circulation, the heart assist device is a nonlinear and multivariable system. In this study, the linguistic description of the system, the optimization of the fuzzy sets and the development of the control rule basis have been realized with regard to the physiological parameters. The fuzzy control algorithm has been proved by an off-line simulation enhanced by the on-line and interactive optimization. The use of the fuzzy logic provides higher robustness and reliability for the medical device, since a fuzzy controller tolerates a certain imprecision in dealing with the controlling problem.

The electromotive and mechanical parameters associated with BLDC motor have provided simulation results according to the literature. Current values are consistent with the data given by the manufacturer in its catalog. At the same time, the real time loads of the model show power values compatible with the application of a ventricular assist device (VAD). The PMSM block was appropriate to present the actuator ICBP and allowed

Cardiac output	Very low	Low	Medium	High	Very High
Very low	Negetive Big	Negetive Small	Negetive Small	Negetive Small	Negetive Small
Low	Negetive Small	Negetive Small	Zero	Zero	Zero
Mediume	Negetive Small	Zero	Zero	Posetive Small	Posetive Small
High	Negetive Small	Zero	Posetive Small	Posetive Small	Positive Big
Very High	Negetive Small	Zero	Posetive Small	Positive Big	Positive Big

FIGURE 3. Basis of fuzzy logic

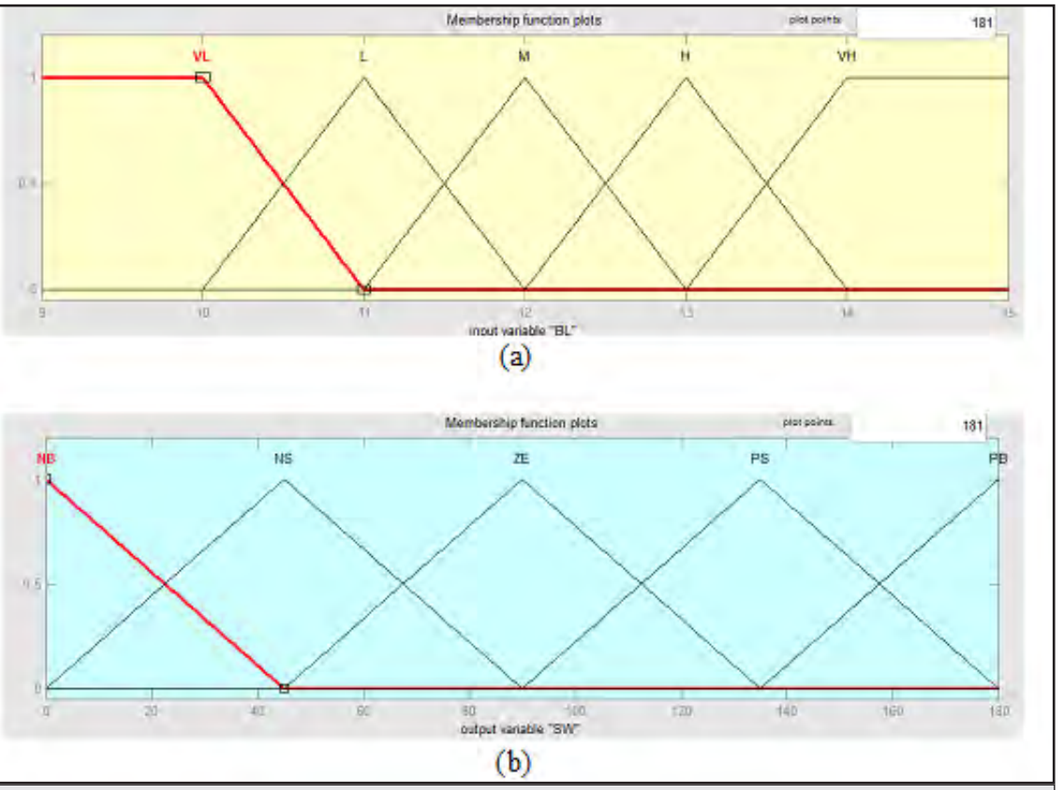

FIGURE 4. (a),(b) heart parameters membership function

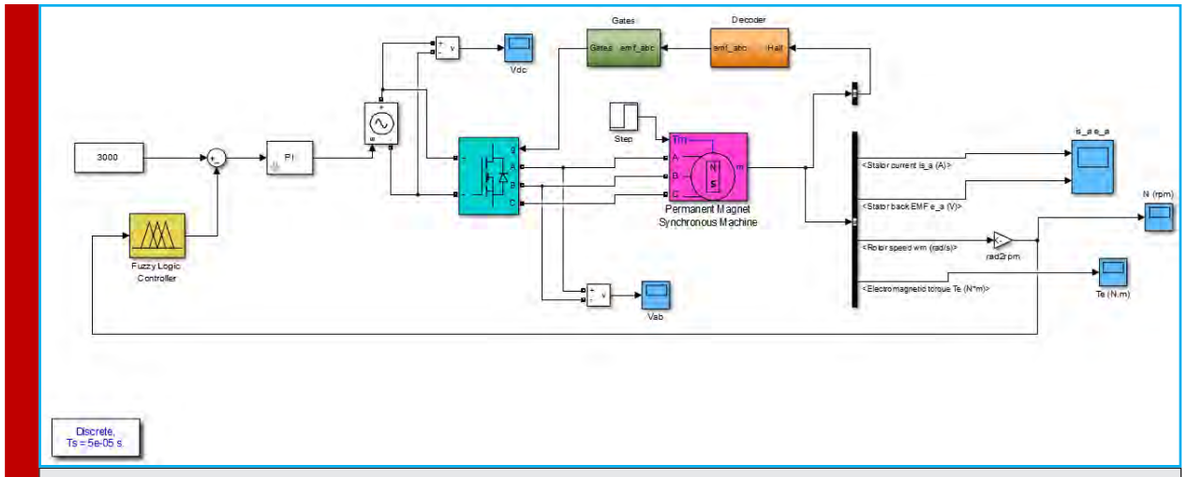

FIGURE 5. The block diagram BLDC

development of a controller. Further studies should be conducted to enhance and improve the dynamic model of control for better power values. Moreover, motor dynamometer tests should be applied for purpose of validating the model dynamic.

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Docking of GSK-3β with novel inhibitors, a target protein involved in Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is a chronic, advancing malady associated with loss of memory or cognition. It is the noted causes of lethality worldwide, there are no such drugs which can cure AD till date and are ineffective in the later stages. Such known drugs only ease the symptoms but do not prevent the onset or progression of the AD. Alzheimer's is caused by the aggregation of the hyperphosphorylated tau which is one of the common characteristics of the neurodegenerative disorder. There are a number of kinases which hosts the excessive phosphorylation of tau protein. One of the kinase extensively targeted in the AD is GSK-3β (Glycogen Synthase Kinase-3β). As indicated by many studies that by applying appropriate docking methods, a number of phyto compounds have shown enhanced target selectivity than the conventional Alzheimer's drugs. This review summarizes the known drug targets in the AD, their conventional inhibitors and also the comparison between the current and future AD therapy based on their binding affinities. As a result, large libraries of compounds with inhibitory effect can be screened. It was also studied that Withanolide-A has the potential to be the future drug for Alzheimer's disease.

KEY WORDS: DOCKING; DRUGS; PHOSPHORYLATION; TAU PROTEIN; WITHANOLIDE-A

INTRODUCTION

Alzheimer's is a type of dementia associated with memory loss and other intellective abilities, severe enough to intrude with regular routine. Alzheimer's disease report for 60 to 80 percent of dementia and the present Alzheimer's disease therapies impaired from in proficient effects on its symptoms such as perception notably in the subsequent stages of the disease (http://www.alz.org). According to the report prepared by Alzheimer's and related disorders society of India in 2010, there are 3.7 million Indians suffering with dementia while the

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numbers are anticipated to bifold by 2030. The number of factors is thought to increase the progression of this disease, some of which are; increasing age, family history, previous severe head injuries etc. Over the past decade, much of the research on Alzheimer disease (AD) has focused on radical-effected oxidative stress and its importance in disease pathogenesis. Oxidative stress increases amyloid beta deposits in the brain which results in the synthesis of neurotoxic aggregates. The net effect of oxygen radicals is damaging as it may lead to neuronal cell death and contribute to AD (Smith et al. 1998). Flavonoids also possess antioxidant activity and they regulate the redox status and prevent damage caused by oxidative stress. Protein Kinases are recognised as encouraging target structures considering their involvement in AD breakthrough pathways like pathophysiological tau protein phosphorylation and amyloid beta toxicity. The sound interdependence of tau phosphorylation and pathology has led to the search for Tau protein kinase inhibitors such as GSK3-β and Tyrosine kinase Fyn, which phosphorylates tau and also plays a causative role in amyloid pathway. Hereafter, acting as potential therapeutic agents (Medina, 2018).

ROLE OF FLAVONOIDS IN THE TREATMENT OF AD

Nature has fascinated us with a lot of natural remedies in the form of fruits, leaves, bark, vegetables, and nuts, etc. The wide varieties of biologically active nutrients existing in these natural products play a vital role in defence and aid of various neurodegenerative diseases. Flavonoids are an array of non-nutrient polyphenolic compounds readily procured from plants. It was realized that the competence of flavonoids to upgrade neurological health was resolved by their antioxidant capability. Flavonoids are endowed with numerous biological activities like anti-inflammatory, anticoagulant, anti-cancer, anti-oxidants, and anti-spasmodic. There is an extensive role of flavonoids and even their metabolites in different signaling pathways by altering the phosphorylation state of target protein put forward their therapeutic potential and beneficial in neurodegeneration(Spencer, 2007). Increasing evidence shows their ability to improve brain function such as memory and learning by interacting with cellular as well as molecular components of the brain resulting in enhanced neuronal function and induce neurogenesis (Spencer, 2010; Baptista et al. 2014).

A study has found the role of plant-derived compounds such as myricetin and epicatechin-5-gallate in abrogating heparin-induced cluster of tau into filaments (Taniguchi et al. 2004). In drug discovery, the dominant secondary metabolites (terpenoids, phenolics, and alkaloids) are of probable remedial relevance. Certain flavonoids such as indirubin and morin are capable of the inhibiting the activity of GSK-3 beta and thereby blocking tau hyperphosphorylation. Kinases are involved in tau phosphorylation and phosphatases reverse this action. Thus, flavonoids also portray a crucial aspect in modulating the activity of phosphatases (Baptista et al. 2014). Genistein (phytoestrogen), a beneficial intermediary for the treatment of AD as it imitates estrogen which is involved in the development of memory and learning along with its neuroprotective activities, (Hussain et al. 2018). It was found that eicosanoyl-5-hydroxytryptamide (EHT), a naturally ocurring component of coffee beans accelerates the activity of serine/threonine protein phosphatase, PP2A and thus provide therapeutic benefits associated with AD (Asam et al. 2017).

MOLECULAR CAUSES OF AD

The key events that lead to AD : Beta-amyloid toxicity. The brain of a patient with the AD is characterized by amyloid toxicity. Amyloid beta denotes peptides of 36-43 amino acids long processed from an amyloid precursor protein (APP) which is digested by beta secretase and gamma secretase to yield amyloid beta (A β). This peptide is found in brains of patients suffering from Alzheimer's (Murphy et al. 2010 Hamley, 2012, Sauer, 2017). Some processes include disruption of amyloid beta aggregates, alterations in the precursor of amyloid beta protein processing through the inhibition of beta-secretase. Thus, modulating the beta-secretase activity is the one suggested a therapeutic avenue to treat AD (Yin et al. 2007). Certain flavonoids may guard to counter the effect of Alzheimer's disease by interrupting with the generation of beta-amyloid peptides into neurotoxic aggregates. It is a matter of contention that interfering with the activity of beta and gamma-secretase enzymes may disrupt their other functional roles besides playing an important part in amyloidogenic pathways.

Thus such interference using γ secretase can result in skin cancers and cognitive dysfunction (Kikuchi et al. 2017). The decades old theory which aims at implicating beta amyloid as the leading cause of Alzheimer's has been questioned by a group of scientists. Researchers have tried and failed to prevent Alzheimer's using drugs targeted at amyloid β protein. Due to the lack of the utility of amyloid-β-aspired approach in Phase III clinical trials, it was prerequisite to conceive substitute drug discovery strategies for alzheimer's (Folch et al. 2016). Solanezumab, a drug which acts on amyloid β protein failed some pivotal clinical trials. However, it is still anonymous whether the disease is caused by plaques or they are just the by- products (Ramsey, 2018).

A number of normal patients have been found with amyloid deposists in their brain. It was anticipated that

amyloid beta deposition is an anomaly of aging and does not correlate with the AD progression(Kametani et al. 2018). Therefore there is a compulsive need to search policies directed at reducing misfolded tau protien which is one of the disease-causing agents (Bruden et al. 2010). Tau is liable to be the more superior target than the amyloid β as it coordinates efficiently with cognitive impairement, provided clinical symptoms are tangible (Congdon et al. 2018).

TAU PROTEIN HYPERPHOSPHORYLATION

For more than a decade, researchers have found 'tau' protein as one of the causes other than the Beta-amyloid plaques (Underwood, 2016). Accordingly, tau hyperphosphorylation and accumulation of insoluble aggregates is strongly related to reduce cognitive performance. Hence, we can affirm that tau is a reliable marker of the neurodegenerative process (Fig.1). Incorporation of phosphate groups into tau depends on; tau's confirmation and equity amidst the activity of kinases and phosphatases (Kremer et al. 2011).

Changes in tau confirmation could lead to excessive phosphorylation resulting in the formation of neurotoxic aggregates and tau-mediated neurodegeneration (Dixit et al. 2008). Tau is the member of family of proteins intricated in stabilizing the microtubules. They are common in neurons of Central Nervous System and also present at low levels in CNS astrocytes and oligodendrocytes (Shin

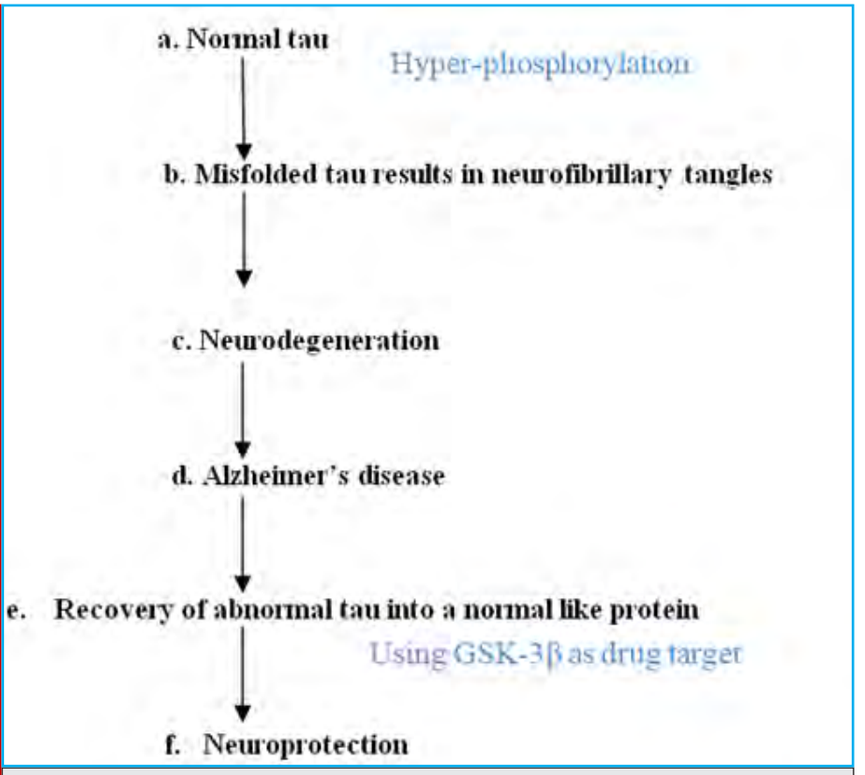

FIGURE 1. Events that are involved in tau mediated neurodegeneration
a. Normal tau is hyperphosphorylated by; 1) Over activation of kinases such as GSK-3β, 2) Inactivation of phosphatases such as PPA, PP2B
b.Tau hyperphosphorylation and accumulation of insoluble aggregates results in formation of paired helical fragments (PHF's) followed by Neurofibrillary tangles(NFT's).
c. Formation of neurotoxic aggregates is the major hallmark in tau-mediated neurodegeneration.
d. Such a pathological event ultimately leads to Alzheimer's.
e. recovery of abnormal tau into a normal like protein by using GSK-3β as a drug target-Inhibiting GSK-3β with the aid of novel therapeutic phytocompounds is the most promising therapeutic intervention.
f. Targeting tau mediated neurodegeneration is one such approach to combat AD and provide neuroprotection

et al.1991). The tau proteins have been formed as a result of alternate splicing of MAPT (microtubule -associated protein tau) gene in humans and is positioned on chromosome 17 (Goedert et al.1989; Jesus et al. 2016).

The hydrophilic nature of the tau protein and its existence as intrinsically disordered protein was unfolded by many biophysical studies. (Porowska et al. 2014).One of the critical function of tau protein is to prevent the depolymerization of microtubules by regulating its stability in two ways: isoforms, and phosphorylation. On the basis of the number of binding domains, six variants of tau protein (352-441 amino acids and apparent molecular weight between 60-74kDa) exist in human brain tissue (Martin et al. 2011). Out of six modifications, three isoforms have 3 tubulin binding domains and other three have 4 tubulin binding domains in the C-terminal half of tau, (Guo et al. 2017).

The domain structure of tau is such that it's positively charged binding domain is located in carboxy-terminal which binds to the microtubule which is negatively charged. Tau is a phosphoprotein (i.e. posttranslationally modified) with 79 probable Serine (Ser) and Threonine (Thr) phosphorylation sites on the extended tau isoform. It has been reported in a study that phosphorylation is possible in about 30 sites in a normal tau protein. PKN, a serine/threonine kinase is one such enzyme among the plethora of kinases which regulates the phosphorylation of tau (Billingsley et al. 1997). As revealed by primary sequence analysis, the tau molecule has three major domains: N-terminal (acidic), a proline-rich region, C-terminal domain (basic). These domains are characterized on the basis of their amino acid character and even on their microtubule interactions. Thus, tau protein acts as a dipole with two domains having the opposite charge (Kolarova et al. 2012; Porowska et al. 2014). Extreme phosphorylation of the tau protein proceeds to the formation of Paired helical fragments (PHF's) due to the loss of affinity with microtubules and they bind with one another which further aggregates in neurofibrillary tangles via. Post-translational modifications. Thus, there is a strong correlation between abnormal phosphorylation and self-aggregation of tau (Guo et al. 2017)

When disorganized, this aside from being very soluble protein forms remarkably insoluble tangles or aggregates which commit to the number of neurodegenerative disorders. The mutations in posttranslational modifications are the main cause of this failure i.e. they form nonfunctional aggregates. One of the studies demonstrated that dephosphorylation of the hyperphosphorylated tau converts abnormal tau protein into a normal like protein which then regulates microtubule assembly(Iqbal et al. 2011). Therefore abrogating the abnormal tau and recovery of the microtubule organization are the most promising therapeutic interventions to combat AD.

GSK-3 BETA AS A DRUG TARGET

GSK-3 is encoded by two genes: GSK-3β, located on chromosome 19 and GSK-3β, positioned on chromosome 2. GSK-3 is ubiquitously expressed in mammals as well as in yeast (Medina et al. 2011). GSK3 mediates the augmentation of phosphate molecules to serine and threonine amino acid residues and for this reason termed as serine/threonine protein kinase. The kinase domain of these 2 isoforms are highly homologous ((Stambolic et al. 1994) but are demarcated in the N- and C-terminal regions. GSK3β has a molecular mass of 46-47 kDa consisting of 433 and 420 amino acids in human and mouse respectively. The protein contains an N-terminal domain, a kinase domain, and a C-terminal domain. The substrate Binding domain (BD) provides GSK-3β specific binding sites for the tumor supressor p53 and other protein complexes (Atlas of Genetics and Cytogenetics in Oncology and Haematology). A number of protein kinases are involved in tau phosphorylation such as CdK5 (Cyclin-dependent Kinase 5), JNK (C-Jun amino-terminal Kinase), CK1 (Casein Kinase1), Dyrk1A, AMPK (Adenosine-monophosphate activated protein kinase), MARK5 (Microtubule affinity-regulating Kinases), PKA (Cyclic AMP-dependent protein Kinase), GSK-3β (Glycogen Synthase Kinase-3β) (Crews et al. 2010). But a study has shown that 31% of the therapeutically favorable phosphorylation sites of tau protein are phosphorylated by GSK3β (Martin et al. 2013).

The classical approach to treat misfolding of tau protein provides inhibition of protein kinases (Glycogen synthase kinase 3β) which hosts tau phosphorylation. According to the 'GSK-3 hypothesis of AD', tau hyperphosphorylation, memory impairment and enhanced β-amyloid production is due to the overexpression of GSK-3, all of which are characteristic features of the AD. If this hypothesis is consolidated then, inhibition of GSK-3β by novel inhibitors provides a better pathway against the effect of this destructing disorder (Hooper et al. 2008). There are two isoforms of GSK-3 gene; GSK-3 alpha and GSK-3β. GSK3β also exist as longer splice variants (Mukai et al. 2002; Schaffer et al. 2003). Moreover, GSK-3β results in a neuronal decline in the AD because of the fact that it is a causal mediator of apoptosis. Increased level of such protein eventuated in the autopsy evaluation of brain of alzheimer's victims (Pei et al. 1997). It is also validated that a spatial and temporal pattern of enhanced GSK-3 expression corresponds with the evolvement of neurofibrillary tangles proceeding towards neurodegeneration (Leroy et al. 2002).

MOLECULAR DOCKING

Drug research is an important tool in the field of medicine. Utility of computers to anticipate the efficiency

of binding of a set of small molecules or ligands with the target is an important element of drug discovery and developmental process. There is an ample realm of software packages used to execute molecular docking such as Dock, Autodock, GOLD, ICM, Glide, AutoDock Vina, FlexX etc . Automated docking is generally used for prognosis of biomolecular complexes, in structure and function examination and in computer-aided drug designing. A dozen of mechanism is available, consolidating varied energy evaluation methods. Due to the enhanced docking speed, AutoDock 4.2 has been widely used for virtual screening. It is the ultimate current version which is based upon the Lamarckian genetic algorithm, a hybrid algorithm comprising of both the genetic as well as local search and is more enhanced and accurate than previous version AD3.0. Unlike AD3.0, Autodock 4.2.6 (henceforth AD4.2) and Auto Dock Vina 1.1.2 (henceforth AD Vina) have upgraded results and improved elucidation, (Collignon et al. 2011, Nataraj et al. 2017 and Alvarez et al. 2017).

Two main programs are involved in AutodockTools: Autodock for docking of the ligand within the set of grids (within the binding site) in the target protein and Autogrid for selection of grid parameters, size of the box, its location etc (http://autodock.scripps.edu/). It is particularly suitable for protein-ligand docking in which we presume the pose and orientation of a small molecule when it is articled to a protein receptor. It is used to select likely drug candidates. Typically, ligands are drug candidates and the macromolecule is the protein or receptor of the known three-dimensional structure. In this docking simulation, the ligand being docked was kept as flexible while target protein was kept as rigid. The graphical user interface i.e. Autodock Tools was used to prepare, run, analyzes the docking simulations.

CURRENT AND FUTURE AD THERAPY

Till date there are no such drugs/treatments available that can cure AD completely. However, there are several medications developed for Alzheimer's disease that can temporarily attenuate the symptoms. The Food and Drug Administration (FDA), U.S. has affirmed two medications-acetylcholinesterase inhibitors and Memantine. Drugs such as tacrine, rivastigmine, galantamine, and donepezilare are the widely used conventional drugs to treat AD (Islam et al. 2013). Memantine is a dissociative hallucinogenic and anesthetic drug of the adamantane class of chemicals that are currently used as an FDA approved drug in the treatment of AD (www.alz.org). Therefore, traditional drugs like memantine and donepezil are being extendedly used as the reference in molecular docking studies. Hence, the objective of eventual AD therapy is to discover such novel compounds which can target the tau protein and so that can be utilized for the recovery of neurodegenerative loss (Schneide et al. 2008).

The study related to the AD is focused more towards the traditional medicinal plants and its components such as *Withania somnifera* (Ashwagandha), *Celastrus paniculatus* (Jyotismati), *Convolvulus pluricaulis* (Shankhpushpi), *Bacopa monnieri* (Brahmi). By analyzing the binding energies of various ligands such as acacatechin, catechin, galangin, scopoletin, silibinin, memantine (as standard), it was observed that flavonoids exhibit binding energy scaled between 7.07 kcal/mol to -4.85 kcal/mol. Silibinin demonstrate prominent binding energy -7.07 kcal/mol than the standard memantine (-5.89 kcal/mol) (Madeswaran et al. 2013). A phytocompound, Catechin (with binding energy -9.7 kcal/mol) was shown to be the potent target of GSK-3β and showed the same drug-likeness as conventional drug Donepzil (with binding energy-8.9kcal/mol), (Alam et al. 2017).

