



Experimental Investigation on Phytochemical Analysis and Antibacterial Activity of *Aegle Marmelos* (Bael) Plants

Kriti Kiran Dawadi^{1,a}, Saugat Khanal^{2,b,*}

¹Department of Chemistry, Institute of Science and Technology, Tribhuvan University, Kathmandu, Nepal

²Faculty of Agriculture, Agriculture and Forestry University, Rampur, Chitwan, Nepal

*Corresponding author

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ABSTRACT

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The present investigation aims to evaluate the phytochemical and antibacterial potential of different parts of *A. marmelos*. Thousands of species are acknowledged to have medicinal value and the use of different parts of several medicinal plants to cure specific ailments has been in vogue since archaic periods. Keeping this point in view, a study was undertaken to analyze the phytochemical and biological activity of a very valuable medicinal plant '*Aegle marmelos*'. Pieces of literature were collected from various journal articles, Government institutes and other relevant reports were studied and the major findings were summarized. Leaves of *Aegle marmelos* has been picked and collected from the local area of Chitwan, Nepal. During the present study, the extraction of the phytochemical was performed by Soxhlet extractor. The leaves were subjected to successive extraction using methanol inclusive of hexane as a solvent to procure extra activity test. Phytochemical screening of methanol extract and hexane extract leaves revealed the presence of alkaloids, terpenoids, tannins, etc. For the antibacterial activity test, the Disc diffusion method was used which showed the presence of *S. aureus* and *E.coli*. The study concluded that *Aegle marmelos* plant incorporated miscellaneous phytochemicals along with the antibacterial activity. Such phytochemicals derived from plant has a great prospect in contributing effective antibacterial agents to treat against intractable life-threatening diseases. Within this view, the present study has explored the efficiency of the *Aegle marmelos* as a valuable natural source.

^a kritikiran11@gmail.com

^b <https://orcid.org/0000-0003-2049-4530>

^b ksaugat506@gmail.com

^b <https://orcid.org/0000-0002-9146-7428>



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Introduction

Many plants are imperative to man for his life. World health organization (WHO) has also figured out that more than 80% of the world population relies on plants to fulfill their basic health care needs (Vines, 2004). Balakumar et al. (2011) has stated that medicinal plants with antibacterial action are local heritage with global importance. Nepal is globally recognized as the botanical garden of the world since it is the largest producer of medicinal herbs. Since time immemorial, plants and their products have been the elementary resource of food and medicines for mankind. The plant-based drugs and chemicals for curing various ailments have been used since antique human civilization. Contrary to synthetic drugs, antibacterial activities of plant origin are confirmed to have insignificant side effects and have an immense therapeutic potential to cure many infectious diseases. Nowadays, several clinically efficacious antibiotics are becoming less effective due to the development of resistance. So, biomolecules of plant genesis appear to be

one of the alternatives for the regulation and control of these antibiotic-resistant human pathogens. The medicinal attributes of *Aegle marmelos* plants have been portrayed in the Ayurveda which translates as "Knowledge of life".

Aegle marmelos, commonly called Bael, a subtropical medicinal plant found up to an altitude of 1400 m from water level, grows well in dry forests of hilly and plain areas. The plant is well-known to own several pharmacological activities like antibacterial, anti-inflammatory, antifungal, antihelminthic, antiprotozoal, antispermatic, antidiabetic, laxative, febrifuge, and expectorant. Also, the plant has been declared to be within the red list in vulnerable status. It is the sole member of the genus *Aegle* and maybe a deciduous shrub or medium-sized tree, reaching up to 13 m height. It has got pale brown or grayish bark, finely fissured, armed with long straight spines, 1.2-2.5 cm in pairs or singly, often with slimy sap oozing out from cut parts. The leaf is alternate and ovate with pointed tip and circular base,

untoothed or with shallow rounded teeth. The flowers are pale green or yellowish, drooping unbranched bundles at the top of twigs and leaf axils. The leaf is meant to be one amongst the topmost accumulator parts of the plant consisting of bioactive compounds that are synthesized as secondary metabolites (Cowan, 1999). *A. marmelos* plants bear varies range of bioactive molecules making them affluence in rich sources of various sorts of medicines. The information collected from extracts of medicinal plants makes pharmacological researches possible. The mode of action of the plants having therapeutic effects may also be inspected if the active ingredients are characterized.

