Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6 Issue 2, 2014

Research Article

EVALUATION OF PHYTOCHEMICAL, ANTIBACTERIAL AND FREE RADICAL SCAVENGING PROPERTIES OF *AZADIRACHTA INDICA (NEEM)* LEAVES

GARIMA PANDEY*, KK VERMA, MUNNA SINGH

Department of Botany, University of Lucknow, India. Email: garima.biochem@gmail.com

Received: 29 Jan 2014, Revised and Accepted: 09 Mar 2014

ABSTRACT

Objective: To analyse and correlate the phytochemical, free radical scavenging and antibacterial attributes of *neem* leaves with an aim to understand its potential for curing skin ailments.

Methods: A 50% ethanolic extract of neem leaves was subjected to qualitative and quantitative estimation of phytoconstituents followed by HPTLC analysis. The DPPH free radical scavenging activity was conducted to understand the antioxidant potentials of *neem* leaves. The antibacterial effect of extract was studied against Gram negative *Escherichia coli* and Gram positive *Staphylococcus aureus* using well-diffusion assay.

Results: The total phenol, flavonoid and tannin content were estimated to be 1.03%, 5.33% and 1.83% respectively. HPTLC studies revealed the presence of β-sitosterol, lupeol, rutin, ellagic acid, ferulic acid and quercetin in 50% ethanolic extract. The extract showed significant free radical scavenging activity with an IC_{50} of 110.36µg/ml. Ascorbic acid was taken as the standard antioxidant and its IC_{50} value was 42 µg/ml. The extract showed significant antibacterial activity against *E. coli* and *S. aureus*, though it inhibited the growth of *S. aureus* more effectively as compared to *E. coli*.

Conclusion: The results are suggestive that leaves of *A. indica* possess significant antioxidant and antibacterial properties, and contain phytoconstituents that may contribute to its medicinal properties.

Keywords: *Neem*, Skin ailments, Antibacterial, Ellagic acid, Ferulic acid, Quercetin.

INTRODUCTION

Azadirachta indica Juss. (Neem) (Meliaceae) is a fast growing evergreen popular tree found commonly in India, Africa and America. It has been used in Ayurvedic medicine for more than 4000 years due to its medicinal properties. Neem is called '*aristha*' in Sanskrit a word that means 'perfect, complete and imperishable'. *Arishtha* is the Sanskrit name of the neem tree meaning 'reliever of sicknesses'. The people of India have long revered the neem tree; for centuries, millions have cleaned their teeth with neem twigs, smeared skin disorders with neem leaf juice, taken neem tea as a tonic and placed neem leaves in their beds, books, grain bins, cupboard and closets to keep away troublesome bugs. The number of benefits of neem is listed in ancient documents like 'Charak Samhita and Susruta Samhita. It is commonly called 'Indian Lilac' or 'Margosa'.Nimbidin, a major crude bitter principle extracted from the oil of seed kernels of *A. india* demonstrated several biological activities. From this crude principle some tetranortriterpenes, including nimbin, nimbinin, nimbidinin, nimbolide and nimbidic acid have been isolated. Nimbidin and sodium nimbidate possess significant dose dependent anti- inflammatory activity against carrageenin induced acute paw edema in rats and formalin induced arthritis. Gedunin, isolated from neem seed oil has been reported topossess both antifungal and antimalarial activities. Mahmoodin, a deoxygedunin isolated from seed oil, has been shown to possess moderate antibacterial action against some strains of human pathogenic bacteria. The antioxidant activity of *neem* seed extract has been demonstrated *in vivo* during horsegrain germination, which is associated with low levels of lipooxygenase activity and lipid peroxides [1]. The chloroform extract of stem bark is effective against carragenin-induced paw edema in rat and mouse ear inflammation [2]. Extracts of leaf, oil and seed kernels are effective against certain human fungi, including *Trichophyton*, *Epidermophyton*, *Microsporum*, *Trichosporon*, *Geotricum* and *Candida* [3]. Oil from the leaves, seeds and bark possesses a wide spectrum of antibacterial action against Gram-negative and Gram-positive microorganisms, including *M. tuberculosis*, *Vibrio cholerae*, *Klebsiella pneumoniae*, *M. tuberculosis, M. pyogenes*, *Streptococcus mutans* and *S. faecalis* [4-5].