Withania somnifera, a potential inhibitor of GSK-3β

Withania somnifera commonly called Ashwagandha, Indian ginseng and wind cherry have been recognizes as an important herb in Indigenous and ayurvedic medical system. Historically, the plant has been used therapeutically for boosting the brain function including memory retrieval. It has a cognition promoting effect in adults and children (Singh et al. 2011*)*. It consists of two components: withanolides and withanamides. Withanolide A is extracted from the roots of the plant and promotes antioxidant properties that protect nerve cells from harmful free radicals. Many clinical trials and excessive research on animals support the use of Ashwagandha for anxiety, cognitive and neurological disorders (Rajasekar et al. 2011). Withanolides have also been used for the treatment of AD (Khan et al. 2016). Withanolide A is used as an inhibitor of acetylcholinesterase activity and reduces beta-amyloid protein formation. Also, it has been involved in the regeneration of pre and postsynaptic neurons. Instead of the root extract, a study also suggested fruits and leaves of Egyptian plant have strong antioxidant activity (Mahrous et al. 2017)

FUTURE PERSPECTIVES

Several new therapeutic approaches are currently under investigation which aims at targeting proteins such as Apolipoprotein E which is also responsible for the accumulation and hyperphosphorylation of tau. Anti-tau immunotherapeutic agents have gained much focus due to their specificity and selectivity to combat AD. But a longer follow up period might be required to test the safety and efficacy as the results were promising .Moreover targeting either tau or amyloid beta individually is

not apparently the satisfactory approach and therefore, combinational therapies might be thought of as a new proposal, (Coman et al. 2017 Bittar et al. 2018).

CONCLUSION

Drug research is of utmost importance in the field of medicine. Consequently, the use of computers to foresee the efficiency of binding of a set of molecules or ligands with the target is an important element of drug development process.To explore potent and effective drugs for the treatment of AD, different phytocompounds were compared against the standard using Autodock4. Appropriate ligands were docked into the active site of the receptor GSK-3β and analyzed for the effective protein-ligand interactions. Therefore molecular docking identified many more promising, efficacious, selective new drugs against Alzheimer's reducing the time span of complex drug discovery process. Appropriate experimental evidences such as ADMET analysis which testifies absorption, penetration and toxicity may also be considered further as a lead in drug discovery process.

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CONFLICT OF INTEREST STATEMENT

Authors state no conflict of interest. All authors have read the journal's Publication ethics and publication malpractice statement available at the journal's website and hereby confirm that they comply with all its parts applicable to the present scientific work.

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The accelerating epidemic of type-2 diabetes in children and adolescents

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ABSTRACT

The occurrence of type- 2 diabetes in youth has increased dramatically over the past 20 years. Adolescents and young adults are fastly entering into the domain of the disease. The emergence of type 2 diabetes mellitus in children in Indian population presents a new challenge. 2048 children had undergone questionnaire and dietary survey and health examination. The scrutiny of the subjects for blood sugar levels along with various other parameters involved in this study revealed that 1.12% subjects were diabetics as compared to 1.56% with impaired glucose level. This shows a total ignorance on the part of parents about their children's' health status.The study brought forth a hard fact that periodic health checkup is necessary to prevent the agony of this disease. It is further pointed out that such surveys are very rare particularly in the underdeveloped and developing countries. It is worthwhile to conduct surveys to detect such cases so that timely remedy can be provided.

KEY WORDS: TYPE 2 DIABETES, CHILDREN, ADOLESCENTS, HEALTH, DISEASE

INTRODUCTION

Type 2 diabetes is rising rapidly amongst children and adolescents worldwide. The incidence of type 2 diabetes in youth has increased dramatically over the past 20 years. Type-2 diabetes is a significant and increasing burden in adolescents and young adults. Overweight is, at present, the most common health problem faced by the children in both develop and developing countries which leads to the development of Type-2 diabetes (Han et al., 2010). This has been attributed to the fact, that the prevalence of obesity is not increasing but the degree of obesity also increases in affected children and adolescents (May et al., 2012). Type-2 diabetes mellitus is a complex metabolic disorder of heterogeneous etiology with social, behavioral, and environmental risk factors

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unmasking the effects of genetic susceptibility (Kiess, 2003). Clear strategies for research, prevention and treatment of the disease in these vulnerable patients are the need of the hour. Understanding the unique pathophysiology of type-2 diabetes in youth, as well as the risk of complications and the psychosocial impact, will enable industry, academia, funding agencies, advocacy groups and regulators to collectively evaluate both current and future research, treatment and prevention approaches. Type-2 diabetes mellitus is still rare in childhood and adolescence, but recent reports indicate an increasing prevalence around the world possibly due to increasing prevalence of obesity in children and adolescents (Thomas, 2013, Kristen et al, 2016).

MATERIALS AND METHODS

2048 children had undergone questionnaire and dietary survey and health examination. Out of these, 1017 were from urban population and 1031 from rural population. Children and adolescents aged 10-19 years were selected randomly for questioning regarding the different aspects of epidemiology and their health examination was done. The permission from parents of the children, undergoing examination and questionnaire survey was also taken on the self-designed consent form. The Centers for Disease Control and Prevention (CDC) suggests two levels of concern for children based on the BMI-for-age charts.

At the 85th percentile and above, children are "at risk for overweight". At the 95th percentile or above, they are "overweight". The cutoff for underweight of less than the 5th percentile is based on recommendations by the World Health Organization Expert Committee on Physical Status 1998. The diagnostic criteria for diabetes mellitus have been modified from those previously recommended by WHO (1985). The revised criteria of report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (2003) for the diagnosis of diabetes was used.

RESULTS AND DISCUSSION

The emergence of type-2 diabetes mellitus in children in Indian population presents a new challenge. The scrutiny of the subjects for blood sugar levels along with various other parameters involved in this study revealed that 1.12% subjects were diabetics as compared to 1.56% with impaired glucose level from the already mentioned area. Such children, along with impaired glucose level and diabetic cases were totally ignorant about their health status viz a viz this disease.

Analysis of Variance (ANOVA)

By subjecting the various observations to statistical analysis, certain factors became quite apparent when comparisons between normal subjects and those having different status of diabetes mellitus were made. When all inter-group comparisons were attempted in order to obtain a clear picture of the status of children among themselves, significant results have been obtained.

The observations given above embodied quite revealing information from 2048 subjects studied. This shows a total ignorance on the part of parents about their childrens' health status. The study brought forth the fact that periodic health checkup is necessary to avoid the agony of this disease. It is further pointed out that such surveys are very rare particularly in the underdeveloped and developing countries. It is worthwhile to conduct surveys to detect such cases so that timely help can be provided. The present work is the first of this nature from Chandigarh in Northern India.

Table 9. Prevalence of impaired glucose levels and diabetes in total population

Sub-Group	N	%
	1993	97.32
110-126	32	1.56
	23	1.12

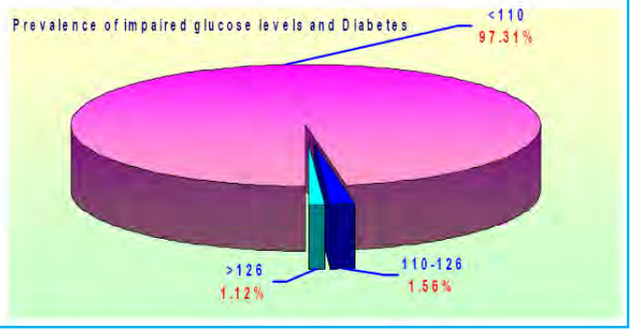
Sub Group	Mean	SD	SEM	SESD	CV	Range	Skew	Kurt.
1 <110	79.532	10.620	0.238	0.168	13.35	1.000	0.139	5.350
2 110-126	114.094	4.276	0.756	0.534	3.75	0.064	0.914	2.850
3 >=126	144.087	22.494	4.690	3.317	15.61	0.210	1.095	2.491

Groups are: <110, 110-126, >=126,

ANOVA Table

Source of Variation	Sum of Squares	df	Mean Sum of Squares	F
Between Groups	130794.198	2	65397.099	565.799
Within Groups	236368.707	2045	115.584	
Total	367162.906	2047		

Conclusion: SIGNIFICANT at 1% (p=0.01) Level of Significance



In India, the incidence of diabetes mellitus is increasing because of intake of high carbohydrate rich food by the children and adolescents. It is therefore necessary to make the general population aware about their health status by conducting periodic health checkups. Such incidents are not restricted to Punjab or other parts of India but are global. It had been noticed that half of the 16 million Americans with diabetes are undiagnosed as had been studied by Harris et al., 1987, Harris, 1993, U.S. Department of Health and Human Services, 1993. Four million Americans with known diagnosis of diabetes are hospitalized annually in this country (Levetan et al., 1998). In the U.S., estimates are as high as 5,000 new cases are added per year (Lawrence et al, 2014).

Prevalence increases with age, tripling from age 10–14 years to 15–18 years (Dabelea et al, 2014). Diagnosis of type-2 diabetes is estimated to be delayed by an average of 10 years after the actual onset of disease. The present study corroborate very well with this observation as 1.56% subjects are having impaired glucose levels which are likely to become diabetics and 1.12% subjects were diabetics. In fact, this group is one which needs an immediate attention of the subject himself, parents and the health authorities. An early study in 1991 of rural areas in Delhi indicated that the prevalence rate for type-2 diabetes ranged from 0.4-1.5% (Ahuja et al. 1991).

This study had not included impaired glucose levels and subjects were only from rural area. Data regarding type-2 diabetes in children and adolescents is very scarce in this area. Over the last decade, it has become apparent that type-2 diabetes extends its wings not only into the young adult population but is also found in adolescents and even, occasionally, in children. The limited data, that is currently available, present a rather uncertain picture, with a rather wide range of prevalence and incidences of type 2 diabetes in children and adolescents. The transition from prediabetes to type-2 diabetes in adults is usually a gradual phenomenon that occurs over 5–10 years (Weiss et al, 2005). Therefore, the early presentation of type-2 diabetes in youth raises the possibility of an accelerated process in pediatric age compared with adults, thus shortening the transition time between IGT and type-2 diabetes. In fact, an interesting report by Gungor and Arslanian (2004) suggested that despite a relatively robust initial insulin secretion, the deterioration in β-cell function in youth with type-2 diabetes is more accelerated than that was observed in adults.

Type-2 diabetes mellitus was reported in children and adolescents from the United States, Canada, Japan, Hong Kong, Singapore, Bangladesh, Libya, the United Kingdom, Australia and New Zealand. The prevalence of type-2 diabetes in children and adolescents ranges from 4.1 per 1000 amongst 12-19 year olds in the United States to 50.9 per 1000 15-19 year old Pima Indians in Arizona. Between 8% and 45% of recently diagnosed cases of diabetes in children and adolescents in the United States were type-2 diabetics (Fagot et al, 2000 and 2001). The emergence of type-2 diabetes coincides with worldwide trends of rising prevalence in overweight and sedentary lifestyle (Troiano et al 1995).

India is poised to be among the world's top four economies by 2020 (Abdul Kalam, 1998) and is under-

going a rapid epidemiological transition: the burden of chronic diseases is overtaking the burden of infectious diseases (Fall and Barker, 1995, and Nath et al, 1998). India already has the highest number of adult diabetes cases (20 million) worldwide and this number is expected to rise to 57 million by 2020 (King et al, 1998 and Narayan et al, 2000). There is only few data available on type-2 diabetes in children and adolescents in India. The prevalence of obesity (body mass index [BMI] exceeding the 95th percentile) among US children and adolescents aged 6–19 years has jumped from approximately 4% in 1963 to 15% in 2000. In some regions in the United States, type 2 diabetes mellitus is as frequent as type-1 diabetes mellitus in adolescents (Arslanian, 2002 and Zeitler, 2015).

Rapid urbanization and economic growth creates social dynamics that promote diabetes risk factors. These include over-weight, decrease in physical activity, increase in sedentary activities such as television viewing, and high fat and high-energy diet among adults and children. Other factors may also make Indian children and young adults more vulnerable to diabetes. These include prenatal factors (e.g., low birth weight, maternal under-nutrition), biological propensity to central obesity and insulin resistance, low lean mass, diabetes during pregnancy, impaired glucose tolerance, and urban stress (Ramachandran et al 1992, 1994, 1997, 1999 and Yajnik 2001).

Type-2 diabetes in children is being increasingly reported from other Asian countries. In Japan, the incidence of type-2 diabetes in children increased over a 20-year period (6-12 years: 0.2/100,000/year in 1976 and 2.0/100,000/year in 1995; 12-15 years: 7.3/100,000/year in 1976 and 13.9/100,000/year in 1995. This increase in incidence correlated with increased reported intake of animal protein and fat(Kitagawa ,1998 and Fagot et al 2000). Type-2 diabetes is being reported in children of Indian origin living in countries such as the United Kingdom (Ehtisham et al 2000). There is an urban-rural gradient in adult diabetes risk in India and when the data are standardized for age and sex differences, the prevalence of diabetes in urban Indians is similar to that of Indians abroad (Ramachandran et al, 1997). This finding suggests that type-2 diabetes in children of Indian origin living abroad may be an early indication of things to come to India.

The biggest challenge India is likely to face in the future is tackling diabetes among children and adolescents. The number of children falling prey to type-2 diabetes has increased manifold over the past two decades.

Rapid urbanization and economic growth have promoted risk factors for diabetes such as obesity, sedentary lifestyle, high fat and high energy diet among adults and children. Unfortunately, no systematic survey has been conducted so far to know the trends in India. Untreated children and adolescents with type-2 diabetes are at much higher risk of cardiovascular disease, kidney failure and vision loss. New health initiatives targeting children and adolescents which are aimed to raise awareness and check the rapidly increasing cases of obesity and diabetes.

Type-2 diabetes in children is probably under-diagnosed because it can exist without symptoms. It may also be under-reported and part of the reason for this may be misclassification (Fagot et al, 2000, 2001 and American Diabetes Association 2000)). The prevalence of childhood diabetes among those younger than 15 years in the early 1990s in an urban population in south India was 0.26/1000 (Ramachandran 1992) and the incidence was 10.5/100,000/year. There are two important implications of the potential emergence of type-2 diabetes in children in India. Obesity and type-2 diabetes in children may be at the epicenter of a much larger diabetes epidemic in India than currently predicted and compulsion to act against the potential diabetes epidemic in an organized and systematic manner. Most of the children having impaired glucose levels and diabetes were ignorant about their status at the time of diagnosis.

The thrifty genotype hypothesis was advanced over 40 years ago to explain the modern emergence of obesity and type-2 diabetes (Neel, 1962). This hypothesis postulates that humans survived by the genetic selection of those whose metabolic storage capabilities permitted survival during periods of famine by taking advantage of episodic periods of plenty in a feast and famine existence. Continuous feasting with an abundance of calorie-rich foods results in fat deposition without the concomitant period of fasting to maintain a normal body weight. Historically, only the prosperous met this condition, but modern food production and marketing have led to low-cost abundance, with obesity now disproportionately affecting those at the less prosperous end of the economic scale.

Perhaps the most important reason for increasing prevalence of diabetes, obesity and type-2 diabetes is the rapidly changing imbalanced dietary habits, both in rural and urban areas, due to several factors–easy availability of convenience foods, frequent snacking on energy dense fast foods, high consumption of packaged food in place of traditional home made food, etc. This transition has resulted in excess consumption of calories, saturated fats, trans fatty acids, simple sugars, salt and a low fiber intake. It is high time to prepare to combat this menace and curb its spread. Indians are genetically more prone to diabetes. On top of this, a rapid shift in our dietary habits and life styles is resulting in a rapid rise in obesity, diabetes, metabolic syndrome and heart disease. Hence, a proper and healthy diet remains the

keystone for the prevention and management of type-2 diabetes.

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Heavy metal contamination in street precipitated dust in Tabriz City, Iran and its ecological risk

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ABSTRACT

Dusts are suspended particulate matters in the air, which are created from different terrestrial and human made sources. Over time, they re-precipitate on surfaces given their size and density. Forty-nine samples of street dusts were collected from the sideways of several main streets from the city center, the Tabriz South passenger terminal, and one sample was collected from the yard inside Tabriz University in the summer and under dry climate conditions. Further, the concentration of iron, manganese, zinc, lead, nickel, chromium, copper, lithium, and cadmium metals was measured in them. The possible sources of contaminants were identified using correlation analysis, cluster analysis, Pearson correlation coefficient, and principal component analysis. In addition, using enrichment factor (EF), the effect of human activities on the concentration of heavy metals was assessed. The results indicated high concentrations of cadmium, lead, copper, zinc, iron, chromium, and nickel compared to the mean concentration of these metals in the Earth's crust. The maximum concentration of copper, lead, chromium, nickel, zinc, and iron was related to Kasaey Expressway, which is one of the most crowded expressways of Tabriz. Analysis of the results indicated that contamination can be due to different human activities including heavy traffic of vehicles, combustion of fossil fuels, additives added to vehicles' fuels, corrosion of metal surfaces of automobiles, and corrosion of construction materials. The enrichment factor values of copper, cadmium, lead, and zinc showed extremely high enrichment, with human origin. The calculations related to the ecological risk were also performed using Hankinson method. All of the studied points show very high ecological risk.

KEY WORDS: STREET DUSTS, HEAVY METALS, ECOLOGICAL RISK, ENRICHMENT FACTOR, TABRIZ

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INTRODUCTION

The growth of population, industries, and vehicles has increased the extent of pollution across cities especially Metropolitan cities. Therefore, recently evaluation of the quality of street dusts as pollution sources has attracted a great deal of attention. The heavy metals in street dusts are one of the major pollutants of urban environment, which can be due to heavy traffic, industries, wear of buildings, wear of rubber and peace is used in vehicles, mineral activities, and combustion of fossil fuels, (Jiries, 2003; Al-Khashman, 2007, Manasreh, 2010 Mukati, 2017).

Over the past few years, large amounts of atmospheric dusts have entered cities including Tabriz through the country's boundaries. Although there is controversy over their accurate origin, it is stated that their main sources are the deserts of the neighboring countries. In any case, significant amounts of them precipitate on the surfaces of urban regions as dust. Combustion of fossil fuels also produces some amount of heavy metals including nickel), chromium, lead, and manganese. These pollutants have aggregation and carcinogenic properties, and can develop various health and environmental problems. Furthermore, exposure to them can bring about low intelligence, kidney problems, and for long-term exposure, it can cause death. Street dusts containing heavy metals can also enter the children's body through hands and mouth (Watt, et al., 1993, Jiries, 2003, Balarak, 2017).

Recently, various studies have been conducted about the concentration and distribution of heavy metals, some of which has been performed in developed countries. In Turkey, Sezgin et al selected one of the expressways of this country to take samples from soil and street dusts. The sampling was performed from both sides of this Expressway and the tunnel. Analysis of the results indicated that the mean lead concentration in the street dusts was 9-11 times as large as its concentration in the soil. Regarding copper and cadmium, the mean concentration of this pollutant in street dusts was twice as large as their concentration in soil. This number is around 9-12 times for zinc.Nickel also indicates a concentration higher than the concentration in the soil. Next, the sources of emission of these metals are attributed to industries and vehicle traffic (Sezgin, et al., 2003).

In another study, conducted in Lebanon by Jiries, the sampling regions were categorized into four regions including city center, tunnels, indoor parking lots for vehicles, and residential areas. The maximum level of heavy metals was observed in the tunnels, while the minimum concentration existed in the residential areas. Thereafter, based on the results, it was found that there is a high correlation between lead and cadmium. Therefore, it can be stated that these pollutants have a common emission source, (Jiries, 2003).

In a number of studies, sources of emission of heavy metals in the soil and street dusts have been examined using cluster analysis and principal component analysis. In 1997, De Miguel used cluster analysis of Method Ward as well as two-dimensional principle component analysis. He considered three sources of vehicle traffic, building construction, and natural resources among the factors for emission of 25 types of rare metals in street dusts in Oslo and Madrid (Miguel et al., 1997). Ordonez et al detected the source for emission of 27 different metals in the studied dusts samples using SPSS and cluster analysis as human sources, natural sources, or a combination of them (Ordonez et al., 2003).

Considering the intensified level of air pollution and suspended particles in recent years in Tabriz, as well as entrance of large dust masses in the past two years along with the adverse effects of polluted dusts on citizens' health and environment, the concentration of heavy metals in this populated city should be investigated. In the present study, the concentration of copper, cadmium, chromium, nickel, manganese, zinc, iron, lithium, and lead present in street dusts of Tabriz was examined to identify the sources of production and the ecological risk resulting from these pollutants was measured, and finally analyzed.

MATERIAL AND METHODS

Tabriz is a metropolitan city in the Northwest of Iran and is the capital of East Azerbaijan province. In 2016, Tabriz population has been around 1558693 people. This city is the largest economic pole of the north west of Iran, and is considered the administrative, communication, trade, political, industrial, cultural, and military center of this region.In recent years, the number of residents and in turn industries in vehicles in this city has increased considerably, such that today Tabriz is considered one of the most polluted cities of the world. Tabriz climate is warm and dry, and precipitation usually occurs during fall and winter. For this reason, sampling was performed in the dry season of summer and in August, when precipitation is minimum. The sampling distribution is as follows:

Ten samples were collected from Imam Khomeini, Azadi streets as well as Kasaei Expressway. Five samples were taken from Shotorbanan and 17 Shahrivar streets, 14 were collected from the southern passenger terminal, and finally one sample was taken from Tabriz University. The sampling from street and expressways was performed to emphasize their vehicles and heavy traffic as one of the most important environmental polluting sources. The sampling was performed using a

sweep and blower taken from the margin of curbs at both sides of the streets, as well as margin of curbs and the walls of the passenger terminal. The samples were then kept inside special bags and transferred to the laboratory at a temperature less than 4°C. To ensure absence of measurement errors and calculate the concentrations and based on the typical method of these studies, the samples were experimented at room temperature (less than 40°C) until reaching a constant dry weight. Thereafter, they were passed through seeves with 10, 35, 60, and 230 scores.

RESULTS AND DISCUSSION

The granulation results of some of the samples have been presented in Table 1. The particles with a diameter of less than 63 micron (the diameter of the sieve pore with score 230), which are easily scattered in the air and become suspended and are more probable to enter the respiratory system and develop risk to the human health as per the method of Zhou et al., (2003) were investigated.

To measure the concentration of heavy metals, acid digestion was performed using HCl, nitric acid, and perchloric gram. The concentration of heavy metals was measured by atomic absorption device (210 VGP). For all of the analyses, the control samples and per each 10 samples, one duplicate sample underwent acid digestion and was analyzed alongside other samples.The error of the experiments was less than 6%. Cluster analysis and principal component analysis were employed using MVSP and SPSS 18.0 software applications to identify the possible sources of metals in street dusts, while enrichment factor (EF) was utilized to investigate the possible effects of human activities on their concentration (Wei et al., 2010). The EF of a metal is obtained by the following relation:

Where, In Eq. (1), [C_x/Cref] *sample* represents the ratio of the intended concentration to the reference metal in the studied sample and [C_x/Cref] *background* shows the ratio of the intended metal to the reference metal as the background values. Five different groups of EF values are defined for analyzing the obtained values, as shown in Table 2 (Yongming, et al., 2006).

To obtain the ecological risk of heavy metals, the following relation was used (Hakanson, 1980):

Where, in Eq. 2, C_s is the concentration of the sampled metal and C_n shows the background values of the metals. E_r denotes the ecological risk of each element and RI reveals the ecological risk of the sum of the elements. Hakanson (1980) has presented T_r value (which is the toxicity index of heavy metals) as 30, 5, 5, 2, and 1 for cadmium, copper, lead, chromium, and zinc, respectively. To analyze the obtained values, four different groups are defined, as presented in Table 3.

The maximum, minimum, mean, and standard deviation values of the concentration of different metals across the 50 studied samples are presented in Table 3.

The maximum concentration of copper, lead, chromium, nickel, zinc, and iron is related to the sampling points in Kasaei Expressway, which is one of the most crowded expressways of Tabriz. Based on this point, it

Table 1. The mass percentage of particles across different sizes of the dusts samples and some of the studied stations

Particle Diameter (mm) / Sample number	0-0/063	0/063-0/25	0/25-0/5	0/5-2
1	%12/6	%43/06	%23/06	%20/63
5	%1/72	%21/41	%39/53	%34/85
9	%8/53	%45/35	%29/6	%15/87
11	%10/58	%62/62	%19/26	%7/49
21	%16/87	%38/17	%34/46	%9/86
23	%9/26	%30/69	%20/92	%38/85
26	%2/6	%23/45	%22/26	%50/43
31	%7/92	%71/28	%15/65	%4/76
33	%17/85	%43/22	%16/68	%21/26
40	%4/31	%51/81	%28/78	%14/32
44	%14/38	%39/38	%20/8	%24/34
48	%10/4	%33/22	%26/96	%28/65

Table 2. Different groups of the range of changes in the EF

EF values	extent of enrichment
Low enrichment	2> EF
Medium enrichment	2≤ EF <5
High enrichment	5≤ EF <20
Very high enrichment	20 ≤ EF <40
Extremely high enrichment	EF≥40

Table 3. The groups of the range of changes in RI and ecological risk value

Ecological risk value	RI value
Low ecological risk	150> RI
Medium ecological risk	150≤ RI <300
Considerable ecological risk	300≤ RI <600
Very high ecological risk	RI ≥ 600

can be stated that the possible origin of these metals includes the sources related to the extensive commuting of vehicles. However, cadmium, manganese, and lithium have different emission sources.

To investigate whether the concentration of the contaminant is measured in this study reveals large values or not, Table 4 compares the different heavy metals studied around the world and in this research. Compared to other cities, especially the cities belonging to developed countries, the concentration of cadmium, manganese, zinc, iron, and lithium reveals larger values. Cadmium and zinc metals show far higher concentration compared to the rest of the points, increasing the concern over the high level of these pollutants in the street dusts of Tabriz. Lead and copper, which are also among the main pollutants on the environment, except for a few cases, have concentrations higher than those around the globe.

Furthermore, except for nickel, chromium, and lithium, other elements have significantly larger concentrations than the mean concentration in the Earth's crust. Considering these three metals, one cannot say that as they have lower values compared to the mean earth crust level, they originate from the nature. This is because according to Table 4, the values of these three pollutants in other cities are also lower than the mean value of the Earth's crust. Since Iran is not considered an industrial country when compared to Canada, England, and Spain, and Table 4 has presented the data of populated cities including London, the high level of some of the pollutants in street dusts of Tabriz is serious and it is possibly due to other sources apart from the natural sources.