The formulation of antibacterial compounds and phytomedicines from higher plants against harmful microbes appear to be rewarding. Numerous extracts of *A. marmelos* are identified to move against various bacterial strains. For example; oil of the leaf restricts the growth of *Escherichia coli*, *Aeromonas species*, *Pseudomonas salanacearum*, and *Xanthomonas vesicatoria*. Bel, being considered as a sacred tree, is additionally employed in the ritual rites of Hindus. In Hindu mythology, it's often considered as an incarnation of goddess Parvati. It is supposed that immortal Lord Shiva is keen on Bel tree and a few of its part is beneficial to worship god. Girls of the Newar ethnic culture have a non-secular reference to bel. There is a conventional practice called "Bel bibaaha (Bel marriage)". Girls are married to the fruits of *A. marmelos* (Bel) and as long as the fruit is kept intact and no cracks at all, the girl will never become widowed, even if her husband passes away. This culture is taken as protection against the social antipathy suffered by widows in the Newar community. For that reason, bel is equally important in religious as well as medicinal values. Against this background information and appreciating the knowledge of use and importance of medicinal plants an effect has been made in this research to determine the antibacterial efficacy of a particular medicinal plant and also distinguishing them by screening preliminary by phytochemical analysis test. The study also associated with inculcates the topic about the economical utilization of natural flora as therapeutic agents. This study was conducted to determine the phytochemical and antibacterial activity of leaves of *A. marmelos* plants.

Aegeline and Liver Injury

Aegeline could be a common constituent of the bel leaf that is consumed as a dietary supplement with the intent to cut back body weight. In the year 2013, Food and Drug Administration (FDA) of USA, Centers for Disease Control and Prevention (CDC), the Department of Defense Armed Forces Health Surveillance Center, and Hawaii state and local health officials diagnosed an outbreak of 97 persons with severe non-viral Hepatitis that first appeared in Hawaii. 72 of these patients had reported taking the dietary supplement OxyELITE pro which had Aegeline. FDA had then taken action against the prior formulation of OxyELITE pro as it possessed dimethylamine, a stimulant that FDA had confirmed to be contaminant when included in dietary supplements and might cause high blood pressure, heart attacks, seizures, psychiatric disorders, and finally death.

Several doctors at the Liver Center at The Queen's Medical Center examining the primary cases in Hawaii reported that between May and September 2013, 8 previously healthy persons presented themselves at their center tormented by a drug-induced liver injury. It was absolutely found that every one of those patients had been using the reformulated OxyElite pro, which had been purchased from various sources, and which had different lot numbers and expiry dates, at doses within the advice of the manufacturer. 3 of those patients were found to develop fulminant liver failure, 2 patients were gone through urgent liver transplantation, and one died. The number of such cases ultimately rose to 44 in Hawaii. Within the starting month of 2014, leaders from the Queen's Liver center informed lawmakers of the state that they were sure that Aegeline was the responsible compound for those cases. However, the mechanism of how aegeline injured the liver has not been isolated.

Chemical Compounds

The bael tree comprises furocoumarins, along with xanthotoxol, and methyl ester of alloimperatorin, as well as flavonoids, essential oils, rutin and marmesin, alkaloids, α -fragarine (allicryptopine), O-isopentylhalfordinol, O-methylharfordinol, aegeline (N-(2-Hydroxy-2-(4-methoxyphenyl)ethyl) cinnamamide, etc. which can be extracted from the leaves of *A. marmelos*. Aeglemarmelosine, the molecular formula of $C_{16}H_{15}NO_{29}$ (α) 27D+7.090(c 0.20, $CHCl_3$), has been segregated as an orange viscous oil.

