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RESEARCH ARTICLES

Bioscience Biotechnology Research Communications

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Biosc Biotech Res Comm is an official publication of an academic, non-profit Society for Science and Nature, Bhopal India, since 2008. It is a peer reviewed journal that publishes original research articles pertaining to exciting applied areas of biology including biomedical sciences. The aim of *Biosc Biotech Res Comm* is to promote quality scientific research in fundamental and applied areas of biological and biomedical sciences and their allied branches.

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Editors Communique

Have we tamed the coronavirus? May be yes,
as pandemics do not die, they can only be faded !

Science and technology has made it possible, in the shortest span of time, it has shown that with firm determination and international cooperation, we can win over the onslaughts of even the worst of the pandemics. COVID-19 is perhaps fading over now, due to our coordinated efforts worldwide. Though we have lost millions, in the two year period, partly due to the mishandling of the viral attacks and somewhat by our own follies and carelessness. Anyway lessons learnt from the past, always make us more stronger and determined. Let us now not relax and work on a better mode, as all is still not well yet. The almost taming of the virus and its cousins have indicated some of the concealed failures, on which we have to focus now. We have to be more vigilant, and even a bit of laxity can spoil the good work done. On societal and governmental parts, utmost care and caution is required on a long term basis.

On behalf of Bioscience Biotechnology Research Communications, we falter at words to express our deep sense of solitude and grief on the catastrophic events of the world wide pandemic, spanning over two years now. We pray for the strength to bear this universal calamity and come up with long lasting fortitude to eradicate it soon.

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Sharique A. Ali, PhD
Editor-in-Chief

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Molecular Biodiversity of Rhizobia Isolated from Root Nodules of Some Economical Important Legumes in Gadarif State – Sudan

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ABSTRACT

Although Gadarif State is an important agricultural area in Sudan, studies must be conducted on rhizobia's genetic or molecular diversity associated with economically important legumes. Therefore, this study was undertaken to isolate rhizobia related to groundnut (Arachis hypogea), Bambara groundnut (Vigna subterranean), and Cowpea (Vigna unguiculata) in different localities in Gadarif State and study their phylogenetic relationships to make the genetic information of the indigenous rhizobia available and establish a molecular database for monitoring future climate change impact on their diversity. Nodules were collected from 11 localities of the Gadarif state, rhizobia were isolated, DNA was extracted, 16S rRNA, recA, glnII, nifH, and nodA genes were amplified, and data were analyzed. The results showed that all isolates obtained from six localities were found to be fast-growing. Isolates obtained from groundnut nodules are Rhizobium sp._Haw1 and Rhizobium sp._G6-11, which are found to be related to Rhizobium leguminosarum. These two strains were found to be associated with Rhizobium etli when BLASTN analyzed the sequences. At the same time, Rhizobium sp._UoG27, which was isolated from the same plant, was found to be related to Rhizobium tropici. There is evidence of new species in Rhizobium sp._UoG30, Rhizobium sp._Sab13 (isolated from Bambara groundnut), Rhizobium sp._Umk34 (isolated from Cowpea), and Rhizobium sp._Taw3 (isolated from groundnut). No symbiotic genes (nifH and nod) were found in all isolates except the strain isolated from Alfalfa grown in Gezira state (Central Sudan). The study concluded that fast-growing rhizobia are dominant in Gadarif state soils, characterized by genetic instability, and may play roles other than nitrogen-fixing.

KEY WORDS: PPHYLOGENY, RHIZOBIUM, ISOLATES, COWPEA, GROUNDNUT, BAMBARA GROUNDNUT.

INTRODUCTION

A For decades, many crop inoculation trials were done in the world to increase legume production and soil fertility at the same time. Many of these trials showed no significant increase in production. The failure may be due to the lack of genetic information about the bacteria used as inoculants, which are supposed to fix atmospheric nitrogen in association with legumes. Nitrogen is the most limiting nutrient for plant growth, including leguminous (Howieson and Committee, 2020; Simon et al., 2014). It is essential in plant cells for synthesizing enzymes, proteins, chlorophyll, DNA and

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RNA, thus essential for plant growth and production of food and feed, (Fahde et al., 2023).

Many times, on-farm activities, such as adjusting crop rotations, boosting the production of legumes in main crops and catch crops, and returning the byproducts to the fields, can fix inadequate N and humus balances. However, as a result, the nutrient cycles may become even more open because of the widespread insufficiency of levels of basic nutrients in the soil that are available to plants. Therefore, deficiencies in the fundamental nutrients P, K, Mg, and S are typically needed for external supplementation by fertilization with organic fertilizers and, in some circumstances, also with mineral fertilizers (Kolbe, 2022).

Rhizobia are Gram-negative bacteria (Proteobacteria) that live in the root nodules of legume plants. They are renowned for being able to fix nitrogen for their hosts' legume species in return for carbon. Members of the family proteobacteria and the genera *Rhizobium, Bradyrhizobium, Ensifer, Phyllobacterium, Mesorhizobium, Devosia, Allorhizobium, Azorhizobium*, and *Microvirga* make up the majority of the bacteria that fix nitrogen in the root nodules of leguminous plants. Rhizobia, or members of the Burkholderiaceae family of proteobacteria, also nodulate legumes (Mukhtar et al., 2020).

According to Vanlauwe et al. (2019), the common bean (*Phaseolus vulagaris* L.), soybean (*Glycine max*), pigeon pea (*Cajanuscajan*), broad bean (*Viciafaba*), chickpea (*C. arietinum*), and cowpea (*Vigna unguiculata* L. Walp.) are the most important legumes in Africa. In the majority of African nations, subsistence farmers grow legumes primarily for food, however any surplus can be sold to generate revenue (Kawaka et al., 2014). Farmers in rural Africa cannot afford nitrogenous fertilizers because of the extreme poverty there; as a result, nitrogen requirements for cultivation are mostly met by native rhizobia. Low legume yields with those isolates have been consistently recorded in East Kenya, albeit certain native isolates there are ineffective at fixing nitrogen (West Africa (Binde et al., 2009), South Africa, as well (Biro et al., 2010 Abubakar, and Yusuf, 2016).

Commercial rhizobia inoculants have had some success in some areas (FAO, 2015), however multiple studies also show that they were ineffective in many agricultural areas in terms of promoting plant growth and final production. [Lack of successful adaptation of the isolates to the local soil conditions may be the cause of the commercial rhizobia's failure in African farms. The majority of formulations (Kawaka et al., 2014) use strains that have been obtained from different continents that may not be well enough acclimated to the local environment. Therefore, it is essential to identify, study, and employ the rhizobial strains in the local soils as commercial inoculants in certain areas of Africa. However, formulations including local elite isolates from the specific localities must be developed and commercialized.

The diversity and biotechnological potential of symbiotic bacteria are high in tropical soils. Nevertheless, tremendous strains' phylogenetic relationships are still poorly understood (Biro et al., 2010). In Sudan, many inoculation trials were achieved, and some of these trials showed that some local isolates have a potential effect on nitrogen fixation. However, few studies concentrate on the genetic characterization of the microbes associated with legumes and responsible for nitrogen fixation.

Gadarif state is located in the eastern part of Sudan; it consists of 12 localities. The people's main job is agriculture, one of the most important agricultural areas in Sudan (Food Net of Sudan). Legumes like groundnut (*Arachis hypogaea*), Bambara groundnut (*Vigna subterranean*), and Cowpea (*Vigna unguiculata*) are grown and used for direct local consumption, and the shoot system residuals are used for animal feeds. Groundnut is also used in oil production and groundnut cake for animal feeding after processing. Despite this, genetic information on bacteria associated with leguminous plants is scarce in the Gadarif State. Although the soil's organic matter and nitrogen content are low in the Gadarif State (FAO, 2015), it necessitates looking for indigenous efficient nitrogen-fixing bacteria to be used in inoculation.

The aims of this study are to: Investigate the molecular differences of rhizobia associated with groundnut, Bambara groundnut, and Cowpea in different localities in Gadarif State. Study the phylogenetic relationship of the isolates associated with the same plants and obtained from different sites. Contribute to the availability of genetic information on the indigenous rhizobia to help in efficient inoculants preparation for restoring and conserving soil fertility. Establish a molecular database to be used as a baseline to monitor future climate change's impact on the molecular biodiversity of rhizobia in Gadarif State.

MATERIAL AND METHODS

Study area: The Gadarif State is located in eastern Sudan between Latitudes 12º N and 13º N and 33º E and 37º E. It covers a total area of approximately 78,000 km². The annual rainfall in the northern part is less than 500 mm. The mean monthly temperature ranges from 26º – 32º C, while the mean maximum temperatures rise to 41º C. Soils are heavy dark-cracking clays; the clay content is very high, 70 % to 80 %. The soil's organic matter and nitrogen content are low, but as there is no deficiency of other plant nutrients, the soil is moderately fertile (FAO, 2015).The state has 12 localities: the Gadarif, Middle locality, Eastern Galabat, Western Galabat, Alrahad, Alfashaga, Almafaza, Albotana, Alfaw, Galaalnahal, Algoraesha, and Basonda. Agriculture is the main activity in the state; the total agricultural area is 8602600 acres. It contributes 54.8% of the state's gross domestic product (GDP).

The main crops are maize, sesame, sorghum, and sunflower, which grow in rain-fed areas. Groundnut was grown in irrigated regions on about 72000 acres in 2014, and the area is increasing (Vincent, 1970); sometimes, it is grown in rain-fed areas. Besides groundnut, leguminous crops like Bambara groundnut and Cowpea are grown in minimal areas for local consumption.

Collecting nodules: Nodules were collected from different localities of the Gadarif State in the autumn (September 2016). The plant roots were carefully removed from the soil, as nodules will be dislodged easily if the plant is pulled from the soil. The nodules were stored and preserved in screw-capped plastic tubes containing silica gel, with a cotton plug separating nodules from the desiccant in the bottom; the tube was marked with a permanent marker to re¬cord the location of the collection sites (Howieson and Committee, 2020).

Bacteria isolation: Bacteria were isolated in the Biofertilization lab, department of Biofertilization, National Center for Research, Sudan. Collected nodules were washed with sterile water, and surface sterilization was done using 70% ethanol and 20 times diluted sodium hypochlorite solution and repeatedly washed with sterile water. After surface sterilization, the nodules were crushed. The resulting suspension was streaked onto yeast extract mannitol agar (YEMA) with or without Congo Red or Bromothymol blue at pH 6.8. The medium contains (g / l): mannitol, 10; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.4; yeast extract, 1; agar (Sulieman et al., 2022).

DNA extraction: A colony of bacteria was grown in AG broth medium in an incubator shaker (150 rev/minutes) at 28°C for two days. Centrifugation was done to collect about 20 ml of the bacterial culture. Bacteria were resuspended in 300 µl TE buffer after washing the bacterial biomass once with TE buffer (10 mM tris, one mM EDTA, pH 8). After that, 100 µl of 5% SDS (Sodium dodecyl sulfate) and 100 µl pronase E (2.5 mg/ml in TE buffer pre-incubated for 90 minutes at 37°C) were added and mixed. The resulting solution was incubated overnight, and the DNA was sheared thoroughly using a syringe. The DNA was purified by two extractions with 300 µl of Tris-buffered phenol and one extraction with methylene chloride. DNA obtained was precipitated with 2.5 volumes of ethanol (Wekesa et al., 2022).

Amplification of the different genes 16S RNA : To amplify 16SrRNA, PCR reaction was set with the following: 5 µl of 10x dream Taq buffer, 1 µl dNTPs, 1 µl forward primer (16Sa 5-cgctggcggcaggcttaaca-3), 1 µl reverse primer (16Sb 5-ccagccgcaggttcccct-3), 1 µl template DNA, 0.5 µl dream taq polymerase, and 40.5 µl double distilled water total volume of 50 µl. To obtain full-length 16SrRNA, it was amplified again with the same reaction using forward primer (16SLoa 5-taacgcattaaacattccgcctgg-3) and reverse primer (16Slob 5-ttaatcttgcgaccgtactcc-3). PCR conditions were initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for the 30s, extension at 72°C for 1.5 minutes, and final extension at 72°C for 10 minutes.

recA: PCR reaction for recA amplification contains 5 µl of 10x dream Taq buffer, 1 µl dNTPs, 1 µl forward primer (recA-a 5- gacgaccttgacgcgsgtctgrttg-3 for all strains and MrecAAI2f 5-ccgaacatgacgccgatcttcatgc-3 for strain Gez1), 1 µl reverse primer (recA-b 5- aaatcggtggayaaaagcaargc-3 for all strains and MrecAAI1r 5-tgtatcatggctcagaattctttgc-3 for strain Gez1), 1 µl template DNA, 0.5 µl dream taq polymerase, and 40. 5 µl double distilled water total volume of 50 µl. PCR conditions were: initial denaturation at 95°C for 3 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for the 30s, extension at 72°C for 45 seconds, and final extension at 72°C for 5 minutes. \

glnII: PCR reaction for glnII amplification contains 5 µl of 10x dream Taq buffer, 1 µl dNTPs, 1 µl forward primer (glnIIetF 5 atgacaaaatataagctcgagtatatttggc-3 for strains Haw1, G6-11, UoG27, and Gez; glnIIAbdF2 5-atcaaccacgaaggcatcaacg-3 for strain Taw3; glnII3for 5-tacaaggacggycgcccgctcggcttcc-3 for strains Sab13, UoG30 and Umk34), 1 µl reverse primer (glnIIetR 5-tcgatgtcgatgccgtatttttcggtcag-3 for strains Haw1, G6-11, UoG27 and Gez; glnIIAbdR 5-gtaggagaacttgttccacgg-3 for strain Taw3; glnII4rev 5- cgcggtctcgtgcttgccgg-3 for strains Sab13, UoG30, and Umk34), 1 µl template DNA, 0.25 µl dream taq polymerase, and 40.75 µl double distilled water total volume of 50 µl. PCR conditions were: initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for the 30s, extension at 72°C for 1.5 minutes, and final extension at 72°C for 10 minutes. To amplify the strain Umk34 glnII gene PCR conditions were initial denaturation at 95°C for 30 seconds, 25 cycles of denaturation at 95°C for 1 minute, annealing at 53°C for 30 seconds, extension at 72°C for 45 seconds, and final extension at 72°C for 5 minutes.

For other strains, gradients PCR was done as follows: initial denaturation at 95°C for 3 minutes, 25 cycles of denaturation at 95°C for minute, annealing (6 cycles at 60 - 55°C for 30 seconds for strains Hw1, G6-11, and UoG27; 6 cycles at 58 - 50°C for 30 seconds for strains Sab13 and UoG30), extension at 72°C for 30 seconds and final extension at 72°C for 30 seconds.

nifH: PCR reactions for nifH contain 5 µl of 10x dream Taq buffer, 1 µl dNTPs, 1 µl forward primer (MnifHAI1f 5- atcggcaagtccaccacctcycaaa-3), 1 µl reverse primer (MnifHAI2r 5- ctccatggtratyggggtcgggatg-3), 1 µl template DNA, 0.5 µl dream Taq polymerase, 1.5 µl DMSO and 39.5 µl double distilled water total volume of 50 µl. PCR conditions were: initial denaturation at 95°C for 5 minutes, 25 cycles of denaturation at 95°C for one minute, annealing at 55°C for 1 minute, extension at 72°C for 45 seconds, and final extension at 72°C for 5 minutes.

nodA: PCR reaction for nodA contained 5 µl of 10x dream Taq buffer, 1 µl dNTPs, 1 µl forward primer (nodA-Sin-F 5- tgtccttaaamgtgcagtggaag-3 for strain 37), 1 µl reverse primer (nodA-Sin-R 5- caatgtacctggcggccattcgt-3 for strain 37), 1 µl template DNA, 0.25 µl dream taq polymerase, and 40.75 µl double distilled water total volume of 50 µl. For amplification, gradient PCR was done as follows: initial denaturation at 95°C for 3 minutes, 25 cycles of denaturation at 95°C for 1 minute, annealing (6 cycles at 58 – 53 °C and 25 cycles at 52 °C for), extension at 72°C for 30 seconds and final extension at 72°C for 30 seconds.

All molecular characterization experiments were done in the Molecular Genetic lab, Institute of Genetics, Faculty of Science, TU Dresden, Germany (Table 1 and 2).

PCR products were purified and sequenced, and then the sequences were analyzed by the algorithm BLASTN to identify similarities

Nucleotide Accession Numbers

RESULTS

According to the 16S RNA phylogeny tree, the isolates obtained from groundnut, Bambara groundnut, and cowpea root nodules were clustered on two main phylogeny branches or main well-defined clusters (Figure 2). One branch was divided into two subclusters comprised of rhizobia isolated from nodules of the three plants with bootstrap support of 99%. These include Rhizobium sp._Sab13, which was isolated from Bambara groundnut grown in Sabarna (Western Galabat locality), Rhizobium sp._Umk34, which was isolated from nodules of Cowpea grown Umkhareet (Basonda locality), and Rhizobium sp._Taw3 isolated from nodules of groundnut grown in Tawareet (Eastern Galabat locality). The second subcluster included Rhizobium sp._UoG30, isolated from Bambara groundnut grown in the Gadarif locality.

Table 1. Different Gene Sequences Accession Numbers

Genes Strain	16SrRNA	recA	glnII	nodA
Rhizobium sp Haw1	MN211542	MN218348	MN218340	-
Rhizobium sp Taw3	MN211543	MN218349	MN218341	-
Rhizobium sp G6-11	MN211544	MN218350	MN218342	-
Rhizobium sp Sab13	MN211545	MN218351	MN218343	-
Rhizobium sp UoG27	MN211546	MN218352	MN218344	-
Rhizobium sp UoG30	MN211547	MN218353	MN218345	-
Rhizobium sp Umk34	MN211548	MN218354	MN218346	-
Rhizobium sp Gez1	MN211549	MN218355	MN218347	MN218356

Table 2. GC% Of The Different Rhizobia Isolated From Different Legumes

Isolates	Plants	16SrRNA	glnII	recA
Rhizobium sp._Haw1	Groundnut	56	62	61
Rhizobium sp._Taw3	Groundnut		59	59
Rhizobium sp._G6-11	Groundnut	56	62	61
Rhizobium sp._Sab13	Bambara groundnut	55	59	61
Rhizobium sp._UoG27	Groundnut	55	61	61
Rhizobium sp._UoG30	Bambara groundnut	55	58	61
Rhizobium sp._Umk34	Cowpea	55	58	61
Sinorhizobiummeliloti	Alfalfa	55	63	61

Figure 1: Phylogeny tree analysis of 16srRNA of Rhizobia isolated from different legumes.

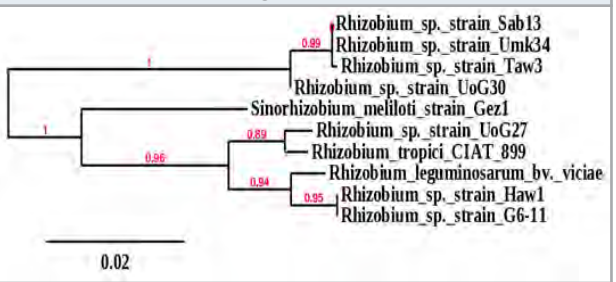
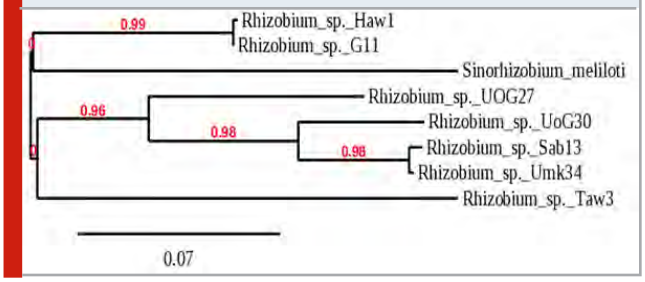


Figure 2: Phylogeny tree analysis of recA of Rhizobia isolated from different legumes



The second branch was also divided into two subclusters with bootstrap support of 96%; one subcluster comprised only *Sinorhizobium meliloti* isolated from nodules of Alfalfa grown in Gazira state (Central Sudan). The second

subcluster branched to two more subclusters; the first contains Rhizobium sp._UoG27 isolated from groundnut grown in Gadarif locality, which was clustered with *Rhizobium tropici* _CIAT_899 (sequences obtained from the gene bank database). The second subcluster contains Rhizobium sp._Haw1 isolated from root nodules of groundnut grown in Al-Hawata (Al-Rahad locality) and Rhizobium sp._G6-11 isolated from groundnut grown in Garia6 (Al-fashaga locality). These last two isolates were found clustered with *Rhizobium leguminosarum*_bv._viciae, whose sequences were also obtained from the gene bank database (Figure 1).

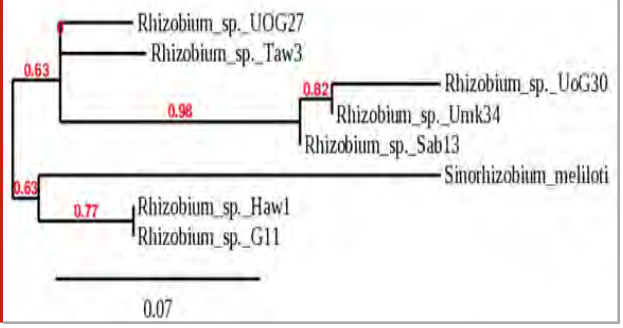
Like16S RNA phylogeny tree, the phylogeny tree of the recA gene also clustered the different isolates into two main groups with bootstrap-support of 99%, and group one branched to two subclusters, one contains Rhizobium sp._Haw1 and Rhizobium sp._G6-11 which were clustered with Rhizobium leguminosarum_bv._viciae in 16srRNA phylogeny tree. The second subcluster contains *Sinorhizobiummeliloti* only. Group two also branched into two subclusters with bootstrap support of 96%, the first subcluster represented by Rhizobium sp._UoG27. The second subcluster branched to two additional subclusters with bootstrap support of 98% containing Rhizobium sp._UoG30 as one cluster, while another subcluster contains Rhizobium sp._Sab13 and Rhizobium sp._Umk34. The second subcluster of group two contains only Rhizobium sp._Taw3, as shown in (Figure 2).

