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Comparative characterization and scientific validation of certain plant extracts from their biomedical importance

Sharique A. Ali,*[a] Ram K. Choudhary[a], Ishrat Naaz[a], Nargis Khan[a], M. Sajid[a], J. Galgut[b], M. Miraj[c], L. Jakkala[d] and Ayesha S. Ali[a]

[a]*Post Graduate Department of Zoology and Biotechnology, Saifia Science College, Bhopal, India*
[b]*671, Asprior Avenue, Mississauga, Ontario, Canada*
[c]*Sai Institute of Paramedical Sciences Dehradun (UK)*
[d]*MacroCare Clinical Research Limited, Makro Towers, Kavuri Hills, Hyderabad, India*
**Corresponding Author*

ABSTRACT

The phyto-chemical research based on ethno-pharmacology is considered as an effective approach in the discovery of novel chemical entities with potential as drug leads. Plant extracts, used by folklore traditions for treating several diseases, represent a source of powerful metabolites but no information is available on their structure related activities.. Therefore scientific validation of herbal extracts is an important step for the establishment of a consistent biological activity, an authentic chemical profile, or simply a quality assurance program for production and manufacturing of herbal drugs. Starting from this viewpoint in the present investigation, a comparative characterization using HPTLC techniques have been employed for the quantitative estimation of active ingredients present in extracts of six plants *Piper nigrum, Aloe vera, Arachis hypogea, Ocimum sanctum, Berberis vulgaris* and *Curcuma longa* as well as their biomedical efficacies have been investigated using animal cell models from pigment cell research point of view. We have reported that the significant quantities of active ingredients are present in the extracts of these plants. Further, it was found that the extracts of three plants *A.vera, A. hypogea* and *O. sanctum* exerted skin lightening effects on the melanocytes of amphibians and B-16 melanoma cell lines, whereas interestingly, extract of other three plants *P.nigrum, B.vulgaris* and *C. longa* elicited an opposite effect: dispersion of the melanocytes leading to skin darkening. It is concluded that characterized and quantified bioactive components of these plants can be used as novel and safe candidates for the treatment of hyper as well as hypo pigmentary disorders, along with a host of other bio medical efficacies.

KEY WORDS: *HERBAL EXTRACTS, PHYTO-CHEMICAL ANALYSIS, BIO MEDICAL USES.*

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INTRODUCTION

Since the beginning of human civilization, medicinal plants have been used by mankind for their therapeutic values. Nature has been a source of medicine for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of the agents in traditional medicine. This plant-based traditional system of medicine continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care and safety, (Kirtikar and Basu, 1989; Hassan, 2012, Singh *et al.*, 2014 and Miraj *et al.*, 2014).

In India, the Ayurvedic system has described a large number of such medicines based on plants or plant products and the determination of their morphological, pharmacological or pharmacognostical characteristics has provided a better understanding of their active principles and mode of action. Although there are thousands of plant species around the globe, only a small proportion has been investigated both phytochemically and pharmacologically. When one considers that a single plant may contain up to thousands of constituents, the possibilities of making new discoveries become evident, (Baker *et al.*, 1995; Agrawal, 2002; Singh *et al.*, 2014).

In recent years, the need for quality assurance tools to ensure the identity, purity and quality of botanical materials has risen dramatically. The crucial factor for the ultimate success of an investigation into bioactive plant constituents is thus the selection of plant material and its qualities. In view of the large number of plant species potentially available for any study, it is essential to have efficient systems available for the rapid chemical and biological screening of the plant extracts selected for any investigation, (Mukerjee, 2002 and Rastogi, 2009).

High-performance thin-layer chromate-graphy (HPTLC) is emerging as a versatile, high-throughput and cost effective technology that is uniquely suited to assessing the identity and quality of botanical materials. Basically HPTLC is a chromatographic technique that can separate a mixture of compounds and is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of the mixture, (Cannell, 1998).