Literature Review

Das et al. (2006) experimented on the effect of aqueous extract of leaf of *Aegle marmelos* on testicular activities in rats. The aqueous extract of leaf of *A. marmelos* plant at the dose of 50 mg/ 100 mg body weight culminated a notable diminution in the activities of key testicular steroidogenic enzymes in conjunction with low levels of plasma testosterone and relative wet weights of sex organs without any mutation in natural body growth. Significant declination in the number of germ cells in different generations at VII stage of seminiferous cell was observed after the treatment of the above extract.

Saenphet et al. (2006) evaluated the safety of aqueous extracts from *A. marmelos* and *S. rebaudiana* on reproductions of female rats. He studied the safety of a Thai medicinal plant, *A. marmelos*, and a non-caloric sweetener, *S. rebaudiana*, on female rat's reproduction. Female rats were treated orally with aqueous extract of *S. rebaudiana* at various concentrations (0.1, 0.2, 1, or 10%) and *A. marmelos* (6%) for 2 months (1 ml/day) before mating. No remarkable deformities were observed in any of the pregnant rats.

Kumar et al. (2008) researched on the insecticidal activity of *Aegle marmelos* (L.) correa essential oil against four stored grain insect pests. Experiments were performed to demonstrate the potential of using essential oil from leaves of *Aegle marmelos* in controlling the insect infestation of stored grain from *callosobruchus chinensis* (bruchidae) and wheat from *Rhyzopertha dominica* (Bostrychidae) and *Sitophilus oryzae* (Curculionidae).

Sabu and Ramadason (2004) demonstrated antidiabetic activity of *A. marmelos* and its relationship with its antioxidant properties. His study examined the reaction of *Aegle marmelos* against experimental diabetes along with the antioxidant potential of the drug. A methanolic extract of *A. marmelos* was proved to lessen blood sugar in alloxan diabetic rats. Declination in blood sugar could be noticed from the 6th day after regular administration of the extract and the sugar levels were observed to be decreased by 54% on the 12th day. Oxidative stress produced by alloxan was found to be much lowered by the administration of *Aegle marmelos*.

Balakumar et al. (2011) appraised the in vitro antifungal activity of *Aegle marmelos* leaf extracts and fractions on the clinical isolates of dermatophytic fungi. Leaf extracts and fractions of *A. marmelos* plant were found to have specific fungicidal action across different isolates of dermatophytic fungi. He concluded that the plant extract considerably inhibits the growth of all dermatophytic fungi studied. He then confirmed the *A. marmelos* plant to be a remedy for dermatophytosis.

Ismail (2009) studied clinical evaluation of the antidiabetic activity of Trigonella seeds and the leaves of *A. marmelos* plant. He clinically scrutinized the antidiabetic activity of fenugreek seeds (FG) (*Trigonella-graceum* Linn.) and bel leaves (*Aegle marmelos*) individually and jointly in non-insulin dependent diabetes mellitus (NIDDM) patients. Considerable changes were noticed in PPBGL of patients who were acquiring these two herbs jointly as compared with the other patients who were receiving these herbs individually.

Sukumaran et al. (2009) has carried out anti-diarrhoeal activity of *Aegle marmelos* unripe fruit for validating its traditional usage. He assessed the hot aqueous extract (decoction) of undried fruit pulp of *A. marmelos* for its antimicrobial action and effect on various aspects of pathogenicity of infectious diarrhoea. The decoction was checked for its anti-giardial, antibacterial and anti-rotaviral activities which showed cidal activity against Giardia and Rotavirus. These observations recommend the varied conceivable modes of action of *A. marmelos* in infectious forms of diarrhoea.

