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PHYTOCHEMICAL AND ANTIBACTERIAL STUDIES OF ANNESLEA FRAGRANS W.

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ABSTRACT
Anneslea fragrans W. belongs to the family Theaceae and it is an evergreen shrub or small tree. The aim of this study was to investigate the Phytochemical constituents and antibacterial activity. The Phytochemical constituents screened by qualitative method. I evaluated the phytochemical analysis for the presence of various secondary metabolites like saponins, tannins, steroids, alkaloids, phenols, terpenoids, inulin, flavanoids, phlobatannin and antibacterial activity of the leaf extracts of *A. fragrans* against pathogenic bacteria like gram positive *Staphylococcus aureus* and gram negative *Escherichia coli* bacteria with significant zone of inhibition by in vitro agar disc diffusion method. Methanolic extract of leaf showed inhibitory action on both bacteria.

KEYWORDS: *Anneslea Fragrans* W., Phytochemical Constituents, Antibacterial Activity.

INTRODUCTION

Since the beginning of civilization, plants are very important for us as they give food, shelter, medicines and many other products like fuel, paints etc. Now a day their chemical and genetic characters are explored for human benefits. Ethnobotany, in broad sense, may be defined as relationship between people and plants. It may also be defined as the scientific study of interaction between human cultures and plants (Turner, 1995). The term Ethnobotany was coined in 1896 by John Harshberger whereas Ford (1978) elaborated its scope. Early herbalists believed that the plant part resembling any part of the human body was considered useful for the ailment of those parts and there is no part of the body without its corresponding herb, a hypothesis known as the "Doctrine of Signature" (Baquar., 2001). It has been estimated that 14-28% of higher plant species are used medicinally and that 74% of pharmacologically active plant derived components were discovered after following up on ethno medicinal use of the plants (Das *et al*., 2010).

Phytochemistry is a distinct discipline somewhere in between organic chemistry, plant biochemistry and closely related to natural products. It deals with a variety of organic substances accumulated in plants. The plant may be considered as a biosynthetic laboratory. Not only their chemical compounds such as carbohydrates, protein, and lipids that are used as food by man, but also a multitude of compounds like glycosides, alkaloids, flavanoids, etc. are used as medicines by him in various ways and means. Since generations, it is an important herbal formulations that are used in traditional systems of medicine.

The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions must be taken to reduce this problem, for example, to control the use of antibiotic, develop research to better understand the genetic mechanisms of resistance, and to continue studies to develop new drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antimicrobial drugs to the patient.

According to World Health Organization (WHO), 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. Now it is essential to investigate newer drugs with lesser resistance. Drugs derived from natural sources play a significant role in the preservation and treatment of human diseases.

***Anneslea fragrans* W.**
Kingdom : Plantae
Order : Ericales
Family : Theaceae
Genus : Anneslea
Species : *A. fragrans*

Phytography (Description)
Anneslea fragrans is a species of shrubs or trees, 3–15 meters tall. Bark dark brown. Leaf blade ovate, elliptic,

obovate-elliptic, oblong or oblong-lanceolate; leathery to thinly leathery, pale green or glaucescent green and reddish brown glandular punctate. Axillary flowers are axillary, pale yellow. There are up to more than 10 in a corymb. Pedicel [(2-3)-(6-7)] cm; bracteoles 2, broadly ovate to triangular-ovate 4 × 3-3.5 mm, margin sparsely glandular. Sepals reddish, broadly ovate, 1-1.5 cm, basally slightly connate, margin glandular. Petals pale yellow, broadly ovate 1.5 cm, basally connate for 5 mm, apex acute. Stamens 30-40, 1.2-1.5 cm; filaments basally connate for 5 mm; anthers linear, glabrous, connective exserted. Ovary half inferior, glabrous, style 1.5-2 cm. The Fruit is ellipsoid 2-3.5 cm in diameter and contains long obovate seeds with 2 or 3 seeds per locule, with a fleshy red outer layer, dehiscent between persistent enlarged sepals. Seeds long obovate, 7-12 × 4.5-7 mm, with a fleshy red outer layer. Flower Oct-Mar, Fruit Jul-Sep. *A. fragrans* is a widespread and somewhat variable species with several varieties having been recognized primarily on leaf character differences.

