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BILOGICAL EEFECT OF AEGLE. MARMELOS (L.) LEAF EXTRACTS AND ITS ANTIBACTERIAL ACTIVITY AGAINST THE WOUND CAUSATIVE ORGANISMS

Biotechnology

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ABSTRACT

Medicinal plant of Aegle marmelos (A. marmelos) leaf extracts and fractions were found to have been used for medicinally and as a dietary supplement. A. marmelos commonly known as bilwa or bael is an important medicinal plant in Ayurveda. Despite this, there are nominal research data on phytochemical properties and pharmacological effects A. marmelos leaf extracts. Hence the current research phytochemical properties and pharmacological effects. This study aimed to explore the preliminary identification of phytochemicals, quantification phytoconstituents and proximate composition along with antibacterial activity wound causative organisms of a solvent extract of leaf from the experimental plant. From the present data A. marmelos leaf extracts contain a little amount of carbohydrates, high ratio of phenols, tannins, flavonoids, saponins etc analysed with both aqueous and acetone solvent confirmed through phytochemical analysis. The antibacterial activity of A. marmelos leaf acetone, hexane extract against the wound causative eight organisms such as Bacillus subtilis, Staphylococcus aureus (Gram Positive), Klebsiella pneumoniae, P. mirabilis, Escherichia coli, Salmonella paratyphi and Salmonella paratyphi (Gram Negative). Results suggest that the hexane extract has significant antibacterial activity against tested wound causative bacteria. The present study justifies the claimed uses of A. marmelos in the traditional system of medicine to treat various infectious diseases. Moreover, the antibacterial compound mainly found in A. marmelos were tannins, phlobatannins, saponins, terpenoids, alkaloids and poly phenols. Hence, the present study clearly showed hexane leaf extract possessed better potential antibacterial activity.

KEYWORDS

Aegle marmelos, Phytocompounds, proximate composition, wound causative organisms

INTRODUCTION

The chemicals obtained from medicinal plant are known as phytochemicals serve as lead compounds in drug discovery and design (Chakravarthy and Gode, 1985). Medicinal plants are valuable for getting novel drugs that forms the ingredients in traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, bioactive principles and lead compounds in synthetic drugs (Ncube et al., 2008). Bael, Aegle marmelos (L.) is one of the medicinally treasured tree species out of the 250,000 living terrestrial plant species on earth. Bael is also known as begal-quince, golden apple, and stone apple in India (Chanda, 2008) and a sacred tree in places where Hindus lives. Bael trees are usually planted near temples dedicated to Lord Shiva and routinely worshiped by the devotees (Singhal et al., 2011). Bael is one of the most appreciated plants used in ayurvedic medicine by the Indian and other South Asian inhabitants in ancient history (Jagetia and Baliga, 2004). According to the historical records, bael is used as a medicinal and food item since 5000 B.C (Baliga et al., 2011) and known to human beings even when writing the famous Sanskrit epicpoem Ramayana (Roy and Sing, 1979). Bael mentioned in the renowned book Charaka Samhita, a comprehensive compilation of all the essential ayurvedic information, which identified bael as a necessary item in ayurvedic medicine (Amit and Rashmi, 2011). A. marmelos (family: Rutaceae, AM) known as bael in Hindi. According to Indian traditional system, it been used to treat pain, fever, inflammation, respiratory disorders, cardiac disorders, dysentery, and diarrhoea. AM have evaluated many pharmacological activities because leaf contains many functional and bioactive compounds such as carotenoids, phenolics, alkaloids, coumarins, flavonoids, terpenoids, and other antioxidants (Davis et al., 1982). The present study has been aimed to assess the following objectives such as quantitative analysis of phytochemicals and estimation of proximate composition in an experimental leaf extract of A. marmelos (L.) plant secondly evaluation of antibacterial activity of two extracts against the pathogenic organisms.

MATERIALS AND METHODS

Preparation of crude extracts:

Ten gm of an experimental sample leaf was soaked in conical flasks containing 100 ml of distilled water separately. Then the conical flasks were placed in orbital shaker for 6 hours at 135rpm. After that, the solutions were filtered through Whatman No.1 filter paper. The filtrate was kept in the refrigerator and used for qualitative and quantitative analysis of phytochemicals and proximate analysis.