The phylogeny tree built with glnII split the isolates into two large groups with bootstrap support of 63%. The first

group comprised three subgroups, subgroup (I) included *Rhizobium* sp._UoG27 only, subgroup (II) included *Rhizobium* sp._Taw3 only, both isolated from groundnut, and subgroup (III) included isolates branched to two subclusters with bootstrap support of 82%; one subcluster contains two isolates, Rhizobium sp._UoG30, and Rhizobium sp._Umk34. The second subcluster in group (III) contains Rhizobium sp._Sab13 only. The second group of the phylogeny tree built with glnII comprised two subgroups with bootstrap support of 77%, the first subgroup included *Sinorhizobium meliloti* only, and the second subgroup included Rhizobium sp._Haw1 and Rhizobium sp._G6-11 as illustrated in (Fig. 3).

The phylogeny tree of 16SrRNA, recA, and glnII confirmed that *Rhizobium* sp._Haw1 and *Rhizobium* sp._G6-11 are the same. However, they were isolated from nodules of groundnut from different localities (Al-Rahad and Al-Fashaga), located in the Western and Eastern parts of the Gadarif State, respectively. *Rhizobium* sp._Sab13 and *Rhizobium* sp._Umk34 were also found the same according to 16SrRNA and recA phylogeny trees despite their different hosts (Bambara groundnut and Cowpea, respectively). However, the glnII phylogeny tree confirmed that they belong to different species. Other isolates related to these last two isolates were *Rhizobium* sp._Taw3 according to 16SrRNA and *Rhizobium* sp._UoG30 according to to16S RNA, recA, and glnII phylogeny trees which were isolated from groundnut and Bambara groundnut, respectively. Accordingly, these results indicate that *Rhizobium* sp._Haw1 and *Rhizobium* sp._G6-11 are related to *Rhizobium leguminosarum*, although BLASTN analysis showed that they are between *Rhizobium etli* and *Rhizobium* sp. N324 depending on the gene sequence analyzed. While Rhizobium sp._UoG27 is *Rhizobium tropici* typically used as BLASTN analysis.

Figure 3: Phylogeny tree analysis of glnII of Rhizobia isolated from different legumes



On the other hand, Rhizobium sp._UoG30, *Rhizobium* sp._Sab13 (isolated from Bambara groundnut), and *Rhizobium* sp._Umk34 (isolated from Cowpea) may be new species as supported by BLASTN analysis which revealed that they are related to *Rhizobium* sp. IRBG74 and found in one subcluster with some differences in the phylogenies built with the three genes analyzed in this study. In addition, *Rhizobium* sp._Taw3 (isolated groundnut) may be a new species because it was clustered with *Rhizobium* sp._Sab13 and *Rhizobium* sp._Umk34 in 16S RNA phylogeny; it appears in a separate group in recA and gene phylogenies. In addition, it is classified between *Rhizobium* etli and *Rhizobium* sp. S41in BLASTN analysis. We did not find 16S RNA, recA, and glnII sequences in the gene bank for fast-growing rhizobia isolated from Bambara groundnut and Cowpea to use in a phylogeny tree to estimate the relationship between already sequenced genes and *Rhizobium* sp._UoG30, *Rhizobium* sp._Sab13, *Rhizobium* sp._Umk34, and *Rhizobium* sp._Taw3.

DISCUSSION

The isolates obtained from the Gadarif State were isolated from different localities with different environmental conditions. Therefore, they can be used to solve poor nitrogen fixation problems, look for strains well-adapted for stresses, use in legume domestication programs, for biodiversity studies, or genetic studies of nitrogen fixation (Howieson and Committee, 2020; Lindström and Mousavi, 2020; Dai et al., 2012). The study of these isolates also can enrich the understanding of the phylogenetic relationships of the different strains, as they are still poorly understood (Biro et al., 2010).

In this study, *Rhizobium* sp._Haw1 and *Rhizobium* sp._G6-11 were the same despite their different site directions (Western and Eastern parts of Gadarif State), respectively. Moreover, *Rhizobium* sp._Sab13 and *Rhizobium* sp._

Umk34 were found the same despite their different hosts (Bambara groundnut and Cowpea, respectively), although they appeared as different species in the glnII phylogeny tree. These results are supported.

partially by other findings, which show that the symbiotic association between rhizobia and legumes may be subject to environmental factors, interactions among rhizobia, legumes, and biogeography. It was found that rhizobia can form nodules in legumes in distinctive geographic regions, and the same rhizobia may form nodules in different legume species (Grönemeyer et al., 2014). Therefore, we expect that each of the isolates mentioned above, which clustered in the same groups, may associate symbiotically with different hosts in different sites due to the minor influence of the plant origin on the relatedness of the isolates (Flores et al., 2005).

The Phylogeny trees built with 16S RNA and recA were found to be nearly the same, with some exceptions. The 16S rRNA sequence analysis agreement with recA was reported before (Grönemeyer et al., 2014). However, glnII phylogeny shows inconsistency with 16SrRNA and recA phylogenies, which agrees with the previous finding that "discordant phylogenies within different loci of rhizobia can result in different phylogenetic tree topolo¬gies for rhizobia species.

The results in this study also revealed that groundnut is associated with different *Rhizobium* species because the isolates were found clustered with *Rhizobium leguminosarum* and *Rhizobium tropici* simultaneously sequence analyses of BLASTN indicate that *Rhizobium etli.Rhizobium leguminosarum* and *Rhizobium etli* are "the symbionts of the common bean plant Phaseolus vulgaris" (Angelini et al., 2011). This supports that "groundnut nodules are a reservoir for different rhizobial lineages (Roughley, 1970). This may be an advantage in inoculants manufacturing in which the inoculants produced from groundnut Rhizobia can be helpful. However, the performance of rhizobia under conditions dissimilar to the original habitat is poor (Law et al., 2007; Botha et al., 2004), and their effectiveness depends on the interaction of environmental factors (Pule-Meulenberg et al., 2010) and host plant variety or genotypes (Keyser et al., 2002 Martínez-Hidalgo and Hirsch, 2017).

Therefore, inoculants of effective indigenous rhizobia adapted to local conditions may perform better (Flores et al., 2005). However, all isolates obtained from groundnut, Bambara groundnut, and Cowpea in this study were found to induce nodules in all three legumes, even in cross-inoculation performed under laboratory conditions directly after the isolates were obtained. In addition, the shoot dry weight of the inoculated plants showed a significant increase (data not shown). After about one year, all isolates failed to form nodules even in their original hosts.

To interpret these findings, we exclude the assumption that these isolates are non-symbionts, and we assume that they lost their symbiotic genes may be due to the storage conditions and repeated sub-culturing, which means that these isolates are genetically unstable. However, maintaining genetic stability is essential in determining the isolate's validity as an inoculant (Zhang et al., 2008).

Even though symbiotic genes were not found in our isolates, the role of rhizobia is not restricted to nitrogen fixation; they also provide plants with plant growth-promoting factors like hormones, which we expect our isolates to play also. Besides that, we isolated rhizobia from six localities of the eleven localities of the Gadarif State surveyed. These findings necessitate conducting more studies to obtain isolates from other localities, searching for the reasons leading to the loss of nitrogen-fixing (nifH) and nodulation (nod) genes, and studying these isolates' roles may play in promoting legumes and growth.

For Cowpea, we obtained only one isolate (*Rhizobium* sp._Umk34), which was found to be related to *Rhizobium* sp._UoG30, *Rhizobium* sp._Sab13 isolated from Bambara groundnut. These isolates may be due to Cowpea and Bambara groundnut affiliation to the same plant genus (*Vigna*). We assumed these isolates were new species because they clustered in one group in the phylogeny tree built with all three genes. We did not find sequences for 16SrRNA, recA, and glnII deposited in the gene bank related to Cowpea rhizobia. Despite its importance as a legume crop, cowpea rhizobia have yet to be well characterized (Ibny et al., 2019).

More than that, recently, it was reported that different isolates obtained from nodules of Bambara groundnut from South Africa, Ghana, and Mali were found to be closely related to different species of *Bradyrhizobium* (Herridge et al., 2002; Keyser et al., 2002), of their isolates belong to fast-growing Rhizobium. In addition, BLASTN analyses of these genes classified them between different species. More than that, rhizobia isolated from cowpea showed unstable relationships. The relationship between Cowpea and Bambara groundnut is a normal phenomenon because they belong to the same plant genus (*Vigna*) as mentioned above. Many studies reported that Cowpea is considered promiscuous, which is modulated by a wide range of rhizobia (Toolarood et al., 2012 Ibny et al., 2019).

Besides groundnut, Cowpea, and Bambara groundnut isolates, we isolated *Sinorhizobium meliloti* from nodules of *Alfalfa* grown in the Gezira state, which was identified by BLASTN analyses of 16S RNA, recA, glnII, nifH, and Noda genes. It was found in a separate branch in the phylogeny tree built with 16S RNA, recA, glnII. No relationship exists between this isolated and the others isolated from the Gadarif State. Previously, "*Sinorhizobium meliloti* is the dominant genus in alfalfa nodules with a relatively high genetic diversity" (Aloo et al., 2022). In contrast to the other isolates in this study, symbiotic genes were found in the isolate obtained from *Alfalfa* nodules. However, it was maintained in the same storage conditions as the isolates obtained from the other three plants. This indicates that the isolate obtained from *Alfalfa* is genetically stable and supports our assumption that the other isolates are characterized by genetic instability.

In all isolates obtained in these fast-growing rhizobia, no slow-growing *Bradyrhizobium* was isolated, although many studies reported fast-growing *Rhizobium* and slow-growing *Bradyrhizobium* from nodules of Cowpea (Flores et al., 2005; Ibny et al., 2019). The advantages of fast-growing rhizobia are that they require a shorter time in inoculants production, contamination occurs at a lower rate during the industrial process, more accessible establishment in the soil, and gene manipulation is easier. However, fast-growing rhizobia lacks competitiveness against *Bradyrhizobium*, which limits their recommendation to use as a commercial inoculant (Kolbe, 2022). Therefore, more studies are required to look for slow-growing rhizobia and its advantages over the fast-growing in the Gadarif State soil.

Finally, studying the diversity and characteristics of soil rhizobia has practical importance for ecology and agriculture. These studies help to enrich agricultural microbial genetic resources (AMiGRs) (Howieson and Committee, 2020), select effective combinations of *Rhizobium*-legume genotypes to increase nitrogen fixation (Grönemeyer et al., 2014), and study the indigenous rhizobia populations adapted to the local environmental conditions contribute to the general understanding of regional species abundance. In addition, they provide a basis for the formulation of a rhizobial inoculant matching local settings (Flores et al., 2005). Moreover, the "variety of rhizobia is a valuable bioresource for the exploitation of bacterial selection in attempts to find bacterial strains with desirable traits that maximize legume crop productivity" (Biro et al., 2010).

CONCLUSION

Fast-growing *Rhizobium* dominates the Gadarif State soils, lose symbiotic genes (unstable genetically), and is related to each according to the host and regardless of the site from which they were isolated. Different species of these fast-growing Rhizobium are associated with groundnut; evidence of new species is found in those associated with Bambara groundnut and Cowpea. Thus, more studies are required to isolate both fast-growing and slow-growing rhizobia, characterize those that seem to be new species and search for symbiotic genes and factors affecting the isolates.

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Conflict of Interest: The authors have no financial conflicts of interest to declare.

Authors contribution: A.S., A.I., M.G, V.N.V developed the concept, A.S., A.I, Z.S designed the experiment and; A.S., A.I. collected data and performed the analyses; A.S., A.I., V.N.V wrote the manuscript.

Data Availability: The data will be made available on request.

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In vitro Evaluations of Anti-inflammatory and Antioxidant Activity of Ethanolic Leaf Extract of *Gymnema sylvestre* R. Br.

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ABSTRACT

Gymnema sylvestre (Apocynaceae) is a well-known anti-diabetic herb used in various traditional Indian medicinal systems including Ayurveda and modern medicine. It is a source of diverse phytoconstituents and was reported to be used to treat various diseases. Hence our study aimed to evaluate the anti-inflammatory and antioxidant properties of *Gymnema sylvestre* by *in vitro* techniques. The ethanolic leaves extract of G. *sylvestre* was preliminarily screened to identify the presence of various phytoconstituents, and further analyzed for total alkaloids, flavonoids, phytosterols, and saponins estimations. The *in vitro* anti-inflammatory activity was assayed by human red blood cell (HRBC) membrane stabilization and protein denaturation assay, while antioxidant activity was assessed by DPPH and nitric oxide scavenging assay. Our study results showed that the ethanolic leaf extract of G. *sylvestre* exhibited dose-dependent anti-inflammatory and antioxidant activity. Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, phytosterols, tannins, phenolic compounds, terpenoids, glycosides, carbohydrates, sugars, proteins, amino acids, and saponins. Quantitative analysis showed high content of saponin 32.44 ± 5.65 mg diosgenin/g, followed by steroidal content 26.30 ± 3.96 mg cholesterol/g, flavonoids 17.66 ± 0.43 mg QE/g, and alkaloids 10.33 ± 0.97 mg AE/g. These results further authenticate the claim and use of G. sylvestre in traditional medicine to treat inflammation and oxidative stress.

KEY WORDS: ANTIOXIDANT, ANTI-INFLAMMATORY, GYMNEMA SYLVESTRE, DPPH, NITRIC OXIDE.,

INTRODUCTION

Oxidative stress has a significant role in chronic inflammatory illnesses such as cancer, diabetes, neurological problems, and cardiovascular ailments. Prolonged exposure to high quantities of pro-oxidants can cause mitochondrial DNA damage and changes in cellular components, which contribute to gene expression anomalies (Sharifi-Rad et al., 2020). The process of oxidative stress that leads to the damage of numerous tissue's physiological and biochemical environments, a minor amount of oxidative stress that helps the immune system withstand microbial infections and intracellular cell signalling, is also thought to be an important physiological activity (Sies et al., 2017). When free radicals outnumber antioxidant defences, oxidative stress, a process that harms the physiological and biochemical balance of tissues, becomes worse (Kopáni et al., 2006). Inflammation increases reactive oxygen species (ROS) generation, which exceeds the body's antioxidant capacity and causes oxidative stress-induced tissue damage (Sies 1997).

Early in the ages, medicinal plants have been extensively used to treat a wide range of diseases, notably in India, where indigenous medical systems such as Ayurveda, Siddha, and Unani have been practiced for centuries. Herbalism, or the medicinal use of herbal plants, is important in modern medicine. While synthetic drugs provide treatments for a variety of illnesses, their large quantity and related side effects sometimes limit accessibility. The use of herbs, on the other hand, is gaining popularity because of low toxicity, low cost, and broad availability, emphasizing the importance of herbs in everyday life (Das et al., 2022).

Nearly 80% of the world's population gets their basic medical treatment from medicinal plants, according to the World Health Organisation (WHO), highlighting the long-standing importance of herbal medicine in treating illnesses affecting human health (Dey et al., 2021). Traditional methods of treating inflammatory illnesses mainly target the fight against bacterial infections, which might not be effective enough. As a result, complementary approaches that target both inflammation and oxidative stress reduction

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have developed as viable therapeutic and preventative treatments. In this context, antioxidant compounds, which may be found in a variety of foods, drinks, plants, vitamins, and minerals are essential. Both conventional insight and contemporary studies have highlighted the anti-inflammatory and antioxidant qualities of medicinal plants. Despite their effectiveness, modern anti-inflammatory drugs can have a variety of negative effects. On the other hand, because of their natural source, herbal extracts are thought to provide a safer substitute. Treating chronic inflammatory diseases is made easier when a single plant extract combines anti-inflammatory and antioxidant effects (Somashekar et al., 2022).

Gymnema sylvestre (G. *sylvestre*), Apocynaceae family, is a well-established antidiabetic herb utilized in various traditional Indian medicinal systems including Ayurveda and modern medicine. Known as "Gudmar," this herbaceous plant is a climbing species found in dry forests up to 600 meters in height. Its leaves are extensively employed for their multifaceted therapeutic properties, encompassing antidiabetic, anti-inflammatory, antiarthritic, anti-obesity, wound healing, astringent, bitter, acrid, thermogenic, anodyne, digestive, antipyretic, stomachic, diuretic, laxative, cardiotonic, and liver tonic effects. Rich in tannins, flavonoids, saponins, and gymnemic acid, G. sylvestre exhibits additional bioactive characteristics such as antimicrobial, larvicidal, antiviral, hypolipidemic, anticancer, and antioxidant activities. particularly gymnemic acids, a blend of at least 17 distinct saponins, acidic glycosides, and anthraquinones (Sudhakar et al., 2018, Pachiappan et al., 2021 Pachiappan et al., 2023).

The research regarding the pharmacological effects of G. *sylvestre* is currently at an early stage and requires further investigation to fully understand the phytochemical advantages of this herb. This *in vitro* study is designed to examine the antioxidative and anti-inflammatory properties of G. sylvestre leaf ethanolic extract.

MATERIALS AND METHODS

Plant material: The fresh leaves of *Gymnema sylvestre* R. Br. (GS) were collected from Kolli Hills Namakkal, Tamil Nadu, India, during January 2021. The plant material was further authenticated by Dr. P. Radha, Research officer (Botany), Siddha Medicinal Plants Garden (Central Council for Research in Siddha), Ministry of AYUSH, Govt. of India, Mettur Dam, Tamil Nadu, India, where the voucher specimen is preserved with the reference number of G180221012S.

Preparation of extract: The collected fresh leaves were washed with running water to remove the sand and dust. Then the shade-dried material was coarsely powdered for extraction. 100 gm of the powdered plant material was loaded in the Erlenmeyer flask and extracted by cold maceration with 95% ethanol for 72 hours at room temperature with occasional shaking. After 72 hours, the filtrate was separated from marc by using a muslin cloth and further filtered by Whatman no. 1 filter paper. The same procedure was performed two consecutive times with the marc material. All three filtrates were mixed and evaporated under reduced pressure and controlled temperature at 40 ^{0}C in a rotary evaporator until all the solvent was removed. The dried material was stored in an airtight container at 4 ^{0}C until further use.

Preliminary phytochemical analysis of ethanolic extract of *Gymnema sylvestre*: The preliminary phytochemical screening of G. *sylvestre* leaves ethanolic extract was carried out using standard procedure to identify the presence of alkaloids, flavonoids, phytosterols, tannins, phenolic compounds, terpenoids, glycosides, carbohydrates, sugars, proteins, amino acids, and saponins (Evans & WC 2009, Kokate et al., 2017).

Quantitative phytochemical analysis: Determination of total alkaloid content: The extract (1 mg/ml), bromocresol green solution, and phosphate buffer 5 ml each were mixed in a separating funnel. It was diluted with chloroform in 10 ml volumetric flask. Atropine was used as standard. The absorbance of both the standard and test solution was observed at 470 nm. The total alkaloid content in the extract was expressed as mg of Atropine (AE)/g of plant extract (Shalini et al., 2021).

Determination of total flavonoid content: The plant extract 3 mg was dissolved in methanol 3 ml for flavonoid content estimation. The quercetin was used as standard. 3 ml of plant extract or standard was mixed with 1 ml of 2% AlCl3 methanolic solution and allowed to stand for 60 min at room temperature. The absorbance was measured at 420 nm. The total flavonoid content was indicated as mg of QE/g of extract (Garg & Garg 2019).

Determination of total steroidal content: The total steroidal content was estimated by Liebermann-burchard colorimetric method with slight modification. The G. *sylvestre* extract was dissolved with chloroform and the freshly prepared Liebermann-burchard reagent was added. The absorbance of standard cholesterol and extract was measured at 650 nm against a reagent blank. The total steroidal content was expressed as mg of cholesterol/g of extract (Kim & Goldberg 1969).

Determination of total saponin content: The 1 ml of diluted G. *sylvestre* extract was mixed with 1 ml of 80% aqueous methanol, followed by 1 ml of 72% H_2So_4 added in the sides of test tube. The mixture was warmed on 60 ^{0}C for 10 minutes. The absorbance of standard diosgenin and extract solution was measured at 544 nm against 80% methanol as a blank solution. The total saponin content was expressed as mg of diosgenin equivalent to gm of dry weight of the extract (Nandhini & Ilango 2020).

***In vitro* anti-inflammatory activity screening : Human red blood cell (HRBC) membrane stabilization assay:** The *in vitro* anti-inflammatory activity of G. *sylvestre* was determined by the Human Red Blood Cell (HRBC) membrane stabilization method. The blood sample was collected from a healthy human volunteer who had not taken any NSAIDs for 2 weeks before the experiment, added with an equal amount of Alsever's solution. This mixture was

centrifuged at 3000 rpm for 15 min. The RBC pellet was washed thrice with sterile saline till the supernatant was clear and colorless.

The packed cellular content was formulated as 10 % v/v suspension with sterile isosaline. 1 ml of different concentrations (100-500 µg/ml) of G. *sylvestre* extract and standard diclofenac was mixed with 1 ml of phosphate buffer, 0.5 ml of HRBC suspension, and 2 ml of hyposaline. After 30 min incubation at 37^{0}C, the reaction mixture was centrifuged at 3000 rpm for 10 min. The supernatant absorbance was observed spectrophotometrically at 560 nm. The percentage of hemolysis was estimated by considering the percentage of hemolysis of control as 100% (Senthil Kumar et al., 2018; Gupta et al., 2021). The percentage of protection/ percentage inhibition of hemolysis were evaluated using the formula

$$\% \text{ protection} = 100 - \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Protein denaturation assay: The *in vitro* anti-inflammatory activity of the G. *sylvestre* extracts by protein denaturation was performed using bovine serum albumin. The increasing concentrations of the extract (100-500 µg/ml) and reference compound diclofenac sodium were incubated with 0.5% w/v of bovine serum albumin at 37◦C for 20 min and the temperature was increased to keep the samples at 57 ◦C for 30 min. After reaching room temperature, the turbidity was measured using UV-Visible spectrophotometer at 660 nm following the addition of 2.5 ml of phosphate-buffered saline (Shanmuganathan et al., 2017; Senthil Kumar et al., 2018). The percentage inhibition of protein denaturation was calculated by using the following formula.

