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Phytochemical Extraction and Antimicrobial Properties of *Azadirachta indica* (Neem)

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Abstract: Aqueous extracts of *Azadirachta indica* (Neem) was subjected to *in vitro* antibacterial assay against human pathogenic *Escherichia coli* and *Salmonella sp* by cup diffusion method. The plant leaves were effective against all the tested organisms. Minimum Bactericidal Concentration (MBC) value of 5mg/l was obtained against *Escherichia coli and Salmonella sp* were found to be resistant with all the solvent extracts except water. A qualitative phytochemical analysis was performed for the detection of secondary plant metabolites [viz., alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins] and reducing sugars. Thin layer chromatography (TLC) was also performed by using different solvent system for the analysis of lipid, alkaloids, flavonoids present in plant extract. The active components separated through TLC were subjected to antimicrobial activity against the pathogens. The present study will be successful in identifying candidate plant with different antimicrobial activity which could be further exploited for isolation and characterization of the novel phytochemicals in the treatment of infectious diseases especially in light of the emergence to produce more effective antimicrobial agents.

Key words: Cup diffusion method · Minimum Bactericidal Concentration · Phytochemical analysis *Escherichia coli* · *Salmonella sp.*

INTRODUCTION

Medicinal plants are part and parcel of human society to combat diseases, from the dawn of civilization. *Azadirachta indica* A. Juss (syn. *Melia azadirachta*) is well known in India and its neighboring countries for more than 2000 years as one of the most versatile medicinal plants having a wide spectrum of biological activity. The sanskrit name of the neem tree is 'Arishtha' meaning 'reliever of sickness' and hence is considered as 'Sarbaroganibarini'. The tree is still regarded as 'village dispensary' in India. The neem tree has been described as *A. indica* as early as 1830 by De Jussieu [1] and its taxonomic position is as follows:

- Order - Rutales
- Suborder - Rutinae
- Family - Meliaceae (mahogany family)
- Subfamily - Melioideae
- Tribe - Melieae
- Genus - *Azadirachta*
- Species - *indica*

Since the early report by Siddiqu [2] on the isolation of nimbin, the first bitter compound isolated from neem oil, more than 135 compounds have been isolated from different parts of neem and several reviews have also been published on the chemistry and structural diversity of these compounds. Antimicrobial effects of neem extract have been demonstrated against *Streptococcus mutants* and *S. faecalis*, a new vaginal contraceptive from neem oil showed inhibitory effect on the growth of various pathogens, including bacteria, fungi and viruses. Oil from the leaves, seeds and bark possesses a wide spectrum of antibacterial action against Gram negative and Gram-positive microorganisms, including *M. tuberculosis* and streptomycin resistant strains.

Clinical studies with the dried neem leaf extract indicated its effectiveness to cure ringworm, eczema and scabies. Lotion derived from neem leaf, when locally applied, can cure these dermatological diseases within 3–4 days in acute stage or a fortnight in chronic case. There have been very few reports on the clinical trials done with bioactive compounds isolated from neem. Sodium nimbidinate, the sodium salt of nimbidin, the main bitter

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principle isolated from neem seed oil, has been shown to act as a potent diuretic under various clinical conditions. Plant produce a wide variety of secondary metabolites which are used either directly as precursors or as lead compounds in the pharmaceutical industry and it is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant microbial pathogens.

However, there has been seldom effective collaboration between the traditional and western medical therapeutics, largely due to the perception that the use of traditional and herbal medicines has no scientific basis. According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, etc. which have been found In-vitro to have medicinal properties. So, our approach involved to explore the antibacterial activity of medicinal plant *Azadirachta indica* and study its antimicrobial constituents.

MATERIALS AND METHODS

Collection of Samples: Leaves were collected from the *Azadirachta indica* tree in the college campus. It was ensure that the plant was healthy and uninfected. The leaves were washed under running tap water to eliminate dust and other foreign particles and to clean the leaves thoroughly and a particular amount of leaves dried under shadow and some fresh leaves kept.

Solvent Extract: The dried and fresh leaves were trodden into small pieces, powdered and mixed in 1:10 ratio with ethanol, methanol, ether, acetone and distilled water separately. The extractions were obtained through continuous grind using mortar and pestle followed by filtration using Whattman No.1 filter paper. Then the filtrates were vacuum dried using rotary evaporator and the concentrates were stored at 4°C for further studies. The residues were redissolved with the appropriate solvents for the antibacterial assay.

Preparation of Standard Culture Inoculum of Test Organisms: *Escherichia coli* and *Salmonella sp,* were used for the study. Three or four isolated colonies were inoculated in 2 ml nutrient broth and incubated till the growth in the broth was equivalent with Mac-Farland standard (0.5%) as recommended by WHO.