Extract Preparation.

The leaves of the plant were properly washed in tap water and rinsed in distilled water. The rinsed leaves were hot air-dried for 3 days. The dried leaves of each plant were pulverized using pestle mortar to obtain a powdered form which was stored in airtight glass containers at 4°C until used. 10g of powdered sample was soaked in distilled water and methanol (200mL and 100 mL) separately for 12 hrs at room temperature. The extracts were then filtered and concentrated to a final volume of 50mL and subjected to phytochemical analysis. For antibacterial screening the leaf extracts were prepared from the experimental plant by following the protocol of Harborne in Soxhlet apparatus using 100mL of distilled water, acetone and hexane solvents. Every extraction was carried out for 24 hrs and the extract was then dried, weighed, and stored in refrigerator at 4°C for further experiment.

Qualitative Estimation of Phytochemical Analysis

Qualitative phytochemical analyses of an experimental plant leaf extracts of aqueous, acetone and hexane were performed by (Harbone, 1998).

Quantitative Estimation of Phytochemicals.

Determination of Alkaloids.

Alkaloids content was measured by following the protocol described by Harborne. A suspension was prepared by dispersing 5gm of the dried leaves in 10% acetic acid solution in ethanol and kept at 28° C for 4hrs which was further filtered through Whatman Number 42.Thereafter alkaloid was precipitated by concentrating the filtrate to one quarter of its original volume and drops of conc. aqueous NH4OH were added. Finally the precipitate was washed with 1% ammonia solution and dried at 80° C in the oven. The content of alkaloid was calculated and expressed as mg/gm of sample.

Determination of Flavonoids.
The flavonoids content was also determined by Harborne method. Briefly, 5 gm of leaves was boiled in 2 M HCl for 30 mins under reflux and filtered after cooling. Equal volume of ethyl acetate was then added drop wise in filtrate. The weight of precipitated flavonoid was determined and reported as mg/g.

Determination of Tannins.
The quantitative estimation of tannins was performed by the method of Swain with minor modifications in our lab. Finely powdered leaves of Aegle marmelos were kept in a beaker containing 20 mL of 50% methanol covered with parafilm and then heated at 80^{o}Cin water bath for 1 hr with continuous stirring. The extract was quantitatively filtered using a double layered Whatman Number 1 filter paper and rinsed by 50% methanol. 1 mL of sample extract was treated with 20 mL distilled water, 2.5 mL Folin-Denis reagent, and 10 mL of 17% Na2CO3 for the development of a bluish-green colour and was allowed to stand for 20 minutes. The absorbance was measured at 760 nm and amount of tannin was calculated by comparing it with standard curve prepared in the range of 0–10 ppm.

Determination of Saponins.
Saponin analysis was performed according to the method described by Brunner. 100 mL Isobutyl alcohol was added to 1 g of finely powdered sample and stirred for 5 hrs. 20 mL of 40% saturated solution of Magnesium carbonate was added to the mixture and filtered. 2 mL of 5% FeCl3 solution and 50 mL volume of distilled water was added to 1 mL of colourless solution and kept for 30 min for colour (blood red) development. The absorbance of the samples along with the standard were read at 380 nm and calculated in mg/gm. Standard saponin solution was prepared in the reference range of 0–10 ppm.

Determination of Total Phenols.
Five grams of the powdered leaves was boiled with 50 mL of ether for 15 mins and distributed in the ratio 1: 2 (extract : distilled water). 2 mL of ammonium hydroxide followed with 5 mL of pentanol was added to it and incubated at the room temperature for 30 minutes. the absorbance was read at 505 nm.

