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Investigation of antibacterial compounds present in different solvent extracts of *Azadirachta indica*

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ABSTRACT

In this article, different solvent extracts of *Azadirachta indica* were tested for antibacterial action. Extraction of *Azadirachta indica* compounds were done using various solvents like ethanol, methanol, benzene, ethyl acetate and toluene that are frequently used as solvents in industries. The compounds showing antibacterial activity were supposed to be soluble in the above used solvents. In the present study, the antibacterial compound present in the different solvent extracts were compared with the standard drug (streptomycin) which is widely used in pharmaceutical industries. Qualitative analysis of the compounds extracted by a particular solvent was done with Thin Layer Chromatographic (TLC) technique which indicates the existence of different compounds. Statistical analysis (One way ANOVA) was performed and ethyl acetate extract showed a significant antibacterial activity against gram positive bacteria compared to other solvents (viz., ethanol, methanol, benzene and toluene). Similarly benzene extract showed a significant antibacterial activity against gram negative bacteria compared to other solvents (viz., ethanol, methanol, ethyl acetate and toluene).

Key words : *Solvent extraction, Gram positive and gram negative bacteria, Chromatographic analysis, Characterization.*

Introduction

Plants are the human best friends providing natural valuable herbal medicines for curing various ailments. *Azadirachta indica* is tree which is over loaded with number of compounds having wide medicinal applications (Biswas *et al.,* 2002 and Talwar *et al.,* 1997). Nimbidin, a major crude bitter principle collected in the leaves has several biological activities. From this crude principle some tetranortriterpenes, including nimbin, nimbinin, nimbidinin, nimbolide and nimbidic acid, ascorbic acid, amino acid, azadirchtin, etc have been isolated qualitatively (Kumar and Gupta, 2002; Sonia Bajaj and Srinivasan, 1999).

Azadirachta indica (*A. indica*) belongs to the botanic family Meliaceae, commonly known as Neem. It has applications in traditional medicine as a source of many therapeutic agents. *A. indica* (leaf, bark and seeds) possess antibacterial and antifungal activities against different pathogenic microorganisms; in addition to antiviral activity against vaccinia, chikungunya, measles, and Coxsackie B viruses. Different parts of neem (leaf, bark and seeds) show a wide pharmacological activities such as antioxidant, antimalarial, antimutagenic, anticarcinogenic, anti-inflammatory, antihyperglycaemic, antiulcer, and anti-diabetic properties. Due to the presence of many bioactive compounds in its different parts it possess variety of biological activities. Aqueous extracts from the leaves of Neem have a good potential in therapeutics as an antihyperglycaemic agent in insulin-dependent and non-insulin-dependent diabetes mellitus (Okemo *et al.,*2001). Hence the work aims in investigating the antimicrobial activity of Neem leaves

against human pathogenic bacteria.

Materials and Methods

Extraction from *Azadirachta indica* leaves

Juvenile and mature leaves from *Azadirachta indica* tree were collected from the campus of Anurag Group of Institutions, Hyderabad. Distilled water was used to wash the leaves and air dried at room temperature. 50 g of dried leaves were macerated in 100 mL of pure solvent and extract was collected after 48 hours and two more consecutive extracts from residue have been collected using same method (Biswas *et al.,* 2002; Subapriya and Nagini, 2005). The collected extracts were then cold centrifuged to remove suspended material and oven dried at temperature corresponding to the boiling point of the solvent used. These extracts were stored at 4 °C in the refrigerator for further use. The solvents used were ethanol, methanol, benzene, ethyl acetate and toluene (Kumar and Gupta, 2002; Talwar *et al.,* 1997).

The extract obtained from different solvents was dissolved in dimethyl sulfoxide in the ratio of 1:1 and used as and when required.

Antibacterial Assay

The gram positive and gram negative bacteria was used for testing of different solvent extracts of *Azadirachta indica* (Awasthy *et al.,* 1999; Okemo *et al.,* 2001; Sonia Bajaj and Srinivasan, 1999).

Peptone medium was used for inoculating microorganisms which are incubated for 3-4 hours at 37 °C. Further they were used as inoculums (Aslam *et al.,* 2009; Subapriya and Nagini, 2005). Muller Hinton Agar of 3.8 g was added to 100 mL distilled water and autoclaved at 121 °C for 15 minutes at 15 lb pressure and poured in sterile petri plates up to a uniform thickness of approximately 4 mm and the agar was allowed to set at ambient temperature and used (Mondali *et al.,* 2009; Raja *et al.,* 2013). Bacterial suspension was inoculated on to the surface of Muller Hinton Agar medium and uniform growth of the bacteria was ensured.