One of the sources for emission can be considered the fuel used in the vehicles of Tabriz, which considering its unsuitable quality, it may show larger values for hazardous pollutants in Tabriz compared to industrial and populated cities of the world. Corrosion of the body of vehicles, different metal surfaces across the city, as well as the tiny particles of rubber and brake pad of the vehicles are also other sources for entrance of these metals as particulate matters in the urban environment.

IDENTIFYING THE POSSIBLE SOURCES

Enrichment factor (EF)

To identify the natural or non-natural sources of the pollutants measured in the study, various analyses have been used. EF values can be used to understand whether the sources of emission of heavy metals are natural or human-made. As shown in the formula related to EF calculation, a value called background values required. Across different studies, the values calculated for heavy metals from previous studies are chosen as the background value (Manasreh, 2010; Zheng, et al., 2010).

In some of the studies, concentration of heavy metals in the Earth's crust has also been used as background values (Tokalioglu, et al., 2003; Kartal, et al., 2006). Therefore, as measuring the concentration of heavy

Table 3. There values of the mean, minimum, and maximum concentration and standard deviation of the studied cases (n=50)

Element	Mean	Minimum	Maximum	standard deviation
copper	223	57/56	779/35	54/56
cadmium	10/12	10/1	12/09	0/65
lead	253/6	63/65	765/8	12/23
chromium	34/5	15/25	57/09	85/32
nickel	33/2	13/55	73/95	444/52
manganese	1211/3	699/25	2322/65	10/98
zinc	865/8	400/34	1934/18	1/65
iron (%)	7/9	2/68	8/69	3/98
lithium	9/8	4/54	17/87	2/55

Table 4. The mean concentration of heavy metals present in street dusts of Tabriz and other parts of the world (ppm)

Heavy metals / City	Diameter of particles (mμ)	Copper	Cadmium	Lead	Nicole	Chromium	Manganese	Zinc	Iron	lithium
Tabriz (this study)	<64	224	10/5	255/4	56/36	33/3	1212/2	863/6	48725/6	9/3
Oman (Jordan) Jiries (2003)	<200	249/6	1/1	976	16/27	18/33	144/6	410	5370/6	1/72
Birmingham (England) Charlesworth, et al	<63	466/9	1/6	48	41/1			534		
Kuala Lumpur (Malaysia)Ramlan and Badri (1989)	<63	35/5	2/9	2466			153	344	1790	
London (England) Schwar, et al (1998)	<500	155	3/5	1030				680	26000	
Mutah (Jordan) Manasreh (2010)	<63	69	1/3	143	1/7		136	132	5362	
Madrid (Spain) De Miguel, et al(1997)	<100	188		1927	44	61	362	476	19300	
Otawa (Canada) Rasmuseen, et al (2000)	100-250	188	0/6	68	19	59	534	184	25660	9
Kawala (Greece) Christoforidis and Stamatis (2009)	<63	172/4	0/2	386/9	67/9	232/4		354/8		
The mean concentration of heavy metals in the Earth's crust Karbassi, et al. (2005) & Niencheski, et al. (2002)	--------	50	0/2	14	80	100	950	75	41000	20

metals present in street dusts of Tabriz is performed for the first time in this study, due to unavailability of previous information and not developing and presenting the background concentration values of elements for different regions of the world by the relevant organs (while these values have been prepared and presented by many countries) (Jien, et al., 2011; Wei, et al., 2010), the mean values present in the Earth's crust have been used as the background concentration of metals.

According to Relation 1, in addition to the background values, some other values are required as the reference metal. Typically, the metal chosen as the reference metal has the minimum correlation coefficient with other heavy metals, and it mostly originates from natural sources. In this study, to select the reference metal in calculations related to the EF, correlation coefficients of heavy metals with each other were calculated and presented in Table 5. The correlation coefficients in this table are Pierson coefficients.

As can be observed in Table 5, manganese, lithium, and cadmium metals do not have a considerable correlation with other metals. In most cases, however, they show negative correlation coefficients with other pollutants. Therefore, they have different possible emission sources from other pollutants. Cadmium is found in trace amounts in the Earth's crust and typically human activities cause elevated concentration of this pollutant in the water, soil, and air.

There are also some studies in which lithium have been used as the reference metal for normalizing the calculations (Niencheski, et al., 2002; Loring, 1991). Therefore, lithium concentration was used for reference values. Using the concentration of the studied heavy metals across the 50 sampling stations, their mean concentration in the Earth's crust in Table 4 and Relation 1, EF related to each metal was calculated and presented in Table 6.

The metals with maximum EF of over 10 may mostly be due to human activities (Yongming et al., 2006). In any case, the high values of this factor represent enrich-

Table 5. The correlation coefficient values of heavy metals with each other

	Copper	Cadmium	Lead	Chromium	nickel	Manganese	Zinc	Iron	lithium
Copper	1/000								
Cadmium	-0/124	1/000							
Lead	0/778	-0/260	1/000						
Chromium	0/653	-0/309	0/675	1/000					
nickel	0/645	-0/351	0/534	0/782	1/000				
Manganese	-0/231	0/211	-0/156	-0/259	-0/401	1/000			
Zinc	0/678	0/129	0/529	0/427	0/342	0/237	1/000		
Iron	0/873	-0/335	0/746	0/709	0/667	-0/117	0/589	1/000	
lithium	-0/167	-0/239	-0/272	-0/007	0/185	0/338	-0/119	-0/154	1/000

ment and possible risks of metals. Accordingly, based on the obtained results, it can be said that copper, cadmium, lead, and zinc are probably a result of human activities. At least it can be stated there is a high risk factor for the human health with exposed to these dusts.

As can be seen in Table 6, it is observed that cadmium shows a large enrichment factor. As the reference concentration of this metal is very low (0.2), thus cadmium is possibly due to human activities and could be the riskiest metal among the studied metals here in the urban dusts of Tabriz. The same situation applies to lead.In calculating enrichment factor which lithium as the reference metal, the EF of nickel and chromium is close to 2, and thus they can be partly due to human activities. In this state, the mean EF of iron and manganese is larger than 2, thus showing a medium enrichment level. To identify the human or natural origin of the studied elements, principal component analysis and cluster analysis have been used, and the results were then compared.

Cluster analysis and Pearson correlation coefficient

To identify the possible emission sources of pollutants, Pearson correlation coefficient was calculated using MVSP software, with the calculation results presented in Table 5. Based on the table, copper, chromium, lead, nickel, zinc, and iron have high correlation coefficients. Therefore, they share common possible emission sources. Lithium has the largest correlation coefficient with manganese. As can be observed, cadmium does not have a considerable correlation coefficient with other pollutants, suggesting a different emission source. To enhance the accuracy of the results, the dendrogram related to these calculations which shows the Pearson correlation coefficient of pollutants with each other can be observed in Fig. 2. Based on this figure, the emission sources can be categorized into three main groups A, B, and C. copper, iron, chromium, and metal lie in Cluster A, with a correlation coefficient of larger than 0.6. Therefore, they may share a common emission source. Zinc, with also a correlation coefficient of larger than 0.5, joins these metals and has almost the same emission source.

Fig. 2. The dendrogram for cluster analysis of the pollutants present in street dusts (source: MVSP software)

Considering the components constituting this cluster, nickel, chromium, iron, lead, and copper may have different emission sources including combustion of fossil

Table 6. The spectrum of the EF values obtained considering lithium as the reference metal across the samples

EF values / Heavy metals	Mean	Minimum	Maximum	Standard deviation	median
Cadmium	42/45	61/29	309/42	48/45	117/01
Proper	7/09	2/32	50/25	7/07	7/49
Chromium	0/39	0/5	1/92	0/35	0/65
Iron	1/25	0/89	7/99	1/22	2/43
Nickel	0/62	0/35	3/25	0/59	0/82
Lead	31/04	8/21	188/34	32/02	31/15
Zinc	12/15	12/17	64/68	12/99	23/35
Manganese	0/95	1/22	4/55	0/96	2/79

Table 7. The matrix of the rotational components of the pollutants present in the street dusts of Tabriz

Heavy metal	Factor 1	Factor 2	Factor 3
Copper	0.915	-0.232	-0.065
Cadmium	-0.202	-0.292	0.705
Lead	0.826	-0.214	-0.122
Chromium	0.800	0.081	-0.381
nickel	0.721	0.092	-0.533
Manganese	-0.005	0.558	0.124
Zinc	0.791	-0.006	0.462
Iron	0.916	-0.147	-0.153
lithium	-0.045	0.890	-0.138

fuels, sources originating from iron alloys (corrosion) and possibly earth sources containing iron element to some extent. As nickel exists in heavy fossil fuels as well as gas oil, it is also possible that some of the elements of this cluster may have originated from combustion of heavier fuels and other heavy hydrocarbons sources such as bitumen for covering the passages.

In the second main branch lies only cadmium. Considering the close-to-zero correlation coefficient it has with Group C, and negative coefficient in conjunction with Group C it has with Group A, its source of emission in street dusts is different from the source of other pollutants. Manganese and lithium are in the branch C, showing a negative and close-to-zero correlation coefficient with other pollutants in other branches.It can be stated that the main source of emission of lithium is the nature. However, regarding manganese, as it has a far larger concentration than the mean concentration in the Earth's crust, in addition to natural origin and local soils, it may also have human sources, which are clearly different from the sources of other heavy metal studied here.

Principal component analysis (PCA)

Using PCA and SPSS 18.0, the accuracy of the results obtained from the previous analyses was examined. The principal factors extracted with a characteristic value of over 0.7 were chosen, with Table 7 revealing the values of the matrix containing the rotational components of these factors. As can be observed, three main factors were obtained from PCA.

The main factors larger than 0.5 in each group of Table 7 have been shown. Copper, lead, chromium, nickel, iron, and zinc have considerable back is larger than 0.7, and thus they share the same emission source, which are human sources. As could be predicted, manganese and lithium in the second group with the factors larger than 0.5 are linked to each other and share the same emission source, which are probably natural sources.

With negative factors values or the very low values it shows with other substances, cadmium has lied in the third group and has a different source compared to other pollutants. Therefore, it can be stated that factors 1 and 3 indicate different human sources, but factor 2 represents natural emission sources.

The three different analysis used for identifying the emission sources of the pollutants presented almost the same results. Therefore, the different sources of production of these pollutants can be categorized into the three following groups:

Group I: copper, lead, chromium, nickel, zinc, and iron lie in this group. These pollutants are most probably due to human activities. Studies have shown that the main sources of emission of lead in street dusts include additives added to vehicle fuels. Chromium, copper, and zinc originated from wear of the alloys used in vehicles as well as other services and metal materials. Iron is also used in coverage of vehicles. Therefore, the wear of

Table 8.

metals / Sampling regions	Zinc	Chromium	Lead	Cadmium	copper	RI	Ecological risk
Kasaei Expressway	15.1	0.91	180.1	1602.55	41.55	1938.1	Very high
Imam Khomeini Street	10.32	0.78	85.5	1697.17	27.51	1718.95	Very high
Azadi Street	10.5	0.61	58.95	1718.5	16.25	1705.8	Very high
Shotorbanan Street	10.1	0.59	75.58	1605	17.45	1706.05	Very high
17 Shahrivar	5.39	0.48	39.02	1748	5.89	1796.4	Very high
Southern passenger terminal	11.09	0.69	100.15	1713	24.91	1848.89	Very high
Tabriz University	12.65	0.53	18.71	1602.34	18.81	1698.96	Very high

the cover used in vehicles can enhance the concentration of this element in street dusts. Industrial activities could also be considered sources for emission of these elements in street dusts. However, as the sampling was performed on Regis inside the city and the margin of streets, where no factory or a special industry existed around the streets, the main source can be considered wear of pieces used in vehicles. Combustion of fossil fuels and the oils used in vehicles are among the main sources of producing nickel. The maximum concentration of the pollutants in this group belongs to Kasaei Expressway, which is more crowded than other regions. Therefore, the high rate of vehicle traffic is the main source for emission of these pollutants.

Group II: cadmium is used in producing batteries, plastic, and construction materials. In this study, administrative and residential buildings were abundant around the streets. Therefore, wear of tires and battery of vehicles as well as construction materials seems to be the main source of cadmium emission. In any case, combustive origin for cadmium is unlikely, but its human origin in the city and considering the illusion intensity is evident.

Group III: manganese and lithium like in this group. Regarding the considerable correlation lithium has with manganese and considering the relatively high concentration of manganese in dusts, it seems that it is the origin of some part of manganese present in the dusts of natural sources containing lithium (such as the regional soil), and the origin of some part of it includes human sources dissimilar to the sources of other metals.

Ecological risk

To investigate the ecological risk of the sampling stations, E_r and RI values were calculated by Relation 2, with the results presented in Table 8.

All of the sampling stations indicate high ecological risk. The maximum ecological risk is associated with Kasaei Expressway, which is one of the most crowded expressways of Tabriz. The minimum risk also belongs to the dusts inside Tabriz University. Considering the minor commute of vehicles in this point, the large space of the University, the extent of the green space, and the distance between the sampling point and the peripheral lines of the University and its surrounding streets in relation to the other sampling points, the obtained results seem to be absolutely logical. Furthermore, although Tabriz University is not considered a crowded region for vehicles, this point of sampling also shows a high ecological risk. Possibly, blow of wind causes displacement of polluted dusts of streets around the University, thereby elevating its ecological risk. The mean RI across the sampling points of the south of Tabriz indicates that the ecological risk and concentration of pollutants in this part of Tabriz are high and serious.

CONCLUSION

In this study, the concentration of nine heavy metals was measured across 50 samples of street dusts in Tabriz city. Furthermore, calculation of ecological risk resulting from emission and identification of different sources of heavy metals in street dusts were performed. Based on the calculations and the analyses, three main sources including high traffic of vehicles (the pieces used in vehicles and combustion of fossil fuels), the pieces used in buildings, and natural sources are among the factors for emission of heavy metals in street dusts. Using the calculations related to the ecological risk, all of the stations indicated high risk. Therefore, the health risks of exposure, inhalation, and possible swallowing of particulate matters of these dusts across Tabriz regions are very high. Thus, more detailed studies are required to investigate the effects and risks resulting from this issue across Tabriz.

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A comparative analysis of overall codon usage pattern of Louping Ill virus with natural livestock host and associated vector

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ABSTRACT

Louping ill is a zoonotic viral disease caused by louping ill virus (LIV) which is a member of genus *Flavivirus* in the family *Flaviviridae*. This febrile illness to livestock can further develop into fatal encephalitis .The virus LIV is closely related to tick-borne encephalitis virus and occurs wherever the primary vector tick (*Ixodes ricinus*) is found. To understand the viral evolution, comparison and analysis of the codon usage of LIV, its vector, and the host is important. The present study reports the pattern of codon usage in LIV, its vector, and the host by calculating the Effective number of Codons (ENC), Codon Adaptation Index (CAI), and Relative Synonymous Codon Usage (RSCU) and other indicators. The results indicate relatively low codon usage bias of LIV. The ENC - plot demonstrates the substantial role played by mutation pressure. The comparative analysis of CAI among virus, vector and its host, indicates that the virus is more adaptive to the host than the vector. A comparative analysis of RSCU between virus, vector, and its host shows that the codon usage pattern of LIV is a mix of coincidence and antagonism. To the best of our knowledge, this is the first report describing codon usage analysis of LIV and findings are expected to increase our understanding of factors involved in viral evolution and fitness toward vector and host.

KEY WORDS: CODON USAGE, EVOLUTION, LOUPING ILL VIRUS (LIV), EFFECTIVE NUMBER OF CODONS, RELATIVE SYNONYMOUS CODON USAGE

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INTRODUCTION

Louping ill virus (LIV) is a tick-born member of the genus *Flavivirus* in *Flaviviridae* family. It is a positive single stranded, 40-50 nm RNA virus whose genome comprises a single open reading frame (ORF) that is approximately 11 kb in length (Grard *et al*.,2007;Jeffries *et al*., 2014). The ORF encodes a polyprotein that consists of three structural and seven non-structural proteins. The virus show high degree of genetic homology to tick-borne encephalitis virus (TBEV) of the same family (McGuire *et al*., 1998; Jiang *et al*., 1993). It is mainly transmitted by ticks and the primary vector is *Ixodes ricinus* (Dobler *et al*., 2010).LIV mainly causes febrile illness in sheep, cattle, horse, pigs and some other animals that may eventually result in fatal encephalitis.

Sheep are the most important reservoir host for LIV. The disease is dominantly detected in animals from upland areas of British Isles (Gao *et al*., 1997) though the disease is also reported in Scotland, Ireland, and northern England where the tick vector *Ixodes ricinus* is found. Infection with LIV was first reported in sheep of Basque region of northern Spain in 1987 (Gonzalez *et al*., 1987). Most of the cases of LI infection occur in spring / early summer when ticks are common. In endemic areas morbidity and mortality depends upon animal's immune status, concurrent infection and other factors. All age group of animal get infected by it and once encephalitis is developed the case fatality rate goes up to 50%. The mortality rate is even higher in animals that are less than two years old. Currently, there is no specific treatment for LIV with only supportive therapies being helpful to some extent (Hyde *et al*., 2007 Mansfield *et al*., 2015 Butt et al., 2016).

The molecular sequence data started to be accumulated nearly 20 years ago. It was observed that the genetic code is redundant and most amino acids can be translated by more than one codon (Wang *et al*., 2011). This redundancy is a key factor regulating the efficiency and accuracy of protein production.Alternative codons within the same group that encode the same amino acid are often called 'synonymous' codons. These codons are not randomly selected within and between genomes. This is referred to as 'codon usage bias' (CUB). CUB are widespread across the tree of life and are influenced by mutation pressure, natural or translational selection, secondary protein structure, replication, selective transcription, hydrophobicity and hydrophilicity of the protein, and the external environment (Xiang *et al*., 2015 Butt *et al*., 2016 Mune *et al.,* 2017).

As viruses are intracellular pathogens they have to co-evolve with host molecular mechanisms. The interplay between the codon usage of the virus and its host is expected to affect the overall viral survival, fitness, evasion of the host immune system and evolution. The knowledge of the codon usage of viruses can provide information about their molecular evolution and extend our understanding of the regulation of viral gene expression. This may also offer significant improvement in vaccine design for which the efficient expression of viral proteins may be required to generate immunity (Tao *et al*., 2009 Velazquez *et al*., 2016). To gain insight into the characteristics of the viral genome and evolution, the codon usage patterns of the three components of transmission cycle, namely - the virus (LIV), vector (*Ixodes ricinus*), and hosts (Sheep (*Ovis aries*), Pig (*Sus scrofa*) and cattle (*Bos taurus*)) were investigated in our study.

MATERIALS AND METHODS

SEQUENCE DATA

The complete genome sequences were downloaded from the National Centre for Biotechnology (NCBI) database (http: //www.ncbi.nlm. nih.gov) in FASTA format. The detailed information (accession numbers, country, sequence length etc.) of the selected genomes were listed [Table. S1]. Open reading frames (ORF) of all the genomic sequences were identified by using NCBI ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). The host (*Ovis aries, Sus scrofa* and *Bos taurus*) and vector (*Ixodes ricinus*) codon usage were obtained from the Codon Usage Data Base (CUD).

CODON USAGE ANALYSIS

The overall frequency of occurrence of the nucleotides (A %, C %, U %, and G %) was calculated along with the frequency of each nucleotide at the third site of the synonymous codons (A_3, C_3, U_3 and G_3).Also the overall GC, AU and GC_3 content were calculated using MEGA7 software to investigate the compositional properties of coding region of LIV. To investigate the codon usage pattern, the RSCU (Relative synonymous codon usage) values for synonymous codons were calculated according to the published equation (Sharp *et al*., 1986). The stop codons (UAA, UAG and UGA) and AUG for Met, UCG for Try were not introduced into the RSCU analysis. Further, ENC (Effective number of codon) values were calculated to measure the magnitude of codon usage bias in the coding sequences of viral genome. The ENC value ranges from 20 (when only one synonymous codon is chosen by the corresponding amino acid) to 61 (when all synonymous codons are used equally). A low ENC value indicates a strong codon usage bias (Wright *et al*., 1990; Zhang *et al*., 2011 Butt *et al*., 2013).

The CAI (Codon adaptation index) was used to estimate the adaptation of LIV to its host and vector codons.

CAI values range from 0 to 1. A higher CAI score for a given gene indicates more similarity between its codon usage and the predefined reference set, using the CAIcal approach (available at: http://genomes.urv.es/CAIcal) (Puigbo *et al.*, 2008).

RESULTS AND DISCUSSION

SYNONYMOUS CODON USAGE IN LIV

The preference for one type of codon over another can be greatly influenced by the nucleotide composition of genome. We first analysed nucleotide composition and observed that the nucleotides A and G were higher and followed by C and U (Table 1) The LIV genome is rich with G content having a mean value of 32.17. For a better understanding we analysed nucleotide composition at third position of codon and observed the dominance of G_3 nucleotide with a mean value of 34.20. Even the percentage of dinucleotide with G is higher compared to dinucleotide with other nucleotides (respective mean values for GC, AU, GC_3 and AU_3 being 54.74, 45.26, 60.74, and 39.26).

To investigate the extent of codon usage bias, the ENC values among LIV genome were calculated. An average value of 53.97 represents stable ENC value (ENC > 40) (Mune *et al.*, 2017) which suggests that the genomic composition of LIV is conserved. The result shows that the codon usage of LIV is slightly biased and mainly affected by the nucleotide composition. To further understand the codon usage pattern, the analysis of ENC - plot (ENC value V/s GC_3 content) was carried out. It is observed that all points lie below the expected curve (Fig.1). This implies that the codon usage bias is mainly affected by nucleotide composition (in other words - by mutation pressure).

To further explore the codon usage preferential optimization and adaptation of LIV in relation to its vector and hosts CAI analysis was performed. CAI values were calculating keeping *Ixodes ricinus, Ovis aries,Sus scrofa* and *Bos taurus* codon usage as a reference set. A mean CAI value of 0.658 was obtained for the LIV ORFs in relation to primary vector *Ixodes ricinus* codon usage reference set and mean CAI values of 0.623, 0.689 and 0.711 were obtained for the LIV ORFs in relation to host pig , sheep and cattle (*Ovis aries,Sus scrofa* and *Bos taurus*) codon usage reference set respectively. In this study we found a tendency for higher CAI values indicating lower efficiency of translation. A comparison between vector and host indicated a lower CAI for LIV in relation to pig, which leads to lower efficiency of protein synthesis in pig. This suggests that the interplay of codon usage between LIV and its hosts may influence viral fitness, survival and evolution.

Table 1. Nucleotide composition analysis of LIV genome (IR: *Ixodes ricinus*, SS: *Sus scrofa*, OA: *Ovis aries*, BT: *Bos taurus*)

Accession no.	U	C	A	G	U3	C3	A3	G3	AU	GC	AU3	GC3	GC12	ENC	CAI^{IR}	CAI^{SS}	CAI^{OA}	CAI^{BT}
NC_001809.1	20.72	22.67	24.47	32.13	18.57	26.53	20.85	34.06	45.19	54.81	39.41	60.59	30.29	53.88	0.658	0.622	0.691	0.711
Y07863	20.72	22.67	24.47	32.13	18.57	26.53	20.85	34.06	45.19	54.81	39.41	60.59	30.29	53.88	0.658	0.622	0.691	0.711
KT224354.1	20.81	22.48	24.57	32.14	18.62	26.47	20.67	34.23	45.38	54.62	39.30	60.70	30.35	54.12	0.657	0.623	0.687	0.711
KP144331.1	20.61	22.59	24.60	32.20	18.16	26.68	20.94	34.23	45.21	54.79	39.09	60.91	30.45	53.89	0.658	0.624	0.689	0.711
KJ495985	20.81	22.48	24.58	32.13	18.62	26.47	20.67	34.23	45.39	54.61	39.30	60.70	30.35	54.15	0.657	0.623	0.687	0.710
KF056331.1	20.74	22.54	24.45	32.27	18.48	26.56	20.59	34.38	45.19	54.81	39.06	60.94	30.47	53.93	0.658	0.622	0.690	0.711
Avg.	20.74	22.57	24.52	32.17	18.50	26.54	20.76	34.20	45.26	54.74	39.26	60.74	30.37	53.97	0.658	0.623	0.689	0.711
Std. D	0.0722	.0890	.0652	.0561	.1780	.0756	.1361	.1234	.0961	.0961	.1532	.1532	.0766	.1261	.0005	.0008	.0018	.0004

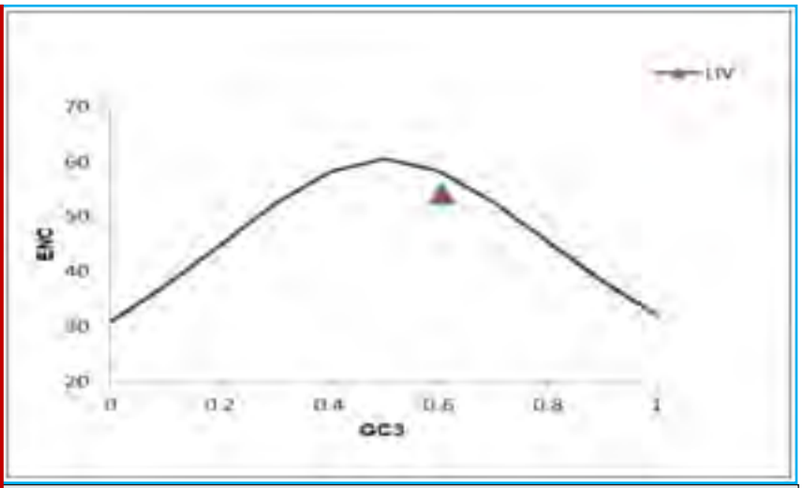

FIGURE 1. Graph showing the relationship between the effective number of codons (ENC) and GC content of the third codon position (GC3).The curve indicates the expected codon usage if GC compositional constraints alone account for codon usage bias.

