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Phytochemical Screening And Antimicrobial Activity Of Fresh And Shade Dried Leaves Of Azadirachta Indica

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Abstract- This study includes the pytochemical detection and antimicrobial activity of the leaves of Azadirachta indica. It was carried out to evaluate the effect of ethanol, acetone and methanol extracts of neem leaves on the growth of some human pathogens such as Aspergillus flavus and Aspergillus niger in in- vitro. Different concentrations (5, 10, 15 and 20%) prepared from these extracts inhibited the growth of the test pathogens and the antifungal activity gradually increased with the concentration. The Antibacterial and Antifungal activity of methanol, ethanol and acetone extracts of leaves of Azadirachta indica were evaluated against the human pathogenic bacteria such as Escherichia coli and Staphylococcus aureus by agar well diffusion method and fungal species such as Aspergillus flavus and Aspergillus niger by disc diffusion method.

Keywords– Aspergillus flavus, Aspergillus niger, Staphylococcus aureus, Escherichia coli, Antimicrobial activity, Azadirachta indica, Antifungal activity.

I. INTRODUCTION

Azadirachta indica (Meliaceae) is commonly known as neem. It is a native of India and it is mostly found in tropical and sub tropical countries. It is widely spread in the world and is of great medicinal value [19]. Some active phytoconstituents are present in neem such as steroids, glycosides, alkaloids and tannins [4]. Eczema, ringworm, acne, antihyperglycemic properties, anti inflammation are treated effectively by neem leaves. It helps to purify our blood and neutralize the free radicals. Neem leaves act as an anticancer agent [10]. A wide variety of secondary metabolites are present in Neem such as tannins, flavonoids, alkaloids, etc. and it is found in-vitro having medicinal property [2]. A mixture of seven isomeric compounds is termed as Azadirachtin. The compound labeled as Azadirachtin A-G, among that Azadirachtin E is more effective [13]. The commonly used chromatography methods such as thin layer chromatography used for fast analysis, provision of semiquantitative information and qualitative deduction of the drug [6]. Neem has a wide range of therapeutic properties such as antifungal, antiviral, anti inflammatory, antibacterial, analgesic and antioxidant [20]. The characteristics metabolites of this family are known as limonoids, which are tetranortriterpenoides that have considerable interest because of structural diversity [3]. Every part of the neem tree is bitter and finds the application indigenous [11]. The medicinal property and biological activity of neem has been reported recently [17]. The application of neem includes healing and antiseptic properties. It is used in medicinal soaps, creams and toothpastes.

Dental caries and oral health/dental health are multifactorial diseases related to diet, oral microbiota, hygiene, salivary characteristics, and are inseparable part of general health, which can lead to considerable pain and suffering. It has an impact on a person's speech, selection of food, quality of life, and general well-being. During the last 20 years neem has been introduced in many countries to overcome afforestation [7]. In view of the prevalence of oral diseases, their impact on individuals and society and the expenses of treatment, they may be considered a major public health problem and they are listed among the most common of the chronic diseases that affect mankind. Neem have attracted entomologists and phytochemists all over the world. Oral diseases are the fourth most expensive diseases to treat in certain countries. Azadirachta indica is a herbal alternative to treat diseases of oral cavity.

Clinical studies with dried neem leaf extract indicated its effectiveness in curing ring worm, 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 cases. There have been very few reports on the clinical trials done with bioactive compounds isolated from neem. Variuos religious documents such as Bible and Quran supported the role of neem in health care and prevention [12]. Sodium nimbidinate, the sodium salt of nimbidin, the bitter component isolated from neem seed oil acts as a potent diuretic under various clinical conditions. Neem is rich with antioxidant and other valuable active compounds such as nimbidin, salannin, quercetin and nimbolinin. Plants 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 have no scientific basis. According to the 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, flavanoids, etc. which have been found in-vitro to have medicinal properties [14]. The present study was aimed to evaluate the phytochemical analysis and the antimicrobial potential of leaves of Azadirachta indica against microbial pathogens.