MATERIAL AND METHODS

Collection of plant material: The fresh sample of leaves of *A. fragrans* were collected from Manipur. The collected leaves were properly washed with tap water, rinsed with distilled water, were dried in a well ventilated room.

Processing of samples: The collected dried leaves of the medicinal plants were pulverized using a sterile electric blender to obtain the powered form. The powered forms of the various medicinal plant samples were then stored in airtight glass containers, protected from sunlight until required for analysis. The powered forms of the samples were used for obtaining methanolic extract.

Preparation of methanolic extract of plant samples

For 25g of powdered sample of the collected plant, 150ml methanol was used i.e. in the ratio of 1:6 sample and solvent. The plant sample was placed in the thimble, whereas methanol was placed in the round bottom flask of the soxhlet apparatus. The temperature was set at 45^0C and the apparatus was run for 48 hours. The solution so obtained at the round bottom flask was the final methanolic extract.

Phytochemical Analysis

Qualitative Analysis: The extracts were subjected to preliminary phytochemical testing to detect for the presence of different chemical groups of compounds. Air-dried and powdered plant materials were screened for the presence of saponins, tannins, alkaloids, flavonoids, inulin, steroids, glycosides, anthraquinones, saponins, reducing sugars, starch and Phlobatannin as described in literature.

Table No. 1: A brief summary of phytochemical screening of secondary metabolites in plants.

Secondary metabolites	Name of test	Methodology	Result(s)	Reference(s)
Alkaloid	Wagner test	Add 2ml filtrate with 1% HCl + steam. Then add 1ml of the solution with 6 drops of Wagner's reagent.	Brownish-red precipitate	**(Chanda et al., 2006).**
Cardiac glycosides	Kellar – Kiliani Test	Add 2ml filtrate with 1ml of glacial acetic acid, 1ml ferric chloride and 1ml concentrated sulphuric acid.	Green-blue coloration of Solution	**(Parekh and Chanda, 2007).**
	Kellar- Kiliani test	Dissolve 50 mg of methanolic extract in 2 ml of chloroform. Add H_2SO_4 to form a layer.	Brown ring at interphase	**(Onwukaeme et al., 2007).**
Flavonoid	Shinoda test	To 2-3ml of methanolic extract, add a piece of magnesium ribbon and 1ml of concentrated hydrochloric acid.	Pink red or red coloration of the solution	**(Kumar et al., 2007).**
Phenol	Phenol test	Spot the extract on a filter paper. Add a drop of phosphomolybdic acid reagent and expose to ammonia vapors.	Blue coloration of the spot	**(Kumar et al., 2007);**
Phlobatannin	-	2 ml extract was boiled with 2 ml of 1% hydrochloric acid HCl.	Formation of red precipitates	**(Edeoga et al., 2005).**
Reducing sugar	Fehling test	Add 25ml of diluted sulphuric acid (H_2SO_4) to 5ml of water extract in a test tube and boil for 15mins. Then cool it and neutralize with 10% sodium hydroxide to pH 7 and 5ml of Fehling solution.	Brick red precipitate	**(Akinyemi et al., 2005)**
Steroid	Liebermann-Burchardt test	To 1ml of methanolic extract, add 1ml of chloroform, 2-3ml of acetic anhydride, 1 to 2 drops of concentrated sulphuric acid.	Dark green coloration	**(Kumar et al., 2007).**
Saponin	Frothing test / Foam test	Add 0.5ml of filtrate with 5ml of distilled water and shake well.	Persistence of frothing	**(Parekh and Chanda, 2007).**
Tannin	Braemer's test	10% alcoholic ferric chloride will be added to 2-3ml of methanolic extract (1:1)	Dark blue or greenish grey coloration of the solution	**(Kumar et al., 2007); (Parekh and Chanda, 2007).**
Naphthoquinone	-	1ml sample+ few drops of 10% KOH	No blue black coloration	
Inulin	-	1ml sample+ α-naphthol+ H_2SO_4	Formation of brownish red color	
Starch	-	2ml sample+iodine solution	No blue coloration	

The present study carried out on the plant sample revealed the presence of medicinally active constituents.