To investigate the codon usage pattern of virus, an RSCU analysis was performed for the 59 sense codons (Table.2). In LIV among the 18 most abundantly used codons, 12 were G/C-ended (five G-ended, seven C-ended) and the remaining six were A/U-ended (five A-ended and one U-ended).

To determine the potential influences of the vector and host on the codon usage pattern of the LIV, the RSCU pattern of LIV coding sequence were correlated with those of *Ixodes ricinus* (vector) and pig, sheep and cattle (hosts) (Fig.2).All the 18 most abundantly used codons of vector and host were G/C ending (In *Ixodes ricinus* twelve C-ended and six G-ended, Pig thirteen C-ended and five G-ended, cattle twelve C-ended and six G-ended, and in sheep eleven C-ended codons six G-ended codons and one U-ended codon) we observed a common pattern of preference towards G/C-ended codons in vector and host. An analysis of over and under - represented codons showed that for LIV 4 out of 18 preferred codons (CUG for Leu, GUG for Val and AGA and GGA for Arg) in *Ixodes ricinus* 11 out of 18 preferred codons (CUG for Leu, AUC for Ile, GUG for Val, AGC for Ser, CCC for Pro, ACC for Thr, GCC for Ala, CAC for His, UGC for Cys, AGG for Arg and GGC for Gly), in cattle 3 out18 preferred codons (CUG for Leu, GUG for Val and GCC for Ala), in sheep 5 out of 18 preferred codons (CUG and CUC for Leu, AUC for Ile, GUG for Val and ACC for Thr), and in pig 6 out of 18 preferred codons (CUG for Leu, AUC for Ile, GUG for Val, AGC for Ser and ACC for Thr, GCC for Gly) had RSCU value >1.6, whereas the remaining preferred codons had RSCU values >0.6 and <1.6. CUG for Leu and GUG for Val are common over-represented codons in virus vector and hosts.

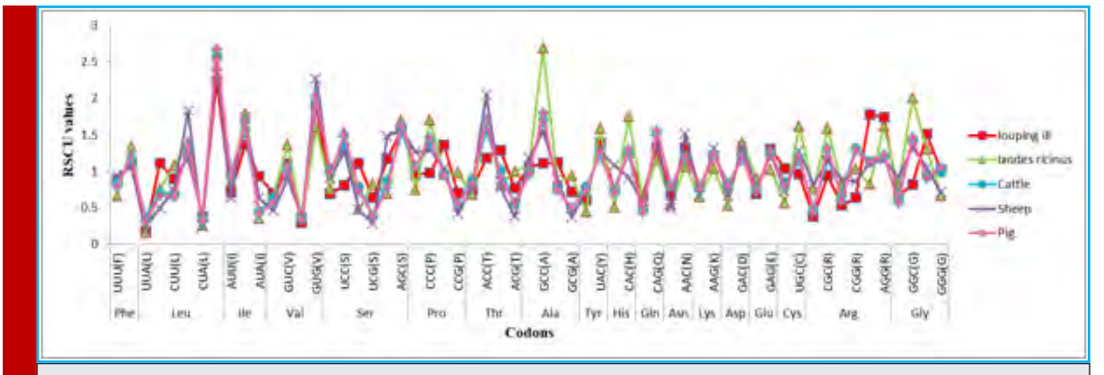

FIGURE 2. Comparative analysis of relative synonymous codon usage (RSCU) patterns between virus, vector and three hosts (cattle, sheep and pig).

Table 2. The relative synonymous codon usage patterns of LIV, its host (cattle, sheep and pig) and primary transmission vector (*Ixodes ricinus*)

AA	Codon	Pathogen	Vector	Host		
		louping ill	Ixodes ricinus	Cattle	Sheep	Pig
Phe	UUU	0.88	0.66	0.85	0.94	0.79
	UUC	1.12	1.34	1.15	1.06	1.21
Leu	UUA	0.18	0.16	0.38	0.24	0.32
	UUG	1.11	0.75	0.71	0.49	0.67
	CUU	0.90	1.08	0.7	0.74	0.65
	CUC	1.2	1.40	1.26	1.83	1.35
	CUA	0.37	0.26	0.36	0.24	0.33
	CUG	2.24	2.45	2.59	2.46	2.68
Ile	AUU	0.71	0.85	0.98	0.63	0.91
	AUC	1.36	1.79	1.57	1.74	1.67
	AUA	0.93	0.36	0.45	0.63	0.42
Val	GUU	0.7	0.68	0.64	0.46	0.57
	GUC	1.1	1.36	1.01	0.91	1.07
	GUA	0.29	0.35	0.4	0.36	0.34
	GUG	1.92	1.61	1.95	2.27	2.03
Ser	UCU	0.69	0.76	1.04	0.91	0.99
	UCC	0.81	1.54	1.37	1.28	1.5
	UCA	1.11	0.48	0.79	0.48	0.73
	UCG	0.64	0.83	0.39	0.28	0.39
	AGU	1.17	0.69	0.87	1.48	0.77
	AGC	1.58	1.70	1.53	1.58	1.62
Pro	CCU	0.96	0.75	1.08	1.26	1.05
	CCC	0.98	1.70	1.39	1.29	1.46
	CCA	1.36	0.96	1	1.03	0.94
	CCG	0.7	0.98	0.53	0.42	0.56
Thr	ACU	0.75	0.68	0.89	0.78	0.83
	ACC	1.18	1.71	1.55	2.05	1.68
	ACA	1.29	0.82	1.01	0.78	0.92
	ACG	0.77	1.00	0.56	0.38	0.57
Ala	GCU	1.06	1.07	1	1.18	0.96
	GCC	1.11	2.69	1.71	1.55	1.8
	GCA	1.12	0.84	0.8	0.9	0.74
	GCG	0.72	0.95	0.48	0.37	0.5
Tyr	UAU	0.61	0.45	0.79	0.72	0.73
	UAC	1.39	1.59	1.21	1.28	1.27
His	CAU	0.75	0.50	0.75	1.08	0.7
	CAC	1.25	1.75	1.25	0.92	1.3

Gln	CAA	0.66	0.60	0.46	0.57	0.44
	CAG	1.34	1.16	1.54	1.43	1.56
Asn	AAU	0.68	0.55	0.81	0.49	0.79
	AAC	1.32	1.07	1.19	1.51	1.21
Lys	AAA	0.79	0.65	0.78	0.68	0.76
	AAG	1.21	1.04	1.22	1.32	1.24
Asp	GAU	0.8	0.54	0.84	0.66	0.8
	GAC	1.2	1.40	1.16	1.34	1.2
Glu	GAA	0.69	0.91	0.78	0.75	0.72
	GAG	1.31	1.02	1.22	1.25	1.28
Cys	UGU	1.04	0.57	0.85	0.72	0.79
	UGC	0.96	1.62	1.15	1.28	1.21
Arg	CGU	0.38	0.75	0.49	0.82	0.44
	CGC	0.94	1.59	1.17	1.15	1.31
	CGA	0.53	0.80	0.68	0.89	0.6
	CGG	0.64	1.04	1.32	0.86	1.29
	AGA	1.78	0.83	1.14	1.12	1.12
	AGG	1.74	1.62	1.2	1.16	1.23
Gly	GGU	0.66	0.78	0.64	0.92	0.57
	GGC	0.82	2.01	1.43	1.33	1.46
	GGA	1.51	1.31	0.95	1.05	0.91
	GGG	1.02	0.67	0.99	0.71	1.05

None of the preferred codons were under-represented (RSCU<0.6). UUA and CUA for Leu and GUA for Val are common underrepresented codons in virus, vector and hosts. Interestingly, a mixture of coincidence and antagonism was observed in the codon usage pattern as LIV showed no complete coincidence or complete antagonism to any of the patterns of its vector and host. Among the 18 most abundantly used codons, the ratio of coincident/antagonist preferred codon was 12:6 between virus vector and hosts.

CONCLUSION

Our analysis has provided an insight into codon usage pattern of LIV virus and its relationship with host and vector. We observed that the codon usage bias of LIV is

Supplementary Table 1. Detail information about the LIV

Strain Name	Virus Type	GenBank Accession	Sequence Length	ORF	ORF Length	Collection Date	Host	GenBank Host	Country
369/T2	LIV	NC_001809	10871	130-10374	10245	-N/A-	Unknown	-N/A-	-N/A-
369/T2	LIV	Y07863	10871	130-10374	10245	-N/A-	Unknown	-N/A-	-N/A-
LEIV-7435Tur	LIV	KT224354	10829	106-10350	10245	-N/A-	Tick	Hyalomma marginatum (tick)	Turkmenistan
LI3/1	LIV	KP144331	10880	133-10377	10245	1962	Sheep	Ovis aries	United Kingdom
Primorye-185-91	LIV	KJ495985	10871	129-10373	10245	07/22/1991	Human	Homo sapiens	Russia
Penrith	LIV	KF056331	10875	132-10376	10245	2009	Sheep	Ovis aries	United Kingdom

slightly biased which reflects that the key role played by mutation pressure and natural selection. Our observations suggest that codon usage of LIV is an evolutionary process However, a more comprehensive analysis with higher sample sizes is needed as this study and subsequent analysis is based on a relatively small sample size.

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A survey on analysis of physico-chemical parameters of flowing water of Langting stream of Dima Hasao Assam, India

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ABSTRACT

Langting stream of Dima Hasao district of Assam originates from Nobdi Langting, the border area of Nagaland, Manipur and Dima Hasao and confluents to Diyung river (originates from Borail range). It is about 38 km from Lumding and is one of the important sources of water for the people living near the river. All the domestic sewage, industrial effluents and solid waste find its way to this stream via channels which may affect the quality of flowing water. In the proposed study an attempt has been made to analyse certain physico-chemical parameters of Langting stream and assess the water quality. From the data of the studies carried out on riparian vegetation it was found that it is at an alarming state and is a subject of great concern. The management of riparian areas is a vital environmental and economic. The values of the physico-chemical parameters of surveyed hill streams are within the permissible limits. Though there are monthly fluctuations in the physico-chemical parameters of surveyed hill streams but the result of the physico-chemical parameters reveals that the water quality is still good and the streams are very productive nature.

KEY WORDS: LANGTING STREAM, WATER QUALITY, PHYSIOCHEMICAL PARAMETERS, POLLUTION

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INTRODUCTION

In general, out of total land water 1% is available for agriculture, drinking domestic, purpose power generation, industrial consumption, transportation and waste disposal (Mishra et al 2002, Gupta et al. 2007). Although Dima Hasao, a hill district in Assam is relatively free from pollution as there are no major industries in the district. However, mining activities and of organic pollution due to household materials gradually polluting in certain areas. Langting stream of Dima Hasao district is a tributary of Diyung river. People living near the river directly pollute the water by taking bath, washing clothes, vehicles and utensils in it. All the domestic sewage, industrial effluents and solid waste find its way to this stream via channels which may affect the quality of water and create health problems, (Raja et al., 2002). The physical and biological characteristics of water determine the quality of water (Diersing, et. al., 2009). The significant changes in these physicochemical parameters lead to assess the water quality of Langting stream through analysis. The salinity, HCO_3, pH, depth, water temperature are responsible for variations in phytoplankton community (Sharif et al 2017).

In eastern Himalayan lotic ecosystem of different environmental factors anthropogenic and other factors may be considered as an components of pollution, (Chowdhury et. al 2017). There is a direct interrelation between physicochemical and biological parameters (Kaur 2017). Water velocity is also an important factor affecting aquatic fauna, (Singh et. al. 2017).

MATERIAL AND METHODS

Langting stream segment was divided into two parts-up and down stream with a length of 8 km. The selected parameters were studied on seasonal basis. The stretches were demarcated into five sampling stations viz: S1, S2, S3, S4 and S5. Water samples were collected from these 5 sampling sites from various locations of river Langting monthly during the study period. From each locality five samples were collected randomly in cans (IL). Except for dissolved oxygen (DO) where 300 ml glass stoppers were used for sample collection and then brought to laboratory for analysis. Following key parameters such as water temperature, pH, depth, water flow and conductivity were determined in the field because of their unstable nature. Water temperature was recorded by mercury thermometer, turbidity with Seccehi disc, pH with pen meter, (model Hanna:H196107) conductivity meter (Hanna H196303) and water current by flow meter. The laboratory analysis of the samples was done using standard methods, (APHA 1998). Alkalinity and free CO_2 were determined by titration method. Dissolved oxygen (DO) was determined by Winker's modified method.

Table 1a. showing details of sampling sites

Sampling site	Name	GPS position
S1	Langting I	25°29˜48N 93°6˜45E
S2	Langting II	25°26˜40N 93°6˜99E
S3	Langting III	25°28˜52N 93°49˜9E
S4	Langting IV	25°26˜50N 93°48˜20E
S5	Langting V	25°30˜25N 93°46˜29E

RESULTS AND DISCUSSION

The analysis of various physico-chemical parameters of Langting stream at stations S1, SII, SIII, SIV, SV, are represented in Table-1(b,c,d e f). Air temperature: The average of air temperature was found to be highest in monsoon. The atmospheric temperature recorded post monsoon varied from 22.67˚C to 25˚C with the lowest mean recorded from S_3 (22.67˚C). Season wise analysis showed that the mean temperature of water surface was highest during monsoon period. The rise in temperature during monsoon declined with the advent of post-monsoon period. Highest mean was recorded from S_1 (28.53˚C) and the lowest mean from S_3 (19.77˚C). Water temperature was optimum during pre-monsoon but the temperature remained lesser than atmospheric throughout the entire period of study. It showed positive correlation with depth, atmospheric temperature. Water temperature showed negative correlation with pH, TDS, electrical conductivity, dissolved oxygen and phosphate. In S_2, it showed negative correlation with pH, DO and PO_4. Water temperature is responsible for variation in Phytoplankton community, (Sharif et. al 2017).

The present study recorded low values of turbidity. The temporal and spatial variation in turbidity showed a similar trend in sampling sites. Turbidity showed positive correlation with depth, flow, Free CO_2. The rain water brought large amount of dissolved and suspended inorganic and organic material that make water turbid and causes lower transparency in the rainy months, (Sawant et al., 2010, Tims and Midglay 1970). The pH of the sampling sites were found to be slightly alkaline during study period and showed range of variation during sampling sites. The pH showed a decreasing trend during the monsoon which gradually increased during post-monsoon season. Maximum value of pH was recorded at S_2 (8.73) and minimum value at S_3 (6.54) in Langting stream. The pH value between 6.5 and 8.5 usually indicate good water quality, (Baruah and Hazarika, 2011). pH values of 5.5 or less are considered as risky,

Table-1(b). Physicochemical parameters of Langting stream (pre-monsoon)

Station	Depth (m)	Flow (m/s)	Air temp. (°C)	Water temp (°C)	Turbidity (NTP)	pH	TDS (µs/cm)	Conductivity (µs/cm)	DO (ppm)	Free CO2	PO4	Alkalinity
S_1	0.5	2.40	28.72	24.5	2.68	7.72	45.2	70.2	6.92	4.51	0.02	48.17
S_2	0.38	1.0	28.54	25.6	3.32	8.12	47.2	72.8	6.50	8.06	0.04	50.20
S_3	0.48	2.3	27.62	23.7	3.29	7.8	48.6	68.8	7.2	7.05	0.07	55.26
S_4	0.41	2.1	27.58	24.2	2.82	7.1	47.5	70.3	6.8	8.05	0.09	54.28
S_5	0.49	1.07	28.01	25.6	2.88	7.3	46.8	71.2	6.6	7.04	0.11	49.26

Table-1(c). Physicochemical parameters of Langting stream (monsoon)

Station	Depth (m)	Flow (m/s)	Air temp. (°C)	Water temp (°C)	Turbidity (NTP)	pH	TDS (µs/cm)	Conductivity (µs/cm)	DO (ppm)	Free CO_2	PO_4	Alkalinity
S_1	1.68	2.9	32.8	28.5	3.52	7.13	28.67	52.33	5.28	7.25	0.02	47.17
S_2	1.38	2.98	31.5	27.8	4.02	7.5	27.14	54.2	4.67	8.49	0.03	44.20
S_3	0.98	2.20	32.2	22.4	3.87	6.7	25.18	53.6	5.1	6.98	0.03	45.26
S_4	1.19	2.13	33.2	24.5	3.82	7.2	26.23	52.13	5.2	7.26	0.02	44.28
S_5	1.25	2.23	32.5	27.1	4.12	7.2	26.29	52.18	5.18	9.01	0.03	44.26

Table-1(d). Physicochemical parameters of Langting stream (post-monsoon)

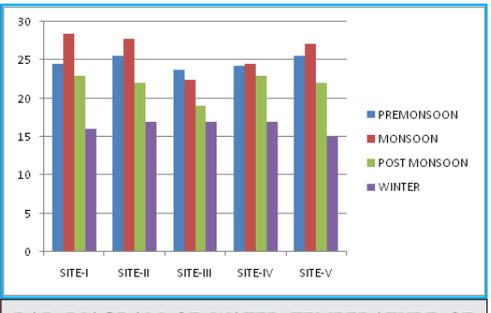
Station	Depth (m)	Flow (m/s)	Air temp. (°C)	Water temp (°C)	Turbidity (NTP)	pH	TDS (µs/cm)	Conductivity (µs/cm)	DO (ppm)	Free CO_2	PO_4	Alkalinity
S_1	0.8	1.7	22.9	23	2.82	7.6	45.8	70.25	8.67	5.3	0.09	66.2
S_2	0.72	1.2	24	22	3.42	8.73	48.6	74.62	8.23	6.2	0.12	67.2
S_3	0.52	1.2	22.67	19	2.62	6.54	44.5	71.28	8.3	5.2	0.08	68.2
S_4	1.02	1.3	23	23	3.45	7.2	47.6	73.28	5.67	5.8	0.07	67.6
S_5	1.03	1.1	24	22	2.82	7.8	23.67	72.66	7.85	5.7	0.09	67.2

Table-1(e). Physicochemical parameters of Langting stream (winter)

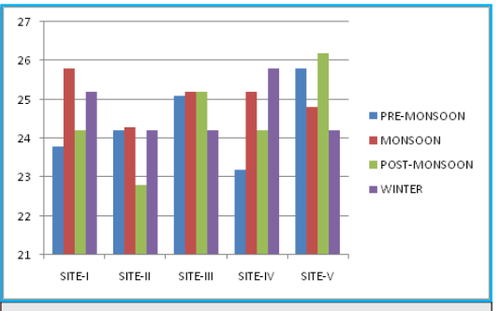
Station	Depth (m)	Flow (m/s)	Air temp. (°C)	Water temp (°C)	Turbidity (NTP)	pH	TDS (µs/cm)	Conductivity (µs/cm)	DO (ppm)	Free CO_2	PO_4	Alkalinity
S_1	0.5	1.2	19	16	1.99	7.2	45.8	70.25	8.2	5.3	0.09	76.1
S_2	0.54	1.1	20	17	1.82	7.8	48.6	74.62	8.4	6.2	0.12	76.2
S_3	0.42	1.1	19	17	1.34	7.7	44.5	71.28	8.3	5.2	0.08	65.2
S_4	.6	1.2	20	17	1.45	7.1	46.2	73.28	7.9	5.8	0.07	67.5
S_5	.9	1.3	18	15	1.76	7.8	46.5	72.66	7.85	5.7	0.09	58.2

Table-1(f). Correlation Coefficient of Physicochemical parameters of Langting stream

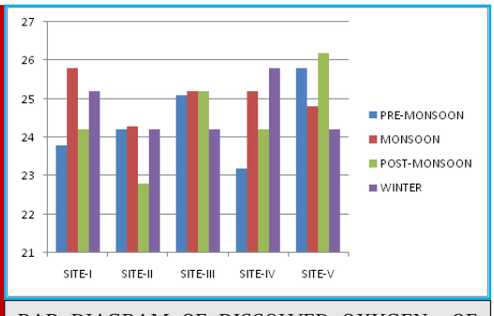
	Depth (m)	Flow (m/s)	Air temp. (°C)	Water temp (°C)	Turbidity (NTP)	pH	TDS (µs/cm)	Conductivity (µs/cm)	DO (ppm)	Free CO_2	PO_4	Alkalinity
Depth (m)	1											
Flow (m/s)	-0.069	1										
Air temp. (°C)	0.521	-0.482	1									
Water temp (°C)	0.315	0.661	-0.516	1								
Turbidity (NTP)	0.585	-0.240	-0.288	-0.246	1							
pH	0.115	-0.249	0.067	-0.032	0.058	1						
TDS (µs/cm)	-0.784	0.192	-0.638	-0.084	-0.573	0.001	1					
Conductivity (µs/cm)	-0.033	-0.782	0.354	-0.582	0.005	0.425	-0.108	1				
DO (ppm)	-0.657	0.540	0.503	0.406	-0.787	0.150	0.644	-0.410	1			
Free CO_2	-0.071	0.468	0.704	0.664	-0.336	0.075	0.142	-0.598	-0.433	1		
PO_4	0.156	0.419	0.458	0.358	-0.554	0.027	0.615	-0.384	-0.275	0.543	1	
Alkalinity	0.238	0.350	0.245	0.067	-0.675	0.170	-0.291	-0.544	0.390	0.579	0.378	1



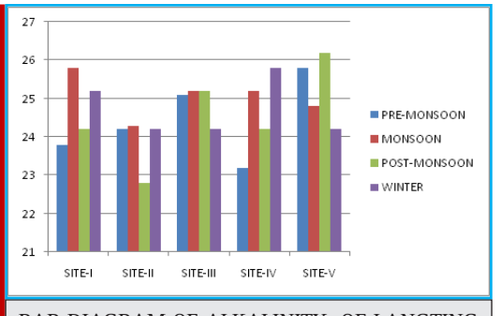
BAR DIAGRAM OF WATER TEMPERATURE OF LANGTING RIVER



BAR DIAGRAM OF FREE CARBON DIOXIDE OF LANGTING RIVER



BAR DIAGRAM OF DISSOLVED OXYGEN OF LANGTING RIVER



BAR DIAGRAM OF ALKALINITY OF LANGTING RIVER

(Sawyer et al 1978). The salinity, HCO3, pH depth are also responsible for variation in phytoplankton community, (Sharif et. al 2017).

Total Dissolved Solids (TDS): The mean of two years data of TDS at the sampling sites showed seasonal cycle, maximum during pre-monsoon and post- monsoon. The values of TDS during pre-monsoon ranged from 43.44 ppm-47.3 ppm with highest mean recorded from S_2(47.2 ppm). During monsoon the values fluctuated between 23.67 ppm-26.65 ppm amongst the sampling sites, lowest mean was recorded from S_3 (24.12 ppm),

Electrical conductivity(EC) showed similar seasonal trend as TDS with high values recorded during pre-monsoon and low values during monsoon. Direct relation was observed between Electrical conductivity and dissolved oxygen in S_1. In S_2 and S_3, it showed positive correlation with DO and phosphate concentration. Inverse relation was observed with depth, flow, atmospheric temperature, Free CO_2 and alkalinity. Free CO_2 recorded from the sampling sites varied from 4.51 ppm -9.01 ppm. During monsoon period relatively high value of FCO_2 was recorded from the sampling sites which ranged from 6.24 ppm – 9.25 ppm and minimum during winter.

Seasonal fluctuation of DO at the sampling sites showed marked variation. Post-monsoon period showed maximum value of DO with highest value 8.67 ppm recorded from S_1 followed by S_2 (8.23 ppm) and S_4 (5.65 ppm). Monsoon period was recorded with low values with minimum value recorded from S_2 (4.67 ppm). Negative correlation was established between DO and depth, Free CO_2 concentration and alkalinity in all the sampling sites whereas DO established positive correlation with pH and phosphate in sampling sites. Dissolved oxygen has been exclusively used as a parameter of delineating water quality and to evaluate the degree of hardness of a river, (Fakayode, 2005). In the present investigation low values of phosphate were recorded which varied within range (0.02 ppm-0.11 ppm). Maximum phosphate content was recorded in post-monsoon from the sampling sites whereas monsoon period showed least phosphate content.

Value of alkalinity recorded from the sampling sites was not very high. Maximum values were sampled in the monsoon months in the sampling sites, while low values were sampled during the post-monsoon and winter period. Alkalinity of water is a measure of weak acid present in it and of cations balanced against them, (Sverdrap et. al 1942). Haemopoiesis is a survival strategy in the characterized habitat specialized fishes inhabiting hill stream, (Chowdhury et. al. 2017).

From various studies carried out on riparian vegetation it was found that it is at an alarming state and is a global concern subject. The management of riparian areas is a vital environmental and economic resource. The finding values of the physico-chemical parameters of surveyed hill streams are within the permissible limits. Though there are monthly fluctuations in the physico-chemical parameters of surveyed hill streams but the result of the physico-chemical parameters reveals that the water quality is still good and the streams are very productive nature.

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Quality assessment of milk samples of Amreli region with nanotechnology based dipstick and other known technology: A comparative study

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ABSTRACT

Milk is important source of nutrient required for the growth human. It is very common to adulterate the milk in supplying chain by the local vendors. This present study is try to explain the hygienic status of milk in Amreli district. Total 100 milk samples were collected from the different region of Amreli district and checked for the presence of urea, detergent, neutralizer and hydrogen peroxide. All the samples were assess by our newly developed nanotechnology based dipstick and REIL EMAT plus for comparative purpose. Later on statistical analysis was also carried out from the obtained result data. Out of 100 collected milk sample, the extent of adulteration is varying from hydrogen peroxide (0 %) to urea (12 %). Due to the malpractice in milk, consumer must aware of this malpractice and they have to be more active and aggressive against the milk adulteration.