Phytochemical Components: Phytochemical analyses were carried out according to the methods described by Trease and Evans [3] of the crude powder of leaves for the identification of phytochemicals like tannins, alkaloid, steroids, saponin and flavonoids.

Antibacterial Activity: Antibacterial activity of the different extracts was determined by cup diffusion method on Muller Hinton agar medium by Anon [4]. Wells are made in Muller Hinton agar plate using cork borer (5 mm diameter) and inoculums containing bacteria were spread on the solid plates with a sterile swab moistened with the bacterial suspension. Twenty micro-liters of the working suspension/solution of plant extracts and same volume of extraction solvent for control were filled in the wells with the help of micropipette. Plates were kept for some time till the extract diffuse in the medium and incubated at 37°C for 24 h. After incubation, the plates were observed for the zone of inhibition (ZI), the diameter of the inhibition zone were measured and recorded.

Separation of Active Compound from Neem Extracts Suspension by TLC

Preparation of Chromaplate: The glass slides were cleaned and dried in hot air oven. Slurry was prepared by mixing silica gel with double the volume of distilled water in a clean beaker. One drop of slurry was placed on the slide by using another slide edge, the drop of slurry was scattered all over to make thin film. The slides were kept as such for few minutes. Then the chromoplates were activated by heating in hot air oven at 120°C for 30 min.

Loading of Sample: The slides were allowed to cool at room temperature and marked about 2 cm from the bottom as the origin. The working suspensions were loaded at the center of each slide above from the edge.

Development of Chromatogram: Eskil Hultin[5] The development tank was saturated with suitable solvent systems as follows.

- Alkaloids: Benzene/ Methanol-80:20
- Flavonoids: Chloroform/Methanol-70:30
- Lipid: Chloroform/Methanol/water-10:10:3

The slides were kept in the tank without touching baseline by solvent. The final solvent front was marked and the slides were dried.

Spot Visualization: For visualization of Flavonoids 1% ethanolic solution of Aluminium chloride was used and viewed under 560nm UV light. Alkaloids were visualized under UV light and they were visible as yellow and orange fluorescent spots. Few pieces of iodine crystals were kept in the tank and covered with glass plate to saturate the tank with iodine vapor for detecting lipids. The plate was then kept in iodine vapor saturated tank and left for few hours and brown colored spots were visualized.

Retrieval of the Active Compound: Bishnu Joshi[6] Spots on the preparative silica gel slides were scratched with the help of clean and dry spatula and collected in beaker containing appropriate solvents and left overnight. The content in the beaker was stirred and filtrated through Whattman No. 1 filter paper. The filtrate was collected in clean and dry beaker. The filtrate containing active compound was used for the determination of antimicrobial effect by cup diffusion method.

RESULTS

A qualitative phytochemical analysis were performed for the detection of alkaloids, saponin, steroids, flavonoids and tannins Table 1). *Escherichia coli and Salmonella* sp were tested for antimicrobial activity. These organisms showed 12mm and 8mm the larger zone of inhibition in ethanol extraction. (Table 2 and Fig. 1). TLC were performed by different solvent system for the detection of alkaloid, flavonoids, lipids (Table 3). The separated active compounds alkaloid, flavonoids, lipid from TLC were found that more effective against all tested organisms in shade dried sample (Table 4) in fresh neem, lipids were ineffective against the tested organisms (Table 4).

Table 1: Phytochemical analysis of Neem [*Azadirachta indica*]

Phytochemical constituents	Acetone	Ethanol	Methanol	Ether	Distilled water
Alkaloids	+	+	+	+	+
Steriods	+	+	+	-	-
Saponin	+	+	+	-	-
Tanin	-	+	+	-	-
Flavonoids	+	+	+	+	+

Table 2: Antimicrobial activity of shade dried neem and fresh neem samples

	Fresh neem (in diameter)		Shade dried neem (in diameter)	
Solvents	*Salmonella*	*E.coli*		*E.coli*
Acetone	4mm	2mm	6mm	8mm
Ethanol	6mm	4mm	12mm	8mm
Methanol	4mm	6mm	8mm	6mm
Ether	4mm	4mm	6mm	4mm
Distilled water	nil	nil	Nil	nil

Table 3: RF values obtained in TLC for fresh and shade dried Neem extracts

	Fresh neem			Shade Dried Neem		
Solvents	Flavonoids	Alkaloids	Lipids	Flavonoids	Alkaloids	Lipids
Acetone	0.96	0.47	0.91	0.95	0.33	0.46
Methanol	0.91	0.56	0.92	0.96	0.28	0.54
Ethanol	0.92	0.52	0.90	0.94	0.45	0.37
Ether	0.86	0.64	0.37	0.93	0.92	0.91
Water	0.60	0.74	0.86	0.95	0.85	0.98