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of Control}} \times 100$$

***In vitro* antioxidant activity screening :DPPH free radical scavenging assay:** The 2, 2 diphenyl-1-picrylhydrazyl (DPPH) 0.2mM solution 1 ml was added to 1 ml of different concentrations (100-500 µg/ml) of G. *sylvestre* extract, the mixture was kept room temperature for 50 mins in dark environment. The antioxidant activity was measured spectrophotometrically at 517 nm. Ascorbic acid was used as standard. The percentage of free radical scavenging was calculated as half minimal inhibitory concentration (IC_{50}). IC_{50} denotes the concentration of the sample required to inhibit 50% of DPPH free radicals (Govindappa et al., 2018). The DPPH radical scavenging capacity was calculated by using the following formula:

$$\text{DPPH radical scavenged \%} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of Control}} \times 100$$

Nitric oxide (NO) radical scavenging activity: The nitric oxide scavenging assay reaction mixture (3 ml) contains 2 ml of 10 mM sodium nitroprusside, 0.5 ml of phosphate buffer, and 0.5 ml of different concentrations (100-500 µg/ml) of G. sylvestre extract. The reaction mixture was incubated at 25 ^{0}C for 150 min. The 0.5 ml of reaction mixture was pipetted and mixed with 1 ml of sulfanilic acid reagent and allowed to stand for 5 min for diazotization. Then 1 ml of naphthyl ethylene diamine dihydrochloride was added and incubated at 25 0C for 30 min. A pink-colored chromophore was formed in diffused light. Ascorbic acid was used as standard. The NO scavenging activity was measured at 550 nm and the results were expressed as a percentage (%) of scavenging using the following formula: (Adebayo et al., 2019)

$$\text{Nitric oxide radical scavenged \%} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of Control}} \times 100$$

Statistical analysis: The statistical analysis was performed in IBM SPSS version 18 (SPSS version 18.0; IBM Corporation, Armonk, NY, USA). The entire assay was performed in triplicate and the values were expressed as mean ± standard deviation (SD). Descriptive statistics were used for continuous variables and expressed in mean and standard deviation. A test of normality was applied.

RESULTS AND DISCUSSION

Extractive yield and preliminary phytochemical analysis: The ethanolic extractive percentage yield of *Gymnema sylvestre* leaf extract was calculated as 22.08 gm% w/w. The preliminary phytochemical analysis of ethanolic leaves extract of G. *sylvestre* showed the presence of alkaloids, flavonoids, phytosterols, tannins, phenolic compounds, terpenoids, glycosides, carbohydrates, sugars, proteins, amino acids, and saponins.

Quantitative phytochemical analysis: Table 1 illustrates the quantification of secondary metabolites alkaloids, flavonoids, steroids, and saponins in the ethanolic leaves extract of G. *sylvestre*. The secondary metabolite saponins content was found to be higher 32.44 ± 5.65 mg diosgenin/g, followed by steroidal content 26.30 ± 3.96 mg cholesterol/g, flavonoids 17.66 ± 0.43 mg QE/g, and alkaloids 10.33 ± 0.97 mg AE/g in the ethanolic leaves extract of G. *sylvestre*.

Table 1. Quantitative phytochemical analysis of *G. sylvestre* leaves ethanolic extract

S. No.	Phytoconstituent	Quantity of Phytoconstituent (mg/g of extract)
1.	Alkaloids	10.33 ± 0.97
2.	Flavonoids	17.66 ± 0.43
3.	Steroids	26.30 ± 3.96
4.	Saponins	32.44 ± 5.65

Values are expressed as mean ± SD, n=3.

***In vitro* anti-inflammatory activity:** The *in vitro* anti-inflammatory activity of G. *sylvestre* was carried out by Human red blood cell membrane stabilization assay and protein denaturation assay techniques.

HRBC membrane stabilization assay: HRBC assay is based on the principle of evaluating lysosomal membrane

protection. During inflammation lysosomes undergo lysis and release specific enzymes into circulation leading to inflammatory diseases. The RBC membrane is similar to the lysosomal membrane, and its stability indicates anti-inflammatory properties (Yesmin et al., 2020). In this study, the hemolysis of RBC was impacted by the hypotonicity of hyposaline, which induces lysis of the cell membrane. The percentage of RBC lysis/protection was taken as an index for anti-inflammatory activity measurement (Kumar et al., 2011 and Kumar et al., 2020).

The treatment of G. *sylvestre* showed dose-dependent membrane stabilization action the maximum activity (72.46 ± 2.58) was observed at the concentration 500 µg/ml. The standard diclofenac showed maximum membrane protection (89.27 ± 1.08) activity at the concentration 500 µg/ml, comparably the treatment of G. *sylvestre* leaves extract showed less percentage inhibition of hemolysis compared to the standard diclofenac.The treatment of G. *sylvestre* ethanolic leaves extract exhibited remarkable anti-inflammatory activity by stabilizing the RBC membrane, preventing the discharge of lytic enzymes and other inflammation mediators (Table 2).

Table 2. Human red blood cell membrane stabilization assay of *G. sylvestre* leaves ethanolic extract

S. No.	Concentration (µg/ml)	% Protection	
		Ethanolic extract of GS	Diclofenac (Standard)
1.	100	23.51 ± 1.01	48.11 ± 2.77
2.	200	34.25 ± 0.98	66.96 ± 2.54
3.	300	40.19 ± 1.50	75.39 ± 7.80
4.	400	56.88 ± 3.55	78.93 ± 4.13
5.	500	72.46 ± 2.58	89.27 ± 1.08

Values are expressed as mean ± SD, n=3

Table 3. Protein denaturation inhibition assay of *G. sylvestre* leaves ethanolic extract

S. No.	Concentration (µg/ml)	% inhibition	
		Ethanolic extract of GS	Diclofenac (Standard)
1.	100	18.88 ± 1.42	24.71 ± 3.31
2.	200	27.12 ± 1.32	39.14 ± 6.04
3.	300	38.41 ± 0.70	52.90 ± 1.11
4.	400	58.62 ± 2.45	67.22 ± 0.83
5.	500	71.89 ± 7.89	78.70 ± 2.81

Values are expressed as mean ± SD, n=3.

Table 4. DPPH radical scavenging activity of *G. sylvestre* leaves ethanolic extract

S. No.	Concentration (µg/ml)	% scavenging activity	
		Ethanolic extract of GS	Ascorbic acid (Standard)
1.	100	27.32 ± 2.56	32.07 ± 1.22
2.	200	38.58 ± 5.92	59.33 ± 5.05
3.	300	46.65 ± 9.02	62.43 ± 3.25
4.	400	68.45 ± 4.52	74.21 ± 3.82
5.	500	77.32 ± 6.29	82.91 ± 6.64
	IC_{50}	289.15 ± 11.45	196.38 ± 8.19

Values are expressed as mean ± SD, n=3.

Table 5. Nitric oxide radical scavenging activity of *G. sylvestre* leaves ethanolic extract

S. No.	Concentration (µg/ml)	% scavenging activity	
		Ethanolic extract of GS	Ascorbic acid (Standard)
1.	100	29.45 ± 0.89	48.33 ± 6.02
2.	200	40.99 ± 1.34	61.73 ± 4.05
3.	300	56.05 ± 2.62	75.00 ± 2.39
4.	400	71.30 ± 3.27	86.19 ± 4.31
5.	500	80.12 ± 6.80	97.68 ± 3.24
	IC_{50}	258.93 ± 9.06	107.07 ± 12.46

Values are expressed as mean ± SD, n=3.

Protein denaturation assay: The denaturation of cellular protein is the most known cause of inflammation and arthritis. The compounds that prevent the protein denaturation have advantageous anti-inflammatory properties (Osman et al., 2016). The results in table 3 shows that maximum (71.89 ± 7.89) percentage denaturation inhibition was observed in the G. *sylvestre* 500 µg/ml treatment, when compared to standard diclofenac it shows maximum inhibition (78.70 ± 2.81) at the concentration of 500 µg/ml which is almost equal. The ethanolic leaves extract of G. *sylvestre* showed dose dependent protein denaturation inhibition property. As results the protein denaturation inhibition capacity confirms the anti-inflammatory property of G. *sylvestre* extract.

In vitro antioxidant assay: The antioxidant potential of G. sylvestre leaves extract was assayed by DPPH free radical scavenging assay and Nitric oxide (NO) radical scavenging assay.

DPPH free radical scavenging assay: DPPH radical scavenging is the most widely used technique for

measuring free radical scavenging. The antioxidant ability of compounds was determined by their capacity to donate hydrogen was assumed to be responsible for DPPH scavenging. The DPPH antioxidant model may have some positive benefits in certain inflammatory disorders (Nagulsamy et al., 2015, Kawra et al., 2020). The G. *sylvestre* leaf extract and ascorbic acid showed a dose-response relationship with the DPPH scavenging activity which was directly proportional to their concentrations. The IC50 value for ascorbic acid was 196.88 µg/ml, which was significantly lower than the G. sylvestre extract 289.15 µg/ml (Table 4).

Nitric oxide radical scavenging assay: Inflammatory diseases have a high production of nitric oxide. Overproduction of NO can cause tissue damage and contribute to inflammatory diseases including atherosclerosis and hypertension. The compounds that can scavenge or inhibit the production of NO are known to have antioxidant properties (Moncada et al., 1991, Adebayo et al., 2019). The G. sylvestre leaf extract and standard ascorbic acid showed a dose-response relationship with the NO scavenging activity which was directly proportional to their concentrations. The IC50 value for ascorbic acid was 107.07µg/ml, which was significantly lower than the G. sylvestre extract at 258.93 µg/ml (Table 5).

CONCLUSION

The results of this study demonstrated that the ethanolic leaf extract of G. *sylvestre* inhibits hemolysis by protecting the RBC cell membrane in HRBC membrane stabilization assay and preventing protein denaturation in protein denaturation assay. Free radical scavenging property was demonstrated through DPPH radical scavenging and nitric oxide radical scavenging in a concentration-dependent manner. These suggest that G. *sylvestre* leaf ethanolic extract has extensive anti-inflammatory and antioxidant potency. The presence of secondary metabolites might contribute to its actions, the free radical scavenging activity of G. *sylvestre* leaf extract can play a vital role in modulating inflammatory reactions. Hence *Gymnema sylvestre* leaf extract may utilized for the herbal medicine development to the inflammation condition associated with oxidative stress. Further, in vivo studies and the specific bioactive compound isolation are needed to explore *Gymnema sylvestre* as a newer therapeutic agent.

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Molecular Characterization and Genetic Diversity of *Blumea* Species Using RAPD Marker

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ABSTRACT

The objective of the present study was to find out the genetic relationship within the species of *Blumea* of family Asteraceae through random amplified polymorphic DNA (RAPD) marker. Genetic analysis was made by using 10 arbitrary primers which revealed a total 292 polymorphic fragments. The genetic similarity was evaluated on the basis of presence or absence of bands. High degree of polymorphism was observed among the samples, suggesting the degree of genetic variability. Collectively all primers data was used for construction of dendrogram. All primers gave amplification products and had from (OPX-07) - 23 bands to 42 bands (OPB-12) with an average 29.9 bands per primers. Thus, these RAPD markers have the potential for assessment of genetic variation and phylogenetic analysis within the species of *Blumea*. The results of the current study have been found to be useful for assessing genetic diversity, genetic relationship and phylogenetic analysis.

KEY WORDS: *BLUMEA*, RAPD MARKER PCR, GENETIC DIVERSITY, POLYMORPHISM, DENDROGRAM.

INTRODUCTION

Genus *Blumea* belonging to the family Asteraceae is one of the important medicinal plant found in the tropical and sub-tropical zones of Asia, especially the Indian Subcontinent and Southeast Asia. A few species are found in Australia and still fewer in Africa. The plants of this genus are mostly relatively small weeds. In India, the species are distributed throughout the country from South to North up to the Himalayas at about 2,000 m elevations. The maximum diversity of the species lies in the North-East region followed by the Peninsular region and Andaman and Nicobar Islands. Many species of genus *Blumea* are used in traditional medicine with higher therapeutic values (Tamilarasi and Thirugnanasampandan, 2014).

Use of DNA markers for the identification of genetic diversity, can be useful in identifying genetic structure as well as diversity among species of genus. Due to their simplicity, reliability and cost effectiveness PCR based markers are in demand (Parita et al., 2018). Different PCR based techniques have been developed during the last two decades. The random amplified polymorphic DNA (RAPD) is simple, cost-effective and a powerful tool in the analysis of plant genome characterization (Bardakci, 2001). RAPD technique has been widely used in many plant species for varieties analysis, population studies and genetic linkage mapping (Prasad, 2014, Mishra et al., 2018, Mostofa et al., 2020. Dobhal1 and Kumar, 2021).

The present work was aimed to assess the genetic diversity of five species of *Blumea* (coded as in Table-I) which are *Blumea axillaris* (Lam.) DC, *Blumea eriantha* DC, *Blumea fistulosa* (Roxb.) Kurz, *Blumea lacera* (Burm. f.) DC and *Blumea oxyodonta* DC. coded as (Table-I) using Random Amplified Polymorphic DNA (RAPD) markers.

MATERIAL AND METHODS

Plant material collection and total DNA extraction: Leaf samples of five selected species of *Blumea* were collected from Amravati, Maharashtra. Fresh and disease-free young leaf samples were taken in Ziplock plastic bag with silica gel and transported to laboratory. The plant materials were stored at -70° C (Remi Queek freezer). DNA was isolated by CTAB extraction method (Murray and Thompson, 1980). 100 mg of leaf tissue was used to extract genomic DNA. The quality of a genomic DNA was checked by agarose gel electrophoresis for this 5 uL of the sample was loaded in each well and quantity with Qubit fluorometer (Invitrogen USA) PCR amplification (Fig.-1). Total 10 primers were used

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for each sample. A 25.0µl reaction mixture for PCR was prepared containing PCR buffer DNTPs, Tag polymerase, primers and sample of DNA.

RAPD analysis :RAPD PCR amplification: The prepared reaction mix was subjected to PCR amplification in which different thermal profile was set for 10 primer types, where total 35 cycle programmed for amplification was run using PCR machine. Thermal profile used for PCR amplification involved an initial denaturing step (95°C) followed by 35 cycles. (Denaturation on at 95°C, primer annealing at respective temperature, and primer execution at 72°C). Final step at 72°C was carried out for polishing the end of PCR products. Finally at the end these PCR amplified products were resolved electrophoretically where 2.0% (w/v) agarose gel was used for RAPD.

Analysis of RAPD data: Analysis of number of base substitutions per site from between sequences were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004). This analysis involved 5 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 402 positions in the final dataset. Evolutionary analysis was conducted in MEGA X (Kumar et al., 2018).

Table 1. Job code for each Blumea species and number of band generated

Sr. No.	Species Name	Job Code	Number of Bands Generated
1	*Blumea fistulosa* (Roxb.) Kurz	11041	88
2	*Blumea laciniata* (Roxb.) DC	11042	38
3	*Blumea eriantha* DC	11043	54
4	*Blumea axillaris* (Lam.) DC	11044	80
5	*Blumea oxyodonta* DC	11045	39

Table 2. Details of RAPD Primers with banding profile used in the present study

S. No.	Primer Code	Nucleotide sequence (5'-3')	TNB	PB	MB	PP
1.	OPA11	CAATCGCCGT	32	31	1	96.875
2.	OPA07	GAAACGGGTG	28	26	2	92.8571
3.	OPB01	GTTTCGCTCC	24	24	0	100
4.	OPB12	CCTTGACGCA	24	23	1	95.8333
5.	OPA04	AATCGGGCTG	34	33	1	97.0588
6	OPM05	GGGAACGTGT	42	41	1	97.619
7	OPM06	CTGGGCAACT	33	33	0	100
8	OPX01	CTGGGCACGA	27	27	0	100
9	OPX07	GAGCGAGGCT	32	32	0	100
10	OPB10	CTGCTGGGAC	23	22	1	95.6522
Total			299	292	7	--

TNB-Total numbers of bands, PB-Polymorphic band, MB-Monomorphic bands, PP-Percentage polymorphism

Dendrogram was drawn using MEGA X software. Briefly all the RAPD bands were scored in binary format. Collectively all primers data was used for construction of UPGMA tree (Iruela et al., 2002). The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973).

RESULT AND DISCUSSION

Total 10 RAPD primers were used for screening of five species of *Blumea*. All primers gave amplification products and had from (OPX-07) - 23 bands to 42 bands (OPB-12) with an average 29.9 bands per primers (Table -2). Among 10 RAPD primers tested all produced bands that were polymorphic across all the samples, 6 RAPD markers OPA07, OPA04, OPB01, OPB10, OPB12 and OPX07 produced monomorphic bands in the sample (Fig.- 2-11). Distance matrix values using Jaccard's coefficient based on RAPD markers ranged from 0.116 between 11045 to

0.689 between 11043 (Table-3). Similarity matrix values ranged from 0.311 between 11043 to 0.884 between 11045 (Table-4).

Table 3. Jaccard's Distance Matrix

11042				
11045	0.116			
11041	0.299	0.337		
11044	0.337	0.361	0.510	
11043	0.200	0.225	0.689	0.349

Table 4. Jaccard's Similarity Matrix

11042				
11045	0.884			
11041	0.701	0.663		
11044	0.663	0.639	0.490	
11043	0.800	0.775	0.311	0.651

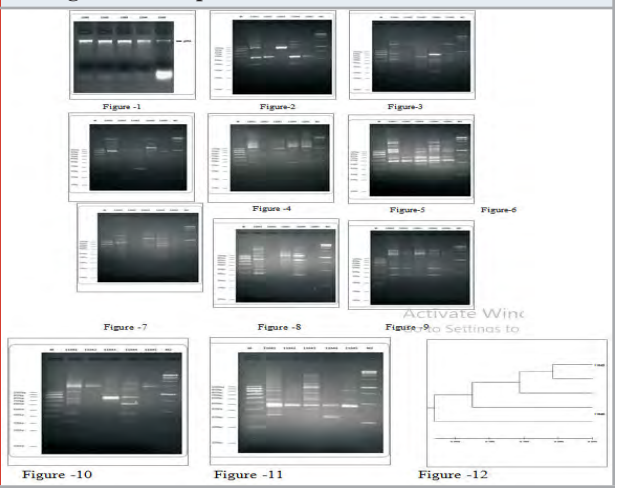
The dendrogram obtained clearly indicated two clusters (Fig. 12). Smaller one having one species-11041 and the larger cluster that could be further divided into four different sub clusters. The optimal tree with the sum of branch length = 0.79751903 was shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. This analysis involved 5 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 402 positions in the final dataset.

RAPD markers have been used in several studies for DNA fingerprinting and phylogenetic analysis (Ramakrishnan et al., 2016; Kumar et al., 2018; Sawsan et al., 2020; Omri et al., 2021). They have been used as effective tools to analyze genetic diversity in many species of family Asteraceae, (Elizabeth et al., 2000 and Geleta et al., 2007). In genus *Blumea* some researchers have made an attempt to evaluate genetic diversity using RAPD markers system. Pornpongrungrueng et al., (2007) studied evolutionary relationship in *Blumea* with sequences of cpDNA and nrDNA. Pang et al., (2014) used AFLP markers to determine genetic diversity of *Blumea balsamifera*.

The genetic distance estimated by authors with Jaccard similarity coefficient index showed low variability among genotypes. RAPD analysis revealed a little genetic variation in micropropagated plants of *Blumea mollis* (Tamilarasi and Thirugnasampandan, 2014). YingBo et al., (2016) did comparative analysis of SRAP and AFLP markers for genetic diversity of *Blumea balsamifera* In their opinion AFLP molecular markers is more suitable to estimate genetic diversity of *Blumea balsamifera* because it has more polymorphic sites and higher markers characteristic index.

Genomic DNA was amplified using ten RAPD primers and all were reproducible however, five primers were used for RAPD analysis out of which primers 2 and 5 showed more bands reported by Tamilarasi and Thirugnasampandan, (2014) in *Blumea mollis*. The findings of the current investigation revealed the successful utilization of RAPD markers for assessment of genetic diversity of *Blumea* species.

Figure 1 to 12: 1% (w/v) Agarose Gel electrophoresis of genomic DNA isolated 11041-11045 samples. 5 uL of the sample was loaded in each well.
RAPD profile for all samples with a single primer in a gel (For ten Primers).
RAPD based dendrogram representing genetic relationship among 5 *Blumea* species



CONCLUSION

The result of the current study found to be useful for assessing genetic diversity, genetic relationship and phylogenetic analysis.

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Method Development and Validation for Fluphenazine in Bulk and Pharmaceutical dosage form using High Performance Liquid Chromatography –Ultraviolet Detection

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ABSTRACT

The present work was carried out to develop and validate a High-Performance Liquid Chromatography (HPLC) technique for the quantification of Fluphenazine HCl injections. The Waters 2965 RPHPLC equipment with PDA detector and autosampler was utilized for both the development and validation of the technique. A Phenomenex column (250 mm x 4. mm, 5 µm) was employed for the detection of Fluphenazine HCl, with a wavelength of 256 nm. The calibration graph exhibited linearity, with a correlation coefficient of 0. 99. The intraday accuracy, expressed as the relative standard deviation (RSD), was determined to be 0. 2 percent. The recovery of Fluphenazine HCl ranged from 99. to 100. percent, with an RSD not exceeding 2 percent. When actual pharmaceutical samples were analyzed using this method, the content of Fluphenazine HCl fell within the permissible limits. Considering its sensitivity, precision, accuracy, and selectivity for the test chemical, this method can be effectively employed for routine quality control testing of Fluphenazine HCl in liquid dosage form. The suggested approach can be employed in quality control laboratories for routine analysis of Fluphenazine HCl in product bulk drugs and in routine application forms of pharmaceutical dose without excipient interference.

