Original Research Article
Phytochemical, Antibacterial, Antioxidant, and Toxicity Analysis of Chloroform Extract of *Aegle marmelos* (L.) Correa Leaf

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ABSTRACT

Phytochemicals are responsible constituents for the therapeutic potential in medicinal plants. In this study, *Aegle marmelos* leaf powder was extracted with methanol using the cold percolation technique. The resulting crude methanol extract was suspended in 1% HCl and neutralized with NH_4OH, and the obtained solution was extracted with hexane and chloroform solvents accordingly. Analysis of the leaf chloroform extract through phytochemical screening confirmed the prescence of alkaloid, glycoside, phenolics, flavonoid, and protein. The GC-MS analysis of the crude chloroform extract revealed nine primary compounds, with the most prevalent constituents being Limonene dioxide, which comprised 27.78%, and GermacreneB, 20.65%. The total phenolic and flavonoid content in the chloroform extract was found to be 58.36 mg GAE/g and 142.29 mg QC/g of dry extract, respectively. Chloroform extract showed antibacterial activity against *Bacillus subtilis* of an inhibition zone of 7 mm. The IC_{50} value of the chloroform extract against DPPH radical was found to be 308.21 µg/mL. The extract demonstrated cytotoxicity against brine shrimp, with LC_{50} of 157.49 µg/mL. Further *in vitro* and *in vivo* experimentation on this plant would enhance the potential therapeutic significance evaluation of the plant.

Keywords: Aegle marmelos, extraction, phytochemicals, antioxidant, toxicity

1. INTRODUCTION

Aegle marmelos (L.) *(A. marmelos)* Correa Rutaceae family, also known as Bael, is a medium-sized, slender, aromatic tree, typically reaching a height of 6.0-7.5 meters with a girth ranging from 90 to 120 centimetres [1]. It is distributed worldwide geographically, including Nepal, Sri Lanka, Myanmar, Pakistan, Bangladesh, India, and China . The fruit, stems, bark, and leaves are among the highest accumulators of bioactive compounds synthesized as secondary metabolites and have been reported to possess various therapeutic values (ref).. *A. marmelos* has numerous ethnomedicinal uses in conventional and folk medicine [3]. The fruit and leaf of the plant have several healing characteristics that can be used in the treatment of ailments such as diabetes, dysentery, jaundice, gastralgia, diarrhoea, gastric problems, constipation, inflammation, febrile delirium, acute bronchitis, snakebite and laxative [4,5]. Through transgenic and metabolic engineering methods, information on the biosynthetic pathways and encoding enzymes present in the

leaves of *A. marmelos* would be beneficial for functional genomics. *A. marmelos* leaf was also employed for the environmental friendly creation of gold and silver nanoparticles [6]. Bioactive compounds found in plants, such as alkaloids, coumarins, carotenoids, phenolics, flavonoids, terpenoids, are likely to protect and cure several diseases . In traditional medicine, Bael pulp was used as an energy drink with milk, which is a rich source of glucose, sugar, fiber, fat, minerals potassium, calcium, phosphate, iron, thiamine, vitamin B1, ascorbic acid, nicotinic acid, riboflavin, are other nutrients found in Bael [9].

In this study, the phytoconstituents and their potential bioactivity of the leaf chloroform extract of *A. marmelos* were evaluated.

Fig. 1. Leaves and flower of *A. marmelos*

2. methodology

2.1. Collection of the Plant Sample

A. marmelos leaves were collected from Dhading, Nepal, at about 1200 m altitude. The identification of the plant was conducted with the help of the Department of Botany at Amrit Science Campus, Kathmandu. The leaves were cleaned, shade-dried, and ground into powder.

2.2. Preparation of Extract

The dried and powdered leaves of *A. marmelos* were subjected to a cold percolation for 30 days using methanol as the solvent. A rotary evaporator was used to concentrate the resulting extract. The resulting crude methanol extract was suspended with 1% HCl in distilled water and neutralized with NH_4OH solution. Then, the resulting solution was washed with hexane using a separating funnel with continuous shaking and release of air. The separating funnel was allowed to stand for some time, and the heavy aqueous layer at the bottom was collected. The light layer of hexane fraction at the top was collected separately. The aqueous layer was further processed for the extraction of chloroform fraction in the separating funnel again with continuous shaking and air release. This time, the heavy chloroform fraction at the bottom of the separating funnel was collected first and then concentrated in the rotary evaporator to obtain the crude

chloroform extract, the aqueous layer at the top was discarded. The chloroform extract was subjected to different phytochemical and bioassay tests.

2.3. Phytochemical Test

Methanol, hexane and chloroform extracts were subjected to phytochemical screening. Several tests were carried out using standard methods [10].