Siddique et al. (2011) reviewed free radical scavenging and hepatoprotective activity of *Aegle marmelos* (Linn.) Corr leaves against carbon tetrachloride. In the analysis hepatoprotective and antioxidant action of the methanolic extract of *A. marmelos* leaves (MEAML) was inspected on carbon tetrachloride (CCl₄) intoxicated rats. The leaves hold an ample amount of phenolic (9.8367±0.0235 mg/kg) and flavonoid (8.248 ± 0.029 mg/kg) contents that proved the antioxidant property of the leaves. He concluded that the leaves possess appreciable hepatoprotective activity by suppressing CCl₄ induced cellular oxidative stress.

Patil et al. (2009) has studied antifungal and antiflatogenic activity of *Aegle marmelos* Linn. The antiflatogenic attributes of ethanolic extract of the leaves of *A. marmelos* experimented on ordinary aflatoxigenic fungal species. The plant depicted antifungal and antiflatogenic action at a concentration range of 0.5 to 2 mg/ml. To assess the antifungal and antiflatogenic activity, the shake flask method was used. The extract revealed varying levels of antifungal

and antiflatogenic action against the test fungi. Exploratory phytochemical tests of ethanolic extracts manifested the presence of dominant phytochemicals like phenols, tannins, flavonoids, and alkaloids.

Singanan et al. (2007) has studied the hepatoprotective effect of bel leaves (*Aegle marmelos*) in Alcohol-induced Liver Injury in Albino rats. Four groups of animals were taken for experiment. Those animals were provided with 30% ethanol for 40 days and the fine crude plant leaves powder was fed for further 21 days. The experimental results illustrate that the Bael leaves have exquisite hepatoprotective effects.

Kothari et al. (2010) illustrated anxiolytic and antidepressant actions of methanol extract leaves of *A. marmelos* in mice. He supplied Albino mice with specific chemicals like AM (75, 150 and 300 mg/kg, PO), imipramine (20 mg/kg, PO), fluoxetine (20 mg/kg, PO), and sub-effective dose of AM with imipramine or fluoxetine in combination. Effects of those chemicals were noticed on (i) time spent on (ii) number of entries into (iii) number of stretch attend postures (iv) number of head dips in arms of elevated plus maze and on the duration of immobility in tail suspension test. He then concluded that AM possess promising anxiolytic and antidepressant activities and also it complements the anxiolytic and antidepressant activities of imipramine and fluoxetine.

Materials and Methods

Materials

Chemicals

Pyridine, conc. /dil. Sulphuric acid (H₂SO₄), CuSO₄, HgCl₂, MHA, lead acetate, ferric chloride, sodium nitroprusside, Mayer's reagent, Benedict's solution, Ninhydrin solution, NaOH, etc.

Glass wares

Beaker, Funnel, Glass, Tubes, Rods, Measuring cylinder, Pipettes, Round bottom flask, Watch glass, Condenser, etc.

Instruments

Grinder, Digital water bath, Electronic heater, Electronic balance, Soxhlet apparatus, Autoclave, Hot air, etc.

Materials

Leaves of *A. marmelos*, *E. coli*, *S. aureus*

Methods

Collection of plant part

Leaves of *A. marmelos* were collected from Nippani, Taandi, and several other areas of Ratnanagar municipality of Chitwan district; central Terai of Nepal during April.

Identification of the plant

The plant *A. marmelos* was identified from the garden of the local area of Ratnanagar municipality.

Drying of leaves

The leaves were then thoroughly cleaned and cut into small pieces with the help of scissors. They were left scattered for shade drying on the floor above the papers for a week. Thereafter the leaves were dried in a hot air oven at around 50 °C for an hour before the extraction process to remove the equilibrium moisture content.

Extraction of leaves

Extraction for the phytochemical screening was performed by Soxhlet extraction to obtain the extract. The leaves were first grinded with the help of a mechanical grinder to obtain the powder of leaves of uniform size. Approximately 28 gm and another of 38 gm of powder was packed in cotton cloth. The cotton cloth impregnated into the Soxhlet apparatus and extraction was carried out by using 260 ml methanol and 500 ml hexane as a solvent and extraction was continued for 36 hours and 48 hours respectively. Then the methanol extract and hexane extract were received in the two different round bottom flasks. Thus received methanolic extract and hexane was dried and yield value of extract was computed. Then dried extract of hexane, as well as methanol, was used for phytochemical screening.