KEY WORDS: FLUPHENAZINE HCL, HPLC, METHOD DEVELOPMENT, METHOD VALIDATION, PDA DETECTOR.

INTRODUCTION

Analytical technique is that the process of choosing an accurate assay procedure to work out on a pharmaceutical product. It's the method of proving that an analytical method is suitable to be used in laboratory to live the chemical analysis of latter samples. Analytical techniques must be established utilising the procedures and acceptance criteria outlined in the ICH guidelines and utilised in GMP and GLP environments (Charles et al., 1985). Methodology development encompasses numerous stages and can take months to complete, depending on the complexity and pretensions of the methodology. The literature has noted multidinous chemical analysis methods for assaying Fluphenazine hydrochloride. These approaches include spectrophotometry, (Reusch 2013), spectrofluorimetry, (El-Houssini and Zawilla 2014) turbidmetry, gas chromatography (GC), HPTLC, force degradation studies (Walash and Wahba 2014 Sistik et al., 2017).

A Numerous high performance liquid chromatography (HPLC) methods using mass (MS) (Belal et al.,2008),(Davis and Fenimore 1983), (Javaid et al.,1981) ,(Sistik et al., 2017), (Sa'sa and Jalal 1988) , (Thummar et al., 2015) and ultraviolet (UV) sensors were also described for this pharmaceutical chemical in conjunction w th Nortriptyline HCl and other Fluphenazine combinations (El-Houssini, and Zawilla 2014),(Mennickent et al.,2010). The literature study reveals that there is no reported simple HPLC-UV (Ashour and Kattan 2012) quantitative analysis methodology for estimating the Fluphenazine HCl in Injections. The goal of the current work was to create a robust, easy, and accurate method for HPLC to measure the amount of Fluphenazine hydrochloride in injection formulation.

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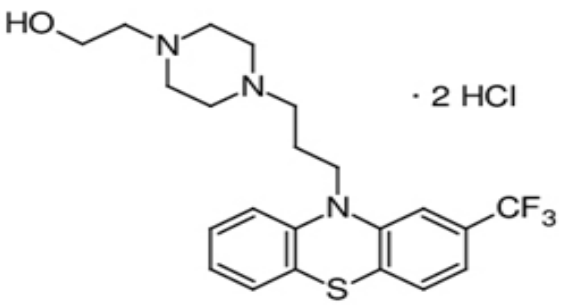
Drug profile: This is the salt of the phenothiazine Fluphenazine hydrochloride, which has antipsychotic and perhaps antineoplastic effects. The antipsychotic drug Fluphenazine 2[4[3[2(trifluoromethyl)phenothiazine10yl] propyl]piperazine1yl]ethanol; Fluphenazinedihydrochloride reduces hallucinations and delusions associated with schizophrenia. It does this by blocking postsynaptic dopamine D2 receptors in the visceral brain, cortical system, and basal ganglia. Additionally, by blocking 5-hydroxytryptamine type 1B (5HT type 1B) serotonin receptors, this drug can stop the growth of lymphocytes and myeloma cells.

Figure1: Structure of Fluphenazine HCl



MATERIAL AND METHODS

Diluent: The diluent is often chosen based on the solubility of the medicines. Fluphenazine hydrochloride's solubility in distilled water, methanol, and acetonitrile were tested to determine the best diluent. water and methanol were selected as the diluent (40:60).

Preparation of standard stock solutions: 10 mg of Fluphenazine were carefully weighed into 10 ml volumetric flasks, then 3/4 of the diluent was added, and the mixture was then sonicated for 10 minutes. The volume was made using diluents, and it was labelled as normal stock solution (1000 g/ml Fluphenazine).

Preparation of working standard solution: From each stock solution, 1 ml of Fluphenazine was pipetted into a 10-ml volumetric flask and the remaining volume was filled with the diluent. Fluphenazine, 100 g/ml.

Preparation of standard stock solutions: To 10 ml volumetric flasks, 10 mg of Fluphenazine was accurately weighed, added 3/4 of the diluent, and then sonicated for 10 minutes. Diluents were used to create the volume, which was then marked as standard stock solution (1000 g/ml Fluphenazine).

Preparation of working sample solutions: A 10 ml volumetric flask was constructed with diluent after 1 ml of filtered stock sample solution was placed there. (Fluphenazine, 100 g/ml)

Selection of detection wavelength: Fluphenazine standard solution 10ppm was scanned in the UV range (200400) and from the overlay spectrum, 256nm was selected as λ (lambda) max.

Selection of detection wavelength: Fluphenazine standard solution 10ppm was scanned in the UV range (200400) and from the overlay spectrum, 256nm was selected as λ (lambda) max.

Figure 1.1 Calibration plot of Fluphenazine

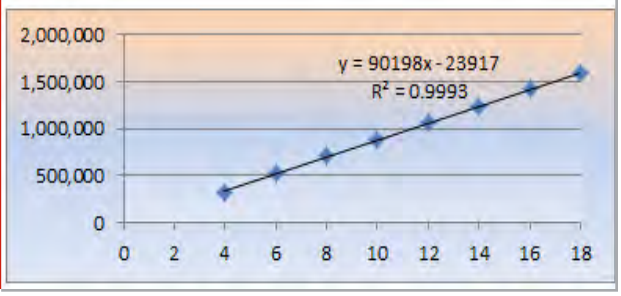


Figure 1.2: Linearity 4% Chromatogram

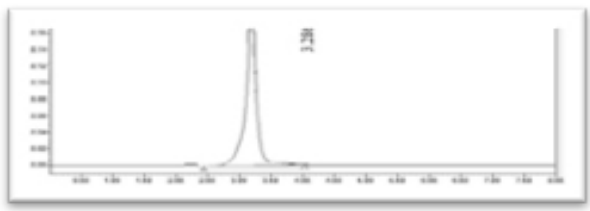


Figure 1.3: Linearity 6% Chromatogram

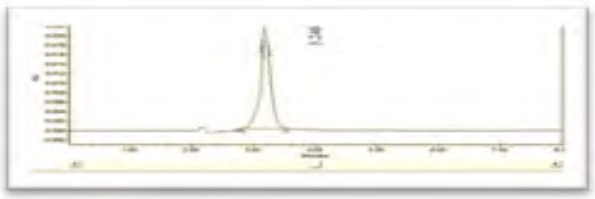
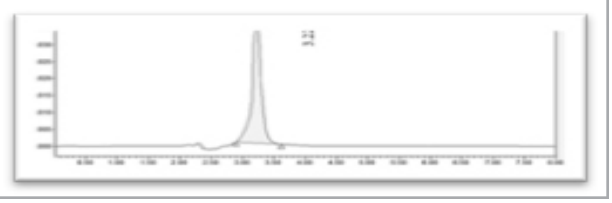


Figure 1.4: Linearity 8% Chromatogram



Method Validation Parameters: System suitability: The preparation of Fluphenazine standard solutions (100 ppm) and injection of the solutions six times were used to establish the system suitability parameters, which included peak tailing, resolution, and USP plate numbers. The RSD percentage for data from six standard injections shouldn't be higher than 2%

Specificity: Check the optimal approach for interferences, to be specific. The retention durations of these medications

using this method shouldn't have any conflicting peaks in the blank or placebo. As a result, this approach was deemed specific.

Figure1.5: Linearity 10% Chromatogram

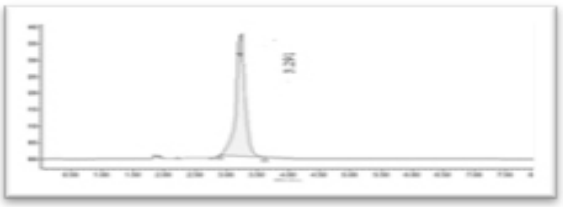


Figure 1.6: Linearity 12% Chromatogram

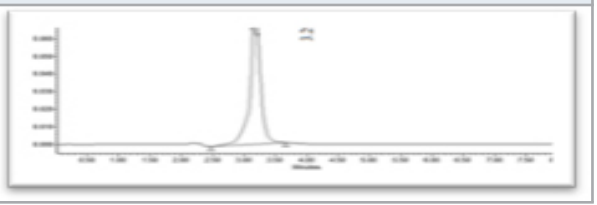


Figure 1.7: Linearity 14% Chromatogram

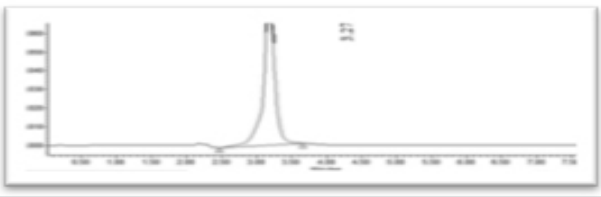
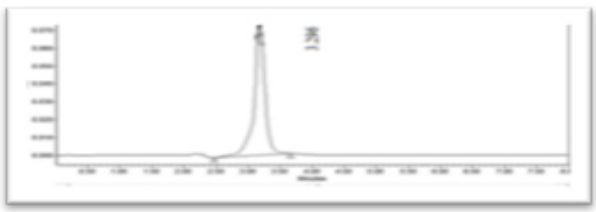


Figure 1.8: Linearity 16% Chromatogram



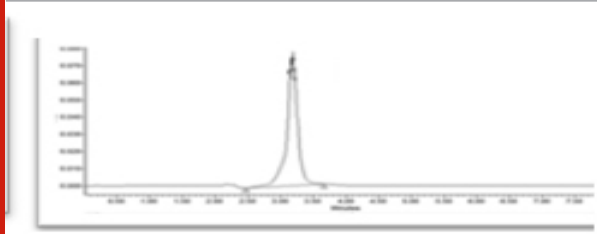
Accuracy: The technique's accuracy: From a single homogenous mixture, six injections of Fluphenazine sample solutions at 10 ppm were created. Calculated was the peak areas' percent RSD for the injections. The percent RSD of the area of six standard injection results cannot be higher than 2%.

Linearity: Standard stock solutions should be made by precisely weighing 10 mg of Fluphenazine into 10-ml volumetric flasks, adding 3/4° of diluent, and sonicating for 10 minutes. Diluting agents were used to create the volume, which was labelled as Standard stock solution (1000 g/ml of Fluphenazine).

Preparation of Standard solution: 1 ml of Fluphenazine was pipetted into a separate 10 ml volumetric flask, where the volume was then filled with diluent. (Fluphenazine, 100 g/ml)4% percent Standard solution: In a 10mL volumetric flask, 0.4ml of the working standard solutions was pipetted out and diluted to 10mL. Similar standard solutions of 6 % ,8%, 10 %,12 %,14 %, 16 %,and 18 % were created using 4g/ml of Fluphenazine.

Accuracy: Preparation of standard solution:10mg of Fluphenazine were accurately weighed and then put to individual 10ml volumetric flasks. 3/4 of the diluents were then added, and the flasks were shaken briefly. Standard stock solution (1000 g/ml of Fluphenazine) was prepared in flasks with diluents.

Figure 1.9:Linearity 18% Chromatogram



Preparation of 50% Spiked Solution: 1.0 ml of each standard stock solution was pipetted out and diluted to the correct concentration before being added to a 10 ml volumetric flask along with 0.5 ml of the sample stock solution.

Preparation of 100% Spiked Solution: A 10 ml volumetric flask holding 1 mL of standard stock solution and 1 mL of sample stock solution was pipetted together to dilute the volume to the required concentration.

Preparation of 150% Spiked Solution: The solution was made in a 10 ml volumetric flask with 1.5 ml of sample stock solution.

Acceptance Criteria: Each level's recovery percentage should range from 98.0 to 102%.

LOD and LOQ sample Preparation: Preparation: Diluents were added to 1 ml of standard stock solution before being pipetted into 10 ml volumetric flasks. Fluphenazine 0.1 mg was transferred to 10 ml volumetric flasks from the aforementioned solution, and the volume was then added using the same diluents.

Assay methodology: By injecting a sample with an equivalent weight into an HPLC machine, the commercial formulation was tested. Furthermore, % To diluted the volume to the appropriate concentration, a 10 ml volumetric flask containing 1 mL of standard stock solution and 1 mL of sample stock solution was pipetted together. Formulas were used to determine purity.

Use the following formula to determine the amount of Fluphenazine that is pure in the injection:

$$\% \text{ of content} = \frac{\text{Peak Area of Sample} \times \text{Concentration of Standard}}{\text{Peak Area of Standard} \times \text{Concentration of Sample}} \times 100$$

RESULTS AND DISCUSSION

Specificity: It is the method to accurately and dependably quantify the target analyte in the presence of contaminants, degradation products, and other chemicals that are expected to be present in the sample matrix and other components. When a blank was injected, no peaks at the Rt of Fluphenazine were seen, demonstrating the method's specificity.

System suitability parameters: System suitability test provides added assurance that the method gives accurate and reliable results on a specific time. System suitability tests are performed each time a method is used before or during analysis. The system suitability parameters evaluated in this study include theoretical plates, tail factor, retention factor, run time and the values are shown in Table 1.1.

Table 1.1: System suitability parameters of Fluphenazine

Parameters	Chromatographic Conditions
Theoretical plates	7280
Tailing factor (Asymmetry)	1.345
Retention Time (Rt)	3.268
Run time	10min

Table 1.2: Method precision of Fluphenazine

Parameters	10 ppm Peak area (HPLC)
1	8,85,532
2	8,87,623
3	8,85,653
4	8,87,324
5	8,83,236
6	8,86,563
STDEV	1592.634
%RSD	0.18 %

Precision:Method precision: Five 10 ppm working sample solutions are injected the day after sample preparation. The standard deviation was calculated and the %RSD was found to be 0.18, which is not more than 2.0. The precision values of the method are presented in the following table 1.2

Linearity:Linearity: Inject 6 standard solutions containing Fluphenazine at concentrations ranging from 4 ppm to 18 ppm to show the linearity of the analytical procedure. Plot a graph showing peak area versus concentration. 90198 was the obtained slope. The correlation coefficient was found to be 0.999 and the intercept was 23917. The various concentrations and associated peak areas examined during the investigation are listed in Table 1.3 below.

Accuracy: Three concentrations of 50%, 100%, 150% are injected tripled and the average percent recovery was calculated as 100.04. The results are listed in Table 1.4, according to the acceptance criteria, the average recovery should be within the range of 98.0% to 102.0% and be within the range; therefore, the procedure is reliable.

Robustness: Methodological tweaks like Minus Flow, Plus Flow, Minus Mobile Phase, and Plus Mobile Phase are little but intentional. Calculated is the above conditions'% RSD. The parameters assessed during the robustness studies are shown in the table below. The findings show that the procedure is reproducible even when the parameters are intentionally changed slightly, there is no deviation.

Table 1.3 Linearity data of Fluphenazine

Concentration (ppm)	Peak Area\
0	0
4	3,12,958
6	5,30,937
8	7,07,916
10	8,84,896
12	10,61,875
14	12,38,854
16	14,15,833
18	15,92,812

Assay of marketed formulation: The system was given separate injections of the standard solution and sample solution. Chromatograms were then taken, and the earlier approach was used to calculate the amount of drug present in the sample.

In order to achieve outstanding retention duration and peak asymmetry, the Phenomenex C18 column (250 mm * 4.6 mm 5.) was employed for the study. Mobile phase A and phase B were also used (methanol: water 40:60). 20 µl of the injection volume were saved while the flow rate was kept at 1.0 mL/min. The temperature in the column was fixed at 30 C. Using a UV detector, the detection wavelength was 257 nm. Fluphenazine HCl had a retention time of 3.286 min. The regression correlation coefficient was found to be 0.9995, indicating that the process was linear in concentration to peak area responses. After conducting precision investigations, it was discovered that the %RSD of the determinations was 0.18%, indicating that the method's precision was within acceptable bounds.

When compared to the overall percentage RSD for recovery, the accuracy of the approach was determined to be high at 80, 100, and 150 percent. This demonstrates that the suggested strategy is the right one. For the detection and quantification of Fluphenazine HCl injectable formulation, all parameters were effectively established. According to the guidelines in the ICH Q2R1 guidelines, the approach has been validated. The HPLC method designed for both

bulk drug and Pharmaceutical Injection Formulation of Fluphenazine HCl has a number of benefits, including reduced retention time, remarkable linearity, good peak symmetry, and speed, accuracy, and robustness. The sample does not need to be prepared using time-consuming processes because the mobile phase may be made rapidly and the diluent is easily accessible.

Table 1.4: Accuracy data of Fluphenazine (HPLC)

% Recovery	Sample Amount (µg/mL)	Standard Amount Spiked (µg/mL)	Peak area	% Recovery	Mean % Recovery
50%	4*	2*	5,29,578*	100.11*	
					100.04%*
100%	4*	4*	6,98,829*	99.94*	
150%	4*	8*	10,10,572*	99.67*	

Table 1.5: Robustness Data of Fluphenazine

Parameter	%RSD
Parameter	%RSD
Flow Minus (0.8mL)	0.4
Flow Plus (1.2mL)	0.5
Mobile phase Minus (30:70)	0.8
Mobile phase Plus (50:50)	0.9

$$\% \text{ of drug content} = \frac{\textit{Peak Area of Sample} \times \textit{Concentration of Standard}}{\textit{Peak Area of Sample} \times \textit{Concentration of Standard}} \times 100$$

$$= \frac{2{,}37{,}324 \text{ X } 10\mu g/ml}{2{,}38{,}432 X 10\mu g/ml} \times 100$$

% Assay of content = 99.53%

Acceptance limit: 90-110% (as per IP)

CONCLUSION

When actual pharmaceutical samples were analyzed using this method, the content of Fluphenazine HCl fell within the permissible limits. Considering its sensitivity, precision, accuracy, and selectivity for the test chemical, this method can be effectively employed for routine quality control testing of Fluphenazine HCl in liquid dosage form. The presently suggested approach can be employed in quality control laboratories for routine analysis of Fluphenazine HCl in product bulk drugs and in routine application forms of pharmaceutical dose without excipient interference.

Conflict of interest: Authors have no conflict of interest

Funding: Nil

Data Availability: All data are available and can be supplied on reasonable request to the corresponding author

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Antibiotic resistance of bacterial Isolates from Food and Environment: *In vitro* and *In silico* Analysis

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ABSTRACT

Manifold types of environmental samples have been known to be contaminated with potential bacterial pathogens stretching the global world with several infections in humans. Among six samples including five environmental samples and one food sample were taken up for *in vitro* study. Applying the disc diffusion method using 10 antibiotics, the isolated bacterial susceptibility to antibiotics was performed and MAR (multiple antibiotic resistance) index was calculated. All the bacterial isolates were sensitive to AK (amikacin), CIP (ciprofloxacin), VA (vancomycin), TE (tetracycline), MRP (meropenem), and IPM (imipenem), and resistant to AMP (ampicillin). The calculated MAR index of the isolates varied from 0.1 to 0.3, where the value was 0.3 for *Bacillus* sp, *Bacillus cereus*, and *Bacillus subtilis. In silico* molecular docking was also performed to know the binding affinity of two antibiotics IMP and AK against bacterial target proteins (gyrase B and sortase A), wherein the binding energies ranged from -5.7 to -6.8 kcal/mol. The current findings revealed the emergence of antibiotic resistance among bacterial strains in environmental and food samples as well as provided information on bacterial contamination of environmental and food samples. The present study also determined the antibiotic susceptibility pattern and the MAR index to know the source environmental quality. Therefore, routine surveillance of environmental and food samples is warranted to monitor antibiotic resistance of the individual bacterial strains associated.

KEY WORDS: COTTON MASK, NOTES, BACTERIA, ANTIBIOTIC RESISTANCE, MOLECULAR DOCKING.

INTRODUCTION

Microorganisms are found to be present everywhere (including soil, water, air even the human body) and they are microscopic single-celled, prokaryotic organisms (Vidyasagar et al., 2015). It is very important and imperatively necessary to characterize the microbiome of various environments inclusive of indoor surfaces as people spend most of the time in an indoor environment (Andualem et al., 2019). Among all indoor environments, laboratories are relatively safe but may be contaminated with bacteria (Zhu et al., 2020). Consequent upon the outbreak of the dreaded COVID-19, the face masks are used on a large scale as they have good effects for averting seasonal virus-causing diseases (Enaigbe et al., 2021). It may serve as a substrate for microbial growth by creating the humid habitat released by breathing, coughing, and sneezing. The reuse of cotton masks, poor filtration, and improper sanitization give rise to the possibility of viral and bacterial transmission (Enaigbe et al., 2021).

Computer keyboards and mouse are the high-touch surfaces, which may act as a vehicle for the transmission of potentially pathogenic bacteria. Most computer keyboards are contaminated with pathogenic bacteria including Gram-positive bacteria, such as *Staphylococcus* sp. and coagulase-negative bacteria such as *Enterobacter* sp. Different locations (laboratories, institutes, hospitals) represent various kinds of potential pathogenic bacteria (*Staphylococcus aureus, Staphylococcus haemolyticus*, and *Escherichia coli* (Nazeri et al., 2019). Currency notes pose a great threat to the public health as it is the most frequently passed item and also act as a vector for disseminating potentially pathogenic microorganisms. Cross-contamination by regular handling of money with poor sanitation practices cause badly the risk of infection by multi-drug resistant bacterial strains. *Staphylococcus* sp., *E. coli* and *Pseudomonas* sp. are the most commonly found species isolated from different note samples (Abdalrahaman et al., 2020).