Antibacterial activity
Antibacterial activity had been tested by agar diffusion methods, by providing a single concentration of the substance in a reservoir having seeded nutritional agar medium. The diffusion of the substance into the medium causes a continuous gradient of decreasing concentrations with increasing distance from the reservoir. Colloidal solutions could be embedded on filter paper discs and applied on the agar surface or placed by filling glass or metal cylinders applied to the agar surface or in wells cut on the agar. Creams or gel substances and fabrics could be placed on the agar surface, usually in circular spots of a defined diameter. After the incubation, there should be a zone of inhibited growth around the reservoir, whose size is related to the anti-microbial capacity of the substance. The result is the diameter of the inhibition zone expressed in mm and is related to the minimal inhibitory concentration of the substance for the bacteria. The most important variables that influence the results of diffusion assays are inoculum density, agar depth, concentration of the substance, diameter of the reservoir and the time intervals between inoculation and application of the substance and start for incubation. These variables must be controlled to ensure reproducibility of the results. It is not applicable to substances that diffuse poorly or to slow growing bacteria under the conditions used.

Proximate Analysis of Experimental leaf extract of A. marmelos
Determination of moisture content:
10 gm of the fresh sample of each plant material was placed in the crucible and heated at 105o C until a constant weight was attained. The moisture content was calculated as a loss in weight of the original sample and expressed as the percentage moisture content (FAO, 1980)3. Hence, Moisture % = (Fresh weight Dry weight/ Fresh weight) ×100.

Determination of Total solids:
A clean dry lined Petri dish along with lid was weighed. 5 gram of the sample was placed in the weighed Petri dish with a lid. The Petridish with the sample was weighed with the lid. The sample was placed in the oven at 105 - 110° C for one hour. After one hour the sample was taken out from the oven and placed in the desiccator for cooling. After cooling, the sample was again weighed4. The moisture content was determined as %

Moisture = Loss in weight /Weight of sample× 100. So, % of total solids = 100 - % of moisture.

Determination of Ash content percentage:
For determination of ash content, a method of AOAC (1984) was followed. According to this method, 3 g of the pulverized plant samples were placed in a a desiccator and weighed at room temperature to get the weight of the ash5. The weight of the ash content was calculated out by using the following formula:

Ash % = (Weight of the ash sample / Weight of the sample taken) ×100.

Different dilutions of BSA solutions were prepared by mixing BSA stock solution (1mg/ml) and water in the test tube. The final volume of each test tube was made up to 5 ml. The value of BSA ranges from 0.05 to 1.0 mg/ml. From these different dilutions, 0.2 ml protein solution was pipette out to different test tubes and 2 ml of analytical reagent was added (alkaline copper sulfate reagent). After mixing the solution, it was incubated at room temperature for 10 min. Then 0.2 ml of Folin Ciocalteau reagent solution was added to each tube and incubated for 30 min. The optical density of the blank was taken in the colorimeter (measure the absorbance) at 660 nm. The values of absorbance were plotted against protein concentration to get a standard calibration curve. The absorbance of the unknown sample was checked and the concentration of the unknown sample was determined using the standard curve.

Determination of nutritive value:
To determine the nutritive value of the medicinal plants, an appropriate amount of crude sample was taken and weighed. Protein, carbohydrate, fats were analyzed by the methods which are discussed earlier in this study. The nutritional value of the plants was calculated as per the formula used by Nile et al., 20099 .Nutritive value = 4 × percentage of protein + 9 × percentage of fat + 4 × percentage of carbohydrate Heavy metal analysis: Test for lead: a) Dilute HCl was added in a sample solution. A white precipitate of CaCl2 is absent indicates the absence of lead. b) KI was added to a sample solution. The yellow precipitate is absent indicates the absence of lead. Test for cadmium: a) NH_4OH was added in a sample solution. The white precipitate is absent indicates the absence of cadmium. b) Potassium ferricyanide was added. The white precipitate is absent indicates the absence of cadmium.

The proximate composition of the plant samples was determined by adopting the official method of analysis by Association of Official Analytical Chemists. The total free Nitrogen content in the extracts was determined as total Kjeldahl nitrogen by microkjeldhal method. The crude proteins were obtained also according to the Association of Official Analytical Chemists procedure. The moisture and ash were determined from the plant materials using weight difference method. Crude fiber was estimated from the extracts by loss in weight on ignition of dried residue following digestion of fat free samples. Crude fat was determined by extracting the samples with petroleum ether in a soxhlet extractor. All the proximate values were reported in percentages (Hazra and Sudipta, 2020).