A. indica is perhaps the most useful traditional medicinal plant in India. During the last five decades, apart from the chemistry of the *neem* compounds, considerable progress has been achieved regarding its biological activity and medicinal applications. The *A. indica* leaves are widely used among the various tribes of India to cure cuts, wounds and other minor skin ailments [6-7].

Tribals of Rajastan use neem leaves for the cure of skin irritation. The Madugga tribes of Siruvani forest, South India, apply crushed leaves externally to cure sore skin [8]. Neem leaves are also used among various other tribes of India, Africa and Burma, to cure ailments of skin, and other parts of body. The present study correlates the phytochemical, antioxidant and antibacterial attributes of *neem* leaves with an aim to understand its potential for curing skin ailments.

MATERIALS AND METHODS

Plant material and preparation of extract

Leaves of *Azadirachta indica* Juss. were collected from Lucknow district, Uttar Pradesh (India) during the month of January (2012). The specimen was identified, authenticated and submitted at CSIR-National Botanical Research Institute, Lucknow (Voucher specimen No. LWG 98571). Leaves of the collected plants were washed thoroughly with distilled water and shade dried for ten days.

A 1000g dried leaves were ground to a fine powder using mixer grinder and subjected to extraction thrice in 50% ethanol, using cold maceration technique. The extract was concentrated in rotary vacuum evaporator and stored at 4^0C until further use (yield= 21.24%).

Qualitative analysis of phytochemicals

The *Azadirachta indica* leaves extract (AILE) was subjected to preliminary phytochemical screening. Presence of alkaloids (Mayer's test), flavonoids (alkaline reagent test), tannins (Braymer's test) carbohydrates (Molischs test), glycosides (Liebermann's test), saponins (Salkowski test), triterpenoids (Liebermann Burchard test), proteins and amino acids (Ninhydrin test) were tested.

Quantitative estimation of total phenol, flavonoid and tannins

The total phenol content was determined using Folin-ciocalteau reagent and the total flavonoid content was estimated using aluminium chloride method [9].The tannin estimation in crude drug followed the method mentioned by Schanderl [10] with slight modifications.

Identification of phytoconstituents in AILE using HPTLC

The HPTLC plates are coated with high performance silica gel which is of very small and uniform in size (about 5m). These high performance silica gels give more efficient and reproducible separation than conventional grades of silica gel. A known quantity of test and standard solutions were applied on a pre-coated silica gel GF254 plate of uniform thickness (0.2 mm) with the help of LINOMAT 5 applicator attached to CAMAG HPTLC system. HPTLC profile of 50% ethanolic extract for various marker compounds were developed in solvent systems, toluene: ethyl acetate: formic acid (9:2:0.1) for β-sitosterol and lupeol; toluene: ethyl acetate: formic acid (5:4:1) for ellagic acid, ferulic acid and quercetin; ethyl acetate: acetic acid: formic acid: water (10:1.1:1.1:2.6) for rutin. The plates were scanned densitometrically by CAMAG Scanner 3 by using software WinCats (3.1.1) and fingerprint profile was recorded. Standard peak of the reference marker compounds were scanned for their spectral analysis at the range of 200-700 nm wavelength and λ max was recorded. Identification of all the marker compounds in extract were confirmed by overlaying absorption spectra at three different levels, *i.e.* peak start, peak apex and peak end position of the spot of the respective marker compounds.

DPPH free radical scavenging assay

The free radical scavenging activity (antioxidant capacity) of AILE on stable radical 1,1-diphenyl -2-picrylhydrazyl (DPPH) was estimated by method mentioned by Brand-Williams et al., [11]. Briefly, 2ml of AALE at varying concentrations (50µg/ml to 250µg/ml) was mixed with 2.0 ml of DPPH solution in methanol (0.004% w/v). The mixture was allowed to stand at room temperature in dark for 20 min. Then the mixture was vortexed and absorbance was recorded at 517nm using spectrophotometer. Ascorbic acid was used as a reference standard and control consisted of DPPH solution without extract. The test was performed in triplicate and percentage scavenging of DPPH free radical by extract was calculated using the equation: (Acontrol- Atest)/Acontrol X100, where Acontrol is the absorbance of control and Atest is the absorbance in presence of extract or standard.

