Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(5): 702-706
Received: 05-07-2017
Accepted: 06-08-2017

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Antibacterial and phytochemical analysis of *Tinospora cordifolia, Azarchita indica* and *Ocimum santum* leaves extract against common human pathogens: An *in vitro* study

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Abstract
In the present study the aqueous, methanol, ethanol and acetone extract of *Tinospora cordifolia, Azarchita indica* and *Ocimum santum* leaves extract were screened for the presence of phytochemical components and tested for antibacterial activity against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Enterobacter aerogenes, Pseudomonas aeruginosa, Salmonella typhimurium, Salmonella typhi, Staphylococcus epidermidis* and *Proteus vulgaris*. Results revealed the presence of anthraquinones, alkaloids, saponins, tannins, glycosides and phenolics. The acetone extracts had wide range of antibacterial activity against bacterial pathogens than the ethanol and methanol extract, whereas aqueous extract were slightly higher antibacterial activity as ethanol extract. Antibacterial activity of various extract of leaves of *Tinospora cordifolia, Azarchita indica* and *Ocimum santum* would lead to the establishment of some compounds that could be used to formulate new and more potent antimicrobial drugs of natural origin. Further work is being carried out to isolate and identify the active constituents of the plants responsible for antibacterial activity.

Keywords: *Tinospora cordifolia, Azarchita indica* and *Ocimum santum,* leaves extracts, phytochemical Screening, antibacterial activity

Introduction
Plants are the richest resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Hammer *et al.,* 1999) [7]. The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization. The earliest mention of medicinal use of plants in Hindu culture is founds in "Rigveda", which is said to have been written between 4500 - 1600 B.C. and is supposed to be the oldest repository of human knowledge. It is Ayurveda, the foundation of medicinal science of Hindu culture, in its eight division deals with specific properties of drugs and various aspects of science of life and the art of healing (Rastogi and Mehrotra, 2002) [17].
Emergence of multidrug resistant pathogens has been reported to be one of the leading causes of death world (Reddy *et al.,* 2009) [10] wide with infectious diseases responsible for 68% of all deaths globally in 2012 (WHO, 2000) [22]. Many infectious microorganisms' are resistant to synthetic drugs and it has become the major concern for health institutions, pharmaceutical companies and governments all over the world; thus there is need for an alternative therapy (Tambekar and Dahikar, 2011) [21].
Azadirachta indica (Neem) is perhaps the most useful traditional medicinal plant. Every part of the tree has been used as traditional medicine for household remedy against various human ailments. The tree is still regarded as "Village dispensary" in India. Most of the parts of the plant such as fruits, seeds, leaves, bark and roots contain compounds with proven antiseptic, antiviral, antipyretic, anti-inflammatory, antiulcer and antifungal properties.
Ocimum sanctum, popularly known as Tulsi is a time-tested premier medicinal herb that is used in ayurvedic medicine since ancient times. It has made an important contribution to the modern research due to its large number of medicinal properties. Different parts of the plant have shown antimicrobial, anti-inflammatory, analgesic, antipyretic, antiulcer, antidiabetic, antioxidant and anticancer activity.
Tinospora cardifolia is a large deciduous climbing shrub found throughout India. The ayurvedic name of the plant is Guduchi, Giloy or Amrita. In India, the extract of the plant is used as a remedy for many diseases including diabetes, hepatitis etc. The plant finds a special mention for its use in tribal or folk medicine in different parts of the country.

The drug has been subjected to extensive phytochemical, pharmacological and clinical investigations and many interesting findings have been reported (Nadkarni, 2005) [14]. Many researchers had studied antimicrobial activity of other parts of plant like bark, leaves and fruits of *Tinospora cordifolia, Azarchita indica* and *Ocimum santum* which are used to cure many infectious diseases in traditional system of medicine but still very, less work has been done on antibacterial activities of leaves of *Tinospora cordifolia, Azarchita indica* and *Ocimum santum*. To prove the validity of traditional medicine the present work has been undertaken to evaluate the antimicrobial screening of leaves of *Tinospora cordifolia, Azarchita indica* and *Ocimum santum* against the human bacterial pathogens.

Materials and Methods

Sample Collection

Tinospora cordifolia, Azarchita indica and *Ocimum santum* leaves were collected from Bhavan's college campus Andheri (W), India in the month of March and authenticated by Botanical Survey of India, Pune (M.S), India.

Preparation of plant material

Leaves were collected and dried at room temperature. The dried samples were powdered separately. 100gm each of the sample was extracted separately with different solvents starting with polar to nonpolar solvents in the order of aqueous, ethanol, methanol and acetone. The crude residues were obtained by removing the solvents in rotary evaporator and each of the extracts were resuspended in the respective solvents for further study.