Statistical analysis and performance of an Experimental Data
The results for each assay have been expressed as the mean ± SE of the percentage values from three independent experiments. The percentage in each experiment was calculated using the formula {(C or E)/T} × 100, where E is the mean value of the duplicate/triplicate readings of the control group and T is mean value of the duplicate/triplicate readings of the test (dilutions of the decoction) groups. Henceforth, the value of control was 100% and the values of the assessment groups have been represented as percentages effective to control.

RESULT
Phytochemical analysis
The result of qualitative phytochemical analysis of A. marmelos was shown in Table-1. In the current study clearly indicates totally ten phytochemicals are analysed Because of the presence of this bioactive chemicals in the leaves, it has the medicinal property to cure almost all common human ailments. From the present study shown leaf extract of A. marmelos possessed enriched amount of following bioactive compounds the presence of Alkaloids, Amino acids, Anthocyanin, Carbohydrates, Cardiac Glycosides, Coumarins, Diterpenes, Fatty acids, Flavonoids, glycosides, Leucoanthocyanin, Phlobatannin, Phytosterol, Proteins, Phenols, Saponin, Steroids, Tannin, Terpenoids. From the above data A. marmelos contain a little

amount of carbohydrates, high ratio of phenols, tannins, flavonoids, saponins etc analysed with both aqueous and acetone solvent confirmed through phytochemical analysis.

Quantitative analysis of bioactive compounds

Thus A. marmelos possessed maximum quantities of flavonoids (69.9 ± 0.053), alkaloids (17.08 ± 0.03), phenol (23.11 ± 0.07), tannins (9.24 ± 0.15), saponins (11.40 ± 0.10). Successive isolation of botanical compounds from plant material was largely dependent on the type of solvent used in the extraction procedure. Phytochemical analysis of the petroleum ether, benzene, chloroform, ethanol and water extracts of A. marmelos, showed the presence of some phytochemical parameters like flavanoids, carbohydrates, saponins. The results of the various phytochemical tests revealed that steroids, tannins, phenols, cardio glycosides, alkaloids, amino acid and protein were present (Table 2)

Antibacterial activity of A. marmelos leaf extracts.

The antibacterial activity of the A. marmelos was tested against eight different wound causative bacterial organisms including a range of gram positive and gram negative bacteria using the paper disc method. (Fig-3) Eight organisms were selected for the present study result was represented in fig-2. The extract such as acetone, hexane and aqueous extract showed varying levels of antibacterial activity with different pathogenic organisms. Initially highest level of zone of inhibirion has been noticed on Klepsiella sp, and Bacillus sp., shown in aquoues and acetone extract of A .marmelos leaf extract. Furthermore, similar level of zone of inhibition was observed in acetone extract against the following organisms such as Staphylococcus sp., Streptococcus sp.,and E.coli. Though, minimum and optimum range of antibacterial activity has noticed in acetone and aqueous extract against pseudomonas sp., Staphylococcus sp and Klepsiella sp., respectively. However, no other antibacterial activity was shown in a an acetone and aqueous extract against Salmonella sp., proteus sp., etc. From the present result has been clearly showed that maximum antibacterial activity has noticed acetone and hexane extract. While it was minimum effect when compared with antibiotics such as gentamycin and rifampicilin data was not revealed but its shown in figure-3.

Table 1:- Preliminary phytochemical analysis of Aegle marmelos from Capecoumarin Region

Test			
	Acetone	Aqueous	Hexane
Molisch's test	-	+	+
Biuret test	-	-	-
Test for phenols	+	+	+
Test for tannins	+	-	+
Test for flavonoids	+	+	+
Test for quinone	+	+	-
Test for terpenoids	+	+	-
Test for steroids	-	-	+
Test for caumarins	-	-	+
Test for saponins	+	+	+

Table 2:- Quantitative estimation of phytochemicals from Aqueous leaf extact of A.marmelous (mg/g).