Determination of in-vitro antibacterial activity

The anti-bacterial activity was tested using agar well diffusion method according to Lino A, *et al* [12] and Arshad H *et al* [13]. The MTCC cultures were obtained from the department of Pharmacognosy, CSIR-NBRI. The AILE was tested for its antibacterial property against *Ecsherichia coli* (ATCC 10536) and *Staphyloccus aureus* (ATCC 33591). A 1 ml of test culture (107 CFU/ml) was inoculated into a sterile plate with 20 ml Muller Hinton agar which was then made to solidify. Three wells of approximately 6 mm diameter were made on the surface of agar plate using a sterile cork-borer. Stock solution of AILE was dissolved in DMSO at varying concentrations (0.50 to 1.50 mg/ml). A 50µl extract of each concentration was pipetted in the well. A 50µl DMSO served as negative control and 10µg of streptomycin served as positive control respectively. The plates were then incubated at 370C for 24 hr and the zone of inhibition was recorded.

RESULTS

Phytochemical studies

The 50% ethanolic extracts of *A.indica* leaves showed presence of saponins, flavonoids, phenols, tannins, alkaloids, glycosides, proteins, triterpenoids,carbohydrates and alkaloids. Phytochemical studies indicate 5.33%, 1.03% and 1.83% of flavonoid, phenols and tannins respectively; in dry leaf powder (Table1). While β sitosterol and lupeol were observed at Rf 0.53 and Rf 0.6 (Fig1) respectively, rutin was observed at Rf 0.57 (Fig. 2).Ellagic acid quercetin and ferulic acid were observed at Rf 0.22, 0.47 and 0.5 respectively (Fig 3).

Table 1: Phytochemical estimations of *Azadirachta indica* leaves

Extraction yield of *A.indica* leaves in 50% ethanol	21.24% ± 1.24
Total phenol content	1.03% ± 0.06
Total flavonoid content	5.33% ± 0.25
Total tannin content	1.83% ± 0.24

Values are mean ± SD of three determinations.

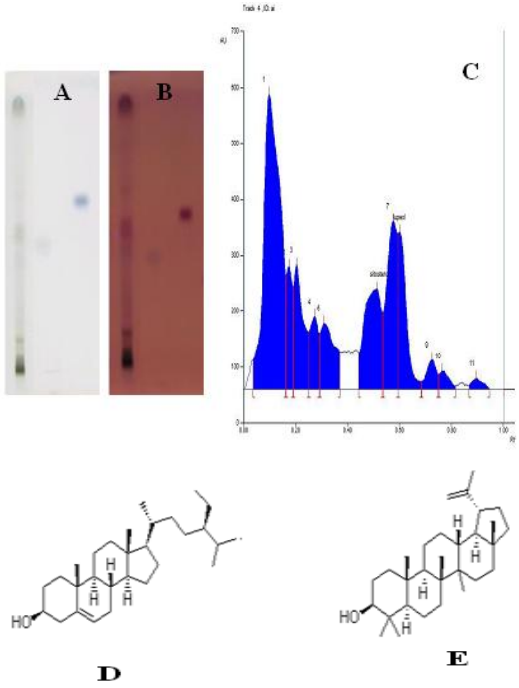

Fig. 1: HPTLC fingerprint profile of *Azadirachta indica* leaves (A) visible light after derivatization ;(B) UV 366 after derivatization ; [Lane 1:50% ethanolic extract; 2: β-sitosterol; 3- Lupeol] (C) densitometric scanning profile along with marker compounds ; (D) Structure of β-sitosterol; (E) Structure of Lupeol

*Source of chemical structures: www.chemblink.com

DPPH free radical scavenging activity

Results of the phytochemical analysis confirmed significant flavonoids (5.33%), phenols (1.03%) and tannin (1.83%) content. The presence of these classes of phyto-compounds is indicative of a significant antioxidant potential. Thus the DPPH radical scavenging activity was performed to quantify the antioxidant potential of *A. indica* leaves. The IC_{50} value of was AILE was 110.36µg/ml. Ascorbic acid was taken as the standard antioxidant and its IC_{50} value was 42 µg/m (Fig 4).