2.4. Gas Chromatography-Mass Spectrometry (GC-MS)

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the chloroform fraction was facilitated by the Department of Food Technology and Quality Control in Kathmandu, Nepal. It was carried out following the protocol mentioned by Shrestha et. al. (2024) [11].

2.5. Total Phenolic Content (TPC)

The total phenolic content was evaluated by using the Folin-Ciocalteau reagent method [12]. Briefly, 1 mg/mL stock solution of gallic acid was diluted to various concentrations (100 to 10 µg/mL). 1 mL of Folin-Ciocalteu phenol reagent and 0.8 mL of aqueous 1 M Na_2CO_3 solution were mixed with 0.1 mL of various concentrations of gallic acid. The reaction mixture was kept in the dark for approximately 15 minutes, after which the absorbance was measured at 765 nm using methanol as the blank. The same process was repeated for the plant extract.

Relation (1) was used to calculate the TPC in the sample as milligrams of gallic acid equivalent,

TPC = …….(1)

Where, c = concentration of Gallic acid from calibration curve (mg/mL)
V= volume of extract (mL)
m= weight of extract (mg)

2.6. Total Flavonoid Content (TFC)

The amount of the total flavonoids in the plant extract was estimated by the aluminum chloride colorimetric method [13]. 1 mL of the sample (0.1 mg/mL in methanol) was combined with 1 mL of AlCl3 (dissolved in methanol) and allowed to react for 1 hour. The absorbance was measured at 415 nm using methanol as the blank. Quercetin was used as standard. The total flavonoid content is expressed as milligrams of quercetin equivalents per gram of dried sample. The amount of total flavonoid content in the sample was calculated as milligrams of quercetin equivalent using relation 2.

TFC = ……..(2)

Where, c= concentration of quercetin from curve (mg/mL)
V= volume of extract (mL)
m= weight of plant extract (mg)

2.7. Antibacterial Activity

The antibacterial potential of chloroform extracts was performed by the agar well diffusion method [14]. The wells were created in the incubated media plates using a sterile cork borer (4 mm) and labeled suitably. The plant extract (15 µL of 25 mg/mL) as a working solution was then added to each well using a micropipette. The solvent, methanol was taken as a negative control. The plates were incubated at 37°C for 6 hours. Following incubation, the plates were inspected for the presence of a zone of inhibition around the well, characterized by a clear area devoid of microbial growth. To ascertain the antibacterial effectiveness, the ZOI was measured using a ruler, and the mean value was noted. The microbial species *Staphylococcus aureus, Bacillus subtilis, Micrococcus leutus, Pseudomonas aeruginosa, Enterobacter cloaceae subsp. Disolvens,* and *Klebsiella pneumonia* were taken to evaluate antibacterial potential of the extract.

2.8. DPPH Scavenging Assay

The antioxidant activity of the extract was evaluated using 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical scavenging. The methodology was used according to the previous research of Poudel et al. [15]. Briefly, the initial stock solution of 1 mg/mL of extract was diluted by a two-fold dilution process in several concentrations. 500 µL of each of the solutions was combined with 1500 µL of 0.1 mM DPPH solution and incubated for 30 minutes in the dark. Their absorbance was measured against a blank at 517 nm after 30 minutes. A mixture of methanol and DPPH was taken as the blank, and standard ascorbic acid was taken as a positive control. Relation 3 was used to calculate % radical scavenging.

% DPPH scavenging = ×100%.....(3)

The IC_{50} (50% Inhibitory concentration) values of the extract were calculated using the logarithm range by plotting the extract concentration vs. the associated scavenging action in Office Excel.

2.9. Brine Shrimp Lethality Assay

The brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of plant extracts [16]. The chloroform extract was subjected to brine shrimp nauplii at varying concentrations (500 to 15.625 µg/mL) for 24 hours. The effectiveness of the chloroform extracts was assessed by quantifying the number of motile nauplii using logarithmic regression analysis of the percent mortality plotted against the corresponding concentration. This method determined the concentration of the chemical that resulted in mortality for 50% of the test subjects after a specific exposure duration. Relation 4 was used to calculate the percentage mortality of the nauplii.

% Mortality = ×100%.....(4)

3. results and discussion

3.1. Phytochemical Screening

The chloroform extract fraction of *A. marmelos* was found to contain alkaloids, phenols, saponins, flavonoids, tannins, cardiac glycosides, carbohydrates and proteins, in the qualitative phytochemical analysis. The results of the phytochemical screening of the extract are shown in Table 1.