In the light of above facts, the present study was designed where we focused various aspects regarding the characterization of plant extracts using HPTLC, which have tremendous medicinal and therapeutic properties and are frequently used in India,

MATERIAL AND METHODS

We have used six commonly known medicinal plants as mentioned in table no. 1 for identification and scientific validation of their active constituents, which are present in them. We have also quantified and studied the relative quantities of the active principles in plant extracts which can be further exploited for their medical efficacies. The highly sensitive HPTLC method has been used to accomplish this goal in which chromatographic separation of plant active constituents was performed on 20 cm ×10 cm aluminium backed HPTLC plates coated with 200 µm layers of silica gel 60F254 (E. Merck, Darmstadt, Germany).

Before use, the plates were washed with methanol and activated at 110°C for 5 min. Both test and standard samples (5 µL each) were applied on to HPTLC plates as 6 mm wide bands and 12 mm apart from middle of bands by spray-on technique along with nitrogen gas supply for simultaneous drying of bands, by means of a Camag Linomat V auto sample applicator fitted with a 100 µL syringe (Hamilton, Bonaduz, Switzerland).

A constant spot application rate of 150 nL/s was used. Plates were developed to a distance of 165 mm, in the dark, with different combinations of various solvent systems in different ratio were used as mobile phase for effective separation of different compounds (Table no. 1). Before development the chamber was saturated with mobile phase for 15 min at room temperature (25 ± 2°C) and 50% relative humidity. Chromatography was performed in Camag's twin-trough chamber.

Densitometric analysis of different active compounds was carried out in the different absorbance modes mentioned in table no. 1. Densitometric scanning was performed with a Camag TLC scanner 3 in reflectance-absorbance mode at wavelength 356 nm, under control of Camagwin CATS planar chromatography manager software version 1.4.4. The slit dimensions were 6 mm × 0.30 comm and the scanning speed was 100 nm/s.

RESULTS AND DISCUSSION

Chromatographic fingerprint analysis has shown to be a rational and feasible approach for the quality assessment and species authentication of traditional medicine. It utilizes chromatographic techniques to construct specific patterns of recognition of medicinal plants. The developed fingerprint pattern of components can then be used to determine not only the absence or presence of markers of interest but the ratio of all detectable analytes as well, (Kamboj and Saluja, 2013).

The present study was designed to identify and quantify some active constituents, which generally

have various medicinal values and have been used in Indian culture since long back. For this purpose HPTLC characterization has been employed for identification and quantification of active ingredients of six selected medicinal plants: *Piper nigrum, Aloe vera, Arachis hypogea, Ocimum sanctum, Berberis vulgaris and Curcuma longa.*

The method for quantitative analysis of different plant extracts was validated with regard to its specificity, precision, accuracy and linearity. The composition of the mobile phase for TLC was optimized by testing different solvent mixtures of varying polarity. The best results were obtained using specific solvent system (mobile phase) and detected under UV at specific wavelength as mentioned in Table no. 1, which showed good resolution of the compounds and visualization of the same with precision.

The specificity of the method was ascertained by analyzing standard and samples. The spots for different active ingredients in the sample were confirmed by comparing the Rf value and the spectrum of the spot with that of standard which is showed in Table No. 1. Compara-

Table 1: Showing HPTLC analysis of extracts of selected medicinal plants and relative concentrations of active ingredients present in them, along with their medicinal efficacies.