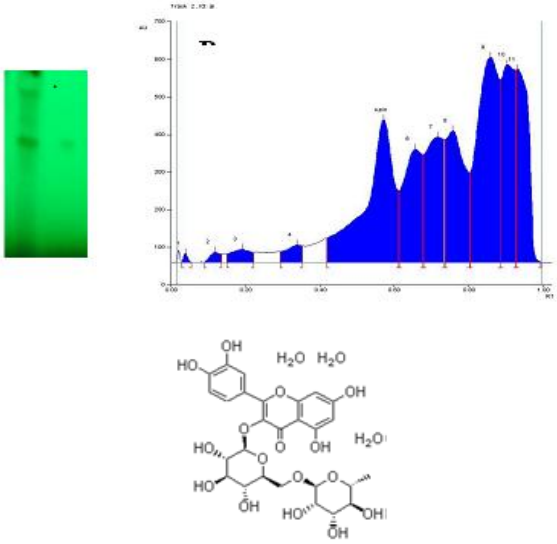

Fig. 2: HPTLC fingerprint profile of Azadirachta indica leaves (A) under UV 254 nm components [Lanes 1: 50% ethanolic extract; 2: Rutin]; (B) densitometric scanning profile along with marker; (C) Structure of Rutin.

*Source of chemical structure: www.chemblink.com

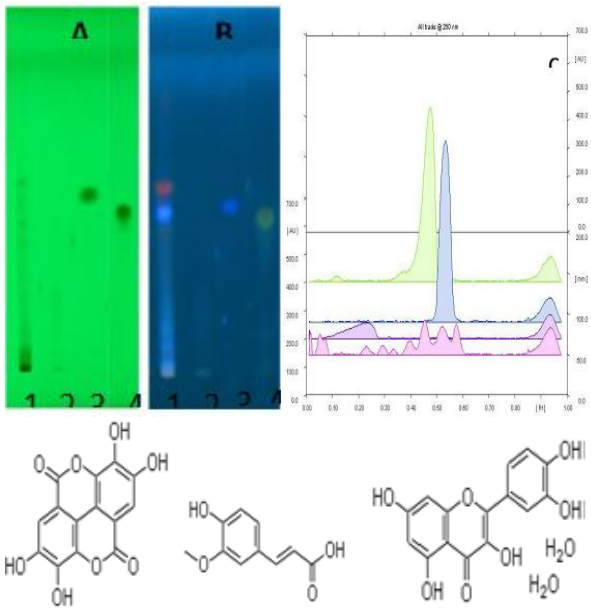

Fig. 3: HPTLC fingerprint profile of *Azadirachta indica* leaves (A) under UV 254 nm ; (B) UV366 nm ; [Lanes 1: 50% ethanolic extract; 2:Ellagic acid; 3:Ferulic acid; 4: Quercetin] (C) densitometric scanning profile along with marker components; (D) Structure of ellagic acid; (E)Structure of ferulic acid; (F) Structure of quercetin

*Source of chemical structure: www.chemblink.com

Antibacterial activity

AILE showed significant antibacterial activity and effectively inhibited the growth of *E.coli* and *S.aureus*. Growth inhibition was concentration dependent and greater inhibition was observed for *S.aureus* as compared to *E.coli*. About 1mg/ml concentration of Gentamycin (standard) inhibited the growth of *E.coli* upto 29 mm and the growth of *S.aureus* upto 26 mm (Table 2).

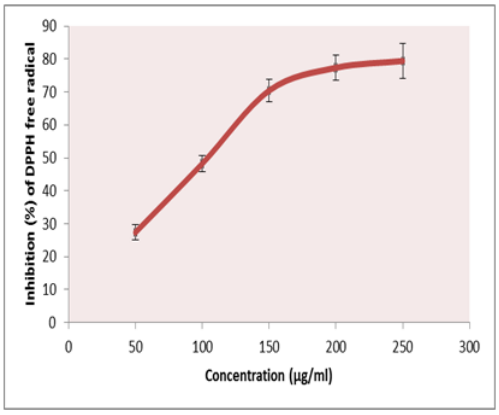

Fig. 4: DPPH free radical scavenging activity of 50% ethanolic extract of *A.indica* leaves. Values are mean ± SD of three determinations.

Table 2: Antibacterial activity of *Azadirachta indica* leaves extract against *E. coli* and *S. aureus*

Concentration (mg/ml)	Inhibition zone (mm)	
	***E. coli* (ATCC 10536)**	***S. aureus* (ATCC 33591)**
0.50	7.2 ± 1.2	10.24 ± 1.25
0.75	8.34 ± 2.31	11.26 ± 2.12
1.25	10.27 ± 2.21	13.25 ± 2.35
1.50	13.23 ± 3.26	16.52 ± 3.28