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Pickling is a traditional method for preserving food by fermentation with the addition of salt, jaggery, oil, and various spices. Multiple ancient civilizations, such as Indians, Chinese, and Egyptians used pickling for food preservation. Pickles are a great source of potential lactobacilli, but poor hygiene and improper storage may be responsible for causing a great risk of high contamination (Behera et al., 2020).

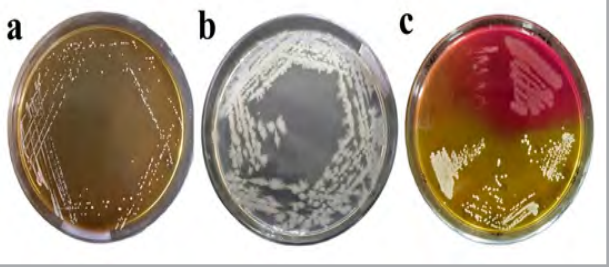
Bacterial antibiotic resistance is nowadays one of the utmost obstacles in health care crisis in (Li et al., 2023). Most of the antibiotics become ineffective against bacteria, since bacteria change themselves by employing various strategies including efflux pump, restrict the access of entryways of antibiotics, destruction of drugs using bacterial enzymes, modification of binding sites of antibiotics, and become resistant (Vila et al., 2020). For clearer close up of antibiotic and bacterial enzymes interaction, *in silico* studies have been very much effective to understand the mode of binding and interaction for the inhibition of bacterial enzyme responsible for pathogenicity and resistance (Budama-Kilinc et al., 2023; Majumdar and Mandal., 2024; Mandal and Mandal., 2024). Therefore, from this study different pathogenic bacterial presence were evaluated in our daily usable items as well as food items, and antibiotic resistance profiles were determined. Also, two antibiotics which were used to check the antibiotic resistance profile of bacteria were used for *in silico* molecular docking study against two bacterial pathogenicity related proteins to analyse the mode of ligand-protein interaction.

MATERIAL AND METHODS

A total six swab samples (five environmental): computer keyboard, currency note, face masks (n=2), and one food: pickle) were collected. Each of the samples was inoculated into the nutrient broth (Hi-Media, India) and MRS broth (Hi-Media, India), and incubated at 37°C for 24 h. From each sample, a loop full of broth culture was taken and then streaked on nutrient agar, MRS agar, and cetrimide agar plate (Hi-Media, India), and incubated for 24 h at 37°C. Nutrient agar stabs and MRS agar stabs were used to preserve single and morphologically discrete bacterial colonies appeared on the plate after subcultures. The isolated bacteria were identified by following gram-staining, gram-reaction, and biochemical tests (TSI, indole, catalase, oxidase, citrate, mannitol salt agar) (Al-Dhabaan., 2019).

The antibiotic susceptibility test was performed for the isolated bacteria, using nutrient agar plate. Pure culture of different bacterial isolates (using the nutrient broth cultures) was swabbed on a different nutrient agar plate. Antibiotic discs: amikacin (AK: 30 µg/disc), ciprofloxacin (CIP: 10 µg/disc), ceftriaxone (CTR), nalidixic (NA), vancomycin (VA: 30 µg/disc), ampicillin (AMP: 10 µg/disc), amoxyclav (AMC), tetracycline (TE: 30 µg/disc), meropenem (MRP: 10 µg/disc) and imipenem (IPM: 100 µg/disc) were placed on each agar plate, which were then incubated at 37°C for 24 h. After incubation, isolates showed zone diameter of inhibition (ZDI) around each antibiotic disc and the results were interpreted following the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2011); the isolated bacteria were categorized as resistant, sensitive, or intermediately susceptible.

Figure 1: (a) *Micrococcus luteus* in MRS agar plate. Isolated characteristic Circular, slightly yellow, convex, and smooth, (b) *Bacillus cereus* in nutrient agar plate. Isolated characteristic grey-white with a less wavy wedge, (c) Mannitol salt agar plate, isolated bacteria from the NA and CA agar plate cultured to determine the particular cocci strain.



MAR index is the ratio between the number of antibiotics for which the bacterial strain had a resistance and the total number of antibiotics exposed to the particular bacterial strain at the time of the antibiotic susceptibility test (Krumperman., 1983). The MAR index of <0.2 was indicated low risk, and ≥0.2 was indicated as high risk.

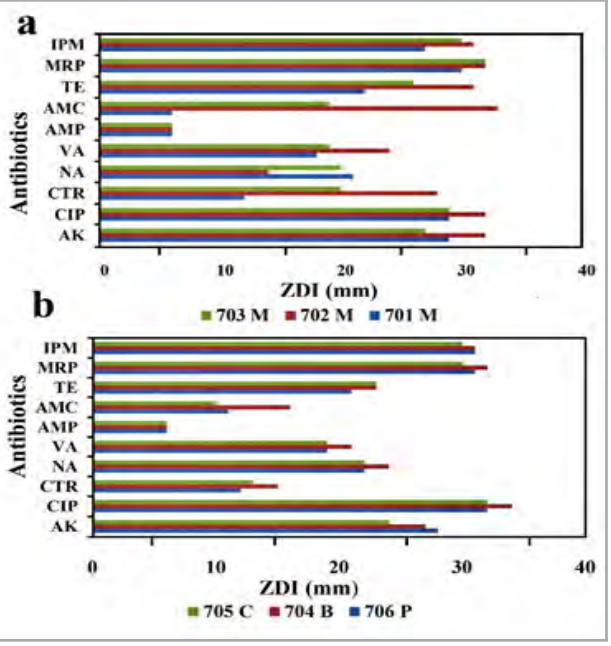
Two 3-D structures of bacterial proteins, ATP binding domains of *S. aureus gyrase* B (PDB ID: 5CPH) and *Listeria monocytogenes* sortase A (PDB ID: 5HU4), responsible for pathogenicity were retrieved from RCSB protein data bank (https://www.rcsb.org/) and used as the targets. 3-D structures two antibiotics IMP (PubChem CID:104838) and AK (PubChem CID:37768) were retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/), and used as the ligands. Both the protein and ligand molecules were refined, and optimized with the help of UCSF Chimera software version 1.15 (https://www.cgl.ucsf.edu/chimera/), by removing solvent, ions, and ligand molecules from the protein structure, and adding Gasteiger charges during ligand preparation.

Two antibiotics were docked with ATP binding domains of *S. aureus gyrase* B (5CPH) and *L. monocytogenes* sortase A (5HU4), using UCSF Chimera inbuilt AutoDock Vina (https://www.cgl.ucsf.edu/chimera/). The active binding sites of the targets were defined using grid box centre: X=15, Y=11, Z= -2, size: X=52, Y=52, Z=52, for protein ATP binding domains of S. aureus gyrase B, and grid box centre: X=12, Y=18, Z= 18, size: X=52, Y=52, Z=52, for *L. monocytogenes* sortase A. Based upon the previous studies, binding energies of molecular docking ≤ -6.5 kcal/mol were considered for favourable binding and protein inhibition by the ligands (Majumdar and Mandal., 2023; Mandal and Mandal., 2021).

RESULTS

Among the isolated bacteria, 7 were round-shaped and the other 10 were rod-shaped (Table 1). In the DNase test, the strains (701M, 704B, 705C, 701M2 and 706P2) showed a transparent zone. In Triple Sugar Iron (TSI) test,10 strains (701M, 702M, 704B, 705C, 705C1, 701M2, 702M2, 703M2, 704B2, 705C2) showed acid butt (yellow) and alkali slant (pink); no strains were found positive for CO_2 and H_2S production. Two bacterial strains (703M and 703M2) showed a positive result for the mannitol test. No strains were found positive for the gram reaction test. The biochemical test results for the isolated bacteria is shown in Table 1. Based upon the cultural characteristics (colony morphology, pigment production), gram staining (cell shape), and biochemical test including TSI test results, and DNase test patterns of the environmental and food bacteria, their identities are represented in Table 2 and Fig. 1.

Figure 2: (a) Antibiotic susceptibility test results for mask samples and (b) Antibiotic susceptibility test results for pickle, notes and keyboard. Abbreviations of antibiotics are mentioned in the text.



All the bacterial strains cultured in the nutrient plate were sensitive to AK, CIP, VA, TE, MRP, and IPM and resistant to AMP. However bacterial strain 701M, 706P, 704B, and 705C were resistant to CTR, in addition, while 702M and 703M were sensitive and intermediately sensitive, respectively. Although strain 701M, 706 and 705C showed resistance to AMC, bacterial isolates 702M and 703M were sensitive, and the isolate 704B was intermediately sensitive. The comparison of all the antibiotic susceptibility or resistances (Fig. 2 and Fig 3).

In the case of MRS agar, all the strains were highly susceptible to antibiotics and the Zone Diameter of Inhibition (ZDI) was >40 mm. The MAR indices of isolated bacteria are shown in Table 3 and Fig. 4.

The interaction between IMP and AK against two pathogenic bacterial proteins: ATP binding domains of *S. aureus gyrase* B (5CPH) and *L. monocytogenes* sortase A (5HU4) were considered to know the mechanism of binding of antibiotics with target proteins. AK showed lowest binding energy of -6.8 kcal/mol against *S. aureus gyrase* B ATP binding domain, and -5.9 kcal/mol against *L. monocytogenes* sortase A. The IMP had binding energies of -6.2 kcal/mol against *S. aureus gyrase* B ATP binding domain and -5.9 against *L. monocytogenes* sortase A (Table 4). During docking, several types of interactions were formed between protein and antibiotics that help in the stability of the complexes and determine binding affinity.

Figure 3: Antibiotic susceptibility test results following the by disc diffusion methods of mask samples.

IMP interacted with *S. aureus gyrase* B ATP binding domain and formed one hydrogen bond and salt bridge with Glu58 (2.26 Å), ten Van der Waals interactions with Ile51, Ser55, Thr173, Pro87, Asn54, Asp81, Ser55, Asp57, Gly85, and Gly83, and three hydrophobic interactions in the form of π-alkayl with Ile86, Ile102, and Ile175 (Fig. 5). AK interacted with *S. aureus gyrase* B, displaying two electrostatic interactions with Glu58, Asp57, seven conventional hydrogen bonds with Ser55 (2.89 Å), Gly85 (3.05 Å), Asn54, (2.14 Å), Ile102 (2.67 Å), Ser128 (2.29 Å), Asp57 (2.41 Å), and Glu58 (2.20 Å), one carbon hydrogen bond with Asn54, and a total eleven Van der Waals interactions with Ser129, Leu103, Asp81, Thr173, Ile86, Gly83, Pro87, Leu60, Ala61, Tyr63, and Arg84 (Fig. 6).

L. monocytogenes sortase A with IMP showed four hydrogen bonds with Arg126 (2.33 Å), Glu98 (2.44 Å), Glu100 (2.35 Å), Asp97 (2.55 Å), ten Van der Waals interactions with Phe148, Pro149, Ser150, Ser12, Leu23, Leu22, Ala35, Val20, Pro67, Leu64, Ser 62, Asp19, and two π-alkayl interactions with Ala54 and Leu33 (Fig. 5). AK formed four hydrogen bonds (carbon hydrogen) with Asn21 (3.15 Å), Asp19 (3.71 Å), Phe148 (3.49 Å), Ala35 (3.75 Å), one conventional hydrogen bond with Leu34 (2.20 Å) and a total of 13 Van der Waals interactions with amino acids Phe148, Pro149, Ser150, Ser150, Ser12, Leu23, Leu22, Ala35, Val20, Pro67, Leu64, Ser62, and Asp19 (Fig. 6).

DISCUSSION

Contamination with bacteria in food and environment is common and isolation of bacterial strains from food such

as homemade pickle and environment samples are not unknown. Different authors from different parts of the world identified various types of bacteria from the environment and food. According to Gund et al. (2021), some species such as *Staphylococcus epidermidis, Staphylococci* sp., *M. luteus, Rothia dentocariosa, Streptococcus oralis*, and *Bacillus* sp. were found in a surgical mask.

Figure 4: MAR indices of isolated bacteria

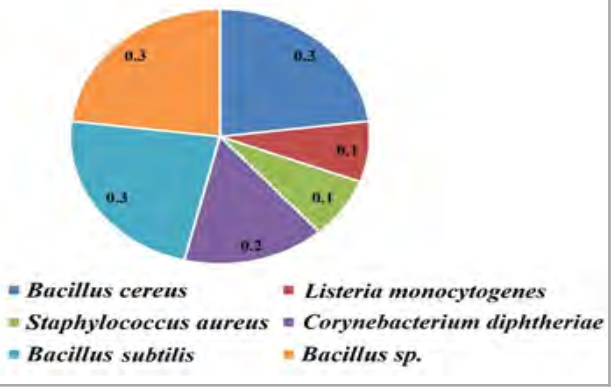
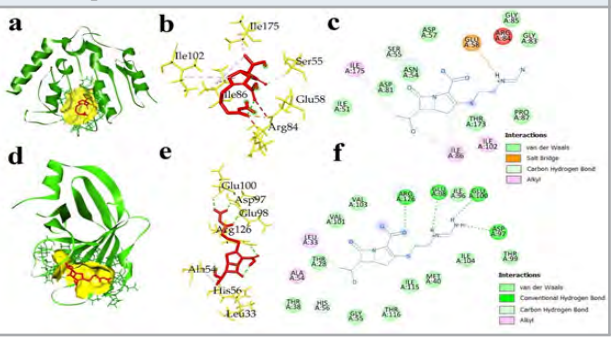


Figure 5: Docked complex of imipenem and two bacterial proteins *S. aureus* ATP binding domain of GyrB (5CPH) and *L. monocytogenes* sortase A (5HU4) (a) 3D docked complex of (5CPH) with imipenem (b) 3D interaction of 5CPH with imipenem (c) 2D representation of interacted amino acids of 5CPH with imipenem (d) 3D docked complex of 5HU4 with imipenem (e) 3D interaction of 5HU4 with imipenem (f) 2D representation of interacted amino acids of 5HU4 with imipenem

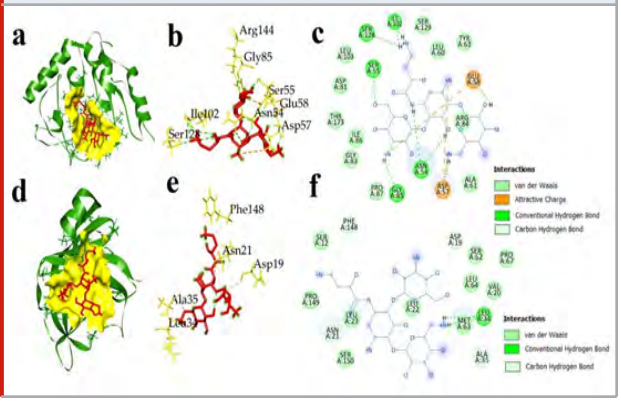


Nightingale et al. (2022) stated that face masks can contain *E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *S. aureus*. But in the current study, bacterial strains in cotton masks such as *Bacillus cereus, S. aureus, S. epidermidis, L. monocytogenes, Micrococcus luteus* and *Corynebacterium* sp. were found. All the bacterial isolates showed sensitivity against 9 antibiotics and resistance to one.

In accordance with the report of Al-Akeedi et al., 2021, *Bacillus* sp., *Corynebacterium* sp., *micrococcus* sp., *Staphylococcus* sp., *B. cereus, Proteus* sp., *P. aeruginosa, Streptococcus viridians*, and *E. coli* were isolated from different computer keyboards. As per the current study, *Corynebacterium diphtheria* and *Staphylococcus epidermidis* were the major bacteria isolated from computer keyboards. In pursuance of the report of Abba and Okoye (2022), bacterial strains such as *S. aureus, Escherichia coli, Pseudomonas* sp., *S. epidermidis, Klebsiella* sp. and *Bacillus* sp. were identified in Naira notes. But our current study showed the existence of *Bacillus* sp. and *M. luteus* in Indian currency, and these two strains showed sensitivity against 9 antibiotics and resistance against to one. Uchino et al. (2020) reported the presence of *Salmonella* sp., *S. aureus, B. cereus* and E. *coli* food spoilage strains that mainly contaminate pickle.

In the current study, bacteria including *Bacillus subtilis* and *Bacillus megaterium* were isolated from the vegetable pickle. Another study, by Aljahani (2020), revealed the isolation of *E. coli, Salmonella enterica* and *L. monocytogenes* in various pickle samples. *B. cereus* is widespread and commonly found in soil, water, and food. It causes foodborne illnesses including vomiting and diarrhoea. It is generally resistant to β-lactam antibiotics such as ampicillin, penicillin, and amoxicillin, but susceptible to vancomycin and erythromycin (Uchino et al., 2020). This bacterial strain was found in eye cosmetics (Nandi and Mandal, 2016) and also found in the face mask, which indicated high contamination. *S. aureus* is a Gram-positive potential pathogenic bacterium that causes skin and tissue infections in humans. By genetic mutations, it becomes multi-drug resistant. The toxic elements of *S. aureus* cause gastrointestinal symptoms (Uchino et al., 2020).

Figure 6: Docked complex of amikacin and two bacterial proteins *S. aureus* ATP binding domain of GyrB (5CPH) and *L. monocytogenes* sortase A (a) 3D docked complex of (5CPH) with amikacin (b) 3D interaction of 5CPH with amikacin (c) 2D representation of interacted amino acids of 5CPH with amikacin (d) 3D docked complex of 5HU4 with amikacin (e) 3D interaction of 5HU4 with amikacin (f) 2D representation of interacted amino acids of 5HU4 with amikacin



B. subtilis also known as hay *bacillus* is a gram-positive bacterium mainly found in soil, the gastrointestinal tract of ruminants, humans, and marine sponges. This bacterium is sensitive to TET and VM but resistant to streptomycin (Colom et al., 2021). *B. megaterium* is an aerobic spore-forming Gram-positive bacteria widespread in nature (John, 2020). In our study, this bacterial strain was found in currency notes. *C. diphtheriae*, the causative agent of

diphtheria, which is the main reason for thousands of deaths per year. This bacterial strain is a toxigenic bacterium, but if we review our study this bacterial strain was found in keyboard (Ott et al., 2022).

Table 1: Biochemical test results for the isolated bacteria from food and environmental samples

Sl no.	Isolated Bacterial Code	Media	Gram staining			Gram reaction (KOH test)	Biochemical test						
			Property	CS	SP		DNS	TSI	CIT	CAT	OXI	MAN	IND
1.	701 M	NA	positive	rod	+	Non-sticky	+	P/Y	-	-	-	-	-
2.	702M	NA	positive	rod	-	Non-sticky	-	P/Y	+	+	-	-	-
3.	703 M	NA	positive	round	-	Non-sticky	-	Y/Y	+	+	-	+	-
4.	704 B	NA	positive	rod	+	Non-sticky	+	P/Y	-	+	-	-	-
5.	705 C	NA	positive	rod	+	Non-sticky	+	P/Y	+	+	+	-	-
6.	706 P	NA	positive	rod	+	Non-sticky	-	Y/Y	+	+	+	-	-
7.	701 M1	MRS	positive	round	-	Non-sticky	-	Y/Y	-	+	+	-	-
8.	702 M1	MRS	positive	round	-	Non-sticky	-	Y/Y	-	+	+	-	-
9.	703 M1	MRS	positive	round	-	Non-sticky	-	Y/Y	+	+	+	-	-
10.	704 B1	MRS	positive	round	-	Non-sticky	-	Y/Y	-	+	+	-	-
11.	705 C1	MRS	positive	round	-	Non-sticky	-	P/Y	+	+	+	-	-
12.	701 M2	CA	positive	rod	+	Non-sticky	+	P/Y	+	+	-	-	-
13.	702M2	CA	positive	rod	-	Non-sticky	-	P/Y	-	+	+	-	-
14.	703 M2	CA	positive	round	-	Non-sticky	-	P/Y	-	-	-	+	-
15.	704 B2	CA	positive	rod	-	Non-sticky	-	P/Y	+	+	+	-	-
16.	705 C2	CA	positive	rod	-	Non-sticky	-	P/Y	-	-	-	-	-
17.	706 P2	CA	positive	rod	+	Non-sticky	+	Y/Y	-	+	-	-	-

Table 2. Identity of isolated bacteria from different environmental and food samples

Bacterial isolates	Source of isolation	Identity
LMEM 701 M	Mask (LMEM-M)	*Bacillus cereus*
LMEM 702 M		*Listeria monocytogenes*
LMEM 703 M		*Staphylococcus aureus*
LMEM 701 M1	Mask (LMEM-M1)	*Staphylococcus epidermidis*
LMEM 702 M1		*Staphylococcus epidermidis*
LMEM 703 M1		*Micrococcus luteus*
LMEM 701 M2	Mask (LMEM-M2)	*Bacilluscereus*
LMEM 702 M2		*Corynebacterium* sp.
LMEM 703 M2		*Staphylococcus aureus*
LMEM 704 B	Computer keyboard	*Corynebacterium diphtheriae*
LMEM 704 B1		*Staphylococcus epidermidis*
LMEM 704 B2		*Corynebacterium* sp.
LMEM 705 C	Note	*Bacillus megaterium.*
LMEM 705 C1		*Micrococcus luteus*
LMEM 706 P	Pickle	*Bacillus subtilis*

From the molecular docking studies, ATP binding domain of S. *aureus* GyrB with IMP showed -6.2 kcal/mol and with AK -6.8 kcal/mol of binding energies, and the *L. monocytogenes* sortase A with IMP and AK showed binding energies of -5.7 and -5.9 kcal/mol. ATP binding domain of S. *aureus* GyrB docked complex with AK, showed intense lower binding energy as compared to others (Terefe and Ghosh., 2022). In all the four complex as formed by different interactions mainly hydrogen bonds, Van der Waals interactions and hydrophobic interactions, but the inhibition potency was

highest in case of ATP binding domain of S. *aureus* GyrB and AK according to docking results (Santha et al., 2022). Less interaction energy might be associated with the formation of hydrogen bonds (Li et al., 2023). The docking analysis provided the insights of the mechanism of binding of antibiotic to their bacterial counterpart responsible for infection, and the protein-ligand interaction suggested the amikacin as well as imipenem both of them were effective inhibitors, as also supported by the *in vitro* study.