KEY WORDS: AMRELI DISTRICT, HYGIENIC STATUS, MILK ADULTERATION, PUBLIC HEALTH

INTRODUCTION

Milk is an important source of nutrient required for growth in infants and children and for maintenance of health in adults. Milk is a perfect food, readily digested and absorbed. It is a sole natural food for infants and children. Milk contains more than 100 substances that are either in solution suspension or emulsion in water, the important being casein - the major protein of milk, lactose - milk sugar, whey and mineral salts. From past few year with the increase of living standard of people and advance technology, there is significantly increased in milk and milk product consumption. Though it is very common in dairy industry to adulterating milk and other dairy product for the cost-cutting purpose or increase the life of dairy product. Addition of such adulterant in

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milk decrease the nutrient value, its quality and flavor and may also cause harm full effect on human health, Karukonda *et al*. (2017) and Brindha *et al*. (2017).

According to the report of Food Safety Standard Authority of India (FSSAI, 2016), in India about 68.4% of milk is adulterated with different adulterants. The common adulterants which can be mixed with milk are starch, urea, hydrogen peroxide, boric acid, detergent, neutralizer, and maltodextrin and ammonium sulphate. These all adulterants mixed with milk for the cost cutting purpose. Consumption of adulterated milk can cause the several health hazardous in both infant and in adult, sometimes it can prove to be fetal, (Makadiya and Pandey 2015).

We have developed innovative method for the instant detection of adulterants in milk. Our developed technique is nanotechnology based dipstick which is quite reliable, portable, instantaneous, cheap, require very low amount of milk sample for the testing. Apart from this, nanotechnology based dipstick can be used at house hold level and it doesn't require any skilled person to handle it. Presence or absence of particular adulterant in milk sample can be detect within second by this dipstick. We have developed total eight individual dipstick for the instant detection of starch, urea, hydrogen peroxide, detergent, neutralizer, maltodextrin and ammonium sulphate.

Unique Selling Point (USP) of our newly developed dipstick is ease of use and instantaneous result. These both the property are very useful for common man to utilize this dipstick for detection of adulterants at house hold level as well as at village cooperative also which is the first entry point of milk collection chain. This dipstick is very simple in which detector pad is adhere at the lower end and reference color tag is adhere at the middle of dipstick. Detector pad of dipstick is dip into milk and if color of detector pad is changed as same as reference color tag, the presence of particular adulterants is confirm.

Public consume fluid milk which has been adulterated and diluted to an extent that there is very little nutritive value left in it resulting to a great extent to general public health concerns and malnutrition Quasid, *et al*. (2007). Keeping in view the above facts, the present study was conducted to achieve the following objectives: To determine the chemical composition of the milk available in local market. To check the hygienic status of market milk. To detect various adulterants in market milk following the method of Rajesh *et al*. (2016).

In this article we have try to explore the comparative analysis between our newly developed nanotechnology based dipstick and standard instrument based technique. We have compare our result with REIL EMAT PLUS (Milk adulteration testing instrument) developed and patented by CSIR & CEERI technology, India. REIL EMAT PLUS is able to detect the presence of hydrogen peroxide, neutralizer, detergent and urea. In this article we have compare our newly developed nanotechnology based dipstick with REIL EMET plus for the detection of hydrogen peroxide, neutralizer, detergent and urea. Generated data from the result were analyzed to check the specificity, accuracy and sensitivity of dipstick compare to REIL EMAT plus and statistical analysis was carried out with help of Medcalc software.

MATERIAL AND METHODS

A total 100 of milk samples were collected from different talukas viz. Babra, Liliya, Dhari, Savar Kundla, Rajula and Bagasara of Amreli district and were preserved at - 4°C temperature in refrigerators. The samples were collected in 100 ml screw capped sterilized plastic bottles. All the possible precautions were taken to avoid external contamination at the time of collection of samples and during processing Baharullah, *et al*. (2013). Milk samples were collected in clean, dry and neatly labelled sample containers and transported to laboratory in cold chain. The milk samples were tested for the following adulterants urea, neutralizers ($NaHCO_3$, $Na2CO_3$, NaOH, etc.), detergents, hydrogen peroxide, (Singuluri and Sukumaran 2014).

Collected milk samples were analyzed for the presence or absence of different milk adulterant from group of hydrogen peroxide, neutralizer, urea and detergent. First our developed nanotechnology based dipstick were dipped into the milk sample and check for the presence/absence of adulterants and then same milk sample was analyzed with help of REIL EMAT plus and compare. Later statistical analysis was carried out to check the specificity, accuracy and sensitivity. In statistical analysis, the comparison of sensitivity, specificity and accuracy of dipstick were compared with standard instrument by using Medcalc software version 13.1 and Medcalc software was basically worked upon the Wilson intervals method.

RESULTS AND DISCUSSION

All these hundred samples were analyzed for the presence of adulterants with the help of our innovative nanotechnology based dipstick and REIL EMAT plus standard milk adulteration testing instrument. The color of all milk samples were observed creamy white in appearance, texture of milk samples were smooth and oily and odour of milk samples were characteristics pleasant and milky.

Collected sample were analyzed to check the presence/absence of different adulterant from group of

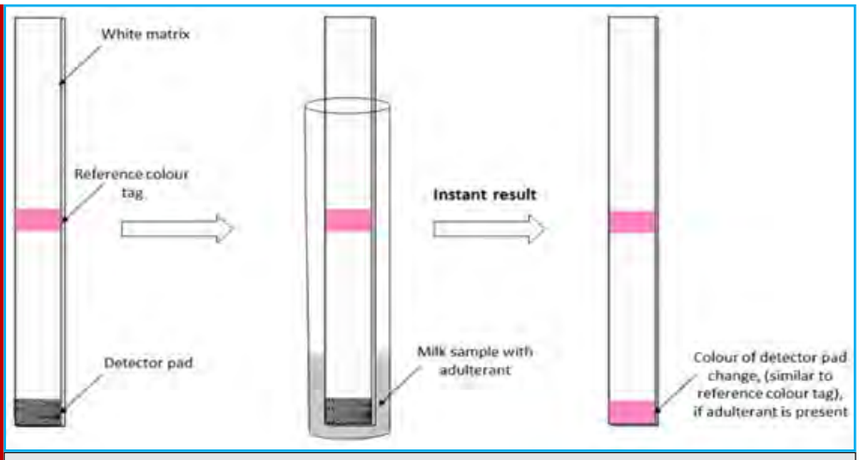

FIGURE 1. Innovative nanotechnology based dipstick

FIGURE 2. REIL EMAT plus standard milk adulteration testing instrument

hydrogen peroxide, neutralizer, urea and detergent. Out of 100 collected milk samples, 28 sample were found to be adulterated with different milk adulterant from the group of hydrogen peroxide, detergent, neutralizer and urea. While checking the presence or absence of adulterants by our innovative dipstick, it was found that 12%, 8% and 8% out of 100 milk samples have been adulterated with urea, detergent and neutralizer respectively. While no sample was found to be adulterated with hydrogen peroxide. Again same milk sample was analyzed by standard milk adulteration testing instrument REIL EMAT plus and same result was found which was found by our innovative dipstick. Table- 1indicate the data of positive and negative adulteration detection in milk collected sample from the Amreli region. Figure 3 shows the percentage wise presence or absence of different adulterants in milk samples.

Fig.3 shows that 12 %, 8 %, 8 % milk was found to be adulterated with urea, detergent and neutralizer respectively while no sample was adulterated with hydrogen peroxide.

Urea added with milk to increase the witness of milk, increase consistency and also for the balancing of content of Solid-Non-fat (SNF) as present in natural milk. Result obtain from comparative analysis of our study, 12 % of milk sample were found to be adulterated with urea while result obtain by Singuluri and Sukumaran (2014) was 60%. Our result is comparatively lower than them. The mixing of urea in milk lead to the overload the function of kidney as they have to filter out more amount of urea from the body. This may lead to the renal failure and impaired vision. Apart from this, urea may also disturb the function of liver and heart.

Another most widely used milk adulterant is detergent. It is used to emulsify and dissolve the oil in water and giving frothy solution, the characteristic white color

Table 1. Shows the total number of positive and negative different adulterant in milk sample

Sr. No	Name of Adulterants	Number of collected samples	Positive	Negative
1	Urea	100	12	88
2	Detergent	100	8	92
3	Neutralizer	100	8	92
4	Hydrogen peroxide	100	0	100

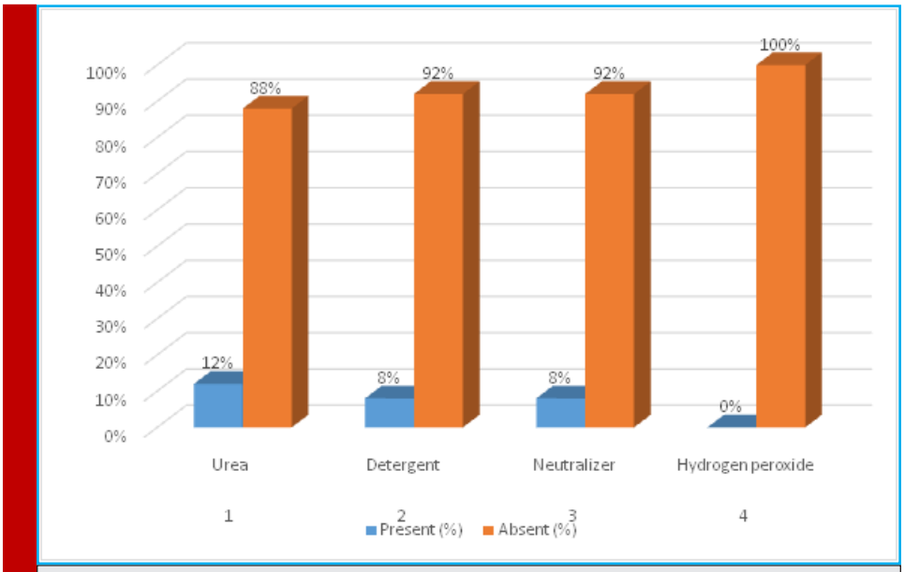

FIGURE 3. Adulterants profile in milk

of milk. Result obtained from the above comparative study, 8 % milk sample were shown positive for detergent. Our result is lower compare to result obtained by Swetha *et al.* (2014). In their result 14 % milk sample were found to be adulterated with detergent. Addition of detergent in milk can cause the gastrointestinal complication. The two major component of detergent, octylphenol and nonylphenol can cause the breast cancer and also decrease the sperm production from testis.

Hydrogen peroxide is added in milk for the long term preservation and freshness of milk. Result obtained from our comparative study, no single milk sample was adulterated with hydrogen peroxide while result obtained by Singuluri and Sukumaran (2014) 32 % milk sample were shown positive for the hydrogen peroxide. Our result is opposite then them. Long term consumption of hydrogen peroxide can damages the cells of gastro intestinal track which can ultimately cause the gastritis and inflammation of the intestine. Neutralizer added in milk can help to neutralize the acidity developed in milk. Consumption of neutralizer containing milk can disturb the hormonal signaling. Result obtained from our comparative, 8 % of milk sample shows the positive test for the presence of neutralizer in milk. Brindha et al. (2017) also did same analysis and they got 20 % of the milk sample shows the positive result for neutralizer. Our result is lower than them.

Medcalc software was performed using ROC (Receiver Operating Characteristic) curve analysis and AUC (Area Under curves) analysis. A ROC curve shows the sensitivity and specificity whereas Area Under curve (AUC) shows an index of accuracy. The ROC curve has been prepared by using Medcalc software for four different adulterant. If the value of AUC is 1 or closer to 1 then dipstick has same accuracy compare to standard compared instrument. Four different adulterants, i.e. hydrogen peroxide, neutralizer, urea and detergent with standard instrument REIL EMAT plus. After statistical analysis and based on ROC curve, AUC value of these four adulterant were found 1.0 while 95% confidence interval (CI) was 0.996 to 1.0. If the value of AUC was 1 or closer to 1 then dipstick have same accuracy compare to standard instrument. ROC curve indicate the 100% specificity and sensitivity. Hence, our nanotechnology based dipstick is excellent as compared to standard instrument (REIL EMAT plus).

CONCLUSION

In a country such as India where milk and milk products play an important role in different foodstuffs, this analysis carried out should bring about more awareness to the general public about the malpractices in milk marketing. On the basis of data obtain from the above result it is conclude that quality of milk sample is not as per the standard and milk adulteration is still in practice. Consumption of lower quality milk may lead to serious human health problems. To eradicate this malpractice by local wanders which is deep rooted in the cities more than rural areas, steps should be taken from the door steps of local consumers. The consumers must be more active against milk adulteration going on in whole country. It is important to have a quality control

system that regularly check and ensure that only good quality milk is sold. The consumers and the milk sellers combined effort will help to decrease the adulteration practice.

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The potential of certain algal species as source of biodiesel

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ABSTRACT

Biofuels are important source of renewable chemical energy, more precisely those that are available in liquid form. Algal biofuel offers the advantage of being a low-emission fuel. Algal biofuel offers a clean source of renewable energy. It burns without smoke and can be easily cultured in areas which are unsuitable for agriculture due to low production and installation costs. It releases only carbon dioxide and water on burning and carbon dioxide is one that is fixed from atmosphere during photosynthesis. In the present work, five algae samples were collected from different sites. According to the maximum growth of algae in BG11 media, it was selected for mass culture of potentially growing samples. They were kept for two weeks and optimized through various confirmatory tests. The flame test and transesterification test showed positive results for all five samples. These samples were mass cultured in pond and oil was extracted using Soxhlet extractor with ethanol as solvent. The oil was recovered and transesterification was performed to convert it to biodiesel.

KEY WORDS: BIODIESEL, BIOFUEL, ALGAE, CULTURE, BG11, BOLDS BASAL, BRISTOL, SOXHLET

Algae are a diverse group of eukaryotic organisms that belong to the phylum Protista. These organisms use light energy to convert CO_2 and H_2O into carbohydrates and other cellular products. During this process, oxygen released. Algae are found anywhere there is water, fresh water, salt water, and in the soil (Brown, 1969). Macro-algae are defined as the multicellular plants also known as seaweeds found in salt or fresh water (Kharkwal, 2012) whereas one of the miniscule plants known as Micro-algae contributes in the production of around 60 percent of the earth's oxygen. Such organisms comprise of twenty five to thirty thousand species representing a range of forms and sizes that can exist from unicellular microscopic organism (microalgae) to multicellular large size (macroalgae) (Ugoala, 2012). Due to the fact that the oceans cover 70% of the earth's surface, aquatic algae

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are major producer of oxygen and important users of carbon dioxide. All algae are primarily made up of proteins, carbohydrates, fats and nucleic acids in varying proportions. While the percentage can vary with the type of algae, some types of algae are made up of up to 40% fatty acids based on their overall mass. It is this fatty acid that can be extracted and converted into biofuel. Due to the high lipid content, algal strains are of great interest in the search for sustainable sources for the production of biodiesel. Reports proclaim that Macro-algae is composed of more than 2400 organic and chemical free products with a great commercial value in industries such as pharmaceutical, biomedical, neutraceutical, etc. (Saranya, 2013).

Fuel derived from plants, animals or algal resources can not only bring an initiative to preserve a healthful global environment but also shall prove to be a substitute in order to reduce our dependency on fossil fuels. High lipid content, ease of cultivation, cost effective and rapid growth rate is the factors that make microalgae to become a desired candidate for biofuel production (Alam, 2015). Over the past decade there have been plenty of advancements in algal technology for biofuel production. Algae is considered as a traditional food or feed and can be cultured in huge open ponds or closed photo bioreactors placed on non-arable land. Certain algal species have a high potential as the oil-producer as compared to oil crops. It can be isolated from various carbon (CO_2) sources and further processed in a wide spectrum of products like biodiesel, gasoline replacements, green diesel, methane, bioethanol, heat, high protein animal feed, bio-oil, etc. (Yang, 2016).

In the future of transport sector various biofuels, including bio-ethanol, -methanol, -diesel- and –hydrogen appear to be appealing options. Therefore, it is essential to look for novel feedstock sources which ar suitable for biofuel production and does not withdraw the supply of edible feedstock. Algae can be an alternative to the conventional crop. Algae or cyanobacteria based third generation technology contains an elevated oil mass fraction grown in ponds (Suganya, 2016). It is a favourable source of third generation renewable fuels. However, the procedure of biofuel production from the growth of microalgae till the last step is still not contemplated economically viable (Laamanen, 2016). Microalgae have various autotrophic capabilities which add an element of flexibility as compared to conventional fuels. They are also supported by fundamental nutrients which capture solar energy to fix the amount of carbon dioxide and split water (Hallenbeck, 2016). The production of biodiesel using algae demands the conversion of algal biomass through transesterfication (Kandiyoti, 2017). It is a process in which lipids are simultaneously separated and trans esterified from microalgae cell walls and trans esterification is generally used for heterotrophic microalgae (Veillette, 2017). Further research is needed to discover the most energy efficient, cost effective and high yield extraction process to amplify the economic viability of global algae biofuels sector. This will address all the crucial technical trials which affect the production processes involved. Hence, these aforementioned research when incorporated with government emission policies will fast-track the date when algal biofuel production can be a commercial enterprise. In the field of science and research to succeed fossil fuels, algae feedstock has appeared as a suitable candidate not only for its renewable and sustainable characteristics but also for its economic reliability based on the prospective to match up with the global demand for transportation fuels (Adeniyi, 2018).

In the present study, fresh water algae were collected from 5 below mentioned sites and denoted as R1, R2, R3, R4 and R5. (Orchid, SHIATS R1, Agriculture Department, SHIATS: R2, Forestry Department, SHIATS:R3, Arail Ghat, Allahabad:R4 and Saraswati Ghat, Allahabad: R5).

Algae culture was carried out in tissue culture bottles with 3 different growth media and one was in distilled water as control. 2 gm of algae was inoculated in each media and further in open land area in polybags, these media were following-Bolds Basal Medium (BBM) (Nichols and Bold, 1965), Bristol Medium, (Bold, 1949) and BG11 medium (Stanier, 1971). Oil extraction from algae using Soxhlet apparatus was performed as per the method of Franz von Soxhlet, (1879). The algae was dried by exposure to hot air oven at 60 ^{0}C for 1 hour. After complete drying, the algae were blended to get powder. A 25 gm sample of the dried algae was placed in the thimble, which was loaded in the main chamber of Soxhlet extractor. The Soxhlet extractor was placed onto a flask containing extraction solvent *i.e.* 95% ethanol. The solvent was heated to reflux, so it forms vapors, which travels up a distillation arm, and floods into the chamber housing the thimble of algae powder. Some of the desired compound will then dissolve in the warm ethanol. When the Soxhlet chamber was almost full, the chamber was automatically emptied by the siphon side arm, with ethanol running back to the distillation flask. This cycle was repeated for three to four times. During each cycle, a portion of the oil is dissolved in ethanol.

Confirmatory Tests for Biodiesel:-Transesterification is the most commonly used method for Biodiesel oil production from algae. Harvested biomass from these algal species was dried, ground and oil was extracted by Soxhlet extractor using ethanol as a solvent. The extracted oil was trans esterified to biodiesel using sodium methoxide as a catalyst.1ml of extracted algal oil was taken with 3ml of methanol and 0.075 g of NaOH and it was shaken vigorously and then kept in a shaker for over-

FIGURE 1. Different sites from where algal samples were collected

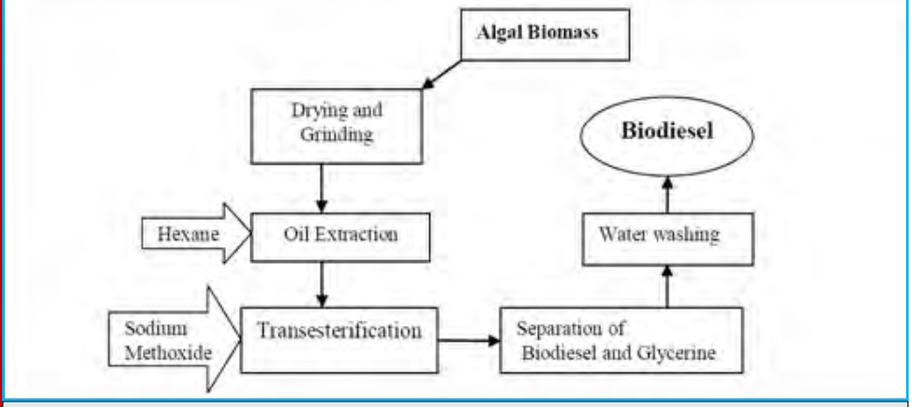

FIGURE 2. Steps involved in biodiesel production from algal biomass

FIGURE 3. Algal growth of R3

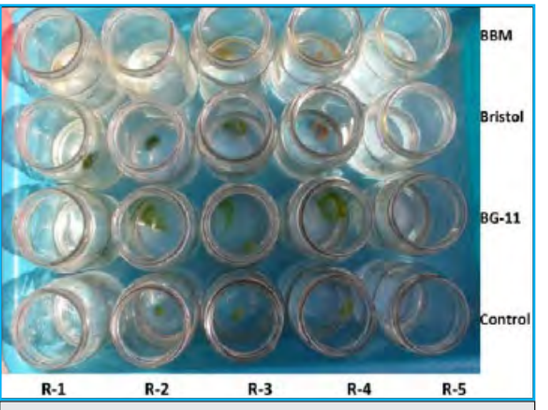

FIGURE 4. Growth of algal sample in first week of inoculation

Table 2. List of the species present in the algal samples

Samples	Present species
R-1	*Microcystis*
R-2	*Vaucheria* *Microcystis*
R-3	*Microcystis*
R-4	*Vaucheria* *Microcystis* *Nostoc*
R-5	*Microcystis*

night. Two layers were observed the next day in which the lower layer was glycerol and the upper layer was biodiesel. The upper layer i.e. biodiesel was extracted for further experiments.

Ideally, there were two distinct layers: an amber (ranging from very light to very dark depending on the oil used) biodiesel layer on top and a darker glycerol layer on the bottom (usually contaminated with catalyst, alcohol, or dust particulates). Sometimes, there will be a third or fourth layer between the glycerol and the biodiesel. These layers are soap from too much catalyst or water and often appear milky or yellowish. The property of a biodiesel is to be highly flammable when in contact of fire. So for the flame test of biodiesel we lit the matchstick in front of the upper layer of solution which was taken out by the process of transesterification.

Growth rate measurement: The tissue culture bottles were kept in open area where proper sun-light and aeration was available for the suitable growth of algal sample as shown in figure 3 and 4.

In all these above 4 growth medium BBM, Bristol, BG11 including one control (distilled water) BG11 media

FIGURE 5. Transesterfication

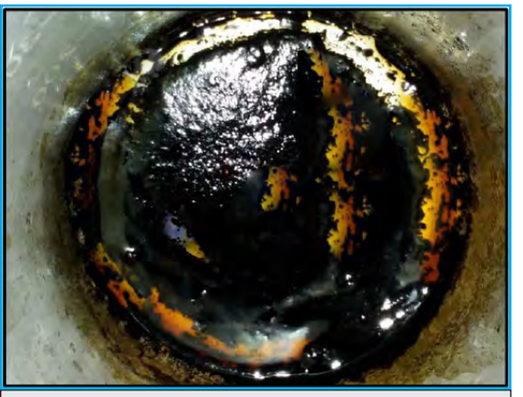

FIGURE 5. The remaining extract after Ethanol evaporation is the desired algal lipid

showed maximum algal growth, so it was taken for the mass culture of algal sample in open ground.

Identification: This process was carried out at department of biological sciences, SHIATS, Allahabad, using a compound microscope for analysis of temporary slides of Algae samples collected from different sites. Different Algae species were identified by observing their unique reproductive structures. The table 2 shows the results of identification of algae samples showing the type of species which constitute every sample.

After the transesterification process, a separate layer of biodiesel was obtained from the extracted algal oil. The upper layer contained biodiesel which was carefully extracted with pipette in a separate flask, and the lower layer was glycerol so it was discarded.

As Biodiesel has the highly inflammable property, it caught fire rapidly when a matchstick was passed over it.In the current study, 5 algae samples were used to extract oil and it was converted to biodiesel. The study revealed that algae are fast growing and effective organism for biodiesel production as these can be grown in

FIGURE 6. Flame Test

normal water as well as in artificial media. Oil extracted from harvested biomass of these algae was trans esterified to biodiesel using sodium methoxide as a catalyst. It was found that properties of biodiesel so it can be blended with fossil fuels or can be used individually as an alternative of fossil fuels.

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Evaluation of anti-hyperglycaemic potential of the ethanolic leaf extract of *Quisqualis indica*

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ABSTRACT

The present study was conducted to evaluate the in vitro inhibitory effect of ethanolic extract of *Quisqualis indica* leaves on the digestive enzyme α-amylase and to characterize the compounds responsible for it as a means of managing hyperglycaemia. The presence of various phytochemicals such as flavonoids, phenols, alkaloids, tannins may be responsible for the plant biological activities. The ethanolic leaf extract of the plant was further subjected to α-amylase inhibitory assay where it showed the dose dependent inhibitory effect on the α-amylase enzyme when compared with the positive control acarbose. GC-MS analysis of the ethanolic plant leaf extract revealed the presence of phytol in the highest concentration and other compounds like linolenic acid, pentadecanoic acid and 9, 12-linoleic acid in moderate concentrations which were further evaluated for their biological activities using PASS and compared with the biological activity profile of the anti-diabetic drug acarbose. The various modes of action of these compounds include α-amylase inhibition, α-glucosidase inhibition, insulin inhibition, etc. *In silico* studies were also done using AutoDock to study the compound's minimum free energy of stabilization in complex with the α-amylase enzyme. Further Ligplot+ was used to study the presence of hydrogen bonding in the complex. Drug parameters of the identified compounds in the extract were evaluated and compared with the acarbose. The results obtained were successfully compared to the pharmacological and toxicological activity information available for the studied compounds.