II. MATERIALS AND METHODS

2.1. Collection Of Raw Materials

The healthy and uninfected leaves of Azadirachta indica (Neem) were collected from Pondicherry. The fresh leaves and shade dried leaves of neem were used for this study. The neem leaves were washed thrice in 150 ml of water and once in 50 ml of sterile distilled water. The neem leaves were shade dried for 5 days. The dried leaves were ground into a coarse powder. The powdered sample was weighed and sealed in separate polythene bags.

2. 2. Preparation Of Extract

One gram of the powdered sample was weighed and the extract was prepared by reflexing it with 10 ml of the ethanol, acetone and methanol solvents each separately. The extracts were filtered and collected in 100 ml beaker and they were wrapped with aluminium foil to prevent evaporation. The extracts of neem leaves were stored in an airtight containers at 4°C.

2. 3. Phytochemical Screening

The phytochemical analysis of the extract of Azadirachta indica for the determination of alkaloids, saponins, tannins, glycosides, flavonoids were carried out by using the methods described by Harborne (1973,1993), Sofowara (1982) and Trease and Evans (1983).

2. 3. 1. Determination Of Alkaloids

2 g of Azadirachta indica of fresh and shade dried leaves were extracted and were treated with 5 ml of 50% methanol, ethanol and acetone solution. Then 3-4 drops of 2% of picric acid solution was added to the methanol, ethanol and acetone extract.

2. 3. 2. Determination Of Flavonoids

4 g of neem extract of fresh and shade dried leaves was taken and it was treated with 6 ml of 50% methanol, ethanol and acetone solution. The solutions were warmed and 1.5 g of metal magnesium wasadded to the neem leaves extract solution. Then 4-5 drops of concentrated hydrochloric acid was added.

2. 3. 3. Determination Of Saponins

6 g of Azadirachta indica of fresh and shade dried leaves were extracted and it was treated with 8 ml of 50% methanol, ethanol and acetone solution. Saponins were detected using the froth test. 10 ml of sterile distilled water was added to 1 g of sample in a conical flask and boiled for 5 minutes. The mixture was filtered and 2 ml of the filtrate was added to 10 ml of the sterile distilled water in a test tube. The test tube was shook vigorously for about 30 seconds. Then it was allowed to stand for half an hour.

2. 3. 4. Determination Of Tannins

8 g of neem extract was extracted from fresh and shade dreied leaves and treated with 10 ml of 50% methanol, ethanol and acetone solution. Add 3 to 4 drops of 10% ferric chloride solution to neem extract.

2. 3. 5. Determination Of Glycosides

10 g of neem leaves extract was taken in a test tube with 12 ml of 50% methanol, ethanol and acetone solution. 25 ml of dilute sulphuric acid was added and boiled for 15 minute. Then it was allowed to cool and neutralized with 3 ml of 10% sodium hydroxide and 5 ml of Fehling solution was added.

IV. DETERMINATION OF EXTRACTS

4. 1. Ethanol Extract

10 g of Azadirachta indica leaves were ground into a fine powder [8] using a stainless steel grinder and dipped in 100% ethanol (200 ml) for 12 hours by using a sterile muslin cloth. The ethanol fraction were separated and filtered through the sterile Wattman filter paper.

4. 2. Acetone Extract

For the preparation of the Acetone extract (150 ml), 10 g of Azadirachta indica leaves were added in acetone and left for 12 hours at room temperature at 37 ºC [5].

4. 3. Methanol Extract

10 g of dried plant material was extracted with 100 ml of methanol. It was kept in the rotary shaker for 24 hours. It was filtered and centrifuged at 5000 x g for 15 minutes. 1 ml of supernatant was collected and the solvent was evaporated to make the final volume to one fifth of the original volume. It was stored at 4ºC in airtight bottles.

V. SOURCES OF MICROORGANISMS

Bacterial species such as Staphylococcus aureus and Escherichia coli and Fungal species such as Aspergillus niger and Aspergillus flavus were used in this study and it was obtained from Krishna Microbiology Laboratory, Cuddalore, Tamil Nadu, India.