Table 3: MAR indices of isolated bacteria from different sources

Isolated bacteria	MAR index	Risk factor
Bacillus cereus	(3/10) = 0.3	Highly risk
Listeria monocytogenes	(1/10) = 0.1	—
Staphylococcus aureus	(1/10) = 0.1	—
Corynebacterium diphtheriae	(2/10) = 0.2	Moderately risk
Bacillus sp.	(3/10) = 0.3	Highly risk
Bacillus subtilis	(3/10) = 0.3	Highly risk

Table 4: Molecular docking results of two bacterial proteins *Staphylococcus aureus* ATP binding domain of GyrB (5CPH) and *Listeria monocytogenes sortase* A (5HU4) with two antibiotics imipenem and amikacin

Protein and ligand	Involved amino acid in hydrogen bond formation with Distance (Å)	Involved amino acid in Van der Waals interaction	Involved amino acid in hydrophobic interaction	Binding energy (kcal/mol)
5CPH and imipenem	Glu58 (2.26)	Ile51, Ser55, Thr173, Pro87, Asn54, Asp81, Ser55, Asp57, Gly85, Gly83	Ile86, Ile102, Ile175	-6.2
5CPH and amikacin	Ser55 (2.89), Gly85 (3.05), Asn54 (2.14), Ile102 (2.67), Ser128 (2.29), Asp57 (2.41),	Ser129, Leu103, Asp81, Thr173, Ile86, Gly83, Pro87, Leu60, Ala61, Tyr63, Arg84		-6.8
5HU4 and imipenem	Glu58 (2.20) Arg126 (2.33), Glu98 (2.44), Glu100 (2.35), Asp97 (2.55)	Thr28, Thr38, His56, Gly55, Met40, Ile104, Thr99, Val101, Ile96, Val103, Ile115	Ala54, Leu33	-5.7
5HU4 and amikacin	Asn21(3.15), Asp19 (3.71), Phe148 (3.49), Ala35 (3.75)	Phe148,Pro149, Ser150, Ser150, Ser12, Leu23, Leu22, Ala35, Val20, Pro67, Leu64, Ser62, Asp19		-5.9

CONCLUSION

All the bacterial isolates were sensitive to AK, CIP, VA, TE, MRP and IPM and resistant to AMP. Isolated bacteria such as *C. diphtheria, S. epidermidis*, and *L. monocytogenes* notable in the current study. The keyboard can cause the spreading of contagious diseases, so the keyboard must be disinfected regularly. Cash notes are the most variable things in the world. So, every time someone used those notes, must sanitize their hands properly. Vegetable pickle is a preserved food, which was found to be contaminated with bacteria such as *B. subtilis* that might potentially cause infection. So, pickle should be prepared carefully following hygienic ways, and should be stored in properly sterilized containers. Bacteria such as *Bacillus cereus, C. diphtheriae,* and *B. subtilis* which were isolated from the tested samples had MAR index ≥0.2, which indicated the bacterial origin from highly antibiotic polluted sources. Besides that, isolated *L. monocytogenes* and *S. aureus* showed comparatively lower MAR index, which was 0.1 indicating their origin from an environment with moderately antibiotic contamination. *In silico* studies further revealed the effectiveness of the antibiotics (AK and IMP) against bacterial target proteins, which implied possible cause of bacterial susceptibility.

Conflict of interest: The authors have no conflict of interest.

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Data Availability: Data will be available on request

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Evaluation of BHMT2 Gene Expression and its Expression Factor Regulating LNC00922 LNCRNA with rs10944 Genotype Determination in Patients with Breast Cancer.

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ABSTRACT

It is predicted that dysregulation of *BHMT2* expression level could have a remarkable effect on Breast cancer status. The absence of methionine and Hyperhomocysteinemia have been linked to a decline in *BHMT2* expression. This mRNA expression and its correlated long non- coding RNA (lncRNA) (LINC00922) have been investigated in our experiment based on the bioinformatics approach. Also, the genotype frequency analysis of *BHMT2* in SNP rs10499 has been demonstrated. The mentioned SNP is correlated with the differences in the binding affinity of miR542-3p. We performed a microarray analysis to find the differentially expressed genes (DEGs). GeneMANIA and miRWalk online databases were used for mRNA-mRNA and mRNA-miRNA interaction analyses. Clusterprofiler package was used for functional and pathway enrichment analyses. dbSNP database was applied for finding the relevant Single nucleotide polymorphism (SNP). The Co-lncRNA database was used for finding the correlated lncRNA.A real-time PCR experiment was performed to count the relative expression level of *BHMT2* and LINC00922. The high resolution melt (HRM) method was performed to identify the SNP genotype frequency.*BHMT2* had a significant down-regulation, and LINC00922 had a significant up-regulation in Breast cancer tissues compared to normal. No significant difference in genotype frequency of rs10944 was observed. The current study revealed that BHMT2 and LINC00922 can be the two novel correlated biomarker of breast cancer. More similar investigations are needed for evaluating more accurate and reliable result, specially about rs10499.

KEY WORDS: BREAST CANCER, RNA INTERACTION, SINGLE NUCLEOTIDE POLYMORPHISM.

INTRODUCTION

Breast cancer (BC) is the second cause of death in developed countries. The prevalence of BC in developing countries throughout the Asia-Pacific region is prognosis to increase in the next few years continuously. Breast cancer differed based on biomolecular features into luminal A and luminal B (expressing the estrogen receptor (ER)), basal-like and human epidermal growth factor receptor 2 (HER2)- enriched (without ER expression). Mainly the ESR1 gene caused the expression of estrogen receptor-α (ER). Triple-negative breast cancer (TNBC) is a heterogeneous disease, and its biomarkers can be used for diagnostic approaches (Sporikova et al., 2018). Though lots of effort has been paid for knowing the BC mechanism, still there is a lack in detecting new visions of biomarkers in transcriptional mechanism and gene regulation for drug discovery and early diagnosis. Overall, several reports about tumorigenesis activities of biomarkers like noncoding RNAs, especially deregulation in mammary cells (D, 2011; Gupta et al., 2019).

Long noncoding RNA (lncRNA) is a single-stranded class of noncoding RNA with more than 200 nucleotides. This molecule is known as one of the critical characteristic biomarkers in BC prognosis (Liu et al., 2016). Reports have indicated that lncRNA has a potential function in intercellular processes (Klinge, 2018). There is not much information about all lncRNAs; nonetheless, Scientists clustered these biomolecules into three types that encoded from different regions in the genome (Yousefi et al., 2020). Besides, LncRNAs can influence the function of microRNA. As an illustration, LINC00460 overexpressed levels have been identified in BC tissue. This lncRNA has interacted with miRNA-489-5p (Zhu et al., 2019). Another example of lncRNA, LINC00324, and LINC00472, have been detected

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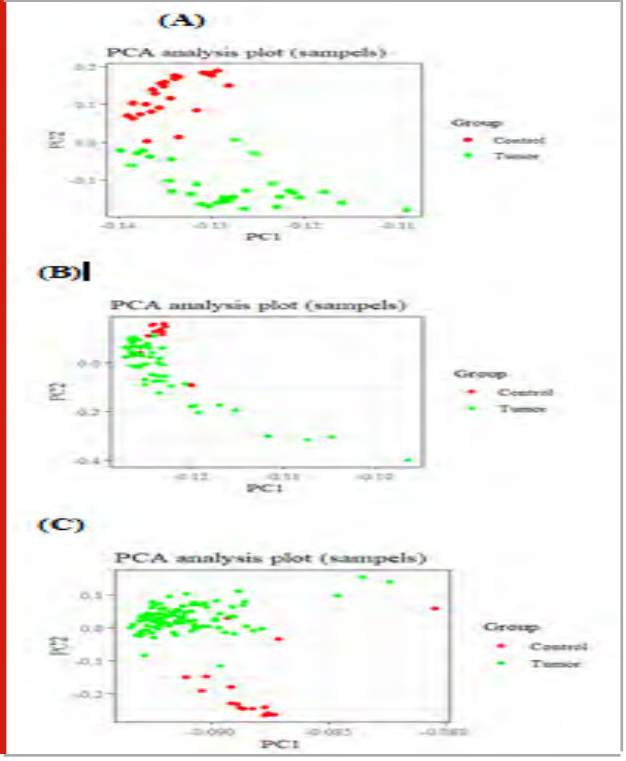
in breast cancer tissue (Soudyab, Iranpour, and Ghafouri-Fard, 2016). Our present study has demonstrated a unique and novel lncRNA, Long Intergenic Non-Protein Coding RNA 922 (LINC00922) placed in16q21 chromosome.

The endogenous subtype of noncoding RNA is microRNA with 18-22 nucleotides length, has different roles; involved translation, post-transcription regulation in genetic and epigenetic areas. It has a crucial role in cancer pathways (Hata and Lieberman, 2015) and controlling protein levels via binding from seed sequence (5' ends) to the untranslated region of target mRNA. Besides, the complementarity of miRNA-mRNA interaction can be affected by single nucleotide polymorphism (SNP). Researchers reported that an SNP, rs1071738 influenced miR-96 and miR-182 by inhibiting the translation product of the PALLD gene, which reduced breast cancer cell migration (Gilam et al., 2016; Moszyńska et al., 2017). our study focused on mir-542-3p placed on the Xq26.3 chromosome area.

Figure 1: Principal component analysis of GSE18018(A), GSE29431(B) and GSE42568 (C) samples, based on the Normalized expression data.



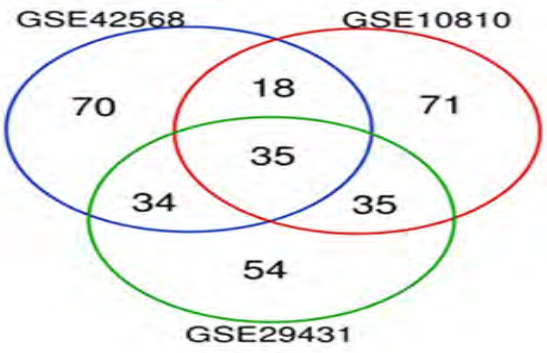
Scientists observed that *BHMT2* (Betaine-Homocysteine2) encodes a zinc metalloenzyme, which has a Methyl-donor role in methionine biosynthesis via homocysteine (Giusti et al., 2008; Mostowska et al., 2010; Ganu et al., 2015). Based on proven studies, Hyperhomocysteinemia (disorder due to increased homocysteine level) happened by preventing remethylation of homocysteine to methionine, which caused methionine dependency disturbance in the methionine pathway (Mohammad et al., 2013). Apart from that, reducing the expression of *BHMT2* has been considered a result of an absence of methionine and Hyperhomocysteinemia. This phenomenon correlates to cancer development and metastasis (Pirouzpanah et al., 2014).

The main purpose of this study was to evaluate the differential expression of the *BHMT2* gene and its expression regulators in breast cancer tissue compared to the standard control. Our results belong to other potential genetic factors could develop more precise diagnostic routes or new therapeutic method for breast cancer.

MATERIAL AND METHODS

Data collection: Gene Expression Omnibus (GEO) datasets analysis was conducted to collect differential expressed (DE) genes in BC tissue samples (https://www.ncbi.nlm.nih.gov/geo/).GSE29431, GSE10810, and GSE42568 datasets were retrieved and downloaded in order to achieve the profile of differential expressed genes in breast cancer tumor tissue compared to adjacent healthy cells. The limma R package was used to obtain altered gene expression profiles between patients and healthy individuals. P-value was calculated for each gene. The False Discovery Rate (FDR) method was applied to measure the Adj.p.Value ($P<0.01$ was considered a significant threshold). Genes were filtered based on $-2\leq FC \geq 2$ (Foldchange) and Adj.p.Value <0.01, then top 200 DE genes were collected from each dataset. Using a Venn diagram, common genes were isolated from the collected sets and based on a common gene's possible role among three datasets in breast cancer development. Finally, among common genes, a gene that has not been evaluated by qRT-PCR before was selected as a novel gene to analyze its expression level in breast tumors. The GSE40525 study was also analyzed to evaluate the differences in miRNA expression in breast tumor tissue using the limma package in the R environment.

Figure 2: The investigation of common genes among the selected datasets by Venn diagram showed that 35 common differential expression genes (DEGs) were identified as significant DE genes whose altered expression can be considered essential factors for early detection of breast cancer.



Network interaction construction: The genes obtained from microarray studies were mapped using the GeneMANIA database and visualized using Cytoscape software (version 3.7.2). the grid was drawn only to show proteins that coexpression with each other (Franz et al., 2018). miRWalk

online software was used for miRNA-mRNA interaction analysis. Then the miRNA-mRNA regulatory network was visualized by Cytoscape.

Functional/pathway Enrichment analysis: Enrichment analysis of genes was performed by the Clusterprofiler package in R. the statistical method of Adj.p.Value was used to confirm the function of genes in the molecular pathways and cellular processes ($p<0.05$ was considered as significant) (Yu et al., 2012).

Figure3: The volcano plots of differentially expressed genes collected from three different datasets of breast tumors as compared with normal samples, BHMT2 gene is highlighted in red.

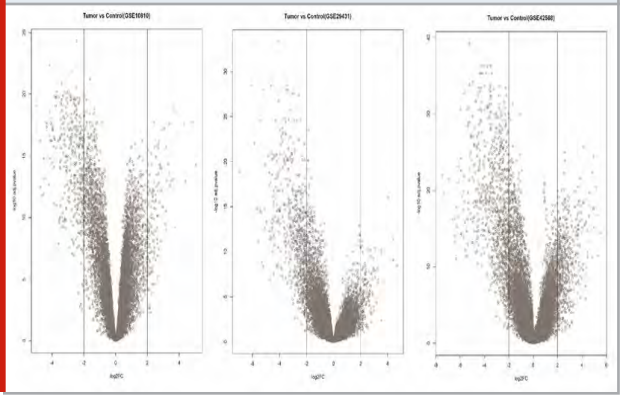
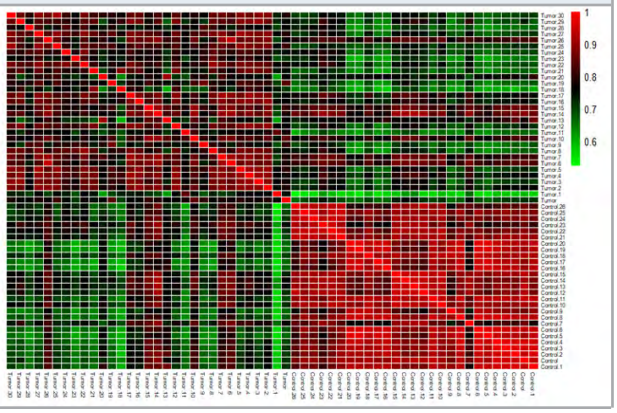


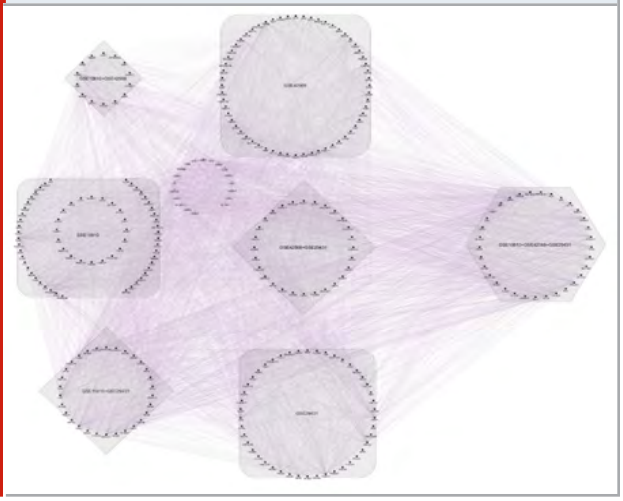
Figure 4: Heatmap of the correlation between the samples of GSE18018(A), GSE29431(B) and GSE42568 (C) dataset.



Noncoding regulatory elements of BHMT2: Using the dbSNP webtool and examining the existing SNPs located in the 3'UTR region of the BHMT2 gene, the SNPs with the MFE>0.1 were isolated. Seed region sequences of miRNAs were then downloaded from the TargetScanHuman database, and the SNP region was searched among seed sequences for human miRNAs. The RNAhybrid database was used to calculate the free binding energy (MFE) to evaluate the effect of SNP on the hybridization of miRNA to the mutant sequence (Krüger and Rehmsmeier, 2006).
Using the co_LncRNA database, the correlation between genes and lncRNAs was examined. This tool is developed based on The Cancer Genome Atlas Program (TCGA) data of various cancer types. Spearman's correlation test was used to estimate coexpression relationships between lncRNAs and the BHMT2 gene in BC samples. Moreover, the p-value of the correlation coefficient was estimated, and a p-value less than 0.01 was considered significant. Finally, a coefficient threshold of 2 was applied. the articles were reviewed to select the most effective lncRNA in the pathogenesis of breast cancer (Zhao et al., 2015).

Sample Collection: Cancerous and surrounding normal breast tissue samples from 24 female patients were used in a case-control study. Also, the samples were kept in liquid nitrogen after being deposited in RNAlater solution (Thermofisher Scientific). The Ethics Committee of Al-Zahra Hospital, Isfahan University of Medical Sciences, certified all of the samples, and all patients signed written consent documents.

Figure5: Network interaction of shared genes among three microarray datasets. This network contains 303 nodes and 9690 edges. Edge in this network represents the co-expression relationship between two interconnected proteins. The square represents the unique genes for each study, the rhombus contains the shared genes between two-by-two datasets, and the hexagon contains 35 common genes between the three evaluated datasets in this investigation.

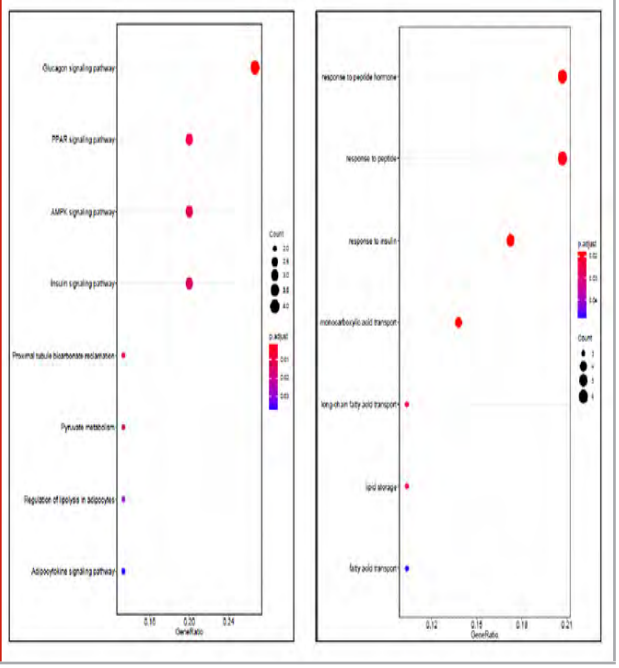


Real-Time PCR: Total DNA was extracted from each sample using the YTA kit according to the manufacture's protocols. Total RNA was extracted from each sample using TRizol (YTZ) following the manufacturer's instructions. Total RNA quality and quantity verified by NanoDrop 1000 spectrophotometer (Thermo Fischer). The cDNA synthesis was done using the RevertAid First Strand cDNA synthesis (ThermoScientific) kit. First, 0.1 ng of extracted RNA was mixed with 1µL Random Hexamer primer; then, the mixture reaches 12 µL volume by adding ddH2O and incubate at 65˚c for 5 min. In the next step, master mix that includes 4µL 5x Reaction Buffer with 1µL Ribolock RNase Inhibitor (20 U/µL), 2µL 10mM dNTP Mix and 1µL RevertAid M-MuLV RT (200 U/µL) was incubated for 5 min at 25˚c following by 60 min at 42˚c.

Using OligoCalc (http://www.basic.northwestern.edu/biotools/OligoCalc.html) and Gene Runner software,

gene-specific primers were designed. The mRNA level of target genes was quantified by real-time PCR analysis on Magnetic Induction Cycler (Mic) PCR Machine using the Quantifast SYBR Green qPCR Master Mix (YTA). PCR reactions contained one µl of gene-specific primers (10pmol), five µl Quantifast SYBR Green PCR master mix, one µl of template cDNA, two µl water in a ten µl reaction volume. The reaction mixtures were subjected to initial denaturation of 95 °C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 15 sec and 72°C for 20 sec. The final melt curve analysis was performed by increasing the temperature from 60 °C to 95 °C with an increase of 0.1 °C per sec followed by the last hold at four °C. The specificity of the amplification was validated by electrophoresis of the PCR product on an agarose gel and examination of the melt curve.

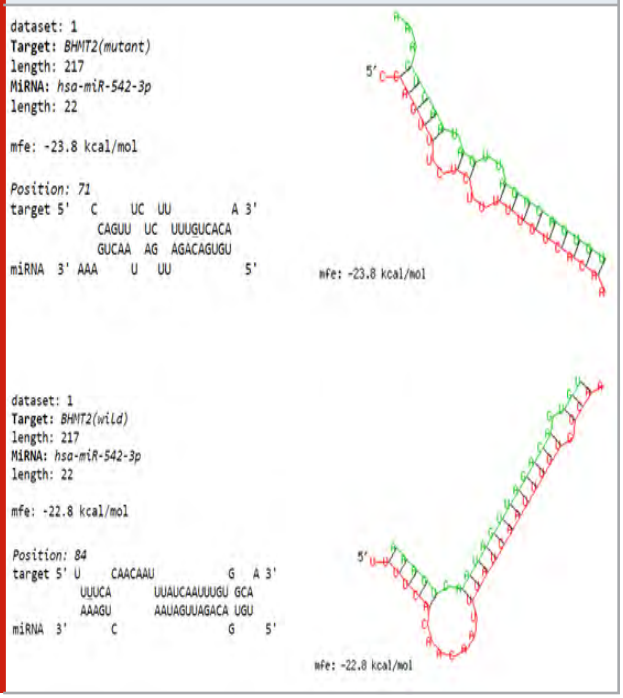
Figure 6: Functional Enrichment (Right image) and pathway enrichment (Left image) analyses.