Phytochemical analysis

Phytochemical tests were performed as follows:

- Alkaloid test: A little chunk of the extract was mixed with a limited amount of dil. HCl and is filtered with various alkaloid reagents like Mayer's reagent to test for the presence of alkaloids which showed the appearance of yellowish or orange insoluble pigments.
- Carbohydrate test: A little portion of the extract was dissolved in 5 ml of distilled water followed by filtration. The filtrate then obtained was treated with Benedict's solution which gave red or orange precipitate to test for the presence of Carbohydrates.
- Saponins test: About 1 ml solution of leaves extract was diluted with distilled water to near 20 ml and was shaken in a graduated cylinder for almost 15 minutes. The process is known as the foam test.
- Phenol test: A few drops of 10 % solution of lead acetate were added to the test solution. This resulted in the formation of a white precipitate which confirmed the presence of phenolic compounds.
- Tannins test: Same as in the phenol test, white precipitate confirmed the presence of tannins.
- Flavonoid test: A little fraction of extract was taken in a test tube and mixed with a few drops of dil. NaOH to test for the presence of Flavonoids which resulted in the formation of intense yellow color precipitate. On the addition of few drops of HCl, the yellow color disappeared and became colorless which represents the presence of flavonoids.
- Proteins and amino acids: A few portions of the extract were treated with drops of conc. HNO₃. This process of testing the presence of proteins and amino acids is known as the xanthoproteic test. Formation of yellow-colored precipitate determined the presence of protein.
- Terpenoids test: A little portion of extracts were treated with chloroform and was filtered. The filtrates were then treated with few a drops of conc. H₂SO₄, shaken well and allowed to stand. The lower layer appeared to be somehow red which indicated the presence of steroids. After adding conc. H₂SO₄ to the side carefully (without shaking), the color changed to reddish brown which indicated the presence of terpenoids.

- Gums and mucilage: The extract was gradually added to a few ml of absolute alcohol under steady stirring to test for the presence of gums and mucilage.
- Phlorotannins test: The sample of plant powder was mixed with distilled water in a test tube. Then it is filtered after shaken it well to take plant extract. 1% aqueous solution of HCl was added to the plant extract and boiled to test for the presence of phlorotannins. The formation of red precipitate indicated the presence of it.

Antibacterial test

- Preparation of solution extract: A few dry extract was dissolved in water to produce a solution. To assure the proper mixing, the solution was kept in centrifuge. Microorganism used: -Gram-positive: *S. aureus* : -Gram-negative: *E. coli*
- Preparation of culture media: At first, Muller Hinton Agar (MHA) in the concentration of 3.8 gm was mixed with 100 ml of distilled water. To dissolve the agar completely it was heated to clear transparent solution with constant shaking. Thereafter, the media was autoclaved at 121°C for 15 minutes at 151 bs/punch square pressure. Sterilized media was then allowed to cool around 50°C and poured in a sterile plate. The media was let to be solidified and then both cultures of *E. coli* and *S. aureus* were swabbed in agar plate. After that, well (bore) was constructed and the methanol extract of bel was poured in the well. Then it was incubated for 24 hours at 37°C and the zone of inhibition was evaluated.

Results and Discussion

Many pathogenic microorganisms that are responsible for causing human health hazards exhibit drug resistance due to the meager use of antibiotics. Along these lines, there is a need for the discovery of new products from natural sources like plants. Antimicrobial activities of *A. marmelos* were assessed *in vitro* against some bacterial species. The plants inspected in this study revealed the antibacterial activity against some of the tested microorganisms with inhibition zone that ranged certain values.