Values are mean ± SD of three determinations

DISCUSSION AND CONCLUSION

The aim of phytochemical screening is to confirm the presence of various constituents of AILE for assessing their biological activity or medicinal uses. The medicinal value of plants is due to the presence of particular chemical substances that have a definite physiological action on the living system. The most important of these are alkaloids, glycosides, saponins, steroids, phenols, flavonoids and tannins. Phytochemical screening of *neem* leaves showed the presence of saponins, triterpenoids, alkaloids, tannins, glycosides and steroids. These class of compounds independently or in combination may be responsible for the broad range of medicinal properties of *neem*. Alkaloids are organic nitrogenous substances. These are alkaline in nature and exhibit an extra ordinary array of pharmacological activities. Certain alkaloids act as cardiac and respiratory stimulants and are used in treatment of many types of cancer (www.enthrogen.com). Glycosides are active and complex substances containing carbon, hydrogen and oxygen. They have characteristic actions on contractile forces of cardiac muscle [14]. Saponins show anti-fungal, antibacterial and anti-protozoal effects [15]. Flavonoids are widely distributed in higher plants. The flavonoids act as antioxidants which provide protection against free radicals that damage cells and tissues. Tannins promote healing of wounds. These are effective in diarrhea, colitis and peptic ulcers. The phytoconstituents i.e., alkaloids, glycosides, flavonoids and saponins are antibiotic principles of plants. These antibiotic principles are actually the defensive mechanism of the plants against different pathogens [16]. The current study was mainly focussed to understand the effect of neem leaves for curing various skin diseases. High flavonoids content indicates the probability of significant antioxidant potential of the *neem* leaves. These phytoconstituents may be mostly responsible for various medicinal properties of *neem* leaves. The total flavonoid content falls in accordance with earlier estimations done by Pongtip and Gritsanapan [17]. Preliminary evidences suggest that β-sitosterol has a role in strengthening the immune system. Some people also apply it on skin for treating wounds and burns (www.webmd.com). Roots of Indian labernum, *Cassia fistula* are known to contain β-sitosterol and are used for curing skin diseases. The phytoconstituents is also a well- known antibacterial agent. A study conducted by Bumrela *et al.,*[18], β-sitosterol along with β carotene in the methanol extract of *Dipteracanthus patulus* was found to be the major antimicrobial agent against a broad spectrum of Gram negative and Gram positive bacteria, including *S. aureus*. Lupeol is a pharmacologically active triterpenoid found in a variety of plants, the ubiquity of this triterpenoid among higher plants implies that it is essential. It has several medicinal properties, one being anti-inflammatory. Lupeol has a complex pharmacology in humans, displaying antiprotozoal, antimicrobial, anti-inflammatory, anti-tumor and chemo preventive properties [19]. Rutin is a natural antioxidant; its antioxidant activity has been studied in various model systems [20]. By reducing the free radical damage to the basement membrane, rutin promotes good communication between skin layers. Rutin aids proper absorption and function of vitamin C, one of the vitamins critical to maintain collagen in a healthy state. Rutin is also known for its anti-inflammatory and vasoactive properties [21]. Due to its vasoactive property rutin ensures that the skin gets its fair share of nutrients, thus it can be said that rutin contributes to the medicinal properties of neem and helps to alleviate skin disorders.

The DPPH free radical scavenging activity was performed to study the antioxidant potentials of neem leaves. Any natural drug that is used as a remedy for skin diseases is assumed to possess antioxidant properties. Phenolic and flavonoid content have been showed to contribute significantly to antioxidant activity [22] Results of the HPTLC studies confirm the presence of a wide array of phytoconstituents with antioxidant properties. Rutin is a glycoside of the flavonoid quercetin. In humans, it attaches to the iron ion Fe2+, preventing it from binding to hydrogen peroxide, which would otherwise create a highly reactive free radical that may damage cells. Ferulic acid, like many natural phenols, is an antioxidant *in vitro* in the sense that it is reactive toward free radicals such as reactive oxygen species (ROS). ROS and free radicals are implicated in DNA damage, cancer and accelerated cell aging. If added to a topical preparation of ascorbic acid and vitamin E, ferulic acid may reduce oxidative stress and formation of thymine dimers in skin. Quercetin is most importantly known for its ability to act as antioxidant. It seems to be the most powerful flavonoid for protecting the body against reactive oxygen species, produced during the normal oxygen

metabolism or induced by exogenous damage [23]. Ellagic acid is a powerful antioxidant, polyphenol that decreases lipid peroxidation and is an effective free radical scavenger. It also protects the skin from radiation-induced chromosome damage (http://www.lef.org/).