All reactions were carried out in duplicate, and the cycle threshold (Ct) values for the target genes were normalized using GAPDH as a reference gene. Table2 lists the primer sequences used according to cDNA synthesis. The fold change in the expression of the target genes was determined using the formula:2−ΔΔCt, where ΔCt= average Ct of target gene−average Ct of GAPDH, and ΔΔCt=ΔCt of the objective sample (cancer tissue) −ΔCt of evaluator sample (normal tissue). The SNP reference sequence was downloaded from the National Center for Biotechnology Information's (NCBI) dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP). Primer3 (http://frodo.wi.mit.edu/) was used to design the primers, which were then tested for specificity using BLAST (http://blast.ncbi.nlm.nih.gov). The primer sequences are described in Table 1.

High-Resolution Melt: High-Resolution Melt (HRM) method (by Mic PCR Machine) was used for genotyping the target SNP.HRM entails PCR cycling followed by a gradual rise in temperature to melt DNA. Fluorescence intensity shows that the individual nucleotide changes had different melting-curve forms. The total reaction volume was 10µl (Eva green 2µl, Forward primer 1µl, Reverse primer 1µl, water 4µl, DNA 2µl). The initial denaturation was at 95°C for 10 min followed by 40 cycles including 95° C for 15 sec and 60 °C for 15 sec and 72° C for 20 sec. The final melt curve analysis was performed by increasing the temperature from 60° C to 95°C with the increase of 0/1° C per 1 sec followed by the last hold at four °C. Furthermore, 5 out of 24 samples were chosen randomly for Sanger sequencing to validate the result of HRM compared with sequencing.

Figure 7: The amount of minimum free energy of hsa-miR-542-3p binding in 3'UTR region of BHMT2 gene in both wild condition and SNP rs10944 was measured by RNAhybrid software, which in the case of occurrence of mutation, the binding rate of this miRNA will be stronger.



Statistical analysis: GraphPad prism (8) was used for real-time PCR data analysis, including expression, correlation, and ROC analyses. SPSS software was used for genotype frequency analysis. The paired t-test and chi-square test were performed to evaluate the RNAs expression level and frequency of rs10944 genotypes in tumor samples compared to standard samples. P-Value less than 0.05 was considered as the significance level. Pearson correlation test was performed on the RNAs expression level to obtain the correlation of gene and lncRNAs

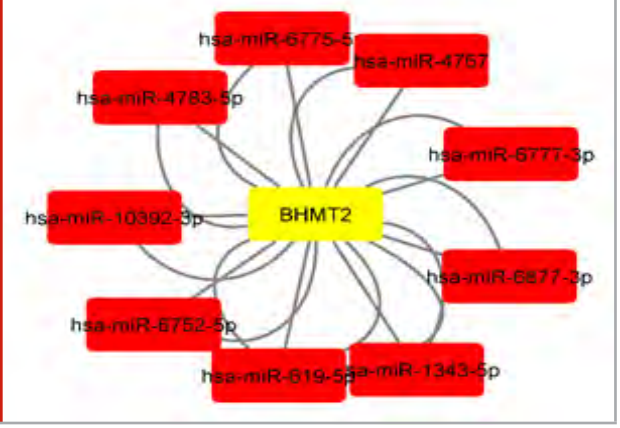
RESULTS

Bioinformatics analyses: Based on our criteria of the microarray study described in the method section, GSE29431, GSE 10810, and GSE42568 studies were identified, and differential expression analysis revealed a

total of 200 genes that had significant changes greater than two and Adj.p.Value less than 0.01 were selected from per dataset (Figure 1). Using a Venn diagram, 35 common genes were extracted from the mentioned datasets (Figure 2).

These genes had significant expression changes in all three studies, therefore very likely that changes in their expression will be helpful in the pathogenesis of breast cancer for the detection of some genes whose expression levels have not been studied yet in detail. In the process of breast cancer, literature mining has been done, and 6 out of 35 genes, including *MYZAP, BHMT2, PPP1R1A, PLAC9, LOC284825*, and *SPX*, was obtained which their role in breast cancer has not been studied yet. Following to evaluation of assays that they support a potential role of the gene in breast cancer, according to the function of the protein expression of *BMHT2* gene in the process of Homocysteine to Methyonin conversation and it is a potential role in the process of the Hyperhomocysteinemia that its importance in the breast cancer progression was confirmed before, investigation of the expression of this gene was selected to continue the study process (Figure 3, 4).Functional enrichment analysis related to the 35 common genes were obtained, revealed the involvement of these genes mainly in response to peptide hormones and pathway enrichment analysis, introduced Glucagon signaling pathway as the top pathway (Figure 6).

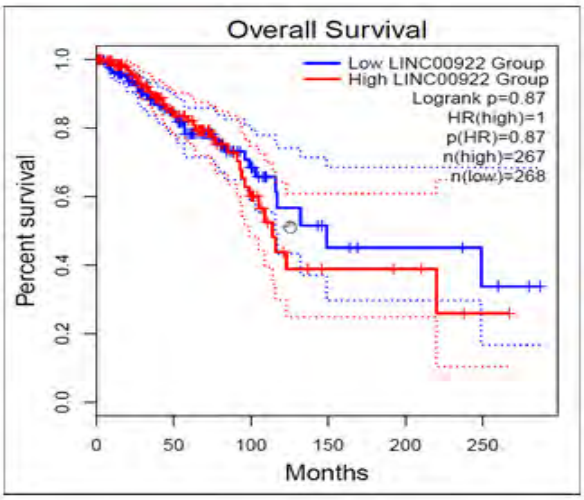
Figure 8: Cytoscape network of mRNA-miRNA interaction analysis.



0.1-0.5, the SNPs which had an effect on 3'UTR of the *BHMT2* gene were obtained. RS10944 demonstrated the highest MAF score. Following the investigation of seed region and nucleotides around the SNP, hsa-miR-542-3p in the case of occurrence of SNP, it seems the nucleotide alteration affects the pattern of the miR function. Also, using the RNAhybrid database, the minimum free energy of miRNA and target hybridization was measured, and the results showed that in the case of occurrence of mutation led to the free energy of the miRNA became more negative, and the probability of binding increased (Figure 7). Investigation of the expression of miRNAs in GSE40525 was performed using the limma package in the R environment, and the expression level of hsa-miR-542-3p was reported to be significantly increased.

Using the Co-lncRNA database, based on TCGA information, analysis has been done to identify the relationship among genes and lncRNAs in breast tumor tissue compared to normal; the complete list of lncRNAs that had an effect on the *BHMT2* gene was collected. Totally 130 lncRNAs were obtained, which among them, lncRNA called LINC00922 was selected based on the confirmation of its role in the process of breast cancer pathogenesis due to previous articles (according to the results of this study, the p-Value of this lncRNA in tumor samples is very acceptable and it was 0.0004). It is noticeable that this lncRNA was measured only by the RNAseq method in breast cancer tissue samples, and it was reported as increase expression. For the first time in the Isfahan BC population, the expression level in breast tumor tissue samples was examined by the real-time method in this study. miRNA-mRNA interaction analysis by miRWalk online software revealed that *BHMT2* could have a remarkable interaction with 9 hub miRNAs shown in (Figure 8).

Figure 9: Survival analysis of LINC00922 RNA-seq data.

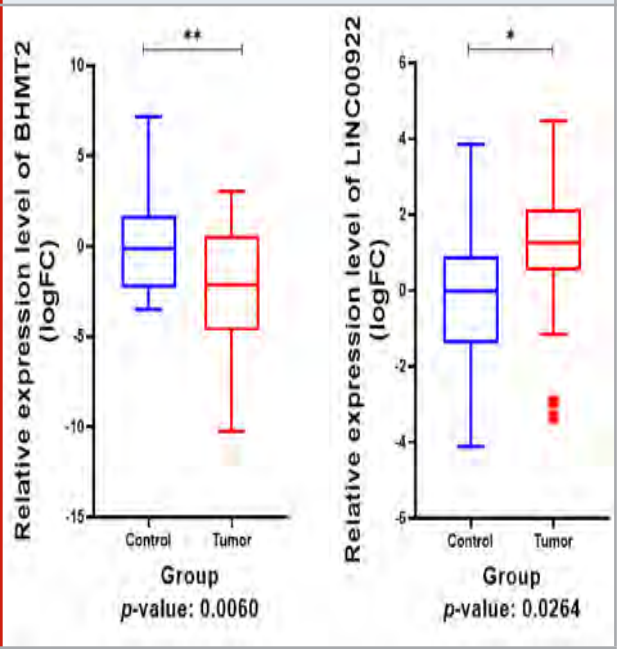


Obtained results from in silico database predicted the association of rs10944 in *BHMT2* 3'UTR within has-mir542-3p and T allele is minor allele. Therefore, G to T replacement single nucleotide in the *BHMT2* transcript could alter binding reaction (affinity), which might change *BHMT2* regulation in transcription level. To prove this, based on the HRM method, we concerned allelic frequencies for rs10944. However, no significant differences were found between case and control. Survival analysis of the TCGA RNA-seq data was performed by GEPIA2 online software. This analysis revealed that higher expression of LINC00922 is correlated with the lower survival rate of this lncRNA (Figure 9). This analysis can confirm the hypothesis that the gene is oncogenic in breast cancer.

Real time PCR expression data analysis: According to real-time PCR data analysis, *BHMT2* had a significant down-regulation in the tumor samples compared to normal samples (P-Value: 0.0060). Also, this analysis revealed that lncRNA LINC00922 had a significantly high expression

in Breast cancer samples (P-Value: 0.0264, Figure 10). Correlation analysis of the tumor expression data of *BHMT2* and LINC00922 revealed that these two RNAs have no significant correlation in the Isfahan Breast cancer population (r: -0.1581, P. Value: 0.4605).ROC analysis revealed that LINC00922 could be a prognosis biomarker for distinguishing Breast cancer samples from normal samples (AUC: 0.7205, P-Value: 0.0088, Figure 11).

Figure 10: Relative expression analysis of BHMT2 and LINC00922.



Genotype frequency analysis for rs10944: Analysis of three different CC, CT, and TT genotypes of rs10944 SNP revealed that there is no significant difference between the frequency of these three genotypes in control and tumor samples (Table 3, Figure 12). P-Value was calculated by the Pearson chi-square test. The clinicopathological characteristics of the patients is provided in the Table 2.

DISCUSSION

All in all, in this research, we investigated the effect of the *BHMT2* gene and its relevant lncRNA and SNP in the Breast cancer status of the Isfahan population. Our bioinformatics analyses predict that the *BHMT2* could have a significantly low expression in Breast cancer tissues as compared to normal tissue. Also, it was predicted that the lncRNA LINC00922 could have a significant relation with the *BHMT2* mRNA. Correlation of hsa-miR-6775-5p, hsa-miR-4767, hsa-miR-6777-3p, hsa-miR-6877-3p, hsa-miR-1343-5p, hsa-miR-619-5p, hsa-miR-6752-5p, hsa-miR-10392-3p, and hsa-miR-4783-5p miRNAs with the *BHMT2* mRNA have been revealed. According to our experiment, *BHMT2* has a significant down regulation in tumor tissues. Also, this mRNA could not be a prognosis biomarker in our samples. About lncRNA, we demonstrated that LINC00922 has a significantly high expression in Isfahan Breast cancer samples. Unlike the previous result about *BHMT2*, LINC00922 could be a suitable biomarker for Breast cancer. Furthermore, we find that *BHMT2* and LINC00922 had no significant correlation. Our investigation about the allele frequency of rs10944 genotypes revealed no significant relationship between the difference in frequency of rs10944 genotypes in the *BHMT2* gene with Breast cancer status.

Table 1: Forward and reverse primer sequences (5′→3′) of BHMT2, LINC00922, GAPDH and SNP rs10944.

	FORWARD	REVERSE
BHMT2	CAGGTAAAGGAGGTGGGTG	GCATTTACATCTTCCCACTTGC
LINC00922	CAGCCTGGGAGACATAATTC	CAAAGACTGCGACATAGTGAG
GAPDH	GCTCTCTGCTCCTCCTGTTC	ACGACCAAATCCGTTGACTC
rs10944	CAGCCTGGGAGACATAATTC	CAAAGACTGCGACATAGTGAG

This result represents that *BHMT2* could have a tumor suppressor effect in Breast cancer. Also, LINC00922 could be a significant oncogenic in the Isfahan Breast cancer population. Previously, several studies have been conducted on the association of genes with various cancers. For example, Jiang et al. reported that *BHMT2* could have an essential role in Osteosarcoma by involving in the Osteosarcoma metabolic pathway. Another experiment confirmed that the amount of *BHMT2* protein has a remarkable change in Breast and Ovarian cancers (Dufresne et al., 2019). Other researchers discussed a correlation between *BHMT2* and colon cancer and proposed that routine exercise can help to avoid aberrant methylation of the *BHMT2* gene and thereby lead to a lower risk of colon cancer (Buehlmeyer et al., 2008).

Our investigation about this gene represents the same result about Breast cancer tissues. By the way, the exact effect of changing the expression of this gene on the status of breast cancer requires more detailed and comprehensive research. About lncRNA LINC00922, previous studies have demonstrated that LINC00922 has a significant overexpression in Osteosarcoma and lung cancer. Especially for lung cancer, this lncRNA could control the expression rate of *CXCR4* and directly targeted miRNA-204/*CXCR4* (Liang et al., 2019).

Furthermore, the role of LINC00922 in BC was investigated in another research. This study represents that this lncRNA could prevent Breast cancer development by linking to miR-424-5p/*BDNF* through the prohibition role of this miRNA (Yue and Wang, 2020). The results of Our experiment could be an approve on these studies about LINC00992. Also, the bioinformatical survival analysis on RNA-seq data revealed that the low expression is directly correlated with the higher survival rate. This survival analysis could validate our experiment and results.

Table 1

Characteristic	Status	Number of patients
Stage	I	2 (8.3%)
	II	6 (25%)
	III	6 (25%)
	IV	0
	Unknown	10 (41.7%)
Tumor size (TS)	< 5 cm	13 (54.2%)
	> 5 cm	5 (20.8%)
	Unknown	6 (25%)
Menopausal status	Yes	11 (45.8%)
	No	12 (50%)
	Unknown	1 (4.2%)
Lymph node	Yes	14 (58.3%)
	No	3 (12.5%)
	Unknown	7 (29.2%)
ER receptor	Positive	9 (37.5%)
	Negative	7 (29.2%)
	Unknown	8 (33.3%)
PR receptor	Positive	7 (29.2%)
	Negative	9 (37.5%)
	Unknown	8 (33.3%)
HER2/neu receptor	Positive	8 (33.3%)
	Negative	8 (33.3%)
	Unknown	8 (33.3%)

Table3: Genotype frequency of BHMT2 gene in relation to rs10944 in control and breast cancer patients.

			Genotype		Total	P.Value
			GG	TT		
Groups	case	Count	9	12	21	
		% within groups	42.90%	57.10%	100.00%	
	control	Count	10	11	21	
		% within groups	47.60%	52.40%	100.00%	
Total		Count	19	23	42	
		% within groups	45.20%	54.80%	100.00%	0.757

Based on GWAS, SNP rs10944 has been investigated as a common SNP along BHMT2, which is related to BC's risk. Other studies indicated rs10944 was among variant alleles related to higher plasma betaine concentrations (Ilozumba et al., 2020). However, our investigation about this SNP on the Isfahan population did not show a significant genotype frequency and relationship with Breast cancer.Furthermore, our explored data estimated the upregulation of mir-542-3p by utilizing miRNA expression data. However, other studies' results differ from the findings presented here. An example is mir-542-3p downregulated in endometriotic tissue adenocarcinoma compared to normal endometrial tissues (Hiroki et al., 2010). Similarly, Li, Shao and Feng, 2019 proposed has-mir-542-3p low expressed in ovarian cancer, thus inhibited targeting CDK. Moreover, it has been believed has-mir-542-3p regulated several mRNAs such as *BIRC5, MTDH*, and *MID1*(Unterbruner et al., 2018).

Our experiment had some remarkable limitations, including the sample size. We suggest that similar experiments be performed on this SNP with a more significant number of samples to obtain more valid and reliable results of this SNP's association with Breast cancer status in the Isfahan population. Analysis of different genotype models like dominant, co-dominant, and recessive models could be performed. Additionally, evaluating the expression level of listed miRNAs that was our bioinformatical result of mRNA-miRNA interaction in Isfahan Breast cancer samples could be performed. Also, the RIP method is suggesting for more RNA interaction analyses.

CONCLUSION

It is predicted that dysregulation of BHMT2 expression level could have a remarkable effect on Breast cancer status. The absence of methionine and Hyperhomocysteinemia have been linked to a decline in BHMT2 expression. The current study revealed that BHMT2 and LINC00922 can be the two novel correlated biomarker of breast cancer. More similar investigations are needed for evaluating more accurate and reliable result, specially about rs10499. We suggest that similar experiments be performed on this SNP with a more significant number of samples to obtain more valid and reliable results of this SNP's association with Breast cancer status in the Isfahan population

Conflict of Interest: The authors have no financial conflicts of interest to declare.

Data Availability: The data will be made available on request.

Authors contribution: Seyed Ali Dibaj Zavareha : Writing- Original draft- Formal analysis- Investigation. Zahra Akhlaghia : Writing - Original draft - Formal analysis- Investigation.Sarina Samiei Esfahania: Writing - Review & Editing- Investigation.Nilofar Mohammadia: Writing - Review & Editing- Investigation.Negin Hadisadeghb: Writing - Review & Editing- Investigation. Samira Rahimiradb: Supervision- Methodology- Writing - Review & Editing.Mansoureh Azadehc: Conceptualization, Validation.

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ICT Usage in Learning and Instruction of Teacher Trainees from Teacher Education Institutions (TEIs) of Bhopal, Madhya Pradesh India.

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ABSTRACT

Teacher education plays a pivotal role in any education system and the same role of School Internship and Teaching Practice is for any teacher education programme. It produces the quality teachers for any country. Integration of ICT in teaching learning process is one of the indicators for quality teaching and learning. Now, ICT integration is inevitable. Hence, the researchers intended to explore which type of technology is used by teacher trainees in their academic endeavors. The present study was carried out to explore the uses of ICT integration in learning and instruction of teacher trainees studying in Teacher Education Institutions (TEIs) of Bhopal city of Madhya Pradesh (India). The descriptive survey method was adopted to accomplish the objectives of the study. Simple Random sampling technique was employed to select the 239 Teacher Trainees from intact classes of six TEIs of Bhopal. Researchers' self-constructed questionnaire was administered to the teacher trainees of selected TEIs. The data was analyzed through Frequencies and Percentages. The findings revealed that 72.8% of teacher trainees had used their Computer/ Laptop/Services/ ICT devices/ tools for accomplishing teaching-learning activities at home; 83.7% of them responded that ICT and Internet helped in preparing lesson plans; 90.8% of them reported that ICT and Internet helped in preparing teaching aids for delivering their lesson; 93.7% replied that ICT and Internet has helped them in preparing their assignments; 98.3% of them used social media for their teaching-learning related activities. Only few (i.e. 10%) of them found difficulties in using computer or laptop. Excessive uses of the ICT and internet have created medical, psychological and socio-emotional problems for 72.8%, 73.2% and 74.5% of teacher trainees respectively.

KEY WORDS: ICT USAGE, TEACHER EDUCATION INSTITUTIONS,TRAINEES,

INTRODUCTION

In recent past, Information and Communication Technology (ICT) usage has become more significant and flourishing in human life. In fact, it is now an inseparable component required for performing routine activities of our lives (Bhattacharjee & Deb, 2016). Nowadays, ICT usages means using of computers (Khan et al., 2011) along-with internet either in household or academic activity or in any occupation. Without internet the computers have now been used rarely (Hrastinski, 2019).

The emergence of Internet has proved a revolution in the field of ICT by providing impetus in its integration. Using internet totally depends upon computers, laptops, smart-phones and tablets through which we are performing each and every task of our daily life. Application of ICT has made our lives more standard and luxurious. We are so dependent on ICT tools/ gadgets/ devices that we feel ourselves handicapped without them. If we do not use ICT and its gadgets for a while, we feel helpless, uneasiness and become restless. Today, these ICT tools/ devices/ gadgets are being used by each and every person in almost every sphere of life (Javed et al., 2020) viz. in performing household activities, academic work, business, recreational activities, online trading, purchasing, selling of almost all goods and commodities even medicines too, and so on irrespective of age, profession and geographical boundaries. It is being now acknowledged that ICT has interwoven with each and every aspect of our life. Teaching profession and teacher trainees are also not exception to it (Singhavi & Basargekar, 2019, Hasan and Mirza 2020, Javed et al., 2020).

Applying and integrating ICT in any field is now considered a basic digital literacy. Rather, digital literacy and ICT skills

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have become essential competencies and proficiencies for 21st Century's teacher (Bhattacharjee & Deb, 2016). Therefore, ICT competencies must be addressed and improved among prospective teachers / pre-service teachers / teacher trainees (Lovianova et al., 2021) because it helps the teachers in planning and executing their roles and duties effectively. Through the ICT, they benefitted in gaining learning experiences themselves; transferring same to the students; and preparing students as independent learners by enabling them self-directed learner. Also, ICT helps to enhance the e-learning capacities and proficiencies among the teacher trainees (Abdallah & Abdallah, 2022).