KEY WORDS: AUTODOCK VINA, DIABETES MELLITUS, GC-MS ANALYSIS, PASS, *QUISQUALIS INDICA*, LIGPLOT+ V.1.4.3.

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INTRODUCTION

Diabetes mellitus is a chronic heterogeneous endocrine disorder characterized by elevated blood glucose level resulting in serious metabolic disturbances in carbohydrate, protein and fat metabolism causing premature fatality (Patel et al. 2011; Warjeet 2011). After cancer, cardiovascular and cerebrovascular disease, it is the third most life-threatening disease posed to the health of mankind (Chauhan et al. 2010). Annually, a rise of 4-5 % in number of diabetic patients is observed (Wagman and Nuss 2001). It is caused due to insufficient insulin production or psychological unresponsiveness to insulin. Pancreatic α-amylase enzyme is an endoglucanase that catalyzes the internal α-1, 4 glycosidic bond hydrolysis in starch and other polysaccharides to yield maltose and maltotriose polysaccharides. Inhibition of this enzyme can help to manage the disorder by lowering the level of glucose released in the blood. There is considerable evidence that lipid peroxidation owing to free radical activity causes induction of oxidative stress that plays a crucial role in the onset of the abnormal condition. Alteration in anti-oxidant enzymes, impaired glutathione metabolism and decreased ascorbic acid levels are the main causes of disturbance of anti-oxidant defence system (Patel et al. 2011). This leads to accumulation of advanced glycation products (AGEs) and sorbitol concentration that cause complications such as retinopathy, neuropathy and renal dysfunction (Safı et al. 2014). The physical symptoms include cycle of heavy thirst and frequent sugar loaded urination along with presence of sugar in mucus, sweat and breath. In the past recent years, natural pharmacologically bioactive compounds derived from terrestrial and marine organisms had received considerable attention to cure potentially vulnerable diseases due to their lesser or virtually no side effects as compared to synthetic drugs (Yuan et al. 2016).

Recently many R&D based pharmaceutical company are employing more time and money on development of herbal therapies rather than formulating synthetic drugs due to their unpredictable adverse effects. Ayurveda, which is the renowned traditional system of medicines native to India, had always promoted the use of plant biodiversity for several therapeutic uses due to their ease of availability and low cost of production (Patwardhan and Hopper 1992). Plant-derived molecules (PDMs) could be chemically elaborated to generate novel leads and to screen molecules from drug-like libraries and hence can be proved effective to systematically extract unique molecular scaffolds. *Quisqualis indica*, commonly known as 'rangoon creeper' is an important medicinal plant of the Indian subcontinent, Africa and Indo Malaysian region (Joshi 2002). It is a vine with pink and white flowers. It is also used as anthelmenic by the inhabitants of North Annan to expel parasitic worms (helminths) and other internal parasites from the body (Kirtikar and Basu 2006). It has also been reported that the extract of flowers of *Q. indica* exhibit hypoglycaemic and hypocholesterolemic activity against animal models (Bairagi et al. 2012). Various parts of the plant are used individually or mixed with other ingredients as a cure for ailments like antiflatulence, coughs, diarrhea (Khare 2007), body pains, toothache (Padua et al. 1999).

With the available therapeutic knowledge, scientists have been thriving hard to explore the safe and effective treatment of the disease which is yet to be achieved. Clinically various oral anti-diabetic drugs are used such as biguanides that increase glucose uptake, sulfonylureas that increase insulin secretion and digestive enzyme inhibitors that delay complex carbohydrate digestion and absorption. Large scale research is being done worldwide, exploiting the known ethno-botanical knowledge and phytochemical interpretations that could be an effective approach for the diabetes treatment. In the designing of drug, the *in silico* analysis using bioinformatics tools is a boon for the researchers as they minimize the time and labour employed during the work. PASS is one such tool that uses algorithms based on the physico-chemical methods, that predicts the possible activity of the drug against a target using the intrinsic property of the compounds (Filimonov et al. 1995; Filimonov and Poroikov 1996). In 1972, the National Registration System of New Chemical Compounds organized in the USSR formulated the computer program PASS which was suggested by researcher V. Avidon (Burov et al. 1990; Poroikov et al. 2003).

Computational based approaches, such as molecular docking, hydrogen bonding analysis, evaluation of drug parameters have been widely used in the modern drug discovery to explore drug-receptor interactions. For designing of novel inhibitors, molecules from a database of organic compounds should be screened based on steric and electrostatic complementarity with the binding pocket of protein. Molecular docking simulation studies were performed using AutoDock Vina. Docking of the individual compounds with the α-amylase enzyme which is the key enzyme involved in the regulation of the metabolic pathway was done using Autodock Tools 1.5.4 package. Ligplot+ v.1.4.3 software can help in refined assessment of docked complexes and in obtaining detailed information on protein-ligand interaction (Laskowski and Swindells 2011).

Chemicalize.org[beta] software tool by ChemAxon was used to study the drug like activities of the compounds identified through GC-MS analysis. Therefore, combination of these methods can be used to study mechanisms of drug-receptor interactions, and provide structural

insights by which molecules interact within binding pocket of the receptor. Further experimental evaluation and validation are required to establish the clinical significance of leads obtained that are promising candidates.

MATERIALS AND METHODS

Plant material and preparation of extract

The plant leaves of *Quisqualis indica* Linn. were obtained from Forest Research Institute (FRI), Dehradun, India. The plant was botanically identified at FRI. Leaves were thoroughly cleaned with tap water to remove any dust particles and further shade dried for 3 weeks to make them crisp and completely devoid of moisture. The dried leaves were finely grinded and 10 g of the powdered material was extracted with 250 mL of ethanol procured from Merck, India using Soxhlet apparatus for 48 hrs. The extract was concentrated using vacuum rotary evaporator at 78 °C. The extract was filtered through Whatmann filter paper No.1 and stored at 4 °C until use. Rest of the chemicals were of analytical grade. Preliminary screening: Phytochemical screening of the leaf extract was done using the methods described by Trease and Evans (1996), and Sofowora (2006).

In vitro α-amylase inhibitory assay

The assay was carried out according to the standard protocol (Hansawasdi et al. 2000), with slight modifications. 2 mg of starch azure was suspended in 0.2 mL substrate solution containing 0.5 M Tris-HCl buffer (pH 6.9) and 0.01 M $CaCl_2$. The substrate solution in tubes were boiled for 5 min and then preincubated at 37 °C for 5 min. Ethanol extract of *Q. indica* was dissolved in DMSO to obtain concentrations of 10, 20, 40, 60, 80, and 100 µg/ mL. Then, 0.2 mL of plant extract of particular concentration was added to the substrate solution in respective tubes. Further, 0.1 mL of porcine pancreatic α-amylase procured from Hi-Media in Tris-HCl buffer (2 units/mL) was added to the tube containing the plant extract and substrate solution. The reaction was carried out at 37 °C for 10 min. The reaction was terminated by addition of 0.5 mL of 50 % acetic acid in each tube. The reaction mixture was centrifuged at 3000 rpm for 5 min at 4 °C. The absorbance of resulting supernatant was measured at 595 nm using spectrophotometer. Acarbose, a known α-amylase inhibitor was used as a standard drug control. The experiments were done in triplicates. The α-amylase inhibitory activity was calculated by using following formula:

$$\text{The } \alpha\text{-amylase inhibitory activity} = [\{(Ac+) - (Ac-)\} - \{(As - Ab)\}/ \{(Ac+) - (Ac-)\}] \times 100,$$

where Ac+, Ac-, As, and Ab are defined as the absorbance of 100 % enzyme activity (only solvent with enzyme), 0 % enzyme activity (only solvent without enzyme), a test sample (with enzyme), and a blank (a test sample without enzyme), respectively. The percentage inhibitory effects of acarbose and plant extract on α-amylase activity were determined. Statistical analysis was performed using Microsoft Excel. All values were expressed as mean ± standard deviation.

GC-MS was carried out at Advanced Instrumentation Research Facility (AIRF) at Jawaharlal Nehru University (JNU), New Delhi, India. The analysis was done on GCMS-QP2010 Ultra under the following conditions to study the phytochemical components present in the extract. Column-Rtx-5 MS (30 m X 0.25 mm i.d. X 0.25µm film thickness) was used. 2 µL of the plant sample was injected in split mode at a constant column flow rate of 1.21 mL/ min with linear velocity 40.9 cm/ sec flow control mode and purge flow of 3.0 mL/ min. The column temperature was programmed to 100 °C, ion source temperature at 220 °C and the injection temperature was 260 °C. Total GC-MS running time was 45 min. Inbuilt libraries WILEY8.lib and NIST11.lib were used for the identification and comparison of the organic compounds. The name, molecular weight and structure of the phytochemical components of the extract were ascertained.

The activity list comprises of names of pharmacotherapeutic effects as well as names of mechanism of action. The compounds present in high concentration in the plant leaf extract were studied using PASS for their biological activity prediction, whose structures were drawn using MarvinSketch v5.10.0 and compared with acarbose. The mean accuracy of prediction is about 85 % in leave-one-out cross-validation (LOOCV), hence it is reasonable using this tool to find and optimize new lead compounds which is a crucial step in pharmaceutical research and development process. The chemical structures of molecules were drawn and edited using MarvinSketch v.5.10.0 software (https://www.chemaxon.com), an advanced chemical structure editor. The structures were saved in 3D MOL2 format. Individual MOL2 files were converted into PDBQT format (acceptable format for AutoDock Vina package (Trott and Olson 2010)), using the python script 'prepare_ligand4.py' available in Autodock Tools 1.5.4 package (Morris et al. 2008). During this conversion, appropriate charges were added to ligands. The commercially available drug acarbose as well as the compounds present in high concentration in the extract were docked with enzyme α-amylase using software AutoDock Vina to analyze their free energy. Further presence of hydrogen bonding were analyzed using software Ligplot+ v.1.4.3 software and molecular interactions between protein and ligands were predicted.

Table 1. Phytochemical screening of ethanolic leaf extract of *Q. indica*

S. No.	Phytochemicals	+ = present; - = absent	S. No.	Phytochemicals	+ = present; - = absent
1.	Anthraquinones	-	6.	Tannins	+
2.	Flavonoids	+	7.	Terpenoids	-
3.	Reducing sugar	-	8.	Phenols	+
4.	Saponins	+	9.	Alkaloids	+
5.	Steroids	-	10.	Quinones	+

Drug parameters were studied using chemicalize.org^{beta} by ChemAxon.

RESULTS AND DISCUSSION

The plant leaf extract was found to contain the phytoconstituents like flavonoids, saponins, tannins, phenols, alkaloids and quinones upon preliminary screening among which flavonoids, tannins and phenols are reported to play a crucial role in the management of diabetes mellitus (Table 1, S. No. 1-10). The percentage inhibitory activity exhibited by the extract and comparison with acarbose is shown in Table 2. (S. No. 1-6). Acarbose at concentration 100 µg/mL showed 71.79 ± 0.55 % inhibitory effects on the α-amylase activity. The ethanolic leaf extract of *Q. indica* at a concentration 100 µg/mL exhibited 56.40 ± 2.35 % of α-amylase inhibitory activity. The plant extract showed potent α-amylase inhibitory activity in a dose dependent manner as compared with acarbose (Fig. 1). Acarbose was used as a positive control which is a secondary metabolite which belongs to the class of drugs called digestive enzyme inhibitors. It is obtained via a multistep batch fermentation process from bacterium *Actinoplanes* species SE50. It is approved for treating type II diabetic patients. This reveals that the extract exhibited a comparable inhibitory effect on the α-amylase enzyme as compared to the standard drug acarbose.

Although the presence of flavonoids and phenols contribute to the anti-diabetic activity, the specific bioactive components of the plant extract were studied through GC-MS analysis (Fig. 2). The peaks clearly show that compounds like phytol, linolenic acid, pentadecanoic acid and 9, 12-linoleic acid were present in subsequently higher concentrations. GC-MS study of the *Q. indica* leaf extract has shown the presence of a number of phytochemical constituents which confer the medicinal property to the plant (Table No 3).

This study depicted the presence of 55 compounds. Among these, 21 compounds with their biological activities have been reported earlier. Compounds like lauric acid, 9, 12-linoleic acid, linolenic acid, vitamin E, stigmasterol, α-sitosterol are reported to posses hypocholesterolemic activity. Compounds like neophytadiene, squalene, heptacosanol, solanesol, α-tocopherol, vitamin E, stigmasterol, α-sitosterol, fucosterol are reported to have anti-oxidant activity. Both of these activities play a direct role in the management of diabetes mellitus by blocking the sugar metabolism. Phytol also known as phytanic acid is the test compound to be studied which was present in the highest concentration in the plant *Q. indica* leaves. Phytol is an acyclic diterpene alcohol molecule which is a precursor of synthetic vitamin E and K1 (Thomas and Netscher 2007; Daines et al. 2003). It was first obtained by chlorophyll hydrolysis and now obtained in the process of chlorophyll separation from alfalfa. It is reported as transcription factors peroxisome proliferator activated receptor (PPAR-α) and retinoid X receptor (RXR) activator. It is already reported to be a cholesterol lowering agent in patients with type II diabetes, obesity and cardiovascular diseases. It is also reported to possess anti-inflammatory as well as metabolic properties.

The predicted biological activity spectrum of the compounds by PASS contributing to their anti-hyperglycaemic potential is shown in Table 4 (S. No. 1-5). The PASS result is obtained in the form of names of biological activity whose probability value ranges from 0.000 to 1.000. Only activity types for which Pa>Pi, are considered possible. Pa and Pi are the probability measures for the compound to be active and inactive respectively for the respective activities in the biologi-

Table 2. α-amylase inhibitory effects of *Q. indica* leaf extract in comparison with standard drug acarbose

S. No.	Concentration (µg/mL)	% inhibition by acarbose	% inhibition by ethanolic leaf extract of *Q. indica*
1.	10	20.19 ± 0.96	14.86 ± 1.93
2.	20	27.04 ± 0.98	24.35 ± 0.88
3.	40	37.17 ± 1.47	26.15 ± 3.35
4.	60	43.91 ± 1.46	38.97 ± 1.17
5.	80	57.68 ± 1.92	53.07 ± 2.03
6.	100	71.79 ± 0.55	56.40 ± 2.35

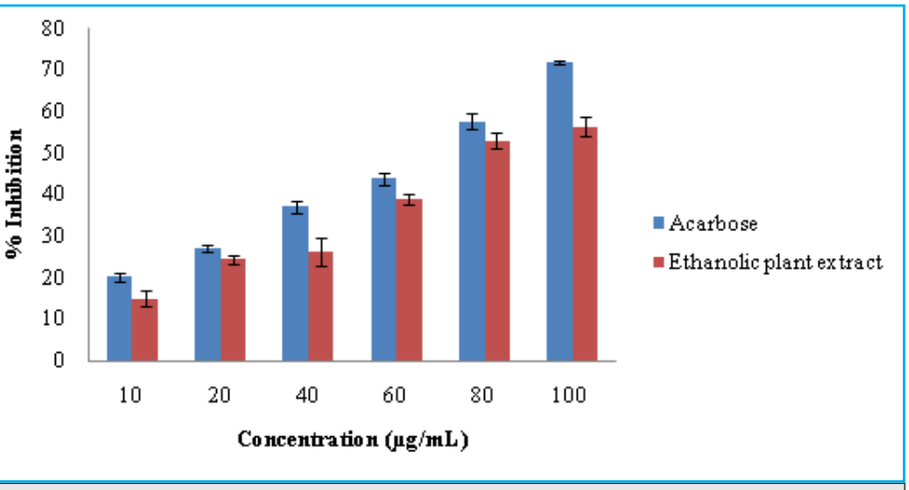


FIGURE 1. Percentage of α-amylase inhibitory effects of acarbose (standard drug) and ethanolic leaf extract of *Q. indica*

cal activity spectrum. The various predicted metabolic pathways of phytol involved in control of diabetes include dextranase inhibition, glycerol-3-phosphate dehydrogenase inhibition, α-glucuronidase inhibition, diabetic neuropathy treatment, etc. It shows 7 possible pathways known to play a role in diabetes management that can be further studied using wet lab experiments to explore its potential to be used as drug for treatment of diabetes. Other compounds like stigmasta-5, 23-dien-3β-ol, squalene, stearic acid, tetracontane, heptacosanol, stigmasterol were also predicted for the presence of their biological activities based on their chemical

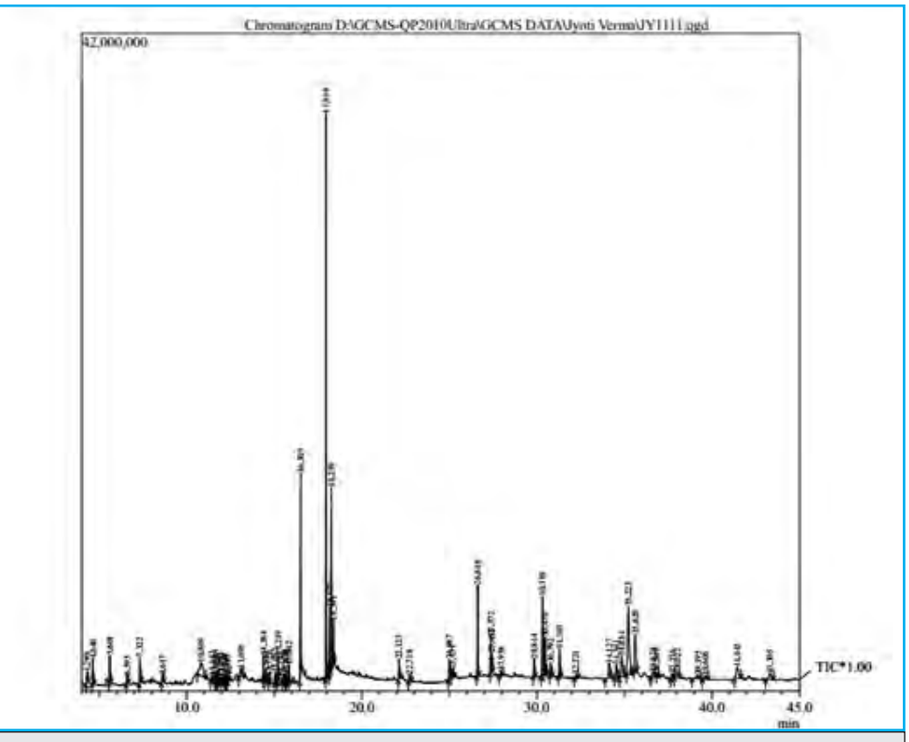


FIGURE 2. Chromatogram of the ethanolic leaf extract of *Q. indica* by GC-MS

Table 3. Phytochemical components identified as major percentage in the ethanolic extract of leaves of *Q. indica* by GC-MS

R.Time	Mol. Wt.	Formula	Name	Area%	Activity already reported
16.509	242	$C_{15}H_{30}O_2$	Pentadecanoic acid	8.73	-
17.959	296	$C_{20}H_{40}O$	(E)-Phytol	18.07	anti-microbial, anti-cancer, anti-inflammatory, anti-diuretic, immunostimulatory, anti-diabetic[a]
18.173	280	$C_{18}H_{32}O_2$	9,12-Linoleic acid	3.48	anti-inflammatory, nematicide, insectifuge, hypocholesterolemic, cancer preventive, hepatoprotective, anti-histaminic, anti-acne, anti-arthritic, anti-eczemic, 5-α reductase inhibitor, anti-androgenic, anti-coronary[b]
18.259	278	$C_{18}H_{30}O_2$	Linolenic acid	9.52	prevent heart attacks, lowers high blood pressure, lowers cholesterol, reverse "hardening of the blood vessels (atherosclerosis)[c], treatment of rheumatoid arthritis (RA), multiple sclerosis (MS), lupus, anti-diabetic, treatment of chronic obstructive pulmonary disease (COPD), migraine headache, skin cancer, depression, allergic and inflammatory conditions such as psoriasis and eczema[d]

[a]Venkata et al. 2012; [b]Sermakkani and Thangapandian 2012; [c]Brouwer et al. 2004; [d]Christensen et al. 2000

Table 4. Biological activity spectrum of standard drug acarbose and peak compounds identified by GC-MS

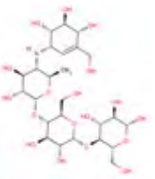
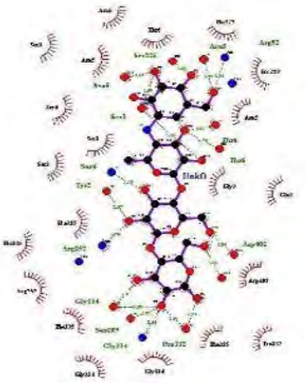
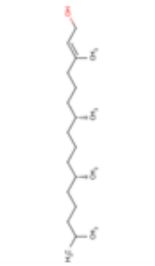
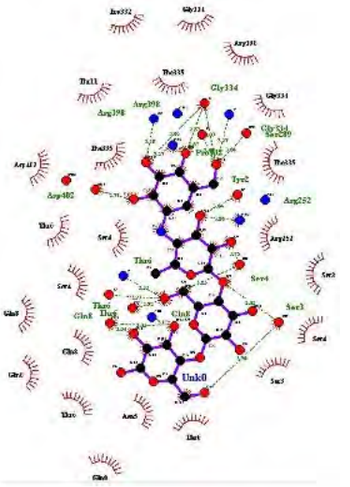
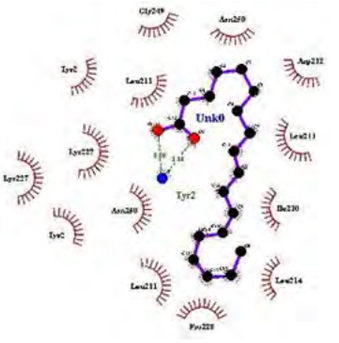
S. No.	Compound	Pa	Pi	Biological activity
1.	Acarbose	0,972	0,000	Sucrose α-glucosidase inhibitor
		0,962	0,000	4-α-glucanotransferase inhibitor
		0,958	0,000	α-glucosidase inhibitor
		0,943	0,000	α-amylase inhibitor
		0,779	0,002	β-amylase inhibitor
		0,752	0,005	Anti-diabetic
		0,691	0,005	Fructan β-fructosidase inhibitor
		0,681	0,002	β-glucosidase inhibitor
		0,636	0,009	β-glucuronidase inhibitor
		0,611	0,001	β-galactosidase inhibitor
		0,512	0,002	Amylo-α-1,6-glucosidase inhibitor
		0,508	0,003	Isoamylase inhibitor
		0,499	0,003	Oligo-1,6-glucosidase inhibitor
		0,447	0,016	Galactose oxidase inhibitor
		0,333	0,002	α-L-fucosidase inhibitor
2.	Phytol	0,541	0,018	Dextranase inhibitor
		0,491	0,013	Sorbitol-6-phosphate 2-dehydrogenase inhibitor
		0,465	0,020	Glycerol-3-phosphate dehydrogenase inhibitor
		0,401	0,030	Fructan β-fructosidase inhibitor
		0,369	0,006	α-N-acetylgalactosaminidase inhibitor
		0,373	0,112	Diabetic neuropathy treatment
		0,349	0,010	α-glucuronidase inhibitor
3.	Linolenic acid	0,902	0,003	Dextranase inhibitor
		0,852	0,004	Cholesterol antagonist
		0,826	0,004	Antihypercholesterolemic
		0,597	0,015	Insulin promoter
		0,574	0,011	Fructan β-fructosidase inhibitor
		0,515	0,004	α-glucuronidase inhibitor
		0,490	0,006	α-amylase inhibitor
		0,491	0,013	Sorbitol-6-phosphate 2-dehydrogenase inhibitor
		0,467	0,006	1,2- α-L-fucosidase inhibitor
		0,466	0,012	Anti-diabetic (type II)
		0,447	0,016	Galactose oxidase inhibitor
		0,464	0,051	β-glucuronidase inhibitor
		0,430	0,021	Diabetic neuropathy treatment

		0,409	0,012	Anti-diabetic symptomatic
		0,405	0,018	α-N-acetylglucosaminidase inhibitor
		0,411	0,041	Anti-diabetic
		0,363	0,015	Antioxidant
		0,348	0,004	Nitric oxide scavenger
		0,346	0,007	β-glucosidase inhibitor
		0,344	0,016	Cholesterol synthesis inhibitor
		0,316	0,005	Diabetic nephropathy treatment
		0,308	0,009	β-D-fucosidase inhibitor
		0,301	0,009	β-amylase inhibitor
		0,336	0,116	Insulysin inhibitor
	4.	Pentadecanoic acid	0,957	0,001	Dextranase inhibitor
			0,753	0,004	Insulin promoter
			0,739	0,004	Fructan β-fructosidase inhibitor
			0,723	0,007	Cholesterol antagonist
			0,699	0,002	α-glucuronidase inhibitor
			0,611	0,013	Antihypercholesterolemic
			0,593	0,004	β-amylase inhibitor
			0,583	0,003	α-amylase inhibitor
			0,540	0,028	β-glucuronidase inhibitor
			0,544	0,037	Insulysin inhibitor
			0,453	0,012	Diabetic neuropathy treatment
			0,422	0,011	Anti-diabetic symptomatic
			0,400	0,004	β-glucosidase inhibitor
			0,387	0,011	Cholesterol synthesis inhibitor
			0,359	0,011	Pancreatic disorders treatment
			0,318	0,005	Nitric oxide scavenger
			0,315	0,027	Free radical scavenger
			0,323	0,070	Anti-diabetic
	5.	9,12-Linoleic acid	0,923	0,002	Dextranase inhibitor
			0,836	0,004	Cholesterol antagonist
			0,801	0,005	Antihypercholesterolemic
			0,573	0,003	α-glucuronidase inhibitor
			0,574	0,011	Fructan β-fructosidase inhibitor
			0,490	0,006	α-amylase inhibitor
			0,491	0,013	Sorbitol-6-phosphate 2-dehydrogenase inhibitor
			0,482	0,008	Anti-diabetic symptomatic
			0,447	0,016	Galactose oxidase inhibitor
			0,438	0,018	Diabetic neuropathy treatment
			0,464	0,051	β-glucuronidase inhibitor
			0,377	0,005	β-glucosidase inhibitor
			0,373	0,013	Cholesterol synthesis inhibitor
			0,348	0,004	Nitric oxide scavenger
			0,334	0,004	Diabetic nephropathy treatment
			0,403	0,086	Insulysin inhibitor
			0,314	0,021	Antioxidant
			0,301	0,009	β-amylase inhibitor
			0,315	0,027	Free radical scavenger

structures that could play a role in the management of diabetes.