Preparation of extracts

Solvent extraction method Thirty grams of dried powder of *Tinospora cordifolia, Azarchita indica* and *Ocimum santum* leaves were extracted with aqueous, ethanol, methanol and acetone using soxhlet apparatus for 48 hrs. The collected extracts were filtered with Whatman No.1 filter paper and used for estimation of phytochemicals and antibacterial activity.

Phytochemical screening

Preliminary qualitative phytochemical screening was carried out with the following methods (Khandelwal, 2001) [9].

Test for Tannins: To 0.5 ml of extract solution, 1 ml of distilled water and 1 to 2 drops of ferric chloride solution was added, observed for blue or green black coloration.

Test for Saponins: Two ml of distilled water was added to 2 ml of the test solution shaken well and observed for frothing.

Test for Flavonoids: A volume of 1.5 ml of 50 % methanol was added to 4 ml of the extracts. The solution and magnesium metal was added and warmed. Then, 5 to 6 drops of concentrated hydrochloric acid was added to the solution and observed for red coloration.

Test for Steroids (Salkwoski's test): Five drops of concentrated sulphuric acid (H2SO4) was added to 2 ml of each extract and observed for red coloration.

Test for Glycosides: To 4 ml of extract solution and add few drops of glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid and observed for a reddish brown coloration at the junction of 2 layers and bluish green colour in upper layer.

Test for Alkaloids: To 4 ml of extract filtrate, a drop of Mayer‟s reagent was added along the sides of test tube. Creamy yellow or white precipitate indicates that the test is positive.

Test for Anthraquinones: One gram of powdered plant material was taken and extracted with 10 ml of hot water for five minutes and filtered. Filtrate was extracted with 10 ml of CCl4 then CCl4 layer was taken off. Five ml water and 5 ml dilute ammonia solution was added. No free anthraquinones were revealed as absence of appearance of pink to cherry red colour. One gram of second sample of the same plant material was extracted with 10 ml of ferric chloride solution and 5 ml of hydrochloric acid then it was heated on water bath for 10 minutes and filtered. Filtrate was cooled and treated as mentioned above.

Test for phenolic compounds: Two ml of extract was diluted to 5 ml with distilled water. To this a few drops of neutral 5 % ferric chloride solution was added. A dark green colour indicates the presence of phenolic compounds

Bacterial cultures

The standard pathogenic bacterial cultures were procured from IMTECH, Chandigarh, India and used in the present study (Table 1). The bacterial cultures were rejuvenated in Mueller- Hinton broth (Hi-media laboratories, Mumbai, India) at 37^0C for 18h and then stocked at 4^0C in Mueller-Hinton Agar. The inoculum size of the bacterial culture was standardized according to the National committee for Clinical Laboratory Standards (NCCLS, 2002) [15] guideline. The pathogenic bacterial culture was inoculated into sterile Nutrient broth and incubated at 37^0C for 3h until the culture attained a turbidity of 0.5 McFarland units. The final inoculum size was standardized to 10^5 CFU/mL with the help of SPC and Nephlo-turbidometer.

Table 1: Bacterial cultures used in study (IMTECH, Chandigarh, India).

Bacterial Pathogens	MTCC Number
Proteus vulgaris	426
Staphylococcus epidermidis	435
Staphylococcus aureus	96
Escherichia coli	739
Pseudomonas aeruginosa	424
Klebsiella pneumoniae	109
Salmonella typhi	733
Enterobacter aerogenes	111
Salmonella typhimurium	98

Preparation of disc for antibacterial activities

The aqueous, ethanol, methanol and acetone extracts were prepared in their respective solvents and the sterile blotting paper disc (10 mm) were soaked in the diluted extract in such concentration that the amount of solution absorbed by each disc was 1mg, 2mg, 3mg, 4mg, 5mg of each extracts of *Tinospora cordifolia, Azarchita indica* and *Ocimum santum* leaves. The prepared disc were dried in controlled temperature to remove excess of solvent and used in study.