The extract obtained from different solvents dissolved in dimethyl sulfoxide in the ratio of 1:1 is then placed on the inoculated agar surface using sterile forceps (Awasthy *et al.,* 1999; Maragathavalli *et al.,* 2011). The concentration of extracts used during this method is 10, 20, 30 µl. All these extracts were tested with the gram positive bacteria and gram negative bacteria. Standard Streptomycin was used for the comparison of results. Incubation was performed at 37 °C for 48 hours. Antimicrobial activity was estimated by measuring zone of inhibition using a scale.

Results and Discussion

Chromatographic analysis

Thin Layer Chromatography (TLC) analysis was performed for all solvent extracts of *Azadirachta indica* which evidences the presence of number of compounds present in the different solvent extracts (Cserhati *et al.,* 2007). The spots of different extracts were observed under UV light as shown in Figure 1. Different compounds identified were Nimbin, Ascorbic acid, Amino acids, Azadirachtin. This shows that every individual solvent has its own capacity to extract different compounds based on their respective polarities (Goutam Brahmachari, 2004; Prus and Kowalska, 2001).

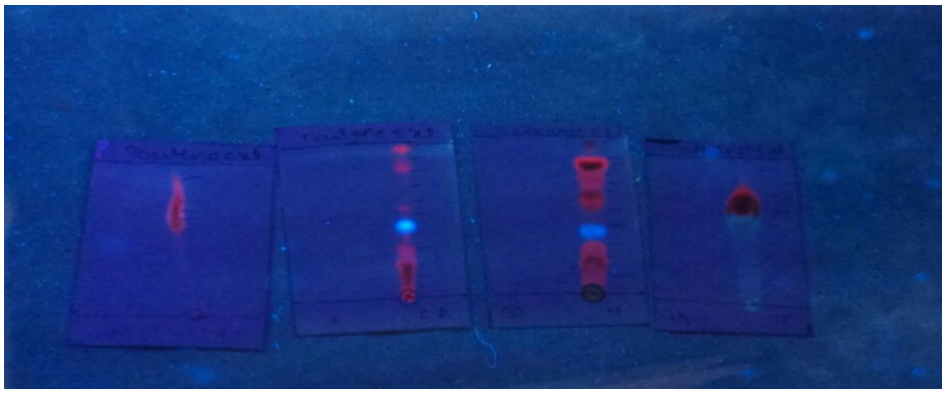

Fig. 1. Thin Layer Chromatographic analysis of different extracts.

Result of antibacterial assay

In this article, different solvent extracts of *Azadirachta indica* were tested which shows the antibacterial activity. Figure 2(a), 2(b) and 2(c) shows zones of inhibition of ethyl acetate and benzene extract of *Azadirachta indica* and standard drug (streptomycin) on gram positive and gram negative bacteria respectively after incubating at 37 °C for 48 hours. Zones of inhibitions in Muller Hinton Agar medium were observed in the respective petri dishes using the gram positive and gram negative bacteria. Standard drug (streptomycin) was used for comparing the results. Different volumes viz., 10 µL, 20 µl and 30 µl of standard drug (streptomycin) and various solvent extracts were tested on gram positive and gram negative bacteria separately and the results were shown in Table 1 and 2.

Table 1. Zones of inhibition for different types of extracts on gram positive bacteria.

Type of Extract	Volume of Extract (µL)		
	10	20	30
	Zone of Inhibition (mm)		
Standard (Streptomycin)	3.2	3.4	3.4
Ethanol	1.4	1.5	1.6
Methanol	1.4	1.4	1.4
Benzene	1.5	1.7	1.9
Ethyl acetate	1.9	1.9	2.2
Toluene	*0*	*0*	*0*

Volume of extract vs zone of inhibition (for gram positive and gram negative bacteria) were plotted for different solvent extracts of *Azadirachta indica* in Figure 3 and 4 respectively and their zones of inhibition were compared with the standard drug for the antibacterial activity on gram positive bacteria and gram positive bacteria. Types of extracts that exhibits antibacterial activity on gram positive bacteria in increasing order are as follows:

Table 2. Zones of inhibition for different types of extracts on gram negative bacteria.