Table No. 2: Preliminary phytochemical analysis of A. fragrance.

No.	Secondary metabolites	Results Present(+)/Absent(-)
1.	Alkaloid	(-)
2.	Glycoside	(-)
3.	Flavonoid	(+)
4.	Phenol	(+)
5.	Phlobatannin	(+)
6.	Reducing Sugar	(-)
7.	Steroid	(+)
8.	Saponin	(+)
9.	Tannin	(+)
10.	Naphthoquinone	(-)
11.	Inulin	(+)
12.	Starch	(-)
13.	Quinoid	(+)

Antibacterial Assay

For Antibacterial activity strain *Staphylococcus aureus* Lyophilized culture was ordered from culture collection center MTCC Chandigarh with MTCC-11949.

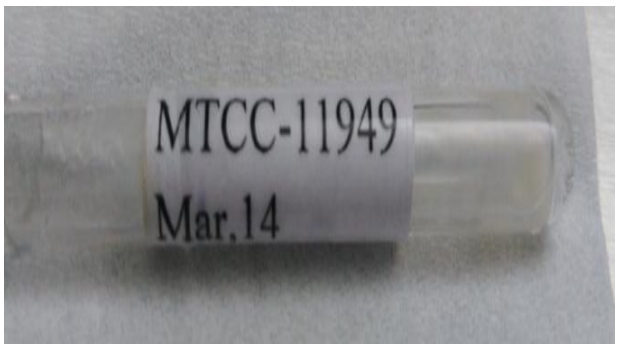

Figure. 1: Lyophilized culture from Microbial culture collection center Chandigarh.

Primary Inoculation
A day before preparing the cells, a single colony of *E. coli* DH5α and *Staphylococcus aureus* was inoculated from a fresh plate into 5ml LB tube and incubated for 16-20 hours at 37°C with vigorous agitation (overnight).

Secondary Inoculation
From the above tube, 100ml LB flask was inoculated with 1% inoculum (i.e. 1 ml in 100 ml) and incubated for 1½ to 2½ hours at 37°C with vigorous agitation monitoring the growth of the culture.

Now with the help of pipette dispense 100µl of inoculums on LB Agar Plate. A reusable glass or metal spreader should be flame sterilized by alcohol (70% isopropyl or ethanol). Then the spreader is allowed to cool. The spreader is brought in contact with the inoculum on the surface of the plate and positioned in such a way that the inoculum is run evenly along the length of the spreader. With the application of even pressure the spreader and the plate is spun by hand. A sterile whatman filter paper discs soaked with 10µl crude methanol extract was placed on it. After spreading keep plates on incubator at 37°C for 12-16 h. Antibacterial activity was evaluated by inhibition zone of bacterial growth. The results are represented as average zone of inhibition of all the isolates of individual species.

Preparation of Agar Plates
2% LB Agar plates were prepared: 500ml distilled water was taken in 1000ml conical flask and to this 25g of LB medium and 20g of agar was added. This medium was heated until the agar and LB medium dissolved completely. This medium was then transferred to the 1000ml measuring cylinder and volume was makeup to 1000ml by adding distilled water. Medium was transferred to the conical flask and covered with cotton plug. Medium was autoclaved for 15 minutes. Autoclaved medium was poured in sterilized petri plates and these were sealed with parafilm.