Hence, the teachers are more willing now for using the ICT tools and digital technologies in the classroom. Findings of the Digital School Survey conducted in India by Ocansey and Sharma (2020) revealed that the usages of ICT in preparing and delivering the lessons by school teachers are 81.5% and 83.3% respectively. It enhances the learning achievement of the students (Khan et al., 2011; Seifert & Feliks, 2021) by improving teaching competencies as well as teaching effectiveness of teachers (Ghavifekr & Rosdy, 2015; Manjunath, 2015; Kundu, 2018; Sharma, 2021).

It provides the opportunity for student-centric-constructivist-approach to the teachers. Hence, it is the need of the hour to introspect our teaching process, to provide institutional support for establishing ICT infrastructure (Avidov-Ungar & Forkosh-Baruch, 2018) and to integrate the ICT in education (Dharwad, 2016) so that the reflective practices can be promoted in teacher education (Killeavy & Moloney, 2010; Kalk et al., 2019). In the present study, effort has been made to explore how the ICT is being used by the teacher trainees in teaching-learning process at Teacher Education Institutions of Bhopal city of the State Madhya Pradesh (India). Since some decades, lot of studies on ICT related variables have been conducted in developed countries; as a result, efforts have also been made to integrate ICT in teaching learning process (Lim & Pannen, 2012; Romeo et al., 2012). However they are relatively rare and new for developing countries, especially in Indian context. In order to develop an understanding on methodology of the research conducted, researchers included the studies those conducted from the year of 2000.

Sources of research database revealed that Sinha (2004) conducted a research in Barak Valley of South Assam, and discovered that access to the internet has improved the standard of research, academic activity for faculty and students or the entire academic community, and e-commerce for corporate sector/business groups. These services have also made it possible for these groups to obtain information instantly for a variety of purposes. This shows that teachers as well as students, nowadays, have favorable attitude towards internet rather they are addicted to it. But, the study of Çınar et al. (2021) revealed that e-learning readiness among in-service teachers was found to be average level. Though, this readiness differed significantly according to gender, school-type, teaching experience, and teaching level, e-learning experience and Internet usage.

Digital divide in terms of access of ICT gadgets have been observed in some researches which is surely related to development of those regions. Availability of ICT infrastructure and digital literacy has a cause and effect relationship,(Cinar et al 2021).

Polat (2021) reported low levels of digital literacy among pre-service teachers. On the other hand, findings of some studies revealed that the students reported issues and problems encountered while integrating and applying the ICT such as, lack of ICT resources/ infrastructure (Agyei & Voogt, 2014) like computers, ICT labs or Smart Boards in their institution (Crawford, 2003; Merc, 2015); using the technology in the classrooms; lack of exposure to ICT integrated lesson plans; lack of ICT infrastructure in cooperating schools and lack of experience of using the Smart Boards (Merc, 2015). Such types of situations are still prevailing in some part of the world like African and South Asian countries. These situations have been exposed more during the recently handled pandemic. Therefore, it is quite pertinent that for increasing the digital literacy, our teacher education institutions must have enhance the ICT infrastructure. It is also an urgent need to equip our prospective teachers with ICT integration skills so that such type of issues may be controlled.

Studies show that the access and uses of ICT infrastructure and internet access has been increased significantly. Hanauer, et al. (2004) conducted a survey at a diverse community college to examine accessibility and usage of the internet. Findings of this study revealed that while all students had free access to the internet at their school, only 97% of them actually used it. Of those who did, 81% said they used the internet primarily for schoolwork and 80% for e-mail and chat. Luambano and Nawe (2004) reported that most of the university students didn't use the internet for academic related activities they also felt the need for enhancement in training of ICT infrastructure and services whereas, Romeo et al. (2012) reported that proficiency programs in ICT education for pre-service teacher have been initiated at broad level in Australia. A Capacity building program in developing ICT competencies among pre-service teachers in Asia-Pacific region has also been reported by Lim and Pannen (2012). Saunders and Pincas (2004) studied the student's attitude towards ICT usage in teaching and learning in the UK. Their findings revealed the opinion of the students towards ICT has a significant role in imparting teaching-learning experiences.

In a study of engineering students from Punjab, Haryana, and Himachal Pradesh, Kumar and Kaur (2006) found that the internet has become an essential tool for their instruction, research, and learning processes. By giving teachers and students access to the most recent knowledge and global information, the internet facility has allowed them to improve their academic performance. Mishra et al. (2005) undertook a research study to find the internet usage habits of first-year students at the Govind Ballabh Pant University of Agriculture and Technology, Pantnagar. The findings revealed that, most students (85.7%) used the internet. Also, 51.6% of women and 61.5% of men used the internet to prepare assignments.

Some studies revealed that ICT usage has boosted the effectiveness of learning and instruction. Maharana et al. (2009) conducted a study on usage of ICT in medical students and revealed that 77% of the students were in favor of having ICT in medical education syllabus. Almost all students had a viewpoint that an ICT lab should be established in their college. Almost 78% of them perceived that their education has become ineffective without ICT. Khan et al. (2011) reported that 48.8% of the students had access of ICT at their institutions and students had great level of ICT usage. Students also opined that ICT accelerate their learning process. About 39% of the students use the internet on daily basis, 20.7% twice a week, 33.5% use weekly and 6.7% of them uses on monthly basis. Their information retrieval skill was found to be good but they use the internet sometimes for academic purposes; only 50.6% of them use it for academic activities. Most of them 76.8% were users of Google search engine whereas 26.2% were Yahoo users. Researcher also revealed that teachers use ICT sometimes during classroom lecture. Sahin (2018) found no significant difference in the use of ICT by male and female teachers but significant difference reported between teachers of rural and urban backgrounds.

Kaur (2019) conducted a study on Role of Teachers' Attitude and Belief regarding use of ICT in Indian classroom and found no gender differences in the use of ICT by teachers. This research was carried out with a major objective to study the ICT usage in learning and instructional activities by teacher trainees of Bhopal city. It is evident from the review of related literature, that the investigators have found numerous studies conducted across the globe on ICT usage and related variables. But the investigators did not come across any specific study where the usage of ICT in learning and instructional process among teacher trainees studying in Teacher Education Institutions of Bhopal, Madhya Pradesh has been carried out or reported. Also the investigators intended to know whether the teacher trainees are using any specific kind of ICT in their learning and instructional activities or not? And if yes, then up to what extent they are utilizing the ICT in their learning and instructional process. All these queries motivated the investigators to plan and execute this study.

Operational definitions of the technical terms: ICT usage: It is defined as the activities performed with the help of softwares, tools and technology of computers or laptops or tablets or smartphones based on internet.

Learning: It is a process of acquiring either of the knowledge, comprehension, application, analysis, synthesis or evaluation about any skill or competency or proficiency or ability through formal or informal way or through experiences or self-study or instruction. It is a cognitive process and outcome that reflected in the learner. It is typically self-directed and may occur spontaneously without any formal instruction. It involves making connections, synthesizing information and internalizing the concepts.

Instruction: Instruction refers to the process of imparting and transacting the knowledge, skills or attitude to others in a formal setting. It is a deliberate act of providing information or guidance to facilitate learning. It is often structured and planned by an instructor or teacher. It involves pedagogy of approaches, methods, strategies, techniques, maxims and activities designed to support and enhance the learning. It may be performed through various modes like; lectures, demonstrations, discussions, questioning, brainstorming, mind-mapping, or hands-on activities.

Teacher Trainees: Those student-teachers of B.Ed. program of Government and Private TEIs who are undergoing internship.

Delimitations of the study: The study confined to the following limitations: The sample for the present study consisted of 239 teacher trainees only. Only two types of TEIs (Government and Private) have been taken for study. The study has been confined to Bhopal city only. Only teacher trainees of B.Ed. program have been taken for the sample.

MATERIAL AND METHODS

Descriptive survey method was followed to carry out the present study. Teacher Trainees studying in the Government and Private Teacher Education Institutes of Bhopal City was the population of the study. To carry out the study, two Government and four Private Teacher Education Institutions (TEIs) were selected through simple random sampling technique and intact classes of those TEIs were taken as sample. In this way, sample comprised of 239 Teacher Trainees out of which 106 were male and 133 were female. In all, there were 92 teacher trainees from Government TEIs and 147 from Private TEIs.

Initially, permission was taken from the Heads/ Principals of identified six TEIs and accordingly teacher trainees were informed about the purpose of data collection. Also, they informed that their participation is completely voluntary and they may quit at any stage. They were assured about keeping their data confidential and shall be used for research purpose only. Afterward instructions were given them to respond the questionnaire.

In order to collect the data pertaining to the usage of ICT and Internet by the teacher trainees, a self-made questionnaire was used. In which there were 30 items. Each item has two responses, "YES" and "NO". Although there was no time limit for completing this questionnaire, but it takes generally 15-20 minutes to complete it. For analyzing the obtained data frequency and percentage were used and result was presented in tabular and graphical form.

RESULTS AND DISCUSSION

The obtained results have been summarized and presented in the table 1 below:

Table 1 shows the increased usage of computers in learning and instructional activities. It also indicates that fear of using computer and computer anxiety has been reduced over a

period of time as reported in earlier studies (Abdullah et al., 2016). This can proved to be helpful in enhancing the confidence and satisfaction among learners and thereby lead to better academic performance (Al-Busaidi & Al-Shihi, 2011).

Table 1. Item-wise responses on ICT usage in learning and instruction by teacher trainees

S. No.	Items	YES %	NO %
1	Do you have computer/ laptop at home?	40.6	59.4
2	Do you have internet access at home?	88.3	11.7
3	Do you use your computer/ laptop/ mobile for accomplishing teaching-learning activities at home?	72.8	27.2
4	Do you have ICT lab with internet connection in your College/ Institute?	95.4	4.6
5	Do you use ICT lab of your College/ Institute for carrying out teaching-learning activities?	92.1	7.9
6	I enjoy doing work through the internet.	79.5	20.5
7	I am tired briskly by using the internet.	31.4	68.6
8	I have learnt a lot of new knowledge by using the internet.	81.6	18.4
9	The ICT and Internet have proved really helpful in preparing and delivering my lesson plans.	83.7	16.3
10	I feel comfortable working with internet.	79.1	20.9
11	Working with internet makes me nervous.	19.7	80.3
12	Using the internet is very frustrating.	16.7	83.3
13	I prefer to do as little work with internet as possible.	12.1	87.9
14	Working through Computer/ Laptop is difficult for me.	10	90
15	I feel sad, frustrated and irritated when I get my internet connection is not working properly.	97.9	2.1
16	ICT and Internet have helped me in selecting and preparing the teaching aids for my lesson plans.	90.8	9.2
17	Working on computer without Internet is boring.	97.5	2.5
18	I am able to send e-mails for communication.	77.4	22.6
19	I can use different search engines to explore the desired content on their web pages.	69	31
20	I feel scared by the internet.	66.9	33.1
21	The excessive usages of the ICT and internet have created medical issues for me.	72.8	27.2
22	The excessive usages of ICT and internet have created socio-emotional problems for me.	74.5	25.5
23	The usages of ICT and internet have created psychological problems for me.	73.2	27.8
24	The use of ICT has enhanced my slandered of living.	66.1	33.9
25	The ICT and Internet have helped me in preparing the assignments.	93.7	6.3
26	The ICT has created unemployment for unskilled persons.	65.3	34.7
27	The internet is a fast and efficient means of gaining information.	79	21
28	Carrying out Teaching-learning activities through ICT has become easier and faster for me.	74.5	25.5
29	I have at least one account on social media platform like; Face-book, Whats-App, Twitter, Linkedin, Instagram, Telegram, etc.	100	0
30	I use social media only for my teaching-learning related activities.	98.3	1.7

However, this study indicates that there is still lack of access of ICT gadgets and internet to a considerable proportion (i.e. 40.6% and 88.3% respectively) of the participants of developing country. It is a positive sign that, these are being used in preparing assignments (93.7%); selecting and preparing instructional aids (90.8%) and preparing the lesson plans (83.7%) by a large number of participants among those who have access to ICT resources and internet either at their home (72.8%) or at their institutions (92.1%). They enjoyed (79.5%) and felt comfortable (79.1%) while using internet despite being tired (31.4%) and scared (66.9%) of using the internet. More than 97% of participants express the feelings of sadness, irritation, frustration and boredom in working without internet or interrupted connectivity. This might be due to our over dependency on internet being used in online activities. But, the positive point which may be inferred that the availability or uninterrupted access of internet can bring joyfulness among students if utilized in systematic way (Gan & Balakrishnan, 2017).

In this regard, smart-phones of students may be utilized for their self-directed learning. With the same psychology of enjoyment and fun, all of the participants have used either of social media platforms. On the contrary, some of them exhibited computer anxiety such as nervousness (19.7%); frustration (16.7 %); escapism (12.1%); difficulties in working (10%). Probably the reason behind this may be most of the participants were first generation learners or belongs to rural areas. In such type of circumstance, it is quite natural to have some difficulties in integrating new ideas and design (Kim et al., 2012).

However, there are certain problems and issues reported by sizable number or participants those who have used the ICT and Internet excessively such as, tiredness (31.4%); medical issue (72.8%); socio-emotional problems (74.5%); and psychological problems (73.2%). The main findings of the study have been presented as below: Form the above Table 1 and Fig. 1, it is evident that almost 40.6% (97 out of 239) of the teacher trainees had their personal computer/ laptop at home whereas sizable number of teacher trainees (59.4% i.e. 142 out of 239) didn't have this.It was also evident that majority of teacher trainees i.e. 88.3% (211 out of 239) had internet access at homes whereas few of them i.e. 11.7% (28 out of 239) didn't had this service at their homes.

Further, it was noted that 72.8% (i.e. 174 out of 239) teacher trainees had used their Computer/ Laptop/ Mobile/ ICT devices for accomplishing learning and instructional activities at home but 27.2% (i.e. 65 of 239) couldn't use it for their academic activities. This finding is substantiated by the study of Gan and Balakrishnan (2017) reporting that (94.2%) of the students of higher education used their mobiles for learning. It was also found that 95.4% of them (i.e. 228 out of 239) reported that their institution has ICT lab with internet service. This finding is substantiated by the report of Ocansey and Sharma (2020) stating that 91.2% of schools have internet facility. It was observed that 92.1% of the sample (i.e. 220 out of 239) used the ICT lab for learning and instructional purposes whereas 7.9% (only 19 out of 239) of them did not use for learning and instructional purposes, (Ocansey and Sharma 2020).

This is in line with the finding of study of Gan and Balakrishnan (2017) which revealed that 95.7% of students of higher education institutions access the internet through their mobile devices. Furthermore, 79.5% (i.e. 190 out of 239) of teacher trainees opined that they enjoyed while working through the internet. Whereas more than one-fifth of them couldn't enjoyed while working through the internet. It was noted that almost one-third (75 out of 239) of teacher trainees (i.e. 31.4% of the sample) were getting tired when using the internet whereas mostly (164 out of 239) teacher trainees (i.e. 68.6%) weren't tired. It was found that more than 4/5 (i.e. 195 out of 239) of teacher trainees (i.e. 81.6%) believed that they gained new knowledge through internet. Further, 83.7% (i.e. 200 out of 239) of teacher trainees believed that ICT and Internet have proved helpful in preparing and delivering their lesson plans whereas 16.3% (39 out of 239) of them didn't believe so. This finding is supported by the report of Ocansey and Sharma (2020).

It was also explored that 79.1% (i.e. 189 out of 239) teacher trainees felt comfortable working with internet. It was noted that almost one-fifth (i.e. 19.7% or 47 out of 239) teacher trainees felt nervous working with internet. This showed their less exposure to the working on internet. Moreover, it was also found that some (i.e. 16.7%) teacher trainees (i.e. 40 out of 239) got frustrated while using the internet. This might be due to low speed of data or other connectivity issues. It was also observed that some (i.e. 12.1%) teacher trainees (i.e. 29 out of 239) intended to avoid the use of internet which shows their low confidence in using the internet. It was explored that a few of them (i.e. 10% of the sample) found difficulties in working through computer or laptop. Therefore, it may be inferred that proper assistance and support should be provided by the teachers so that they may enhance self-directed learning with technology (Sumuer, 2018).

It was noted that almost all (i.e. 97.9%) 234 teacher trainees felt irritated when internet connection gets interrupted. This shows the wider usability of internet. It was found that most (i.e. 90.8%) 217 teacher trainees reported that ICT and internet proves helpful in preparing instructional aids for their lesson plans. Further it was also noticed that almost all (i.e. 97.5%) 233 teacher trainees felt boring in working on computer without Internet. It was also noted that 77.4% (i.e. 185 out of 239) teacher trainees were able to send communication through emails. It shows that most of the teacher trainees are now learnt to communicate through emails.

It was also observed that 69% (i.e. 165 out of 239) teacher trainees were able to use different search engines to explore the desired content which proves that the necessity is the best teacher. Moreover, it was also found that 66.9% (i.e. 160 out of 239) teacher trainees felt scared of internet. This shows that every technology possess some threats. The only thing that matter is how ethically and judiciously we are utilizing these technologies. It was explored that excessive uses of the ICT and internet have created medical issues for 174 out of 239 (i.e. 72.8%) of teacher trainees.

This indicates that one should use the technology in a balanced way. It was also noted that excessive usages of ICT and internet have created socio-emotional problems for almost three-fourth (i.e. 74.5%) or 178 out of 239 teacher trainees. Again, it implies that we should take some measures and adopt some strategies to avoid excessive use of technology like, taking short-break during the usage of ICT or exploring some alternative ways to do that work. It was observed that excessive usages of ICT and internet have created psychological problems for 175 out of 239 (i.e. 73.2%) of teacher trainees.

This finding suggests that we should appoint full time or hiring services part time to take consultations from the psychologist on regular basis. It was noted that 158 out of 239 (i.e. 66.1%) teacher trainees opined that use of ICT has enhanced their standard of living. This finding reiterated the strength of ICT uses and this may be enhanced if its negative effects are controlled judiciously. It was found that most (i.e. 93.7%) of teacher trainees (224/239) believed that ICT and Internet have helped them in preparing their assignments. This shows that ICT has multifarious benefits including the teaching-learning process. Furthermore, a 65.3% of teacher trainees (156/239) believed that ICT has created unemployment for unskilled persons. This shows the urge to learn new technology and institution may provide training to imbibe new skills required for any technology.

It was opined by 79% of teacher trainees (189/239) that the internet is a fast and efficient means of gaining information. It shows the economical and cost effective aspect of technology. Further, it was also opined by three-fourth (i.e. 74.5%) of the teacher trainees (178/239) that carrying out the learning and instructional activities through ICT made their task easier and faster. This also highlights the strength of the technology provided when used ethically. It was also noted that all (239) the teacher trainees (i.e. 100%) has at-least one account on social media platforms like; Face-book, Whats-App, Twitter, Linkedin, Instagram, or Telegram. This implies that ICT tools can be used constructively in learning and instruction. These technologies possess great potential of individualized learning and instruction and some of them being widely used by the teaching community in instructional and training processes like face-book (Demiraslan, et al., 2014).

It was also found that almost all i.e. 98.3% of teacher trainees (235/239) are using social media for their learning and instructional related activities which is a good indicator of stepping towards the digital India campaign. Whereas very little (only four) of them i.e. 1.7% of them are not using social media for their learning or instructional related activities. Several researches indicate that advanced technology based social media are being used widely during recently ended pandemic.

The implications of this study are very useful for teacher trainees as well as teacher educators and policy makers. On the basis of this study it may be recommended that teacher trainees should ensure the use of ICT gadgets like smart-phones, laptops, computers, smart boards, ICT tools and internet for their effective teaching practices, preparing and delivering the lesson plans as per the requirements of the classroom delivery and planning and preparing at home. But, due to various limitations and challenges faced by both the teacher trainees and the teacher educators, they are supposed to explore, learn, apply and integrate ICT tools with pedagogy and content knowledge themselves so that their self-directed learning can be enhanced (Yan et al., 2012).

This may help them to enhance and update their technological, pedagogical, content knowledge and their integration as well. The teacher educators should motivate the trainee teachers and give assignments, projects, tasks, activities them in such a way that they may best utilize the infrastructure of ICT lab and their own devices. The administration of TEIs should ensure the internet access and other ICT based infrastructural facilities, like Computer labs, Smart-boards and Projectors in the institutions and congenial environment or learning supported culture for utilizing them as this was the major barrier which demotivates the learner (Agyei & Voogt, 2014). Finally, the role of curriculum developers and policy makers as suggested by Javed et al. (2020) is very crucial in incorporating the ICT advancement in teaching and learning. They should include the latest ICT based content in the curriculum by revising it from time to time. They should try to evolve and implement the latest and futuristic technology based curriculum at the possible extent.

CONCLUSION

The present study suggests that ICT, its tools and softwares in combination with internet has been now proved to be an inevitable component for any learning and instructional process By virtue of these ICT skills, we can prepare a student as life-long learner so that she/ he may retrieve and apply the information from digital resources. Policy makers should design the curricula in such a way that it enable the teacher as well as student to learn and apply new technologies (Javed et al., 2020). Also, administrators must provide the accessibility of ICT infrastructure and opportunities, rather it should be made mandatory, for teachers' training to acquire the knowledge and skills required for transacting the ICT integrated education. Administrators should provide moral and logistic support and facilitate the teachers as well as students to make use of the technology in their respective areas in a best possible way. Then it becomes the responsibility of teachers to utilize the ICT gadgets and tools in the classroom so that digital divide can be minimized and students' performance can be optimized. In this way we can create a digitally empowered society and turned out India into knowledge economy globally as envisaged in its National Educational Policy (NEP) 2020.

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