Type of Extract	Volume of Extract (µl)		
	10	20	30
	Zone of Inhibition (mm)		
Standard (Streptomycin)	2.3	2.6	3.0
Ethanol	1.4	1.5	1.7
Methanol	1.2	1.4	1.5
Benzene	1.7	1.85	2.1
Ethyl acetate	1.5	1.9	2.0
Toluene	0	0	0

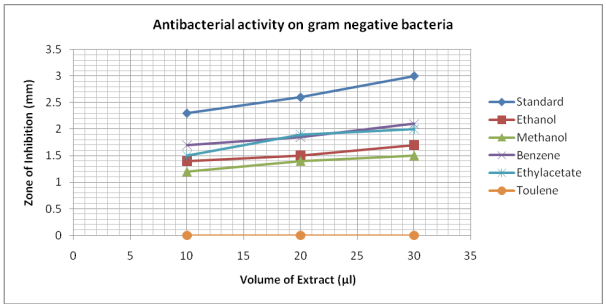

Fig. 3. Antibacterial activity of different solvent extracts of *Azadirachta indica* on gram positive bacteria.

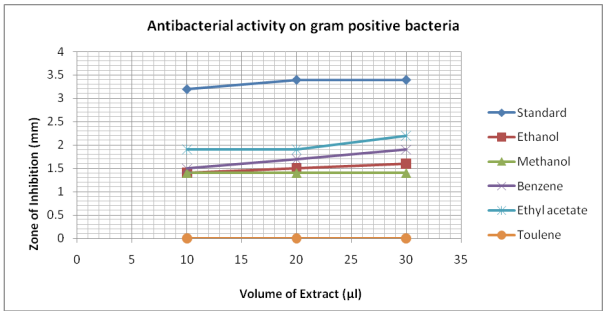

Fig. 4. Antibacterial activity of different solvent extracts of *Azadirachta indica* on gram negative bacteria.

Toluene< Methanol< Ethanol<Benzene<Ethyl acetate<Standard

(a) (b) (c)

Fig. 2(a). Zones of inhibition of Ethylacetate (b) Zones of inhibition of Benzene (c) Zones of inhibition of Standard drug (streptomycin).

And for gram negative bacteria in increasing order are as follows:

Toluene < Methanol < Ethanol < Ethyl acetate< Benzene < Standard

The above results indicate that the extracts exhibit almost similar antibacterial activity for both gram positive and gram negative bacteria. The minute difference in the activity between gram-negative and gram-positive bacteria may be due to the variation in their cell wall structure.

Statistical Analysis

One-way ANOVA was performed to check whether any significant effect on antibacterial activity was observed against gram positive bacteria by using the solvents viz., ethanol, methanol, benzene, ethyl acetate and toluene. Zones of inhibition (mm) against gram positive bacteria were tabulated for different volumes as shown in the given Table 3.

Table 3. Zones of inhibition against gram positive bacteria

	Zones of Inhibition (mm)			
Ethanol	Methanol	Benzene	Ethyl acetate	Toluene
1.4	1.4	1.5	1.9	0
1.5	1.4	1.7	1.9	0
1.6	1.4	1.9	2.2	0

From the above Table 3 null hypothesis and alternate hypothesis can be framed. They are:

Null hypothesis (H0): There is no significant antibacterial activity observed on gram positive bacteria among different extracts of the solvents.

Alternate Hypothesis (H1): There is a significant antibacterial activity observed on gram positive bacteria among different extracts of the solvents.

One-way ANOVA was performed using Microsoft Excel 2007 and the results were shown in Table 4.

From the above Table 4 it was observed that the probability value was less than 0.05 ($p<0.05$) and even $F_{cal} > F_{crit}$ for source of variation between the groups (i.e between different solvent extracts). Hence Null hypothesis (H0) is rejected and alternate hypothesis (H1) is accepted as shown in Figure 5.

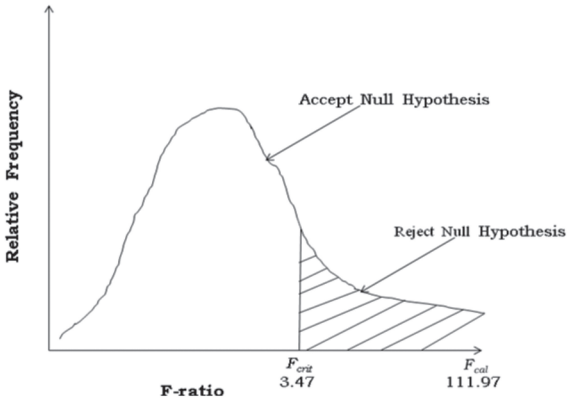

Fig. 5. Effect of antibacterial activity on gram positive bacteria by different solvent extracts.