Screening of Plant Sample by Disc Diffusion Method
Disc diffusion screening was performed in the laminar air flow. LB Agar plate was taken and 20µl bacterial culture was poured and spread on it. Sterilized disc (whatman filter paper was cut into similar size discs and these disc were autoclaved) was then dipped in the plant extract and placed in center of the agar plate. Then Petri plates were sealed with parafilm and these were incubated overnight at 28°C in oven. Observations were taken next day.

Antibacterial Screening

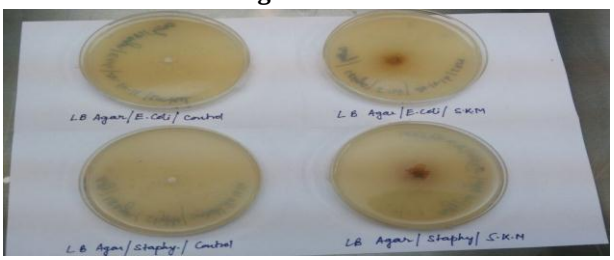

Figure. 2: Antibacterial activity of *A. fragrans* methanolic extract against *Staphylococcus aureus* and *Escherichia coli* bacteria by disc diffusion method.

RESULTS AND DISCUSSION
In the present study, the Phytochemical screening and antibacterial activities were performed with methanol extract of the leaf of *A.fragrans*. The study was made against one gram positive pathogenic bacteria and one gram negative bacteria using the standard disc diffusion method. The leaves of *A. fragrans* were rich in Alkaloid, Glycoside, flavonoid, phenol, phlobatannin, reducing sugar, steroid, saponin, tannin, naphthoquinone, inulin, starch and quinoid (Table no.2). These phytochemicals confer antimicrobial activity on the leaf extracts (Figure 2).

The presence of antibacterial substances in the higher plants is well established (Srinivasan, 2001). We used methanolic extract of leaves of *A. fragrans* for antibacterial study. The results showed (figure 2) the extract possesses antibacterial activity against the tested gram positive *Staphylococcus aureus* and gram negative *Escherichia coli* bacteria with significant zone of inhibition i.e. 12mm and 10 mm respectively. The results of this study are very encouraging and indicate that this herb should be studied more extensively to explore its potential in the treatment of many infectious diseases. Further trials using solvents of various polarities will explore the effects of solvent composition on extract efficacy (Romero et al., 2005).

The various phytochemical compounds detected are known to have beneficial importance in medicinal sciences. Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. Phytomedicine can be used for the treatment of diseases as is done in case of Unani and Ayurvedic system of medicines or it can be the base for the development of a medicine, a natural blueprint for the development of a drug (Didry et al., 1998). Successive isolation of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure.

The traditional healers use primarily water as the solvent but we found in this study the plant extracts by methanol provided more consistent antimicrobial activity compared to those extracted by water. We used methanol solvent for the extraction of active components from leaves of *A. fragrans*. *A. fragrans* shows significant

antibacterial activity on both pathogenic bacteria like gram positive *Staphylococcus aureus* and gram negative *Escherichia coli* bacteria (figure 2). Preliminary phytochemical analysis revealed the presence of many potent compounds (Table no.2). It is not surprising that there are differences in the antibacterial effects of plant groups, due to phytochemical properties and differences among species. Thus this study on the leaf extracts of *A. fragrans* reveals that many phytochemical compounds have been identified and the compounds present in it have a high antibacterial activity. So the leaf extracts can be used as a drug in the Indian system of medicine.

CONCLUSION

A. fragrans is a plant of great medicinal importance. *In vitro* study indicates that these plant extracts is a significant source of natural antibacterial, which might be helpful in preventing the bacterial diseases. *A. fragrans* can serve as potential source of bioactive healthy compounds in the diet and their consumption could be useful in the prevention of diseases. Further research is needed toward isolation and identification of active principles present in the extracts which could possibly be exploited for pharmaceutical use.

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