S.NO.	Name of the phytochemicals	Quantity (mg/g)
1.	**alkaloids**	17.08 ± 0.03*
2.	**Saponins**	11.40 ± 0.10
3.	**flavonoids**	69.9 ± 0.053**
4.	**tannins**	9.24 ± 0.15
5.	**phenol**	23.11 ± 0.07**

*--statistically significant at 0.05% level

**-- statistically highly significant at 0.005% level

Table 3: Proximate Composition of highlighted Aqueous Experimental leaf extract of (Aegle marmelos) plant

S.NO	Name of the test	Quantity (%)
1.	Carbohydrate (%)	64.01
2.	Fats (%)	0.82
3	Protein (%)	1 9.58
4.	Moisture (%)	10.33
5.	Crude fibre (%)	4.26
6.	Calorific value (kCalories/100g)	331.74
7.	Starch (%)	36.60
8.	Vitamin C (mg/100g)	Neg
9.	Total Solids (IU)	1.22
10.	Phytic acid(mg/100g)	05.0
11.	Ash content	15.83

From the proximate analysis, it is observed that A. marmelos shows maximum of 331.74 calorific value, carbohydrate, vitamin C. and a minimum of fats, phytic acid and crude fibre. Total solids content is negligible.

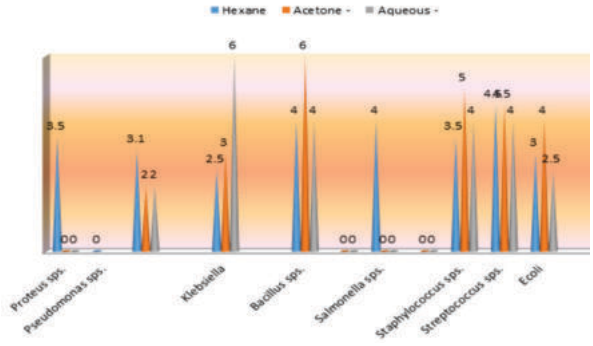

Fig:-1:- Antibacterial activity of experimental leaf extract of A. marmelos and A. indica against the wound causative agents

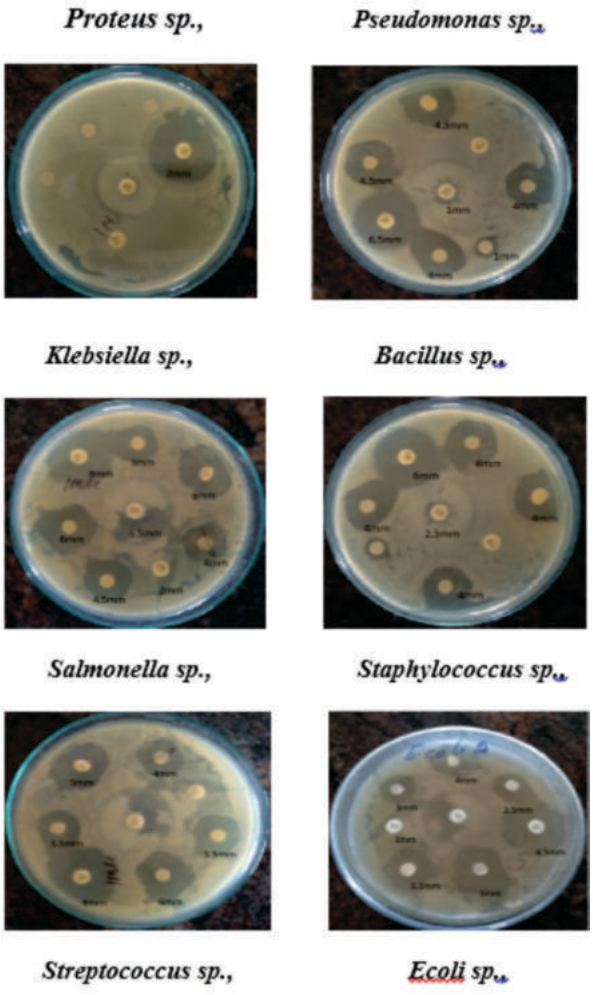

Figure-2:-Antibacterial activity of hexane, acetone and aqueous leaf extract against selected pathogenic bacterial organism of wound causative agents