In an earlier study Khan *et al.,* [3] reported that neem leaves extract had a characteristic effect on dermatophytes especially for lower polar extracts over high polar ones. The author suggested that one possible explanation for this is the flavonoid quercetin contained in the extract. In another experiment conducted by Subapriya *et al.,* [24] *neem* leaves extract was found to have interesting inhibitory action on a wider spectrum of microorganisms including *C. albicans*, *C. tropicalis, Neisseria gonorrhea*, multi drug resistant *S. aureus, E. coli* and *Herpes simplex*. Singh *et al.,* [25] owed the fungicidal and bactericidal properties of extracts from neem leaves either in *in vitro* or *in vivo* trials to the presence of several antimicrobial active ingredients in the leaves of neem tree, such as desactylimbin, quercetin and sitosterol. Whereas others researchers explain this activity by the presence of active ingredients like triterpenes or limonoids such as meliantriol, azadirachtin, desactylimbin, quercetin, sitosterol, nimbin, nimbidin, nimbinin, nimbosterol and margisine [26] and/or to different bitter substances such as alkaloids, phenols, resins, glycosides, terpenes and gums [27]. Lyer and Williamson [28] attributed antifungal properties of neem extract to the inhibition in protease activity of dermatophytes induced by neem organic extract. It should also be mentioned here that in a study conducted by Arima *et al.,* [29] it was found that the antibacterial activity of the combinations of quercetin and quercitrin, quercetin and morin, and quercetin and rutin were much more active than either flavonoid alone. It is interesting to know that though rutin did not show any activity in itself, the antibacterial activity of quercetin and morin were enhanced in the presence of rutin. The presence of rutin in neem leaves extract was confirmed by HPTLC in the current study. It can be suggested that rutin enhances the antimicrobial activity of flavonoids and various other phytochemical groups and thus contributes to the medicinal properties of the plant.

The phytochemical and biological experiments performed during the current study confirm the antioxidant and antibacterial properties of neem leaves. Though neem based products from *Azadirachta indica* have been successfully used for pest control in agriculture since long, the registered neem products for control of pathogens or disease vectors affecting human, still need to be explored. In line with the above findings it is suggested that the further researches on neem should be directed towards identification and quantification of active principles responsible for curing skin ailments and patenting of findings thereby making these accessible to mankind.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

