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Research Article

Antimicrobial activity and phytochemical constituents of combined extracts of *cissusquadrangularis* and *aegle marmelos*

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ABSTRACT

This study is a part of a screening project has been dedicated to investigate the antimicrobial and phytochemical constituents of combined plant extracts. Antimicrobial activity and phytochemical constituents of ethanolic and ethylacetate extracts of *Cissus quandrangularis* (stem) and *Aegle marmelos* (fruit pulp) was investigated. The crude extracts were analysed for the presence of some phytochemical constituents. Phytochemical screening of the crude extracts revealed the presence of flavonoids, carbohydrates, cardiac glycosides, tannins, phenolic compounds, saponins, fat and oils, gums and mucillages and terpenoids. And the extracts were tested for antimicrobial activity against certain organisms such as *Staphylococcus aureus, klebsiella pneumoniae, Escherichia coli and Bacillus subtillis*. They showed significant antimicrobial activity against the above mentioned organisms. The zone of inhibition in mm was recorded. Since ancient ages plants have served human beings as a natural source of treatments and therapies amongst them medicinal herbs have gained attention because of its wide use and less side effects. The results lead to the conclusion that the combined extracts of Cissus quadrangularis and Aegle marmelos possesses effective antimicrobial activity.

Keywords: *Cissus quadrangularis; Aegle marmelos;* Antimicrobial activity; phytochemical constituents

INTRODUCTION

During the last ten years the pace of development of new antimicrobial drugs has slow down while the prevalence of resistance has increased astronomically (Hugo and Russell, 1984). Herbal medicine has a long history in the treatment of several kind of diseases (Holm et al., 1998)Their use for the treatment of the disease has been practised by man for many years and is still being practiced even today (Kokwaro 1993). According to (Iwu et al., 1999)the first generation of plant drugs were usually simple botanicals employed in more or less their crude form. Medicinal plants have been traditionally used for different kinds of ailments including infectious diseases. Plants are very rich in awide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids which have been found *in-vitro* to have antimicrobial properties. Laboratories around the world are engaged in the screening of biological activity with therapeutic potential. The potential of higher plants for new drugs is unexplored (Hostettman et al., 1996). Among more than 250 000 species of higher plants only about 5-10% has been investigated chemically for the presence of biological active compounds (Balandrin et al., 1993; Ayensu and De fillips 1978). Random screening as tool in discovering new biologically active molecules has been most productive in the area of antibiotics. (Gerhertz, W, et al., 1985)*Cissus quadrangularis* is a medicinal plant belonging to family Vitaceae, the ancient system of medicine such as Ayurveda and used to treat various diseases and disorders. Furthermore many of the licensed drugs are toxic as well as expensive (Pillay D, et al., 2000). India has a rich heritage of traditional knowledge and is home to several important time honored systems of health care like Ayurveda, Siddha and Unani. (Kala CP, et al., 2006) It has been estimated that the proportion of medicinal plants in India (7500 of the 17000 higher plant species are medicinal plants) is higher than any country of the world with respect to the existing flore of that respective country. (Shiva MP, 1996) *Aegle marmelos* is commonly known as Bael/Bilva belonging to the family Rutaceae has been widely used in indigenous systems of Indian medicine due to its various medicinal pro [perties. *Aegle marmelos* tree is held sacred by hindus and offered in prayers to deities lord shiva and parvati and the tree is also known by the name Shivaduma (the tree of shiva). (Chemexcil, 1992)

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MATERIALS AND METHODS

Collection of plant materials and extraction of active ingredients

Cissus quadrangularis and *Aegle marmelos* were collected from in and around area of Nandyal, Andhra Pradesh. The above mentioned plants were examined, identified and authenticated by Dr.Prasad Rao,Professor, Department of Botany, P.S.C & K.V.S.C. Govt. Degree College, Nandyal. The stem part of *Cissus quadrangularis* and the fruit pulp of *Aegle marmelos* were air dried and pulverized into powder. About 25gm of the powdered sample of each medicinal plant were weighed into 100 ml of ethanol and ethyl acetate extract in a Soxhlet apparatus separately and the process is carried out for 7 days at 40-50˚c. The filtrate was evaporated to dryness at 40˚ c in a rotary evaporator. And the above process was repeated for several times, until the sufficient amount of extract is produced. The concentrated extract of each plant was stored at 4˚ c until when required for use.

Qualitative Phytochemical Screening

The different qualitative chemical tests were performed for establishing profile of given extracts for its chemical composition. Qualitative phytochemical analysis were done using the procedures of Kokate(1994) and Kokate et al(1995).The following tests were performed on extracts to detect various phytoconstituents present in them.

Detection of alkaloids

Solvent free extract50 mg, was stirred with few ml of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloidal reagents as follows.

Mayers test: To a few ml of filtrate, a drop or two drops of mayers reagent were added by the side of the test tube. A white or creamy precipitate indicated the test as positive.

Wagner's test: To a few ml of filtrate, few drops of wagners reagent were added by the side of the test tube. A reddish-brown precipitate confirmed the test as positive.