S. No.	Plant species and part used	Active Ingredient identified	Solvent system (Mobile Phase)	Selected Wave length	Rf Value	Concentration Obtained (in %)	*Medicinal Importance
1.	*Piper nigrum* (seeds)	Piperine	Benzene-Ethyle acetate (8 : 4)	354	*0.51*	5.29	Antidepressant, hepato protective, antihypertensive and antiplatelet agent, antioxidant, antitumor, antiasthmatic, antipyretic, analgesic, anti-inflammatory, anti-diarrheal, antispasmodic, antixyolytic, immunomodulatory, antibacterial, antifungal, anti-thyroidal, antiapoptotic, anti-metastatic, antimutagenic, anti-spermatogenic, anti- colon toxin, insecticidal and larvicidal agent.
2.	*Aloe vera* (leaves)	Aloin	Ethylacetate-Methanol-Water (10:1.4 : 1)	356	*0.76*	44.41-65.56	Melanolytic and tyrosinase inhibitory, wound healing, anti- diabetic, anti-inflammatory and anti-arthritic compound.
3.	*Arachis hypogaea* (seed skin)	Resveratrol	Chloroform Ethylacetate–Formic acid (2.5 : 1 : 0.1)	313	0.31	26	Melanolytic,useful in atherosclerosis, coronary heart disease, postmenopausal problems, platelet aggregation inhibitory and having chemopreventive properties
4.	*Ocimum sanctum* (leaves)	Eugenol	Toluene-Ethyl acitate-Formic acid (90:10:01)	280	0.59	98.39	Melanolytic and tyrosinase inhibitory, anticardiopathic, haemopathic, antileucoderma, antiasthma, antibronchitis, anticatarrhal fever, antiotalgia,anti hepatopathy,anti vomiting, antilumbago, antiantihiccups, ophthalmia, antigastropathic, antigenitourinaric and used for ringworm, verminosis and skin diseases
5.	*Berberis vulgaris* (roots)	Berberine	n-Propanol-Water-Formic Acid (90:8.0:0.4)	348	0.56	3.8	Melanogenic,antimicrobial, hepatoprotective, ionotropic, antiarrhythmic, hypolipidemic and anti inflammatory
6.	*Curcuma longa* (tubers)	Curcumin	Chloroform-Methanol (95 : 5)	422	*0.47*	28.10	Antioxidant, antimutagenic, antitumorigenic, anticarcinogenic, antiinflammatory, antiarthritic, antimicrobial, and hypocholesterolemic

*Reported by us and other previous researchers

tive study of different biomarker compounds of different plants has shown that they are present in higher amounts in the specific part of that particular plant species and the activity of a plant extract is always influenced by the quantity of active principles present in the extract.

PIPER NIGRUM

HPTLC analysis of seed extract of *P. nigrum* against standard piperine revealed that best results were obtained using Benzene- Ethyle acetate in 8:4 ratio. Rf value of standard aloin was matched with the Rf value of the extract which was found about 0.51. The contents of piperine were quantified using TLC densitometric methods and were found to be 5.29 % in seed extract of *P. nigrum* (Table-1).

Previous literature showed that *P. nigrum* is a valuable medicinal plant, which is one of the most commonly used spices and considered as "The King of spices" among various spices and is also used as an important component of many Ayurvedic treatments. Donata *et al*., (1990) have used *P. nigrum* fruits orally with other ayurvedic herbs including *Psoralea corylifolia* for the treatment of vitiligo where majority of subjects showed positive response.

Lin *et al*., (1999a) also reported that *P. nigrum* dried fruits and its pure active ingredient piperine induced melanogenesis in cultured mammalian melanocytes.

Later on, melanogenic properties of *P. nigrum* extract, piperine have been further rediscovered and scientifically validated by Sajid and Ali, (2011) using piperine, being the main active ingredient of *Piper nigrum*, on animal melanocyte models. Their data clearly showed that in tadpoles and adults of the frog, *Rana tigerina* and the toad, *Bufo melanostictus*, the melanin dispersion within the black pigment cells, the melanophores is stimulated by piperine-like receptors, similar to that of the cholinergic ones.

Sajid and Ali, (2011) have concluded that piperine can be used as a novel cellular moderator in activating the cholinergic or piperine-like receptors for their varied melanogenic actions. The pioneering study of Sajid and Ali, (2011) also signifies the evolutionary aspect of the receptors of the lower vertebrate melanophores, their phylogenetic development which is homologous to melanocytes in a more evolved mammalian-melanocyte receptor system.