VI. DETERMINATION OF ANTIBACTERIAL ACTIVITY

6. 1. Preparation Of Culture Medium

1000 ml of distilled water was boiled in a round bottom flask. 3.5 g of NaCl, 10 g of agar, 7.5 g of beef extract and peptone were added into the flask and continuously stirred. The flask was wrapped tightly with aluminium foil. Further the medium was sterilized at a pressure of 15 lbs at 121ºC for 20 mins in autoclave. The pH of the nutrient agar medium was adjusted to 7.2.

6. 2. Well Diffusion Method

The antibacterial activity of the obtained neem leaves extract was determined using agar well diffusion method. The nutrient agar medium was inoculated with the selected bacterial species such as Staphylococcus aureus and Escherichia coli. Then the wells were punched and filled with neem leaves extracts. Control wells containing the solvents (negative control) were run parallel in the same plate. The plates were incubated at 36º C for 15 hours and the antibacterial activity was analyzed by measuring the diameter of the zone of inhibition [18].

VII. DETERMINATION OF ANTIFUNGAL ACTIVITY

7. 1. Disc Diffusion Method

Antifungal test was carried out by disc diffusion method. 80 µl of suspension which contains 90 spores per ml of fungi is spread on sabouraud dextrose agar. The solidified agar plates were swabbed by uniform spreading of 0.2 ml of fungal culture. In each plate, 5 mm diameter of a non- contaminated sterile disc was placed. With the help of micropipette, 10 µl (1 mg/ml) of the extract was taken and placed on the disc. Then it was subjected to the formation of the zone of inhibition.

7. 2. Minimum Inhibitory Concentration

100 µl of Sabouraud Dextrose Agar (SDA) broth was taken and placed in well. Freshly grown microorganism were maintained in sterilized condition. It was placed in microtitre plate and labelled properly. Serial dilution of the extracts were placed in an appropriate microtitre plate. Methyl orange indicator was added in each well and made sure that the mixture is well mixed. Then the microtitre plate was placed in a dark room.

VIII. RESULTS AND DISCUSSION

8.1. Phytochemical Screening

8. 1. 1. Determination Of Alkaloids

After the performance of test for the determination of alkaloids, the extract solution changed into orange colour. The appearance of orange colour indicates the presence of alkaloids.

8. 1. 2. Determination Of Flavanoids
After the addition of concentrated hydrochloric acid to the neem leaves extract solution, it turns into red colour. Red colour was observed for flavanoids and orange colour for flavones.

8. 1. 3. Determination Of Saponins
Saponins were determined using the Froth test. By adding 10 ml of sterile distilled water to neem leaves extract, the honey comb froth was observed. This indicates the presence of saponins.

8. 1. 4. Determination Of Tannins
By adding 10% of ferric chloride solution to neem leaves extract, the two colours blue and green were observed. Blue colour was observed for gannic tannin and green colour for catecholic.

8. 1. 5. Determination Of Glycosides
Glycosides were determined by adding 10% of sodium hydroxide and 5 ml of Fehling solution. Brick red precipitate was observed which indicates the presence of glycosides.

8. 2. Antibacterial Activity
The phytochemical activity of both fresh leaves and shade dried leaves extract resulted in the presence of alkaloids, glycosides of methanol extract. The components such as alkaloids, flavanoids, saponins, tannins and glycosides were present in ethanolic extract and components such as alkaloids and glycosides were present in acetone extract respectively.

Table- 1 Phytochemical screening of the fresh and shade dried leaves of Azadirachta indica

	Fresh leaves extract			Shade	dried leaves extract	
Chemical constituents	Methanol	Ethanol	Acetone	Methanol	Ethanol	Acetone
Alkaloids	+	+	+	+	+	+
Flavanoids	_	+	_	_	+	_
Saponins	_	+	_	_	+	_
Tannins	_	+	_	_	+	_
Glycosides	+	+	+	+	+	+

In Table- 1, there is a presence of different phytochemicals with various phytochemical activities which has valuable therapeutic index [9] [15]. In the methanol extract of Azadirachta indica, it has been observed that biologically active phytochemicals were present. Staphylococcus aureus is a gram positive, round shaped bacterium which is found in respiratory tract, skin and nose [16]. Escherichia coli a gram negative, rod shaped bacterium normally found in intestines of healthy people and animals. The antibacterial activity of the obtained leaves of Azadirachta indica was tested by using the well diffusion agar method against bacterial species Staphylococcus aureus and Escherichia coli.