Hagers test: To a few ml of filtrate 1 or 2 ml of Hagers reagent (saturated aqueous solution of picric acid) were added. A prominent yellow precipitate indicated the test as positive.

Detection of flavanoids

Lead acetate test: The extract (50 mg) was dissolved in distilled water and 3 ml of 10% lead acetate solution was added. A bulky white Lead precipitate indicated the test as positive.

Detection of Carbohydrates

Molisch test: To 2ml of filtrate, 2 drops of alcoholic solution of α- naphthol were added, the mixture was shaken well and 1ml of concentrated sulphuric acid was added slowly along thesides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

Fehlings test: 1 ml of filtrate was boiled on water bath with 1 ml each of Fehlings solution A and B. A red precipitate indicated the presence of sugar.

Barfoeds test: To 1ml of filtrate, 1ml of Barfoeds reagent was added and heated on boiling water bath for 2 minutes. Red precipitate indicated the presence of sugar.

Benedicts test: To 0.5 ml of filtrate, 0.5 mlof Benedicts reagent was added. The mixture was heated on a boiling water bath for 2 minutes.A characteristic coloured precipitate indicated the presence ofsugar.

Detection of Cardiac glycosides

50mg of extract was hydrolysed with concentrated hydrochloric acid for 2 hours on water bath, filtered and the hydrolysate was subjected to the following tests.

Borntrager test: To 2ml of filtered hydrolysate, 3ml of chloroform was added and shaken, chloroform layer was separated and 10% ammonia solution was added to it. Pink colour indicate the presence of glycosides.

Legals test: 50mg of the extract was dissolved in pyridine, sodium nitro prusside solution was added and made alkaline using 10% sodium hydroxide.presence of glycoside was indicated by pink colour.

Detection of tannins

Ferric chloride test: The extract(50mg) was dissolved in 5ml of distilled water . To this, few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of tannins.

Detection for Phenolic compounds

Lead acetate test: The extract (50 mg) was dissolved in distilled water and 3 ml of 10% lead acetate solution was added. A bulky white Lead precipitate indicated the test as positive.

Gelatin test: The extract(50mg)was dissolved in 5ml of distilled water and 2 ml of gelatin solution containing 10% sodium chloride was added to it.White precipitate indicated the presence of phenolic compounds.

Detection of Saponins: The extract (50mg) was diluted with distilled water and made upto 20ml. The suspension was shaken in a graduated cylinder for 15minutes. A 2cm layer of foam indicated the presence of saponins.

Detection of Fats and oils

Saponification test: A few drop of 0.5N alcoholic potassium hydroxide solution were added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on water bath for 2 hours. Formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

Table 1: Qualitative Phytochemical Screening

S.NO	Phytochemical tests	COMBINED ETHANOLIC EXTRACTS (Cissus quadrangularis and Aegle marmelos)	COMBINED ETHYLACETATE EXTRACTS (Cissus quadrangularis and Aegle marmelos)
1.	Test for alkaloids Mayers test, Wagners test Hagers test	 – – –	 – – –
2.	Test for flavonoids Lead acetate test	 +	 +
3.	Test for Carbohydrates Molisch test Fehlings test Barfoeds test Benedicts test	+ + – + –	+ + + + +
4.	Test for Cardiac glycosides Borntraggers test Legals test	+ – –	+ + +
5.	Test for Tannins Ferric chloride test	+	+
6.	Test for Phenolic compounds Lead acetate test Gelatin test	 + +	 + +
7.	Test for Saponins Foam test	 +	 +
8.	Test for Fats and oils Saponification test	 +	 +
9.	Test for Proteins and aminoacids Millons test Ninhydrin test Biuret test	 + + + +	 – – – –
10.	Test for Phytosterols Libermannn burchards test	 +	 –
11.	Test for Gums and mucillages Distilled water and alcohol	 +	 +
12.	Test for Terpenoids Chloroform and dilute sulphuric acid	 +	 +

Detection of proteins and amino acids

Millons test: To 2 ml of filtrate, few drops of Millons reagent were added. A white precipitate indicated the presence of proteins.

Ninhydrin test: Two drops of ninhydrin solution (10mg of ninhydrin in 200ml of acetone)were added to 2ml of aqueous filtrate. A characteristic purple colour indicated the presence of amino acid.

Biuret test: An aliquot of 2ml of filtrate was treated with 1 drop of 2% copper sulphate solution. To this 1ml of ethanol(95%) was added followed by excess of potassium hydroxide pellets, pink colour in the ethanolic layer indicated the presenceof proteins.

Detection of phytosterols

Libermann- Burchards test: The extract (50mg) was dissolved in 2ml acetic anhydride. To this, one or two drops of concentrated sulphuric acid were added slowly along the side of the test tube. An array of colour changes showed the presence of phytosterols.