Phytochemical Analysis Test

The phytochemical screening showed the presence of alkaloids, saponins, phenols, tannins, flavonoids, terpenoid in methanol. There is the absence of Carbohydrates, Gums and mucilage, Phlorotannins, Proteins, and amino acids. While in Hexane extract sample there is the presence of Alkaloids, Saponins, Tannins, Flavonoids, and Terpenoids. However, there is the absence of Carbohydrates, Gums and mucilage, Phlorotannins, Phenols, and Protein and amino acids.

Antibacterial Activity

The extract of *Aegle marmelos* plants was tested against two pathogenic bacterial strains *E. coli* and *S. aureus*. The antibacterial activity was recorded by calibrating the diameter of clear inhibition zone around each disc.

In the disc diffusion method, a thin film of tested bacteria applied on a plate was subjected to different antibiotics. Zone of inhibition (ZOI) is a circular region around the spot of the antibiotics in which the bacteria colonizes does not grow further. We evaluated the susceptibility of those bacteria towards antibiotics. This work illustrates an effective approach of measuring the Zone of Inhibition by measuring the radius of the zone.

Zone of inhibition (ZOI) of *E. coli* and *S. aureus* (in diameter):

Activity; *ZOI in (mm): Zone of inhibition expressed in millimeter, including the diameter of the filter paper disc; **Microorganism: *S. aureus* and *E. coli*; ***MHA: Muller Hinton Agar as cultured media; ****DMSO: Dimethyl sulphoxide as solvent; +A: Active; -N: Non-active

In consonance with the results of the disc diffusion method, the significant antibacterial effect was observed in *A. marmelos* plants. The sensitivity of pathogenic bacteria against the extract of *A. marmelos* was noted and evaluated. Muller Hinton Agar was used as cultured media. DMSO was consumed as a negative control without any antimicrobial effect on the tested microorganisms. Whereas, Methicillin for *S. aureus* and Ampicillin for *E. coli* was used as positive control. The standard ZOI for both microorganisms was 10 µg/ml that show 10 mm of inhibition. In *S. aureus* the obtained ZOI was 9 mm of diameter.

Table 1. Phytochemical screening test for methanol and hexane extracts

S.N	Phytochemical test	Methanol	Hexane
1	Alkaloids test	+	+
2	Carbohydrates test	-	-
3	Saponins test	+	+
4	Gums and mucilage test	-	-
5	Phlorotannins test	-	-
6	Phenols test	+	-
7	Tannins test	+	+
8	Flavonoids test	+	+
9	Protein and amino acids test	-	-
10	Terpenoids test	+	+

Table 2. Results of the screening of the plant extracts through the disc diffusion method

	*ZOI in (mm)	
	**S. aureus	**E. coli
**Microorganism	**S. aureus	**E. coli
Media	***MHA	***MHA
Solvent	****DMSO	****DMSO
Positive control	Methicillin	Ampicillin
Negative control	****DMSO	****DMSO
Standard ZOI	10 µg/ml shows 10 mm inhibition	10 µg/ml shows 14 mm inhibition
Obtained ZOI	9 mm	-
Results	+A	+A
Remarks	+A	-N

In this study, phytochemical screening of methanol extract acquired from Soxhlet extraction was carried out. The extract collected from the leaves of *A. marmelos* plant was used in the antibacterial test. Antibacterial

activity of methanol extract was appraised in vitro against two harmful bacterial species *E. coli* and *S. aureus* which are known to cause dysentery, diarrhea, and other several infections in the human body.

Conclusion

This study contributes to the present knowledge of the presence of different active phytochemical compounds of *Aegle marmelos* plants possessing momentous broad-spectrum antibacterial efficacy. Based on the above investigation, it is confirmed that the plant extracts possess antibacterial activity against tested bacteria, the difference in the zone of inhibition suggesting that the varying degree of efficacy of various phytoconstituents of plant on the target organism. The antibacterial action of the plants may be due to the presence of various active principle chemicals in their leaves. It is concluded that the *A. marmelos* plant can be considered as an ideal for holistic medical application. Nonetheless, comprehensive research has not been carried out on their biological activity. For that reason, an in depth investigation is required to exploit bioactive compounds from the plants for medicinal purposes.

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