Binding simulation studies revealed stable complexes of enzyme and the respective compounds with their energy minimization values shown in Table 5 (S. No. 1-5). AutoDock studies have revealed the minimum free energy needed to stabilize the complex of the α-amylase enzyme with the studied compounds. Interestingly, in our docking studies, we found that phytol could be a potent α-amylase inhibitor with quite a strong binding affinity and made remarkable inhibitory interactions with critical residues. It was observed that the chemical interactions established between phytol and α-amylase binding pocket residues were the most stable as compared with other studied compounds like linolenic acid, pentadecanoic acid and 9, 12-linoleic acid. Hydrogen bondings were visualized using Ligplot+ v.1.4.3 software to study the stability of the complex of the enzyme and the target compounds. Drug parameters of the target compounds were analyzed and compared with acarbose,

Table 5. Computational analysis of peak compounds identified by GC-MS and anti-diabetic drug acarbose

S. No.	Compound name	Chemical structure drawn using MarvinSketch v5.10.0	Affinity calculation with enzyme α-amylase (kcal/mol) using AutoDock Vina	Hydrogen bonding visualization with enzyme α-amylase using Ligplot+ v.1.4.3
1.	Acarbose		-8.3	
2.	Phytol		-8.2	
3.	Linolenic acid		-4.7	

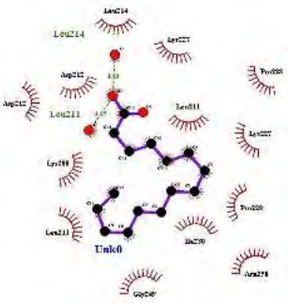
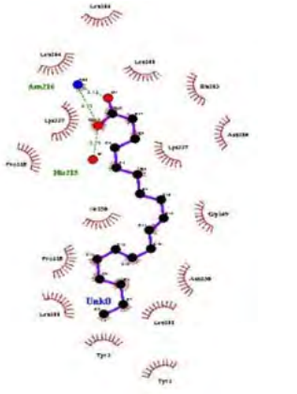
4.	Pentadecanoic acid		-4.4	
5.	9,12-Linoleic acid		-4.2	

Table 6. Evaluation of drug parameters of peak compound identified by GC-MS analysis and comparison with the anti-diabetic drug acarbose

S. No.	Drug parameters	Acarbose	Phytol	Linolenic acid	Pentadecanoic acid	9,12 linoleic acid
1.	Mass	646	296	267	227	267
2.	Formula	$C_{25}H_{43}NO_{18}$	$C_{20}H_{40}O$	$C_{18}H_{30}O_2$	$C_{15}H_{30}O_2$	$C_{18}H_{32}O_2$
3.	Log P	-7.61	-7.04	-6.06	-5.81	-6.42
4.	Polar Surface Area	321.17	20.23	37.30	37.30	37.30
5.	Molar Refractivity	136.524780	95.561760	79.009491	69.104492	80.161491
6.	Lipinski Rule of 5	No	No	No	No	No

as shown in table 6 (S. No. 1-6). The molecular mass, polar surface area and molar refractivity of phytol, linolenic acid, pentadecanoic acid and 9, 12-linoleic acid were found to be in range in compliance with Lipinski rule. The results of the conducted study clearly indicate the anti-hyperglycaemic property of the plant *Q. indica*. All the test compounds studied above may contribute to the anti-diabetic potential of the plant.

CONCLUSION

In the context of already known mechanisms central to diabetes and its complications, we proposed that bioactive compounds of *Q. indica* could be prospected for, in search of potential α-amylase inhibitors. To the best of our knowledge, this is the first report of screening α-amylase inhibitor from *Q. indica* leaves for alleviation of diabetes and related complications. The molecular interaction patterns observed in the conducted study may enable the designing of novel drug structures with considerable inhibitory action. We believe that the study conducted could provide leads for designing novel drug inhibitors of α-amylase with better efficacy and comparatively lesser side-effects. Such a formulation could be exploited for the development of an effective dosage form for drugs that inhibit the digestive enzymes, helping to attain maximum therapeutic efficacy at reduced dose and minimum toxicity.

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CONFLICT OF INTEREST

Authors have no conflict of interest regarding the publication of paper.

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Transcript analysis of the known moisture stress responsive gene orthologs among different genotypes of Little millet, *Panicum sumatrense*

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ABSTRACT

Among different abiotic stresses (cold, temperature, salinity, drought, oxidative stress etc.) moisture stress is the most important limiting factor for crop production and is becoming an increasingly severe problem in many regions of the world. The aim of the current study is to identify some keys genes that are responsible for drought tolerant related traits, in the selected genotypes of Little millet (BL-8, MM-23, MM-10, BL-15-1, RLM-37, OLM-203, BL-4, JK-8). Genotypes belonging to diverse genetic background were grown under stress and control conditions for the identification of moisture stress tolerant traits. A set of known moisture stress related gene orthologs were selected for expression analysis using semi quantitative RT-PCR analysis. Expression analysis of these drought responsive gene orthologs (Amino-transferase, Thionin-osthi, Aquaporin, Synaptotagmin, CDPK, Scythe protein, Ta NAC-2, Ec NAC-67, NAM, U2-SnRNP, Hv NAC, Os NAC-29-2, MPK 17-1, DQP1, DQP 2, DQP 3, DQP 4, DQP 6) had given a differential expression under moisture stress as compared to controlled traits. Majority of these genes were up-regulated in the genotypes RLM-37, MM-23, MM-10, BL-4, BL-8 and BL-15-1 under moisture stress condition and these findings was found to be in correlation with the estimated biochemical traits (Proline, Chlorophyll, Carbohydrate and Protein). This can be taken as a base for drought tolerance response of the crop, which may be useful for further validation studies of the candidate genes for drought tolerance in the millet species as well as other crop plants.

KEY WORDS: ABIOTIC STRESS, DROUGHT, SEMI QUANTITATIVE RT-PCR

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INTRODUCTION

The exceptional tolerance of millets toward diverse abiotic stresses including drought, salinity, light and heat makes them a tractable system to study their stress responsive traits at the cellular, molecular and physiological levels (Bandyopadhyay *et al.,* 2017). Several morpho-physiological and biochemical studies in millets have shown their stress adaptation strategies. Little millet is grown to a limited extent in India, up to altitudes of 2,100 m. It occurs wild in northern India and south East Asia. It belongs to the subfamily Panicoideae, tribe Paniceae, genus *Panicum*, species *P. sumatrense*, with chromosome number 36 (tetraploid) (Hiremath *et al.,* 1990). Little millet is a domesticated form of the weedy species *Panicum psilopodium* (De Wet *et al.,* 1983a). Introgression of genes between the two species is common (Hiremath *et al.,* 1990). Little millet is comparable to other cereals in terms of fiber, fat, carbohydrates, and protein, and rich in phytochemicals including phenolic acids, flavonoids, tannins, and phytate (Pradeep and Guha 2011). Improved varieties of small millets could play a role in the "New Green Revolution"- a term coined to reflect novel strategies which will be required to deal with complex challenges in developing nations including increasing population and ever diminishing arable land. Like many other small millets, it is drought tolerant, pest and salt resistant,(Sivakumar *et al.,* 2006b, Herder *et al.,* 2010, Bhaskara and Panneerselvam 2013, Ajithkumar *et al.,* 2014 Tang et al., 2017, Jaiswal et al., 2018).

Nagarjuna *et al.,* (2016) have reported the identification and characterisation of an abiotic stress responsive protein kinase called CBL Interacting Protein Kinase (EcCIPK31-like) from drought tolerant crop, finger millet. Where, the upregulation was reported for first time under salinity, desiccation, oxidative and temperature stresses at seedling level in finger millet. The stress responsive nature of EcCIPK31-like to diverse stresses indicates that the gene could regulate multiple cellular tolerance traits and its further functional validation can highlight the relevance in abiotic stress. Similarly, it has been reported that Kodo millet is known to be highly drought and salt tolerant crop as ascertained by antioxidants and antioxidant enzymes levels. cDNA library was constructed from 6 days' drought stressed seedlings. 5 ESTs differentially expressed under drought stress were characterized by DDRT-PCR and their expression profile was assessed by real time RT-PCR. Drought stress in Kodo millet led to the characterization of three up-regulated ESTs compared to two down-regulated, (Siddappa et al 2016).

Experimental results by Hittalmani, *et al.* (2017) revealed that, from whole genome sequencing and assembling process of ML-365 finger millet cultivar yielded 1196 Mb covering approximately 82% of total estimated genome size. Transcriptome analysis of low moisture stress and non-stress samples revealed the identification of several drought-induced candidate genes, which could be used in drought tolerance breeding. This genome sequencing effort had strengthened the plant breeders for allele discovery, genetic mapping, and identification of candidate genes for agronomically important traits.

In a study, physiological and transcriptomic comparisons between drought tolerant *S. italica* cultivar 'Yugu1' and drought-sensitive 'An04' were conducted by Tang, *et al.* (2017). They identified 20 candidate genes that contributed to germination and early seedling drought tolerance in *S.italica*. Finally their analysis provided a comprehensive picture of how different *S.italica* genotypes respond to drought, and may be used for the genetic improvement of drought tolerance in Poaceae crops.

Jaiswal, *et al.,* (2018) reported de novo assembly-based transcriptomic signature of drought response induced by irrigation withdrawal in pearl millet. They found 19,983 differentially expressed genes, 7,595 transcription factors, gene regulatory network having 45 hub genes controlling drought response. They also reported 34652 putative markers (4192 simple sequence repeats, 12111 SNPs and 6249 InDels). This Study had revealed the role of purine and tryptophan metabolism in ABA accumulation mediating abiotic response in which MAPK acts as major intracellular signal sensing drought.

The molecular biology of Little millet has been explored to a limited extent. Little millet is perhaps the least studied of the small millet species and there is much that requires investigation, including the establishment of a genetic map and sequenced genome. It is important to dissect the transcriptome information under stress condition for the identification and characterization of the key genes for moisture stress tolerance. The identified genes which were up-regulated under the moisture stress condition, can be taken as a base for drought tolerance response of the crop, which may be useful for further validation studies of the candidate genes for drought tolerance mechanism in the millet species as well as other crop plants.

MATERIAL AND METHODS

Sowing of Little millet (*Panicum sumatrense*) was done in trays. Moisture stress was imposed after 30 days of sowing, at the vegetative stage before panicle initiation for a set of eight Little millet genotypes under the controlled environmental conditions as shown in figure 1.

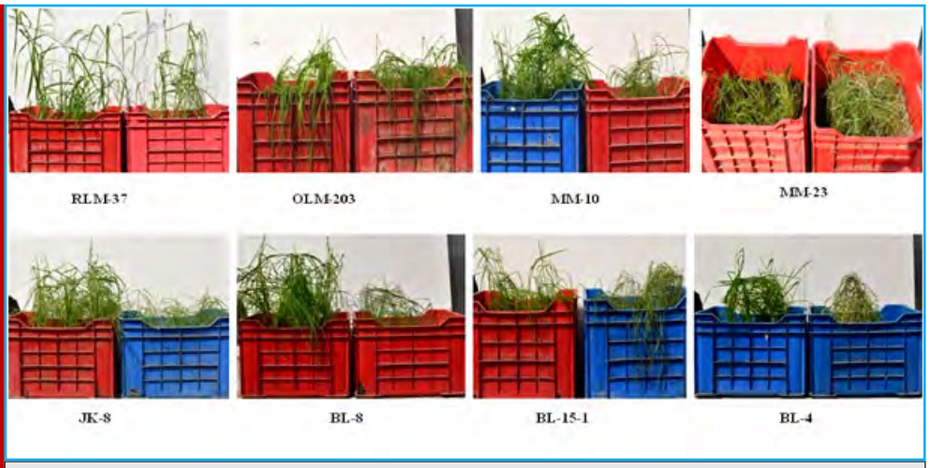


FIGURE 1. Plant morphology of Eight Little millet Genotypes (RLM-37, OLM-203, MM-10, MM-23, JK-8, BL-4, BL-8, BL-15-1) under Control and Stress condition at Vegetative stage before Panicle Initiation.

Temperature was maintained around 30±2. Plants were watered normally once in a day before the stress imposition and the leaf samples are harvested when the soil moisture content in the stress trays as reached below 10%. The harvested samples were stored immediately in liquid nitrogen for RNA isolation. The RWC was calculated based on the formula suggested by Barr and Weatherley (1962) as follows:

$$RWC\% = (FW\text{-}DW/TW\text{-}DW) \times 100$$

Where FW = fresh weights of leaf taken immediately after excision TW = Turgid weight of leaf DW = Dry weight of leaf dried at 70 °C for 48 h. Leaf carbohydrate content was estimated by phenol sulphuric acid method proposed by Krishnaveni et al., (1984). The total carbohydrate present in the sample solution was calculated as given below using the standard graph. Absorbance corresponds to 8 ml of the test = 'x' mg of glucose. 100 ml of the sample solution contains = x / Sample volume X 10 mg of glucose. Leaf proline content was estimated by Acid ninhydrin method described by Bates *et al.*, (1973). Free proline content in the sample was estimated by referring to a standard curve made from known concentrations of Proline by taking following formula:

$$\mu \text{ moles per g tissue} = 5 \times \mu g \text{ proline/ml} \times \text{ml toluene } 115.5 \text{ g sample}$$

Where, 115.5 = Molecular weight of Proline.

Leaf Chlorophyll content was estimated by acetone method developed by Arnon (1949). The amount of Chlorophyll present in leaf sample was calculated by using following equation:

$$\text{Total chlorophyll (mg/ml)} = (0.0202) \times (A.645) + (0.00802) \times (A.663)$$

$$\text{Chlorophyll 'a' (mg/ml)} = (0.0127) \times (A.663) - (0.00269) \times (A.645)$$

$$\text{Chlorophyll 'b' (mg/ml)} = (0.0229) \times (A.645) - (0.00468) \times (A.663).$$

The values were expressed in milligram per gram of fresh weight. Where, A = Absorbance at specific wavelength, V = final volume of chlorophyll extract in 80% acetone, Wt = fresh weight of tissue extract. Leaf Protein content was estimated as per the method given by Lowry *et al.*, (1951). From the standard graph the amount of protein in the unknown solution was calculated. The amount of protein present in the unknown solution is mg (µg of protein). The effect of moisture stress under stress and control condition in genotypes of Little millet was analysed statistically by calculating factorial CRD using OP-STAT, an online computerized software developed at BHU. RNA was isolated using TRIzol (Invitrogen, USA) and the concentration was determined using Nanodrop spectrophotometer ND-1000® (Nanodrop technologies USA). cDNA was prepared by using BIORAD iScript cDNA synthesis kit as per manufacturer's instructions.

Semi-Quantitative RT-PCR reactions were carried out in 20 µl of the solutions using gene specific primers and Actin gene primer as internal control. The reaction was performed by adding following components in order into the PCR tubes for amplification: cDNA of 2.0 µl, 10X PCR buffer of 2.0 µl, (2Mm) dNTP of mix 1.0 µl, Primer Forward of 1.0 µl, Primer Reverse of 1.0 µl, (5U/ul) Taq polymerase of 0.2 µl, Nanopure water 1,500 ng/µl of 11.8 µl. Amplifications were performed by a cycles of 2 min at 95^{0}C followed by 35 cycles each of 15 sec at 95^{0}C, 30 sec at 56-62^{0}C, and 30 sec at 68^{0}C and final extention of 1 min at 72^{0}C.

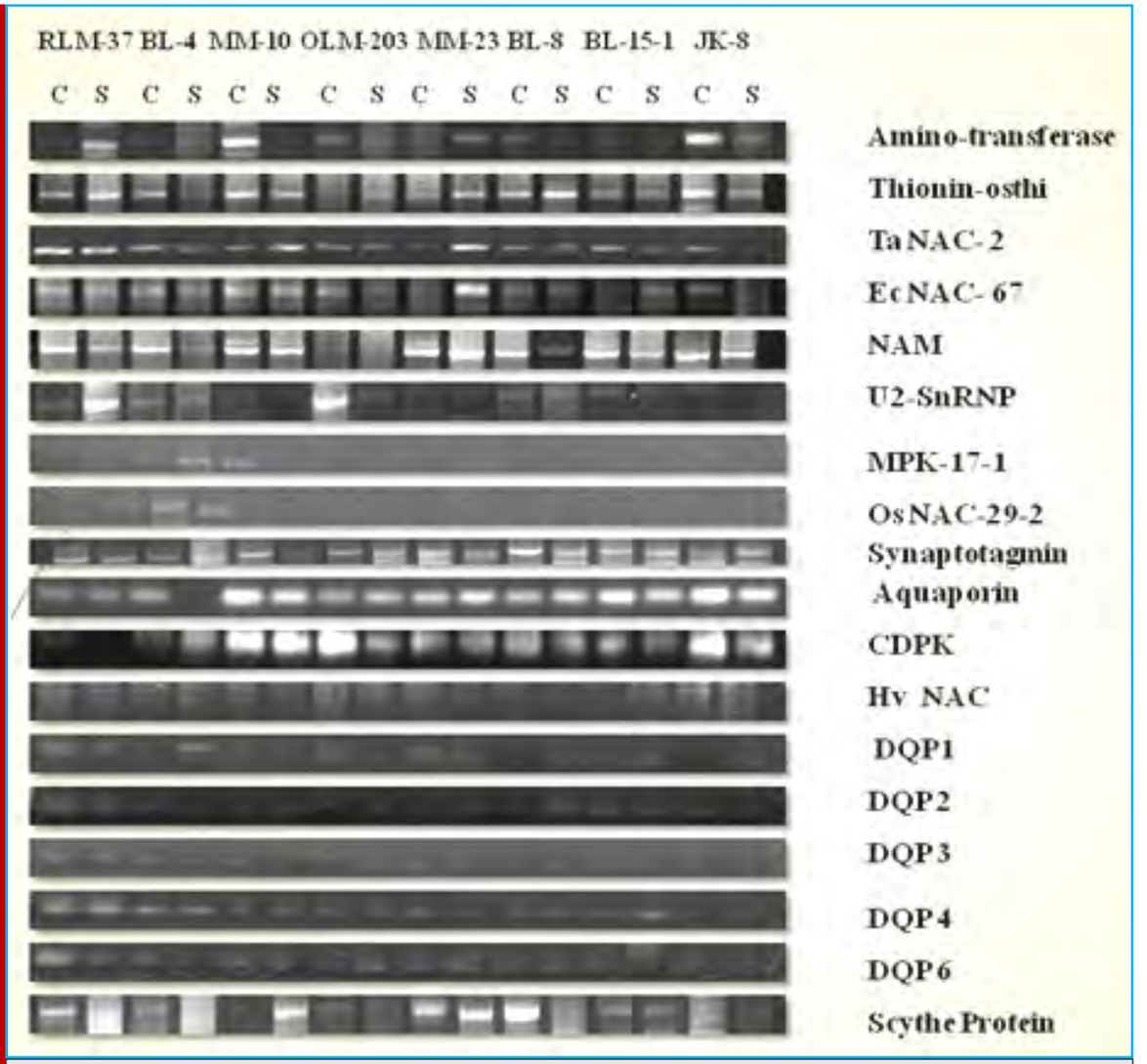


FIGURE 2. Semi quantitative RT- PCR Analysis of 18 Drought Responsive genes for Eight Genotypes of Little millet (BL-8, MM-23, MM-10, BL-15-1, RLM-37, OLM-203, BL-4, JK-8) under Control and Stress condition.

Separation of amplified fragments was carried out using Bio-Rad gel electrophoresis assembly. PCR amplification products were analyzed by Agarose gel electrophoresis on 1.5% agarose gel stained with Ethidium Bromide solution (0.5 µg/ml). The gel was run in 1X TBE buffer at 70-80 Volts for 45 minutes to 1.5 h. Standard ladders of 100bp size were used. The resultant PCR product was then resolved on 1.5% Agarose gel followed by digitalization of fluorescence data to numerical values using GelQuant.NET Analyze. The relative expression of genes was expressed in terms of fold change (Increase/ Decrease) under water stress with respect to their control.

RESULTS AND DISCUSSION

Wide variation for Relative water content values was recorded in stress tissue compared to control one's among all tested Little millet genotypes. RWC of leaf samples ranged from 14.711% to 67.9% in stress plant leaf tissues and 67.821% to 95.073% in control plant leaf tissues. The drought tolerant Little millet genotype OLM-203 has the highest RWC value (67.9%) in stress tissue followed by MM-23 (64.83%), JK-8 (64.255%), RLM-37 (64.028%), MM-10 (52.966%), BL-4(35.48%). whereas, susceptible genotypes BL-15-1 (14.711%), BL-8 (14.194%) showed the minimum RWC in stress tissues. Two Little Millet genotypes RLM-37 and OLM-203 had shown lower decrease

Table 1. Percentage change (Fold Increase or Decrease) in the RWC, Proline, Chlorophyll, Carbohydrate and Protein content for Eight Genotypes of Little millet (BL-8, MM-23, MM-10, BL-15-1, RLM-37, OLM-203, BL-4, JK-8) under Control and Stress condition.

Genotype	Decrease in RWC %	Fold increase proline	Fold decrease Total Chlorophyll	Fold increase Carbohydrate	Fold increase Protein
RLM-37	13.85	1.343	1.042	1.274	1.481
OLM-203	0.141	1.670	1.229	1.522	1.821
JK-8	27.275	3.514	1.334	1.502	2.875
MM-23	28.702	9.121	1.221	2.202	1.431
MM-10	14.855	42.200	2.011	1.633	3.604
BL-4	39.63	13.264	1.583	1.671	8.746
BL-8	56.82	52.789	1.368	1.043	8.457
BL-15-1	80.362	63.460	1.463	2.705	1.750

in RWC with values of 0.141% and 13.85% respectively as given in table 1. This clearly indicates that the two genotypes have the ability to retain more water during moisture stress which helps in sustaining the metabolic and physiological activities of plants. A wide variation for proline content was recorded in stress tissue compared to that of control one's for eight diverse Little millet genotypes (BL-8, MM-23, MM-10, BL-15-1, RLM-37, OLM-203, BL-4, JK-8). The proline content ranged from 0.192 to 7.869 µ mole/tissue under stress; whereas under control condition proline content ranged from 0.015 to 0.204 µmole/tissue. A significant increase in proline has been observed in response to water stress, favouring osmotic adjustment. When comparing fold increase in proline content under stress over control among eight genotypes BL-15-1 was found with (63.460) higher fold increase followed by BL-8 (52.789), MM-10 (42.200), BL-4 (13.264), MM-23 (9.121), JK-8 (3.514), OLM-203 (1.670) and RLM-37 (1.343) as given in table 1. Enhanced proline accumulation in leaf tissues of plants exposed to water stress condition is considered as one of the major trait for the phenotypic characterization of plants for abiotic stress tolerance (Zhu *et al.,* 2006).

A wide variation for chlorophyll content was recorded in stress tissues for eight Little millet genotypes (BL-8, MM-23, MM-10, BL-15-1, RLM-37, OLM-203, BL-4, JK-8). Chlorophyll a, Chlorophyll b and Total Chlorophyll content ranged from 0.783 to 2.441 mg/tissue, 0.403 to 1.332 mg/tissue, 1.330 to 3.811 mg/tissue respectively for stress leaf tissue where as under control condition it ranged from 1.223 to 3.075 mg/tissue, 0.597 to 3.006 mg/tissue, 1.819 to 6.047 mg/tissue respectively. The genotype MM-10 had the highest fold decrease of 2.011 mg/tissue followed by BL-4 (1.583), BL-15-1 (1.463), BL-8 (1.368), JK-8 (1.334), OLM-203 (1.229), MM-23 (1.221) RLM-37 (1.042) in the total chlorophyll content, Where as in case of chlorophyll a, the genotype MM-23 showed highest fold decrease of 2.00 followed by BL-4 (1.749), BL-15-1 (1.641), MM-10 (1.619), JK-8 (1.571), BL-8 (1.319), OLM-203 (1.208), RLM-37 (1.016) and in chlorophyll b, the genotype JK-8 showed the highest fold decrease of 3.00 followed by MM-10 (2.665), BL-8 (1.481), MM-23 (1.360), BL-4 (1.314), OLM-203 (1.270), BL-15-1 (1.159), RLM-37 (1.149) as given in the table 1.