Apart from its melanogenic activity, piperine also exhibits various other pharmacological activities like being an antidepressant (Li *et al*., 2007), hepato-protective (Matsuda *et al*., 2008), antihypertensive (Taqvi*et al*., 2008), antioxidant, antitumor (Manoharan *et al*., 2009), antiasthmatic, (Parganiha *et al*., 2011), antipyretic, analgesic, anti-inflammatory, anti-diarrheal, antispasmodic, anxiolytic, immuno-modulatory, antibacterial, anti-fungal, anti-thyroids, antiapoptotic, anti-metastatic, antimutagenic, anti-spermatogenic, anti- colon toxin, insecticidal and larvicidal activities, (Damanhouri and Ahmad, 2014) .

ALOE VERA

HPTLC analysis of leaf extract of *A. vera* against standard aloin revealed that best results were obtained using ethylacetate - methanol - water in 10: 1.4: 1 (v/v) ratio. The Rf value of standard aloin was matched with the Rf value of extract which was found about 0.76. The contents of aloin quantified using TLC densitometric methods were found to be 44.41 to 65.56 % in leaf extract of *A. vera* (Table-1).

Literature suggests that *A. vera* is an important medicinal plant belonging to family Liliaceae, of which there are about 360 species. The peripheral bundle sheath cells of *A. vera* produce an intensely bitter, yellow latex, commonly termed as aloe juice, or sap, or aloes which mainly contains aloin, responsible for their strong laxative effects.

A. vera has huge demand and is traded in medicinal drug markets of the world for a wide range of therapeutic applications such as wound healing effect, reduction of blood sugar in diabetes, for soothing burns, for easing intestinal problems and for reducing arthritic swellings. Various cosmetic products are also made from the mucilaginous tissues of A. vera leaves, commonly called as aloe gel (Dal'Belo *et al*., 2006).

Tan *et al*., (2002) demonstrated that aloin can bind not only to the enzyme tyrosinase but also to the enzyme–substrate complex, leading to inactivation of the enzyme resulting lightening of skin. Similarly Cheng *et al*., (2002) have also reported that aloin is a potent inhibitor of tyrosinase which plays an important role in melanogenesis.

Ali *et al*., (2012) have also reported anti melanogenic and melanin aggregatory potential of *Aloe vera* leaf gel extract containing aloin in which they found that the leaf extract of *A. vera* and its active ingredient aloin induced powerful, dose-dependent, physiologically significant melanin aggregating effects in the isolated tail melanophores of *B. Melano-stictus* leading to the lightening of the skin.

Their data suggests that the active ingredients of *A. vera* leaves, particularly aloin, stimulate the abundantly present adrenergic receptors of $\alpha 2$ type in tadpole tail skin leading to skin lightening effect. These findings also point to the novel role of aloin as a new sympathomimetic compound, which can have clinical application as nontoxic melanolytic agent, for the treatment of hyper pigmentation, or can be used as a skin fairness agent without any toxicological implications.

ARACHIS HYPOGAEA

HPTLC analysis of seed skin extract of *A. hypogaea* against standard resveratrol revealed that optimum results were obtained using Chloroform: Ethylacetate: Formic acidin in 2.5 : 1 : 0.1 ratio. The Rf value of standard aloin was matched with the Rf value of extract which was found about 0.31. The contents of quantified using TLC densitometric methods were found to be 26 % in seed skin extract of *A. hypogaea* (Table-1).

It is well known that *A. hypogaea* is an important plant which is widely used for the treatment of various ailments in different countries, and is a rich source of resveratrol. Because of the significant pharmacological activities exhibited by the resveratrol, several researchers have focused on the development of various analytical methods to determine resveratrol in different matrices such as plant extracts, wine and serum.

Resveratrol is a strong antioxidant and has been reported to have protective effects against atherosclerosis, coronary heart disease, postmenopausal problems, inhibits platelet aggregation and a broad spectrum of degenerative diseases and also possess cancer chemopreventive properties, (Jang *et al.*,1997 and Bagchi, 2000;).