Detection of Gums and Mucillages

The extract (100mg) was dissolved in 10ml of distilled water and 25ml of absolute alcohol was added with

Table 2: Anti-Microbial Activity

S.No	Name of the strains	Zone of inhibition(mm) Combined ethanol extract	Combined ethyl acetate extract	Combined ethanol extract	Combined ethyl acetate extract
1.	*Escherichia coli*	15.5	14.8	15.4	15.8
2.	*Staphylococcus aureus*	17.8	18.2	18.2	17.8
3.	*Bacillus subtilis*	17.6	19.2	18.2	19
4.	*Klebsiella pneumoniae*	18.2	19.2	18.2	18.5

constant stirring. White or cloudy precipitate in dicated the presence of gums and mucillages.

Detection of Terpenoids

The extract was treated with chloroform and dilute sulphuric acid.Red violet colour is observed for terpenoids.

Anti Microbial Activity

Source of Inoculate

The microoorgnisms used for this work were collected from the stock culture from the department of Microbiology, Santhiram College of pharmacy, Nandyal. The bacteria used were *Escherichia coli, Staphylococcus aureus, Bacillus subtilis and Klebsiella pneumonia.*

Disc Diffusion Assay

The strains used were; *Escherichia coli, Staphylococcus aureus, Bacillus subtilis and Klebsiella pneumonia*. The standard strains were from stocks of culture collections maintained in our laboratory. Bacteria were maintained on nutrient agar slants, and subcultured monthly. Each extract (900 mg) was dissolved in 4 ml ethanol and ethyl acetate separately. Discs of Whatman No 1 filter paper (ϕ 6 mm) were soaked with 2 drops of the extract using a sterile Pasteur pipette and allowed to dry at room temperature. Two colonies of a 24-hour plate culture of each organism were transferred aseptically into 10 ml sterile distilled water in a test tube and mixed thoroughly, using an electric shaker, for uniform distribution. A sterile cotton swab was then used to spread the resulting suspension uniformly on the surface of oven-dried Isosensitest agar (Oxoid) and Sabouraud dextrose agar plates (Sterillin) for bacteria respectively. These were incubated for an hour at 37^{o}C for bacteria, respectively. Sterile forceps were used to place each of the discs on the agar plates aseptically and the plates were then refrigerated for 30 min at 4^{o}C following which, the inoculated plates were incubated at 37^{o}C for 24 hours for bacteria strains and at 25^{o}C. Antimicrobial activity was evaluated by noting the zone of inhibition against the test organisms. (Murray et al., 1995) Those extracts showing any inhibition at all were noted for further tests for the quantitative assessment of their activity.

Agar diffusion assay

Dilutions of 40, 20, 10 and 5 mg/ml were prepared from 225 mg/ml stock solutions of the four extracts that inhibited the microorganisms. Volumes (20ml) of molten nutrient agar were seeded with 1 ml portions of overnight cultures of microorganisms and poured into sterile Petri dishes (85mm) and allowed to set. Holes of diameter 9 mm were made in the agar plates using a sterile metal cup-borer. Two drops of each extract were put in each hole under aseptic condition, kept at room temperature for 1 hour to allow the agents to diffuse into the agar medium and incubated accordingly (Reeves et al., 1979). The plates were then accordingly incubated at 37^{o}C for 24 hours for the bacterial strains and at 25^{o}C. The zones of inhibition were measured and extracts that gave significant activity against *Escherichia coli, Staphylococcus aureus, Bacillus subtilis and Klebsiella pneumoniae* were noted down.

RESULTS AND DISCUSSION

The quantitative phytochemical analysis reveals the presence of carbohydrate, proteins and aminoacids, flavonoids, tannins, cardiac glycosides, gums and mucillages, and saponins. The active constituents such as flavonoids, terpenoids are the most important chemical constituents which is responsible for the antimicrobial activity.

Results obtained in the present study revealed that the combined extracts of both the medicinal plants possess potential antibacterial activity against *Escherichia coli, Staphylococcus aureus, Bacillus subtilis and Klebsiella pneumoniae.* When tested by the disc diffusion method the combined plant extracts showed significant activity against Escherichia coli and Staphylococcus aureus. The highest antibacterial activity of *Bacillus subtillis and klebsiella pneumonia*e of 19.2mm for both extracts respectively and the least activity recorded in *Escherichia coli* 14.8 mm and 15.4 mm respectively. The combined extracts showed almost similar zone of inhibition against all the tested bacteria. The results of present investigation clearly indicates that the antimicrobial activity vary with the species of plants and plant materials used. Thus the study ascertains the value of plants used in Ayurveda which could be of considerable interest to the development of new drugs. Plants are

important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the invitro antibacterial assay (Tona et al.,) Many reports are available on the antiviral, antibacterial, antifungal, anthelmintic and anti-inflammatorry properties of plants. (Samy, R.P.et al., Palombo, E.A. et al., Kumaraswamy, Y. et al., Stepanovic, S. et al., Bylka,W. et al.,Behera, S.K, et al.,Govindarajan,R. et al.,)

CONCLUSION

The extracts and active fractions of these plants may be used directly in ethnomedicine as anti-microbial agents. The minerals and phytochemical constituents of the medicinal plants may have been responsible for the antimicrobial activities of the plants.

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