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Antifungal and phytochemical properties of *Tinospora cordifolia, Azadirachta indica* and *Ocimum sanctum* leaves extract

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Abstract
In the present study the aqueous, methanol, ethanol and acetone extract of *Tinospora cordifolia, Azadirachta indica and Ocimum sanctum* leaves extract were screened for the presence of phytochemical components and tested for antifungal activity against *Candida albicans, Aspergillus niger, Aspergillus fumigatus, Microsporum gypseum and Trichophyton rubrum* 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, where as aqueous extract were slightly higher antibacterial activity as ethanol extract. Antifungal activity of various extract of leaves of *Tinospora cordifolia, Azadirachta indica* and *Ocimum sanctum* would lead to the establishment of some compounds that could be used to formulate new and more potent antimicrobial drugs of natural origin.

Key words: *Tinospora cordifolia, Azadirachta indica* and *Ocimum sanctum,* leaves extracts, phytochemical Screening, antifungal activity

Introduction
The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization. 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) [14] . 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) [6].

Emergence of multidrug resistant pathogens has been reported to be one of the leading causes of death world (Reddy *et al.,* 2009) [15] wide with infectious diseases responsible for 68% of all deaths globally in 2012 (WHO, 2000) [19]. 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) [18].

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) [11]

Many researchers had studied antimicrobial activity of other parts of plant like bark, leaves and fruits of *Tinospora cordifolia, Azadirachta indica* and *Ocimum sanctum* 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, Azadirachta indica* and *Ocimum sanctum*. To prove the validity of traditional medicine the present work has been undertaken to evaluate the antimicrobial screening of leaves of *Tinospora cordifolia, Azadirachta indica* and *Ocimum sanctum* against the human bacterial pathogens.

Materials and Methods

Sample Collection

Tinospora cordifolia, Azadirachta indica and *Ocimum sanctum* 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 non polar 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, Azadirachta indica* and *Ocimum sanctum* 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) [8].

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

Fungal cultures: The standard pathogenic fungal cultures were procured from IMTECH, Chandigarh, India and used in the present study (Table 1). The fungal culture rejuvenated in Sabouraud dextrose media (Hi-media laboratories, Mumbai, India) at 37 ^{0}C for 18h and then stocked at 4 ^{0}C in SDA. Subcultures were prepared from the stock for bioassay. A loopful of culture was inoculated in 10 ml of sterile Potato Dextrose broth and incubated at 37 ^{0}C for 24h. Turbidity of the culture was standardized to 10^5 CFU with the help of SPC and turbidometer.

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

Fungal Strain	MTCC Number
Candida albicans	183
Aspergillus niger	478
Aspergillus fumigatus	870
Microsporum gypseum	7675
Trichophyton rubrum	296

Antifungal activity using disc diffusion method: The modified paper disc diffusion [17] was employed to determine the antifungal activity of solvent extract of leaves of *Pongamia pinnata* (L.). For antifungal properties, 0.1 ml fungal suspension of 10^5 CFU ml^{-1} was uniformly spread on PDA plate to form lawn cultures. The petroleum ether, chloroform, ethyl acetate and methanol extracts were prepared in their respective solvents in such a manner that ultimate amount (in dry form) in each disc came to 10mg, 8mg, 6mg, 4mg and 2mg. The blotting paper discs (10mm diameter) were soaked in various diluted extract, dried in oven at 60 ^{0}C to remove excess of solvent and tested for their antifungal activity against fungal pathogens by disc diffusion technique. After incubation of 24 h at 37 ^{0}C, zone of inhibition of growth was measured in mm. The antifungal activity was classified according to the zone of inhibition such as strong (19-22mm), moderate (15-18mm) and mild (11-14mm). Griseofulvin 10mcg (Hi-Media disc) was used as positive control while discs soaked in various organic solvents and dried were placed on lawns as negative control.

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. 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. 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. The neem oil, also known as oil of Margosa, is believed to have medicinal properties, such as antibacterial (Singh and Sastri, 1981) [17] antifungal (Kher and Chaurasia, 1977) [9].