Antibacterial activity using disc diffusion method

The modified paper disc diffusion method was employed to determine the antibacterial activity of aqueous, ethanol, methanol and acetone extracts. Turbidity of inoculums was matched with McFarland turbidity standard (NCCLS, 2002) [15]. Inoculums were spread over the Nutrient agar plate using

a sterile cotton swab in order to get a uniform microbial growth. Then the prepared antibacterial disc were placed over the lawn and pressed slightly along with positive and negative controls. Ampicillin 10 mcg/disc (Hi-Media, Mumbai) were used as positive control while disc soaked in various organic solvents and dried were placed on lawns as negative control. The plates were incubated for 18h at 37^0C. The antibacterial activity was evaluated and diameters of inhibition zones were measured. Experiment was carried out in triplicate and the averages diameter of zone of inhibition was recorded. The antibacterial activity was classified as strong (>20mm), moderate (16-19mm) and mild (12-15mm) and less than 12mm was taken as inactive.

Results and Discussion

The medicinal plants like Giloy, Neem and Tulsi are being used traditionally for the treatment of inflammation, wound healing, carminative, cough, toothache, antiseptics expectorant, stomatitis and some fungal infection like candidaisis. The antibacterial activity has been attributed to the presence of some active constituents in the extracts. The phytochemical analysis of A. indica extract had earlier been reported (Kraus *et al.,* 1981) [11]. Phytochemical screening of the stem bark extract of *A. indica* in the present study also revealed presence of terpenes and glycosides. Study suggested a number of active constituents might be present in the neem bark extract to control gastroduodenal ulcers. However, a glycoside appeared to be the major bioactive component that offers antisecretory and antiulcer effects (Bandyopadhyay *et al.,* 1998, 2002) [2, 3]. Phytochemical screening of the stem bark extract of *A. indica* in the present study also revealed presence of terpenes and glycosides. Plant glycosides, which are not normally toxic when ingested orally, are known to inhibit chloride transport in the stomach (Machen and Forte, 1979) [12]. The neem oil, also known as oil of Margosa, is believed to have medicinal properties, such as antibacterial (Singh and Sastri, 1981) [20] antifungal (Kher and Chaurasia, 1977) [10].

Table 2: Phytochemical analysis of leaves extract of *Tinospora cordifolia*

Sr. No	Phytochemical Constitutes	Aqueous extract	Ethanol extract	Methanol extract	Acetone Extract
1	Alkaloid	+	+	+	+
2	Flavonoids	+	++	++	+++
3	Glycosides	+	+	+	+
4	Saponins	-	++	++	+
5	Steroids	-	+	+	+
6	Tannins	+	++	+++	+++
7	Anthroquinones	-	+	+	+
8	Phenolic compounds	-	+++	+++	+++

Table 3: Phytochemical analysis of leave extract of *Azarchita indica*

Sr. No	Phytochemical Constitutes	Aqueous extract	Ethanol extract	Methanol extract	Acetone Extract
1	Alkaloid	+	+	+	+
2	Flavonoids	-	-	-	-
3	Glycosides	+	+	+	+
4	Saponins	-	++	++	+
5	Steroids	-	+	+	+
6	Tannins	+	++	+++	+++
7	Anthroquinones	-	-	-	-
8	Phenolic compounds	-	+	+	+

Table 4: Phytochemical analysis of leaves extract of *Ocimum santum*

Sr. No	Phytochemical Constitutes	Aqueous extract	Ethanol extract	Methanol extract	Acetone Extract
1	Alkaloid	+	++	++	+++
2	Flavonoids	-	-	-	+
3	Glycosides	+	+	+	+
4	Saponins	-	++	++	+
5	Steroids	-	+	-	-
6	Tannins	+	++	++	++
7	Anthroquinones	-	-	-	-
8	Phenolic compounds	-	+	+	+
- : absent, +: present in low concentration, ++: present in moderate concentration, +++: present in high concentration					

Table 5: Antibacterial activity of *Tinospora cordifolia*, *Azarchita indica* and *Ocimum santum* extracts against bacterial pathogens (Zone of inhibition of growth in mm, average of 3 readings)

Medicinal Plants	Solvent extract	*P. vulgaris*	*S. epidermidis*	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. typhi*	*E. aerogenes*	*S. typhimurium*
Tinospora cordifolia	Aqueous	20	26	27	-	21	-	-	-
	Ethanol	22	26	25	21	21	19	17	23
	Methanol	22	32	27	22	23	17	19	18
	Acetone	22	32	27	21	21	17	19	17
Azarchita indica	Aqueous	17	32	27	-	20	17	-	-
	Ethanol	22	32	26	-	15	20	19	16
	Methanol	17	32	30	15	16	19	18	16
	Acetone	22	32	24	17	17	20	17	16
Ocimum santum	Aqueous	-	17	14	16	-	-	-	-