Recently, Galgut and Ali, (2011) have also reported that the active ingredient of *A. hypogaea* such as resveratrol can act as a sympathomimetic compound and can induce aggregation of melanophores of the tadpole, *Bufo melanostictus* via the induction of beta type of the adrenoceptors. Their study has suggested a novel way for the use of *A. hypogaea* and its active ingredient, resveratrol for clinical application as a nontoxic melanolytic compound for the treatment of hyperpigmentation or making the skin fairer in complexion.

In this regard the authors have also filed a patent for the quantification, specific use and its mechanism at the cellular level, through National Research Development Corporation, New Delhi, Patent No. 2895/ MUM / 2012 (Ali SA & J Galgut).

OCIMUM SANCTUM

HPTLC analysis of leaf extract of *O. sanctum* against standard eugenol revealed that best results were obtained using Toluene: Ethyl acitate: Formic acid in 90:10:01 ratio. Rf value of standard was matched with the Rf value of extract which was found about 0.59. The contents of eugenol quantified using TLC densitometric methods were found to be 98.39 % in seed extract of *O. sanctum* (Table-1).

O. sanctum is a member of the Lamiaceae family, commonly known as 'Tulsi' in Hindi and 'Holy Basil' in English and is used as food seasoning. Tulsi has been well documented for its therapeutic potentials in Ayurveda described as Dashemani Shwasaharni (anti-asthmatic) and antikaphic drugs (Kaphaghna) (Gupta *et al.*, 2002; Yanpallewar *et al.*, 2004).

Species of tulsi are also valuable due to their pharmaceutical properties i.e. antipyretic, anti-inflammatory, cardio-protective, central nervous system (CNS) depressant, chemo preventive, antiulcer and anticancer properties. Leaves of Tulsi contain a bright yellow volatile oil, which is useful against insects and bacteria. The principal constituent of this essential oil is eugenol (4-allyl 2-methoxyphenol) which is responsible for medicinal properties of tulsi (Prakesh and Gupta, 2005).

Many scientific studies have showed that sweet basil extract is a strong radical scavenger and can be considered as a good source of natural antioxidants, (Pandey and Madhuri, 2010;Kumar *et al.*, 2012). Recently, from our laboratory, the powerful melanolytic role of extract of *O. sanctum* has been demonstrated on B16 melanoma cell lines, thus describing the extract as a possible candidate for its commercial use in making the skin fairer, Nargis and Ali, (2015).

BERBERIS VULGARIS

HPTLC analysis of whole plant extract of *B. vulgaris* against standard berberine revealed that best results were obtained using n-Propanol-Water-Formic Acid in 90:8.0:0.4 ratio. Rf value of standard aloin was matched with the Rf value of extract which was found about 0.56. The contents of eugenol quantified using TLC densitometric methods were found to be 3.8 % in whole plant extract of *B. vulgaris* (Table-1).

Berberis vulgaris L. (barberry) belongs to the Berberidaceae family and it is a small shrub which grows in Europe, but also in Africa and Asia, especially to the forest edge, in shining places. It is cultivated for its valuable biological properties. The main bioactive compounds from *B.vulgaris* are alkaloids (berberine, berbamine, jatrorrhizine, columbamine, berberubine, oxicanthine, palmatine; figure 1), vitamin C, resin, and tannins, but also flavonoids like quercetin and kaempferol (Singh *et al.*, 2011).

Literature showed that berberine possesses a wide range of medicinal property including antimicrobial, hepatoprotective, ionotropic, antiarrhythmic, hypolipidemic and anti inflammatory (Dehar *et al.*, 2012). Various clinical studies have established the efficacy of hydrochloride of berberine in the treatment of oriental sore (Dhar, 1980), trachoma (Babbar *et al.*, 1982; Mohan *et al.*, 1982), CHF18 and Type 2 diabetes mellitus (Yanxia, 1995; Yin *et al.*, 2008).

Chiou *et al.*, (1991) reported that berberine vasodilates the rat mesenteric artery in part by indirectly releasing EDRF, but mainly by directly blocking the release of

Ca2+ from internal stores. Recently it has been revealed that the extract of *Berberis vulgaris* exert skin darkening effect on pigment cells, i.e. melanohores of *Bufo melanostictus* (Ali *et al*., 2014).