Table 4: Antimicrobial activity of active compounds from TLC for fresh and shade dried Neem extracts

		Fresh neem (in diameter)		Shade dried neem (in diameter)	
		Test organism		Test organism	
Active compounds	Solvents	*Salmonella*	*E.coli*	*Salmonella*	*E.coli*
Lipid	Acetone	nil	nil	6mm	nil
	Methanol	nil	nil	nil	2mm
	Ethanol	nil	nil	4mm	2mm
	Ether	nil	nil	3mm	nil
	Water	nil	nil	nil	nil
Flavonoids	Acetone	2mm	2mm	2mm	4mm
	Methanol	2mm	2mm	6mm	nil
	Ethanol	4mm	2mm	4mm	nil
	Ether	nil	nil	2mm	nil
	Water	nil	nil	nil	nil
Alkaloids	Acetone	nil	2mm	2mm	2mm
	Methanol	nil	nil	nil	nil
	Ethanol	nil	2mm	nil	3mm
	Ether	nil	nil	nil	nil
	Water	nil	nil	nil	nil

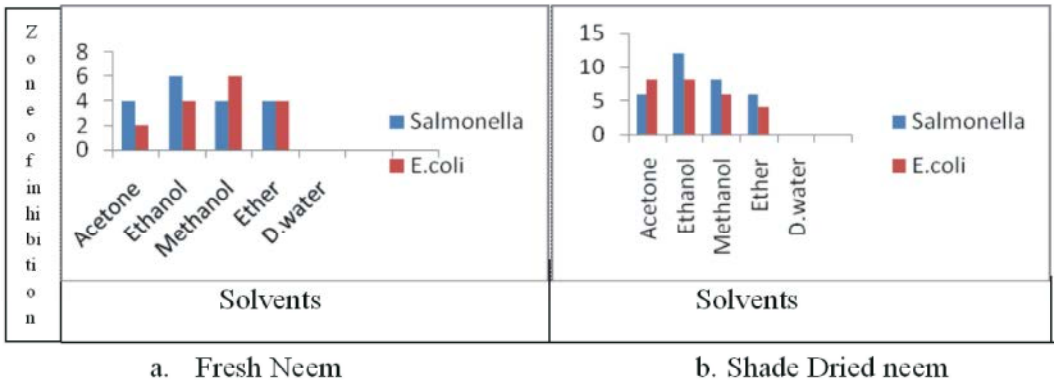

Fig. 1: Antimicrobial activity comparison

DISCUSSION

Plant essential extracts have been used for many thousands of years, in food preservation, pharmaceuticals, alternative medicine and natural therapies. Plant extracts are potential sources of novel antimicrobial compounds especially against bacterial pathogens. *In vitro* studies in this work showed that the plant extracts inhibited bacterial growth but their effectiveness varied.

The medicinal values of the secondary metabolites are due to the presence of chemical substances that produce a definite physiological action on the human body. The most important of these substances include, alkaloids, glucosides, steroids, flavonoids, fatty oils, resins, mucilages, tannins, gums, phosphorus and calcium for cell growth, replacement and body building (Kubmarawa *et al*. [7]). The phytochemical analysis of *A. indica* extract had earlier been reported by Kraus [8]. Qualitative analysis of phytochemical properties listed in Table 1.

The antimicrobial activity of many plant extracts had been previously reviewed and classified as strong, medium or weak (Zaika[9]). The inhibition produced by the plant extracts against particular organism depends upon various extrinsic and intrinsic parameters.

Due to variable diffusability in agar medium, therefore Zone of inhibition value has also been computed in this study (Table 2).

Chromatographic profiles of crude extracts obtained through different solvents were similar. The visualization of chromatographic profiles for each extraction technique and solvent used permit to evaluate the qualitative and quantitative variations in secondary metabolites content (Cristiane[10]). In addition, these data present compound profiles related to the biological effects and medicinal use (Table 3).

Salmonella sp which infects a number of animal species (Furowicz and Terzolo[11]) against plant extract and found to effective (Table 4). Intensive use of antibiotics often resulted in the development of resistant strains (Sydney[12]). These create a problem in treatment of infectious diseases, furthermore antibiotics sometimes associated with side effects (Cunha[13]) whereas there are some advantages of using antimicrobial compounds of medicinal plants such as often fewer side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature (Vermani and Garg, [14]).

CONCLUSION

These findings support the traditional knowledge of local users and it is a preliminary, scientific, validation for the use of these plants for antibacterial activity to promote proper conservation and sustainable use of such plant resources. Study suggested a number of active constituents might be present in the neem bark extract to control pathogens.

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