	Ethanol	19	18	17	20	16	-	15	-
	Methanol	14	20	19	17	14	14	15	-
	Acetone	16	24	23	20	15	15	21	-
Negative control	Water	-	-	-	-	-	-	-	-
	Ethanol	-	-	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-	-
	Acetone	-	-	-	-	-	-	-	-
Positive control	Ampicillin (10mcg/disc)	16	25	24	11	16	18	30	19

According to antibacterial profile (Table 5), maximum inhibitory effect of the aqueous extract observed only on *Staphylococcus epidermidis, Staphylococcus aureus,* and moderate antibacterial against *Escherichia coli, Pseudomonas aeruginosa, Enterobacter aerogenes,* but mild inhibitory effect on *Salmonella typhi, Salmonella typhimurium, Proteus vulgaris*. Methanol and ethanol extract showed strong antibacterial effect against *Staphylococcus epidermidis* and *Staphylococcus aureus* and moderate antibacterial against *Proteus vulgaris, Escherichia coli, Enterobacter aerogenes, Salmonella typhi* and *Salmonella typhimurium* but mild effect on *Pseudomonas aeruginosa.* Acetone extract showed maximum inhibitory effect on *Staphylococcus aureus, Proteus vulgaris, Staphylococcus epidermidis, Pseudomonas aeruginosa, Salmonella typhi, Salmonella typhimurium,* but moderate inhibitory effect on *Escherichia coli, Enterobacter aerogenes.* Several researchers have reported on the medicinal properties of plants derived compounds. These classes of compounds are known to show curative activity against several bacterial and it is not surprising that these plants extracts are used traditionally by herbalist to cure bacteria related ill-health.

Tinospora cordifolia also exerted considerable antibacterial effect against tested pathogens. However, it is ineffective against *E. faecalis* and *S. aureus* at lower concentrations with MIC value of 500 µg. This plant has been subjected to chemical investigations extensively and a number of chemical constituents belonging to different groups such as trepenoids, alkaloids, lignans and flavonoids, tannins, cardiac glycosides and steroids have been reported (Bansal *et al.,* 2012) which may account for the antimicrobial property of this agent.

Azarchita indica extract has shown antimicrobial activity against *Staphylococcus epidermidis, Staphylococcus aureus,* and moderate antibacterial against *Escherichia coli, Pseudomonas aeruginosa, Enterobacter aerogenes*. Prashant *et al*., demonstrated that Neem stick extract produced maximum zone of inhibition against *S. mutans* at 50% concentration. Even at 5% concentration, Neem extract was effective against all four species of microorganisms tested in their study (Prashant *et al.*,2007) [16] Bohora *et al*., concluded that Neem leaf extract has a significant antimicrobial effect against *E. faecalis*, *Candida albicans* and mixed culture (Bohora *et al.*,2010) [4]. Our study has shown the leaf extract of Neem is very effective against *S. mutans* and *S. aureus* with MIC value of 125 µg. The maximum antimicrobial activity was observed on *S. mutans* at 3 mg. concentration with zone of inhibition of (24.67 ± 2.517) mm. Neem contains different active phytoconstituents such as alkaloids, glycosides, trepenoids, steroids and tannins (Prabhat *et al.,* 2010).

Ocimum santum leaves extract have strong antibacterial effect against *Staphylococcus epidermidis* and *Staphylococcus aureus* and moderate antibacterial against *Proteus vulgaris, Escherichia coli,* The results of our study are in agreement with previous studies where different concentrations of Tulsi have been used against all three tested microorganisms (Geeta *et al*., 2001: Sharma *et al.,* 2009: Agrawal *et al.,* 2010: Mishra and Mishra, 2011: Joshi *et al.,* 2011) [5, 19, 1, 13, 8]. The biological properties of the plant has been attributed to the presence of active compounds like Ursolic acid, flavonoids and phenolic compounds (Gupta *et al.,* 2002) [6].

In the present study the biological activity of the acetone extract of *Tinospora cordifolia* can be attributed to the synergistic effect of the combination of flavonoids, steroids, terpinoids and saponins.

Conclusion

The results obtained in this study thus suggests that the identified phytochemicals may be the bioactive constituents responsible for the efficacy of leaves extract of *Tinospora cordifolia, Azarchita indica* and *Ocimum santum* against fever, syphilitic, ulcer, inflammatory disease wounds, conjunctivitis etc. Based on this, it suggested that the traditional medicinal use of *Tinospora cordifolia, Azarchita indica* and *Ocimum santum* be continued and scientific evaluation of its active constituents given serious attention. It is hoped that this study would lead to the establishment of some compounds that could be used to formulate new and more potent antimicrobial drugs of natural origin.

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