Hence even statistically it can be concluded that ethyl acetate showed a significant change in the antibacterial activity on gram positive bacteria among different solvent extracts used (viz., ethanol, methanol, benzene and toluene)

Similarly One-way ANOVA was performed to

Table 4. ANOVA (One-way) with different solvent extracts against gram positive bacteria

Groups	Count	Sum	Average	Variance
Ethanol	3	4.5	1.5	0.01
Methanol	3	4.2	1.4	7.4E-32
Benzene	3	5.1	1.7	0.04
Ethyl acetate	3	6	2	0.03
Toluene	3	0	0	0

ANOVA

Source ofVariation	SS	df	MS	F_{cal}	P-value	F_{crit}
Between groups	7.164	4	1.791	111.9375	2.93E-08	3.47805
Within groups	0.16	10	0.016			
Total	7.324	14				

SS: Sum of Squares, df : degrees of freedom, MS: Mean of Squares, F_{cal}: Fcalculated value, P-value: Probability value, F_{crit}: F-critical value

check whether any significant effect on antibacterial activity was observed against gram negative bacteria by using the solvents viz., ethanol, methanol, benzene, ethyl acetate and toluene. Zones of inhibition (mm) against gram negative bacteria were tabulated for different volumes as shown in the given Table 5.

From the above Table 5 null hypothesis and alternate hypothesis can be framed. They are:

Null hypothesis (H0): There is no significant antibacterial activity observed on gram negative bacteria among different extracts of the solvents.

Alternate Hypothesis (H1): There is a significant antibacterial activity observed on gram negative bacteria among different extracts of the solvents.

One-way ANOVA was performed using Microsoft Excel 2007 and the results were shown in Table 6.

From the above Table 6 it was observed that the probability value was less than 0.05 ($p<0.05$) and even $F_{cal} > F_{crit}$ for source of variation between the groups (i.e between different solvent extracts). Hence Null hypothesis (H0) is rejected and alternate hypothesis (H1) is accepted as shown in Figure 6

Hence even statistically it can be concluded that benzene showed a significant change in the antibacterial activity on gram negative bacteria among different solvent extracts used (viz., ethanol, methanol, ethyl acetate and toluene).

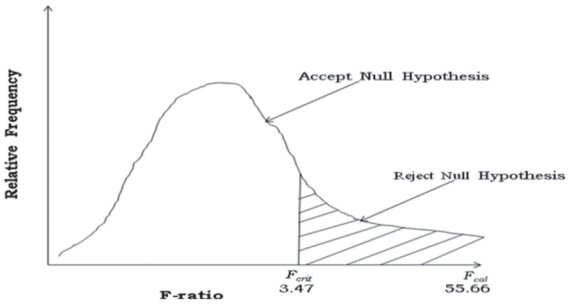

Fig. 6. Effect of antibacterial activity on gram negative bacteria by different solvent extracts.

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Table 5. Zones of inhibition against gram negative bacteria

	Zones of Inhibition (mm)			
Ethanol	Methanol	Benzene	Ethyl acetate	Toluene
1.4	1.2	1.7	1.5	0
1.5	1.4	1.85	1.9	0
1.7	1.5	2.1	2	0

Table 6. ANOVA (One-way) with different solvent extracts against gram negative bacteria

Groups	Count	Sum	Average	Variance
Ethanol	3	4.6	1.533333	0.023333
Methanol	3	4.1	1.366667	0.023333
Benzene	3	5.65	1.883333	0.040833
Ethyl acetate	3	5.4	1.8	0.07
Toluene	3	0	0	0

ANOVA

Source ofVariation	SS	df	MS	F_{cal}	P-value	F_{crit}
Between groups	7.013333	4	1.753333	55.66138	8.49E-07	3.47805
Within groups	0.315	10	0.0315			
Total	7.328333	14				

SS: Sum of Squares, df : degrees of freedom, MS: Mean of Squares, F_{cal}: Fcalculated value, P-value: Probability value, F_{crit}: F-critical value

Conclusion

It was investigated from the above results that ethyl acetate and benzene extracts were showing maximum antibacterial activity compared with all other extracts. It concludes that benzene extracts are having maximum concentration of compounds from chromatographic analysis which are antibacterial in nature. Inhibition zone depends on the concentrations of extracts used during the testing of samples.

Therefore from the above discussion, it can be concluded that *Azadirachta indica*, a common medicinal plant can be exploited as a source of potent biocide as it contains large number of compounds that can be used for variety of applications in all types of industries especially for medicinal purposes.

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