The carbohydrate content ranges from 234.221 to 612.222 mg/tissue under stress condition whereas 153.907 to 302.313 mg/tissue under control condition. BL-15-1 (2.705) had the highest fold increase followed by MM-23 (2.202), BL-4 (1.671), MM-10 (1.633), OLM-203 (1.522), JK-8 (1.502), RLM-37 (1.274), and BL-8 (1.043) as given in table 1. The wide variation for protein content was recorded in stress tissues for eight Little millet genotypes (BL-8, MM-23, MM-10, BL-15-1, RLM-37, OLM-203, BL-4, JK-8). The protein content ranged from 0.040 to 0.586 mg/tissue under stress condition, whereas 0.027 to 0.080 mg/tissue under control condition. BL-4 (8.746) had the highest fold increase followed by BL-8 (8.457), MM-10 (3.604), JK-8 (2.875), OLM-204 (1.821), BL-15-1 (1.750), RLM-37 (1.481), MM-23 (1.431) as given in table 1.

Expression pattern of drought stress responsive genes in little millet genotypes under moisture stress condition

Semi quantitative RT-PCR was performed to analyze the expression pattern of eighteen differentially expressed transcripts in Little millet under moisture stress versus control condition. The genes include Amino-transferase, Thionin-osthi, Aquaporin, Synaptotagmin, CDPK,

Table 2. List of Eighteen Genes, with their Primer sequences, GenBank Acc. No./Locus ID's and Annealing temperatures used for Expression Analysis for Eight Genotypes of Little millet (BL-8, MM-23, MM-10, BL-15-1, RLM-37, OLM-203, BL-4, JK-8) under Control and Stress condition.

Gene	GenBank Acc. No./ Locus ID	Forward and Reverse sequence (5'-3')	Tm
Amino Transferase	LOC_Os08g39300	CTGAGTGGAGTGGAGATGGT GTTCGTTGTGCTTCAGATCC	610C
Thionin osthi	GT090938	TCAACGCTGCTCTGGGAAAT GGCTTGGTCGCAACTCTCAA	580C
Synaptotagmin	GT090932	TCTTGCAAGGTGCCAAATCTG GGCTGTGGCGTCCACTTAA	580C
U2 Sn RNP	GT090867	TGTGACCGACTTCCGTGAAG CCACGGTTGCAGCTGTTCT	590C
Scythe protein	GT090877	CCAGACACTAGCAGCACACATG CATCCCTTGCTCTGTTTGCA	590C
Aquaporin	GT090849	CCCGTTCAAGAGCAGGTCTTA CCTGTTTGGACTGGCATCTCA	610C
CDPK	GT090918	CAGAATTGACAGAGAATGAAATCCGATGGTTCCGCTGTTGTCAATA	580C
Os NAC 29-2	NC_029266	AAAGAAGGAGCAACGTGCATTCTTGTGGATTCTGCACAGC	560C
MPK 17-1	GT090884	TGTCGATGGATTGTCTGAAAAAGT TGCCGCGGTCTTTGGA	560C
Ta NAC-2	JN621240	GATTTGGTCGGGATTTCAGA GCTCCATCATCGTCTCCTCT	570C
Ec NAC-67	KU500625	CACTGCAAAGGAGGAGGAAG CTTCTTGGGCACCATCATCT	580C
Hv NAC	JX855805	CTACGACGACATCCAGAGCA GTCATCCATTCCGCTTCTGT	580C
NAM	LOC_Os03g21060	CAAGACCAACTGGATCATGC TTCTTGTAGATCCGGCACAG	620C
DQP1	LOC_Os08g36920	AGTACATGATCCGATTCGAC GTCCTGTAGCCGGAGATGAC	65.40C
DQP 2	LOC_Os11g26760	GTGAAGGAGGAGCACAAGAC TTGATCTTCTCCTTGATTCC	640C
DQP 3	LOC_Os01g44390	CGATGTCGGTGAGCTCGT GGTCTCGATGCGCTTGAC	63.50C
DQP 4	LOC_Os03g20550	ATCAATCACACCATCTAGGC GTATCTGGGGAAATTACAGTTG	610C
DQP 6	LOC_Os04g49550	GAGCTAGAGAGGAAGACGATG ATGATGACGATGTCCCTGTC	64.10C

Scythe protein, Ta NAC-2, Ec NAC-67, NAM, U2-Sn RNP, Hv NAC, Os NAC-29-2, MPK 17-1, DQP-1, DQP-2, DQP-3, DQP-4, DQP-6. Semi quantitative RT-PCR analysis showed differential expression of these eighteen transcripts in Little millet genotypes under stress with respect to the control condition. The results are discussed below in detail. **Note:** The increase or decrease in the Fold value was calculated my measuring the band intensity and size using GelQuantNET software.

You J., Hu H., Xiong L.(2012) have confirmed that OsOAT is a direct target gene of the stress-responsive NAC transcription factor SNAC2 (Li *et al.,* 2008). In addition, OsOAT over expressing plants show significantly increased tolerance to oxidative stress mainly through enhancing ROS-scavenging capacity and pre-accumulation (You *et al.,* 2012). The RT-PCR of Amino-transferase showed up-regulation in the transcript level by 2.25, 5.65 fold in the genotypes OLM-203, MM-23 respectively as shown in graph 1. The transcriptional analysis revealed that PvOAT was strongly induced by drought stress and it has also been reported that the expression of PvOAT was higher in leaves than that in root and stem of common bean (*Phaseolus vulgaris* L.) by drought stress (Ji-bao *et al.,* 2016). Thus the up-regulation of this transcript under water stress suggests that it may play a key role in identification of different transcription factors which are responsible for different drought tolerant mechanisms in Little millet.

Thionin Osthi belongs to oxidative stress category of genes. A report by Yamakawa et al. (2007) reveals

that this gene showed 2.0 fold up-regulation under high temperature stress in *Medicago truncatula*. The RT-PCR of Thionin-osthi in this study showed up-regulation in the transcript level by 10.15, 16.6 fold in the genotypes RLM-37, MM-23 respectively as shown in graph 1. Hence its induction in moisture stress in Little millet may be attributed due to the presence of the cis acting elements, suggesting an important role of this gene in combating oxidative stress.

Aquaporin belongs to major intrinsic protein super family which functions as a membrane channel. Over expression of a Panax ginseng tonoplast, aquaporin enhances drought and salt tolerance ability in transgenic Arabidopsis plants (Yanhui *et al.,* 2007). But the RT-PCR of Aquaporin showed a negligible up-regulation in the transcript level by 1.16, 1.26, 1.42 fold in the genotypes BL-8, MM-23, OLM-203 respectively as shown in graph 1.

It has been shown that Synaptotagmin imparts calcium dependent freezing tolerance via membrane resealing and also loss of function of this gene reduces cell viability and plasma membrane integrity in Arabidopsis (Yamazaki *et al.,* 2008). RT-PCR of Synaptotagmins showed an up-regulation in the transcript level by 2.92 fold in the genotype JK-8 as shown in graph 2. Identification and up-regulation of Synaptotagmin under moisture stress indicates its role in stress signal transduction and tolerance which needs to be further elucidated.

Calcium-dependent protein kinases play important role in signalling pathways for various stress responses (Ray *et al.,* 2007, Sheen J. 1996). The RT-PCR of CDPK showed up-regulation in the transcript level by 1.21, 5.25 fold in the genotypes MM-10, BL-4 respectively as shown in graph 2. In several previous studies, induction and expression of CDPK(s) have been reported to be higher in tolerant cultivars in different abiotic stresses (Kawasaki *et al.,* 2001, Li *et al.,* 2008). The CIPK family of 26 protein kinases regulates the function of several ion transporters near the cell membrane to restore ion homeostasis under stress situations (Chaves-Sanjuan *et al.,* 2014).

The differentially induced expression of OsCIPK genes by different stresses and the examples of improved stress tolerance of the OsCIPK transgenic rice suggest that rice CIPK genes have diverse roles in different stress responses and some of them may possess potential usefulness in stress tolerance improvement of rice (Xiang *et al.,* 2007). Thus, suggesting its putative role in cell signalling pathway and in combating drought stress.

Scythe protein has been observed as a novel reaper-binding apoptotic regulator in vertebrates. Research by Thress *et al.,* suggests that the Scythe protein might work by regulating the folding and activity of the molecules that make up the signaling pathway that controls apoptosis (Thress *et al.,* 1998). The RT-PCR of Scythe protein showed up-regulation in the transcript level by 2.01, 29.20 fold in the genotypes MM-23 and MM-10 respectively and a negligible level of up-regulation was found in BL-4 by 1.31 fold as shown in graph 2.NAC (NAM, ATAF, and CUC) is a plant specific gene family of transcription factors. A few NAC genes from Arabidopsis and Brassica have been reported to be responsive in various environmental stresses (Shao *et al.,* 2015). Over expression of various NAC genes have also been reported to significantly improve drought tolerance in transgenic rice Shao *et al.,* (2015).

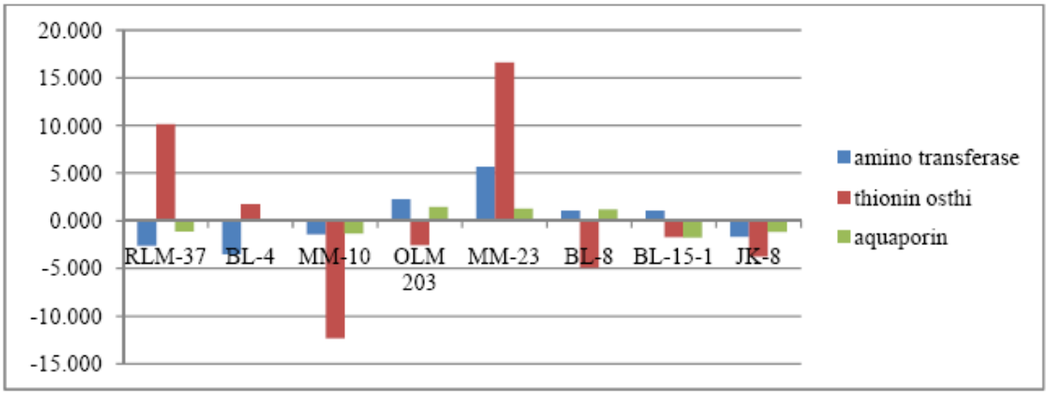
RT-PCR of NAM showed a negligible up-regulation in the transcript level by 1.07, 1.07, 1.20 fold in the genotyes BL-8, BL-15-1, MM-23 respectively, Where as a significant up-regulation was found in the genotype OLM-203 by 3.75 fold as shown in graph 3. The RT-PCR analysis of the up-regulation of this transcript under moisture stress suggests that it may play an important role in the cross-linking of different signalling pathways in Little millet.

In wheat, NAC TFs are known to be involved in processes such as senescence and nutrient remobilization as well as responses to abiotic and biotic stresses, ranging from stripe rust to abiotic stresses including drought and salt tolerance (Xia *et al.,* 2010a). Out of four genes Ta NAC-2, Ec NAC-67, Hv NAC, Os NAC-29-2, The RT-PCR analysis of Ta NAC-2 showed up-regulation in the transcript level by 2.35, 8.66 fold in the genotypes MM-10, MM-23 respectively and RT-PCR analysis of Ec NAC-67 showed up-regulation in the transcript level by 4.44, 10.76 fold in the genotypes BL-15-1, MM-23 respectively as shown in graph 3. The RT-PCR analysis of Hv NAC showed negligible up-regulation in the transcript level by 1.30, 1.75 in the genotypes RLM-37, BL-4 respectively and RT-PCR analysis of Os NAC-29-2 showed a significant up-regulation in the transcript level by 13.99 fold in the genotype RLM-37 as shown in graph 4.

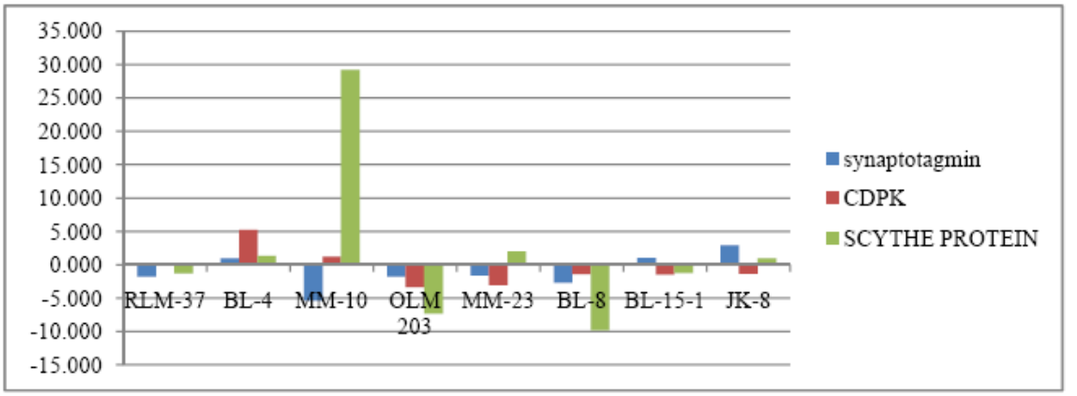
Alternative splicing takes place in highly specialized structures within nucleus called spliceosomes consisting of five small nuclear ribonucleoprotein particles, SnRNPs (U1, U2, U4/6, and U5) and other non-SnRNPs (Reddy ASN. 2001). RT-PCR of U2-SnRNP showed an up-regulation in the transcript level by 3.12, 4.65 fold in the genotypes MM-23, RLM-37 respectively shown in graph 4. Similar gene induction of U2-SnRNP has been reported among tolerant and susceptible cultivars of Foxtail millet (Charu Lata *et al.*, 2010). These results suggest that U2-SnRNP may play a significant role in alternative splicing in Little millet and thus regulating gene expression.

MAP kinase signaling is one of the most important and conserved pathways in most cellular process as well as environmental stress responses (Lee *et al.,* 2008,

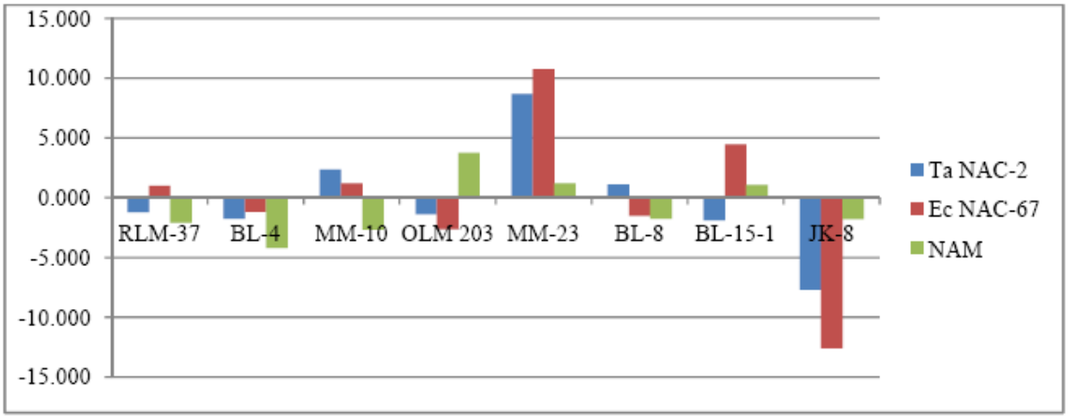
GRAPH-1 for Amino transferase, Thioninosthi, Aquaporin



GRAPH-2 forSynaptotagmin, CDPK, Scythe protein genes

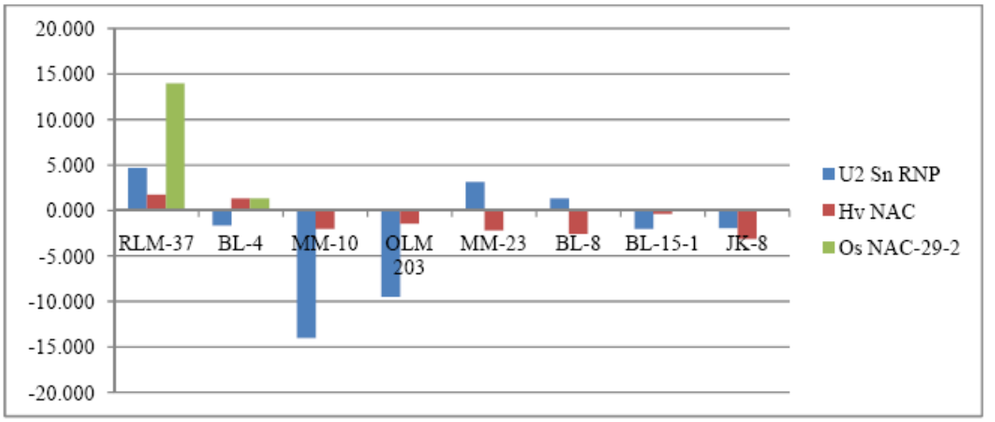


GRAPH-3 for Ta NAC-2, Ec NAC-67, NAM genes

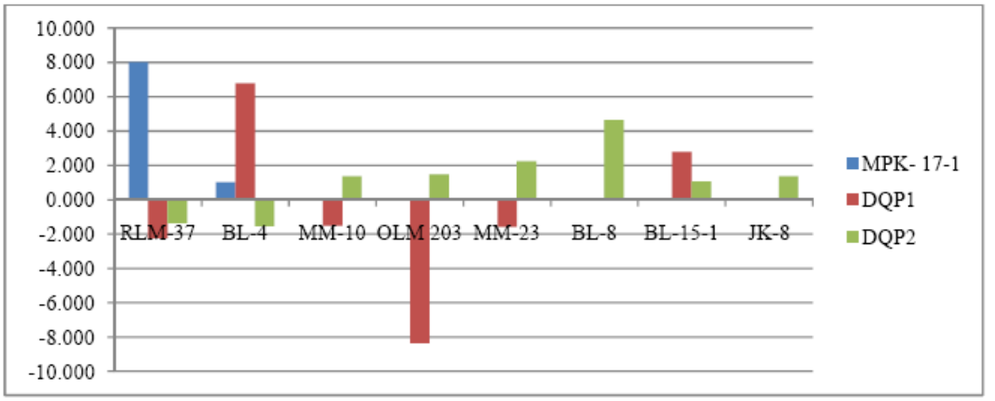


GRAPHS 1-6. Shows the Fold Increase/Decreaseamong Eight Genotypes of Little millet (RLM-37, BL-4, MM-10, OLM-203, MM-23, BL-8, BL-15-1, JK-8) due to the effect of Moisture stress on Gene Expression under Control and Stress condition.

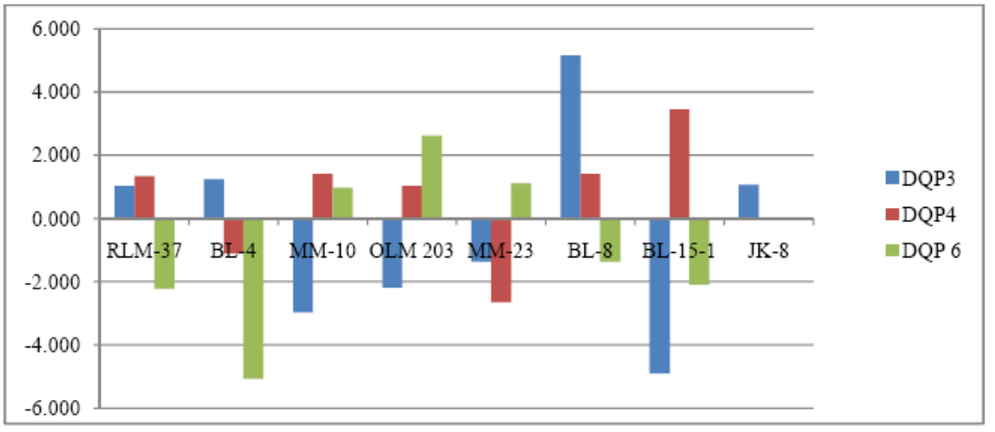
GRAPH-4 for U2-Sn RNP, Hv NAC, Os NAC-29-2 genes



GRAPH-5 for MPK 17-1, DQP-1, DQP-2 genes



GRAPH-6 forDQP-3, DQP-4, DQP-6 genes



GRAPHS 1-6. (*Continued*)

Moustafa *et al.,* 2008). The RT-PCR analysis of MPK 17-1 showed an up-regulation in the transcript level by 7.99 fold in the genotype RLM-37 as shown in graph 5. MAP kinase gene has been reported to be induced due to dehydration, salinity and hyper-osmotic stresses (Moustafa *et al.,* 2008). The activation of MPK 17-1 gene in moisture stress suggests that it may play an important role in the cross-linking of different signalling pathways to activate plant defense mechanisms in Little millet.

The AP2/EREBP genes play crucial roles in plant growth, development and biotic and abiotic stress responses and is one of the largest and specific transcription factor (TF) families in plants. Liu and Zhang have reported in *G.hirsutum* that, 151 non-repeated genes of the DREB and ERF subfamily genes were responsive to different stresses: 132 genes were induced by cold, 63 genes by drought and 94 genes by heat (Liu and Zhang 2017). The RT-PCR of DQP 1 showed an up-regulation in the transcript level by 2.78, 6.79 fold in the genotypes BL-15-1, BL-4 respectively as shown in graph 5.

Three of the four rice genes [(OsBIERF 1–4) *Oryza sativa* benzothiadiazole (BTH)-induced ethylene responsive transcriptional factors (ERF)] with a single conserved ERF domain were found to be up-regulated by salt, cold, drought, wounding as well as in an incompatible interaction between rice and fungal pathogen suggesting their role in biotic and abiotic stress (Jisha *et al.,* 2015). In studies dealing with drought stress, Pelah et al. found a correlation between drought tolerance and accumulation of dehydrin proteins in Populus popularis (Pelah *et al.,* 1997). The RT-PCR of DQP 2 showed an up-regulation in the transcript level by 2.23, 4.65 fold in the genotypes MM-23, BL-8 respectively. There was a negligible up-regulation found in the genotypes JK-8, MM-10, OLM-203 by 1.37, 1.37, 1.47 fold respectively as shown in graph 5.

A total of 44.67% and 47.21% MYB genes were found up and down-regulated in *Arabidopsis* under cold stress, respectively in the case of drought stress, many MYB genes have been isolated and demonstrated to be involved in drought responses in plants (Mmadi *et al.,* 2017). The transcriptional activation of cuticular wax biosynthesis by MYB96 contributed to drought resistance in *Arabidopsis thaliana* (Seo *et al.,* 2011). The RT-PCR of DQP 3 showed a significant up-regulation in the transcript level by 5.15 fold in BL-8 genotype as shown in graph 6. Altogether, these evidences demonstrated the versatility and importance of this gene family in plants.

Members of the large family of WRKY transcription factors are involved in a wide range of developmental and physiological processes, most particularly in the plant response to biotic and abiotic stress. RT-PCR analysis of Yu Y., Wang N., Hu R, Xiang F. showed that in whole soybean plant, 66 GmWRKYs exhibited distinct expression patterns in response to salt stress (Yu *et al.,* 2016). The RT-PCR of DQP 4 showed a negligible up-regulation in the transcript level by 1.33, 1.40, 1.41 fold in the genotypes RLM-37, BL-8, MM-10 respectively and a significant level of up-regulation in the transcript was observed in the genotype BL-15-1 by 3.44 fold as shown in graph 6.

In a study by Liu K. et al. showed over-expression of OsCOIN protein, a RING finger protein in transgenic rice lines significantly enhanced their tolerance to cold, salt and drought, accompanied by an up-regulation of OsP5CS expression and an increase of cellular proline level (Liu *et al.,* 2007). Salt and drought-induced RING FINGER1 (SDIR1), is involved in abscisic acid (ABA)-related stress signal transduction in *Arabidopsis thaliana* (Zhang *et al.,* 2007). SDIR1 is expressed in all tissues of Arabidopsis and is up-regulated by drought and salt stress, but not by ABA (Zhang *et al.,* 2007). The RT-PCR of DQP 6 showed an up-regulation in the transcript level by 2.61 fold in the genotype OLM-203 and a negligible level of up-regulation was noticed in the genotype MM-23 by 1.11 fold as shown in graph 6.

Out of 18 transcripts under control and stress condition for eight genotypes of Little millet, a significant level of up-regulation was observed among the following:

- Genotype MM-23 showed a higher level of up-regulation for the genes Aminotransferase, Ta NAC-2 Ec NAC-67 and Thionin osthi by 5.65, 8.66, 10.76, 16.60 fold respectively.
- Genotype RLM-37 showed a significant up-regulation for the genes U2 Sn RNP, MPK-17-1, Thionin osthi and Os NAC-29-2 by 4.65, 7.99, 10.15 and 13.99 fold respectively
- Genotype BL-4 showed an up-regulation in the transcript level by 5.25 and 6.79 fold for the genes CDPK and DQP1 respectively.
- Genotype MM-10 showed a greater level of up-regulation for the gene scythe protein by 29.20 fold.
- Genotype BL-8 showed an up-regulation for the genes DQP 2 and DQP 3 by 4.65 and 5.15 fold respectively.
- BL-15-1 genotype showed a significant up-regulation for the transcript Ec NAC-67 by 4.44 fold.

CONCLUSION

Current study helps us to identify the key genes expressed in response to moisture stress in the selected Little millet genotypes. Induction of transcripts Amino-transferase, Thionin-osthi, Aquaporin,Synaptotagmin, CDPK, Scythe protein, Ta NAC-2, Ec NAC-67, NAM, U2-Sn RNP, Hv NAC, Os NAC-29-2, MPK 17-1, DQP1, DQP 2, DQP 3,

DQP 4, and DQP 6 suggests that these genes may impart drought avoidance capacity to the tolerant genotypes. Genes which were up-regulated suggests their function in positive regulation in adaptation of the moisture stress under the drought condition, this can be taken as a base for drought tolerance response of the crop, which may be useful for further validation studies of the candidate genes for drought tolerance in the millet species as well as other crop plants.

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