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 4: Phytochemical analysis of leaves extract of *Ocimum sanctum*

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: Antifungal activity of *Tinospora cordifolia, Azadirachta indica* and *Ocimum sanctum* extracts against fungal pathogens (Zone of inhibition of growth in mm, average of 3 readings)

Medicinal Plants	Solvent extract	*C. albicans*	*A. niger*	*A. fumigatus*	*M. gypseum*	*T. rubrum*
Tinospora cordifolia	Aqueous	23	18	27	-	22
	Ethanol	20	16	25	16	24
	Methanol	24	22	27	20	26
	Acetone	24	26	27	21	26
Azadirachta indica	Aqueous	17	26	17	-	20
	Ethanol	22	28	16	-	15
	Methanol	17	32	25	16	16
	Acetone	22	32	28	18	17
Ocimum sanctum	Aqueous	-	17	14	16	-
	Ethanol	14	18	17	20	14
	Methanol	18	20	19	22	16
	Acetone	18	24	23	24	18
Negative control	Water	-	-	-	-	-
	Ethanol	-	-	-	-	-
	Methanol	-	-	-	-	-
	Acetone	-	-	-	-	-
Positive control	Griseofulvin (10mcg)	30	25	22	18	20

According to antifungal profile shown (Table 5), the petroleum ether extract exhibited strong inhibitory activity against *Candida albicans* and *Aspergillus niger,* but had a moderate antifungal activity against *Aspergillus fumigatus, Microsporum gypseum* and mild antifungal activity against *Trichophyton rubrum.* Chloroform extract showed moderate antifungal activity against *Candida albicans*, *Aspergillus fumigatus,* and mild antifungal activity against *Aspergillus niger, Microsporum gypseum, Trichophyton rubrum.* Ethyl acetate extract showed moderate antifungal activity against *Candida albicans, Aspergillus fumigatus,* and mild antifungal activity against *Aspergillus niger, Microsporum gypseum* and *Trichophyton rubrum.* Methanol extract showed strong antifungal activity against *Candida albicans* and *Aspergillus niger* but had moderate antifungal activity against *Aspergillus fumigatus* and mild antifungal activity against *Microsporum gypseum* and *Trichophyton rubrum.*

Plant extracts of *Tinospora cordifolia* (TC) have been reported to have potential against microbial infections. The anti-bacterial activity of Tinospora cordifolia extracts has been assayed against various Gram positive and Gram negative organisms. The antimicrobial activity of TC stem extracts was investigated against bacteria causing UTIs viz. uropathogens, Escherichia coli and Staphylococcus aureus. The study conducted using disc diffusion method showed that all three solvent extracts of TC reveal different antibacterial activity against both uropathogenic isolates with decreasing order as ethanolic (maximum) > methanolic (moderate) > aqueous (poor) (Priyanka *et al*., 2015) [13]. The larger zones of inhibition exhibited by *Tinospora cordifolia* extract against *A. niger* may be due to the presence of variety of active compounds. This is well known, since tannins and saponins are important plant metabolites which is responsible for their antimicrobial activity. From the results obtained, the stem extract of *Tinospora cordifolia* showed antifungal activity among the entire fungal organism. This suggests that *T. cordifolia* contains more of the active compounds and has

high potency. 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.

The methanol and ethanolic extract of *Azadirachta indica* against *Candida albicans, Aspergillus niger Aspergillus fumigates and Microsporum gypseum* was found growth inhibitory, as the zone of inhibition were observed and measured size of ZOI has been incorporated in table, also presented in Table 5 . Among all the extracts the most effective extract - methanolic extract of Azadirachta Indica against *Aspergillus niger* has been observed.

Azadirachta indica leaves possessed good anti fungal activity, confirming the great potential of bioactive compounds and is useful for rationalizing the use of this plant in primary health care (Saradha jyothi, Subbarao 2011) [16]. The extracts of Neem when used as medicinal plant, could be useful for the growth inhibition of the harmful fungus. The phyto-constituents alkaloids, glycosides, flavanoids and saponins are antibiotic principles of plants. These antibiotic principles are actually the defensive mechanism of the plants against different pathogens (Hafiza, 2000) [5].

Tulsi extract has also been shown to be effective against filamentous fungi such as *Aspergillus niger, Aspergillus fumigatus* (Dharmagadda *et al.,* 2005; Bansod and Rai, 2008) [3, 2], *Aspergillus flavus* (Kumar *et al.,* 2010) [10]. Other clinically important filamentous fungi like *Fusarium solani, Penicillium funiculosum, Rhizomucor tauricus and Trichoderma reesi* are also susceptible to Tulsi extract. (Dharmagadda *et al.,* 2005) [3]. The leaf extract has also been effective against fungi such as Rizopous, Cladosporium, Curvularia and Lunata. Finally, dermatophytic fungi were also found to be susceptible to Tulsi extract (Balakumar *et al.,* 2011) [1].

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, Azadirachta indica* and *Ocimum sanctum*. It may be concluded from this study that *Tinospora cordifolia* leaves extract creping on *Azadirachta indica* has potential antimicrobial activity similar to that of neem tree when compared to *Tinospora cordifolia* creping on fencing. This can explain that the host plant will gain some of the activities when they survive on medicinal plants. It is essential that research should continue to isolate and purify the active components of this natural herb and use in experimental animals

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