REFERENCES

1. Rao AD, Devi KN, Thyagaraju K. Mechanism of antioxidant activity in neem. Journal of Enzyme Inhibition. 1998; 14: 85-86.
2. Tidjani MA, Dupont C, Wepierre J. *Azadirachta indica* stem bark extract anti-inflammatory activity. Planta Medica Phytotherapia.1989; 23: 259-266.
3. Khan M, Wassilew SW, Schmutterer H, Ascher KR. Natural Pesticides from the Neem Tree and Other Tropical Plants.Proceedings of 3rd International Neem Conference, Eschborn, Germany. 1987; 460-466.
4. Maragathavalli S, Brindha S, Kaviyarasi NS, Annadurai, B, Gangwar SK. Antimicrobial activity in leaf extract of Neem (*Azadirachta indica* Linn). International Journal of Science and Nature. 2012; 3: 110-113.
5. Almas K. The antimicrobial effects of extracts *Azadirachta indica* (Neem) and *Salvadora persica* (Arak) chewing sticks. Indian Journal of Dental Research. 1999; 10: 23-26.
6. Jain DL, Baheti AM, Jain SR, Khandelwal KR. Use of medicinal plants among tribes in Satpuda region of Dhule and Jalgaon districts of Maharashtra-An ethnobotanical Survey. Indian Journal of Traditional Knowledge. 2010; 9:152-157.
7. Padal SB, Sandhya B, Chandrasekhar P, Vijayakumar Y. Folklore treatment of skin diseases by the tribes of G. MadugulaMandalam, Visakhapatnam District, Andhra Pradesh, India; Journal Of Environmental Science, Toxicology And Food Technology.2013; 4: 26-29.
8. Soudahmini E, Senthil G M, Panayappan L, Divakar Madhu C. Herbal remedies of Madugga tribes of Siruvani forest, South India. Natural Product Radiance. 2005; 4: 54-62
9. Narendhirakannan RT, Nirmala JG, Caroline A, Lincy S, Saj M, Durai D. Evaluation of antibacterial, antioxidant and wound healing properties of seven traditional medicinal plants from India in experimental animals. Asian Pacific Journal of Tropical Biomedicine. 2012; 1245-1253.
10. Schanderl SH (1970). Methods in Food Analysis. Academic Press, New York, London Pp709.
11. Brand-Williams W, Cuvelier, Berset C. Use of free radical method to evaluate antioxidant activity. Lebensm Wiss Technology. 1995; 28:25-30.
12. Lino A, Deogracious O. The *in vitro* antibacterial activity of *Annona senegalensis, Securidacca longipendiculata* and *Steanotaenia araliacea*-Ugandan medicinal plants. African Health Science. 2006; 6:31-35.
13. Arshad H, Shadma W, Iffat Z, Sarfaraj H. Antibacterial activity of the leaves of *Coccinia indica* (W. and A) Wof India. Advanced Biological Research.2010; 4:241-248.
14. Hikino H, Kiso Y. Natural products for liver diseases in Economics and Medicinal Plants Research. Vol. 2nd. In: H Wagner, H Hikino and NR Famsworth (Eds.). Wagner, Academic Press. 1988:39-72.
15. Morisaki N, Watanab S, Tezeka M, Zenikaya M, Shina R, Koyema N, Kanzaki T,Saitto Y. Mechanism of angiogenic effects of Saponin from ginseng dixruber in human umbilical vein: Endothelial cells. British Journal of Urmacology.1995; 11: 1188-1193.
16. Hafiza RE. Peptides antibiotics. Lancet. 2000; 349: 418-422.
17. Pongtip S, Wandee G. Free radical scavenging activity and total flavonoid content of siamese neem tree leaf Aqueous extract from different locations. Mahidol University Journal of Pharmaceutical Sciences. 2005; 32:31-35.
18. Bumrela SB, Naik SR. Identification of β-carotene and β-sitosterol in methanolic extract of Dipteracanthus patulus (Jacq) nees and their rolein antimicrobial and antioxidant activity. International Journal of Phytomedicine. 2011; 3: 204-215.
19. Margareth BC, Miranda JS. Biological activities of Lupeol. International Journal of Biomedical and Pharmaceutical Sciences.2009; 3(Special issue 1): 46-66.
20. Balsundaram A, Rathna Kumar P, George J, Selvakumar BM. Antimicrobial activity of the leaf extract of two medicinal plants against MRSA (Methhicilin Resistant *Staphylococcus aureus*) from Human Urinary Tract Pathogens. Research Journal of Microbiology. 2011; 6 625-631.
21. Casa C La, Villegas I, Alarcón C, La Lastra De, Motilva V, Martín CMJ. Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions. Journal of Ethnopharmacology. 2000; 71: 45–53.
22. Mahmood G, Mohamad S, Ali PG. Variation in phenolic compounds, ascorbic acid and antioxidant activity of five coloured bell pepper *Capsicum annum* fruits at two different harvest times. Journal of Functional Foods.2011; 3: 44-49.
23. Grace PA. Ischemia-reperfusion injury. British Journal of Surgery. 1994; 81: 637-647.
24. Subapriya R, Nagini S. Medicinal properties of neem leaves: a review. Current Medicinal Chemistry: Anticancer Agents.2005; 5: 149-160.
25. Singh PP, Junnarkar AY, Reddi GS, Singh KV. *Azadirachta indica* neuro-ycho pharmacological and antimicrobial studies. Fitoterapia. 1987; 58:233-238.
26. Bhatnagar D, McCormick SP. The inhibitory effect of neem (*Azadirachta indica*) leaf extracts on aflatoxin synthesis in *Aspergillus parasiticus*. Journal of the American Oil Chemists Society.1988; 65: 1166-1168.
27. Joshi PC, Prakash OM, Prakash O, Tauro P, Narwal SS. Allelopathic effects of litter extract of some tree species on germination seedling growth of agricultural crops. Proceedings First National Symposium. Allelopathy in agroecosystems (Agriculture & Foresty), CCS Haryana Agricultural University, Hissar, India.1992; pp. 127-128.
28. Lyer SR, Williamson D. Efficacy of some plant extracts to inhibit the protease activity of Trichophyton species. Geobios Tadhpur.1991; 8: 3-6.
29. Arima H, Ashida H, Danno G. Rutin-enhanced antibacterial activities of flavonoids against *Bacillus cereus* and *Salmonella enteritidis*. Bioscience Biotechnology and Biochemistry. 2002; 66: 1009-1014.