Figure 1. This shows the antibacterial activity of samples by using well diffusion method

In the Figure 1, the result shows that the ethanolic extract of fresh and shade dried leaves at concentration of 200 µl/disc, provides various inhibition zone Staphylococcus aureus and Escherichia coli. Zone of inhibition is higher in the shade dried leaves (19 mm in Staphylococcus aureus and 20 mm in Escherichia coli). Ethanol extract showed an

increased zone of inhibition compared to the Methanol extract. Acetone extract did not record any antibacterial activity.

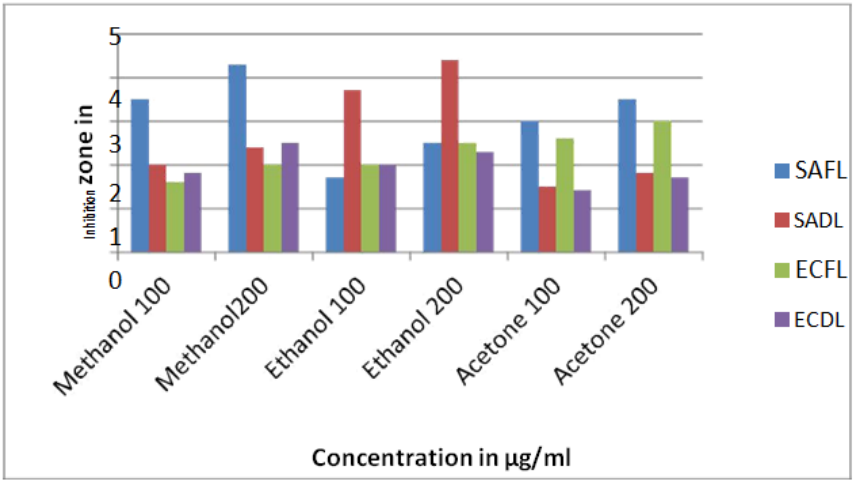

Figure 2 Antibacterial activity of fresh and shade dried leaves extract of Azadirachta indica

SAFL- Staphylococcus aureus on fresh leaves
SADL- Staphylococcus aureus on shade dried leaves
ECFL- Escherichia coli on fresh leaves
ECDL- Escherichia coli on shade dried leaves

These phytochemical components are present which may be responsible for the observed antibacterial activity of the neem leaves extract.

8. 3. ANTIFUNGAL ACTIVITY

The present study of Azadirachta indica shows the antifungal activity in methanol extract, ethanol extract and acetone extract. The zone of inhibiton was observed against Aspergillus niger and Aspergillus flavus. Ethanol extract was having the maximum efficacy in all the Aspergillus niger and Aspergillus flavus. The maximum zone of inhibition was observed in ethanol extract of Azadirachta indica at 600 µg/ml is 12.30 mm diameter and the maximum zone of inhibition was observed in methanol extract of Azadirachta indica at 600 µg/ml is 10.54 mm diameter but the acetone extract at 300 µg/ml is 10.02 mm diameter zone of inhibition against Aspergillus niger. The maximum zone of inhibition was observed in ethanol extract of Azadirachta indica at 600 g/ml is 11.69 mm diameter and the maximum zone of inhibition was observed in methanol extract of Azadirachta indica at 600 g/ml is 12.07 mm diameter but the acetone extract at 300 g/ml is 9.65 mm zone of inhibition against Aspergillus flavus.

(a) (b)

Figure 3 (a) and (b) shows the antifungal activity by well diffusion method

IX. CONCLUSION

From the present work, it was can be indicated that the crude methanol extracts of Azadirachta indica have a great potential as antimicrobial agent. This work shown that the Neem leaves extract of both fresh and shade dried leaves of Azadirachta indica against two bacterial species such as Staphylococcus aureus and Escherichia coli and against two fungal species such as Aspergillus flavus and Aspergillus niger. The antimicrobial property of the Neem leaves extract may be due to the presence of above mentioned phytochemicals detected in ethanol, methanol and acetone extract during phytochemical screening.

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