DISCUSSION

The value of medicinal plant in today's world is that they become a potential source for bioactive compounds Plants are known to have beneficial therapeutic effects in traditional Indian system of medicine (Jagetia and Baliga, 2004). Much work has been done on ethnomedicinal plants in India. It has been suggested that phytochemical extracts from plants are used in allopathic medicine as they are potential sources of antiviral, antitumor and antimicrobial agents (Nair et al., 2005; Ramya et al., 2008). The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of the world. Plants have been reported to possess antimicrobial, antifungal and other activities. This has been elucidated by various workers. In this present study the antibacterial activity was found to be best in leaf extracts of the aegle marmelos against bacterial pathogens current study expressed the similar results also agreed by several aurhors (Baliga et al., 2011; Rajan. et al., 2011). The presence of these phytochemical compounds favours for anti-bacterial and anti-diabetic property. Since ancient times, plants have been a veritable source of drugs.

However, medicines derived from plants have made immense contribution towards the betterment of human health and act as a source of inspiration for novel drug compounds. From the above research it can be concluded that this plant has immense potential to be used in the area of pharmacology and as a prospective source of valuable drugs. Due to the presence of various compounds that are essential for good health, it can also be used to improve the health status of society (Roy and Sing, 1979). The extracts showed a significantly high antibacterial activity against the tested bacterial pathogens particularly wound causative agents. The data clearly depicts the presence of compounds were used for treating various bacterial diseases, indicating its use in the traditional system of medicine since ancient times. Further, the broad spectrum activity of aqueous, methanol, and aqueous: ethanol extracts proves to be encouraging in the development of novel antimicrobial formulation in the near future Prusti, 2006. A spectrum of compounds showing strong antibacterial, antioxidant, and anti-inflammatory activities was revealed the methanolic extract of A. marmelos. Antimicrobials derived from plants possess vast curative properties since they have fewer side effects as compared to synthetic antimicrobials drugs. Aegle marmelos is of utmost importance for ethnobotanical purposes, and it has been placed in the priority list of thirty-two medicinal plants. (Sudharameshwari and Radhika, 2007) The present study contributes to the current knowledge of presence of various phytochemical active compounds A. marmelos possessing significant broad spectrum antibacterial efficacy (Rajan.et al., 2011). The traditionally important plant leaves provide the baseline data to establish a connection between the traditional health practioners and scientific communities, which could be substantial in novel drug discovery (Mohan et al., 2005). Furthermore, ethnobotanical data is of significant value for conservation managers and policy makers for sustainable management of medicinal plant species, which are under threat due to over exploitation.

CONCLUSION

Present assessment revealed that a number of medicinal plant species are used by indigenous people of the study area to treat various ailments. The indigenous community still relies on traditional medicine although; the modern health-care services are available, which indicates the significance of plant based traditional recipes. From this study it has been concluded that A. marmelos possess antibacterial and wound healing properties, which help them to play a principle role in the prevention and treatment of many diseases. Relative study of both phytochemical as well as the antimicrobial and proximate analysis of plant extract shows that the chemical constituents present in the A. marmelos were responsible for the therapeutic and antibacterial action of the drug. The hexane extract shows significant antibacterial activity against the wound causative organisms such as B. subtilis, S. aureus, K. pneumonia, P. mirabilis, E. coli and S. paratyphi. Thus the plant extract with known antimicrobial properties remarkably contributes in modern therapy. So the extract of A. marmelos can be used as a source of drugs against various infections in respiratory, excretory, circulatory and nervous system, etc. The important bio-compound potential value of this experimental plant was an indication of their preference by local inhabitants to treat specific ailments. Therefore such popular plant of A. marmelos could be further analyzed for bioactive constituents, in vivo/in vitro biological activities, which may leads to identify new potential drugs.

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