Recently Ali *et al*., (2014) stated that extract of *Berberis vulgaris* exert skin darkening effects on pigment cells, i.e. melanohores of *Bufo melanostictus*. They reported berberine as a powerful melanogenic agent, as it induced a physiological melanophore dispersion effect, leading to darkening of the *B. melanostictus* skin.

Ali *et al*., (2014) have pharmacologically demonstrated that the berberine isolated from roots of *B. vulgaris* behaves like isoprenaline, the well known sympathomimetic agonist, in activating the dominantly present β_2 adrenoceptors of the neuronmelanophore junction of this species, to induce distinct and marked melanin dispersion of the *B. melanostictus* melanophores.

CURCUMA LONGA

HPTLC analysis of rhizome extract of *C. longa* against standard curcumin revealed that optimum results were obtained using chloroform and methanol in 95:5 ratio. The Rf value of standard aloin was matched with the Rf value of extract which was found about 0.47. The contents of curcumin quantified using TLC densitometric methods were found to be 28.10% in rhizome extract of *C. longa* (Table-1).

The rhizome of turmeric (*Curcuma longa* L.) has a rich history in India as spice, food preservative, and coloring agent and has been used for centuries in the Ayurvedic system of medicine. Its use as a remedy for hypercholesterolemia, arthritis, indigestion and liver problem has been known since long back (Srimal, 1997).

The continuing research indicates that turmeric and its active principle curcumin have unique antioxidant, antimutagenic, antitumorigenic, and anticarcinogenic, antiinflammatory, antiarthritic, antimicrobial, and hypocholesterolemic properties as reviewed elsewhere (Majeed *et al*., 1995; Kapoor, 2001; Miquel *et al*., 2002). Recently from the authors laboratory, Miraj *et al*., 2014, Miraj and Ali (2014, 2015) have recently described the amelioration of metal toxicity using plant extracts and also have demonstrated for the first time anti rheumatic properties of *C.longa* via iontophoresis, a unique transdermal delivery system using low current for the treatment of rheumatic arthritis in a rat model.

Present outcomes gained from the study clearly demonstrate characteristic HPTLC fingerprint of particular active compound of a particular plant species which will not only help in the identification and quality control of a particular species but also provide basic information useful for the isolation, purification, characterization and identification of marker chemical compounds of the species. Thus the present communication will provide sufficient information about therapeutic efficacy of certain herbal based drugs and also in the identification, standardization and quality control of medicinal plants, from the Indian herbal treasure.

CONCLUSION

HPTLC is one of the sophisticated instrumental techniques which has been widely used for the qualitative and quantitative analysis of the herbs and herbal drugs. In the present investigation we have taken crude extracts of different parts of six plants namely *P. nigrum, A.vera, A. hypogea, O. sanctum, B. vulgaris* and *C. longa* for the quantitative determination of bioactive compounds present in them and have reported that the significant quantities of active ingredients are present in the extracts of these plants.

The pharmacological efficacies of these six plants have been further investigated using various animal models. It has been found that crude extracts of three plants i.e. *A.vera*, *A. hypogea*, and *O. sanctum* exert powerful melanolytic responses, hence these plant extracts can be exploited as novel skin lightening agents in cosmetic industry for the treatment of hyperpigmentation or dark skin which has considerable social implications. However, the other three plant extracts of *P. nigrum, B. vulgaris* and *C. longa* exhibited skin darkening effects, which suggest that active ingredients from these plant extracts can serve as promising therapeutic candidates for the treatment of hypopigmentary disorders like vitiligo, which again is a social evil and has no proper treatment till date. Similarly, curcumin can be used as a powerful anti rheumatic agent if properly applied as in the present case we have used iontophoretic transdermal delivery system, envisaging maximum efficacy with a minimum dose. From these findings, it is concluded that bioactive components of these plants can be used as novel and safe candidates for the treatment of hyper as well as hypo pigmentary disorders, along with a host of other bio medical efficacies, like that for the untreatable dreadful rheumatic disease.

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