Table 1. Phytochemical screening of chloroform extract

Phytochemical constituents	Extract	Test
	Chloroform	
Alkaloids	+	Mayer's Test Dragendorff's Test Wagner's test
Flavonoids	+	Lead acetate test Shinoda test
Phenols	+	Ferric chloride test
Glycosides	+	Molisch's test Fehling's test
Tannins	+	Gelatin test
Quinones	-	Sodium hydroxide and H_2 SO_4
Saponins	+	Froth test
Protein	+	Xanthoproteic test
Carbohydrates	-	Molisch's test Benedicts test

'+' indicates presence, and '-' indicates the absence

3.2. Quantification of Phenolic and Flavonoid Content

The Total Phenolic Content (TPC) was quantified in milligrams of Gallic acid equivalent, employing the calibration curve of Gallic acid. The TPC was tested as gallic acid equivalent (y = 0.0083x – 0.0014, R2 = 0.996) with reference to a standard curve (Figure 2(A)). The phenolic content in the chloroform extract was found to be 58.36 mg GAE /g of dry extract.

Likewise, the total content of flavonoid was demonstrated by relation to a standard curve (y = 0.0253x-0.071, R^2 = 0.9982) (Figure 2(B)). *A. marmelos* leaf extract was found to have 142.29 mg QC/g of TFC in the leaf extract. Table 2 presents the detailed observations during the calculation of the phytochemicals found in chloroform extract.

Phenolic compounds are organic substances produced as byproducts in plant metabolic pathways such as pentose phosphate, shikimate, and phenylpropanoid [17]. Phenolic substances exhibit a broad range of physiological properties, including anti-allergic, anti-inflammatory, antimicrobial, antioxidant, cardioprotective, antimutagenic, anticarcinogenic, and gene expression modifying capabilities. The health advantages associated with consuming abundant fruits and vegetables have been linked to the presence of phenolic compounds [18]. These compounds provide protection against ultraviolet radiation, bacteria, and pathogens in plants. The most significant health benefits of flavonoids are their antioxidant properties, ability to treat diabetes, chelating capabilities and reduction in the incidence of heart disease [19]. Flavonoids demonstrate pharmacological effects such as inhibiting histamine release, preventing blood platelet adhesion, and reducing the action of lens aldose reductase. They also can counteract the inflammatory effects of hepatotoxins and act as heart stimulants. [20,21]. In this plant, flavonoid content in the leaf was found to be higher than the phenolic content which would help to reduce potential reactive oxygen species and enhance the therapeutic value of this plant.

Table 2. Quantitative estimation of phytochemicals of TPC and TFC in chloroform extract

Evaluations	Sample concentration taken (mg/mL)	Absorbance	contents
TPC	1	0.483	58.36 mg GAE /g
TFC	0.1	0.289	142.29 mg QC /g

Fig. 2. Calibration curve of standard (A) Gallic acid (B) Quercetin solution

3.3. GC-MS Spectra Analysis

The GC-MS chromatogram of the chloroform extract of *A. marmelos* is presented in Figure 2.

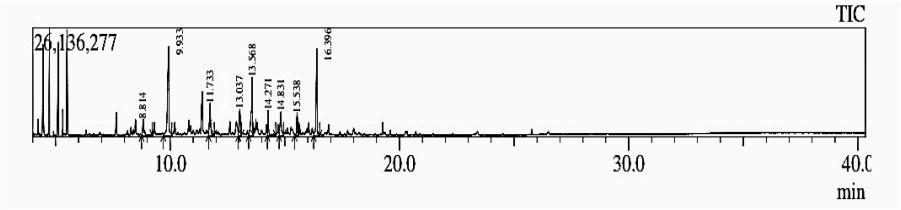
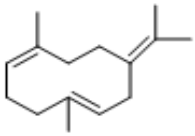


Fig. 3. GC-MS chromatogram of chloroform extract of *A. marmelos*

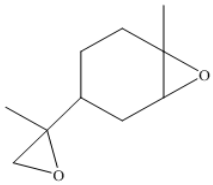
The composition of the chloroform extract was analyzed by GC-MS and it revealed the presence of 9 major compounds, among which Limonene dioxide (27.78%) and Germacrene-B (20.65%) (Figure 3) were found to be the most abundant. Table 3 shows the compound detail obtained from GC-MS analysis.

Table 3. List of Compounds detected in the chloroform extract

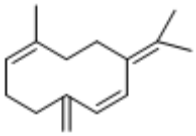
S.N.	Name of Compound	Retention time	Molecular formula	Molecular Weight	Area(%)
1	4(10)-Thujen-2-ol, acetate	8.814	$C_{12}H_{18}O_2$	194	4.85
2	Limonene dioxide	9.933	$C_{10}H_{16}O_2$	168	27.78
3	Cyclohexanone,2-(1-methyl-oxopropyl)	11.733	$C_{10}H_{11}O_2$	168	8.38
4	(1S,2S,3R,5S)-(+)-Pinanediol	13.037	$C_{10}H_{18}O_2$	170	6.40
5	Cis-caryophyllene	13.568	$C_{15}H_{24}$	204	15.14
6	1,4,7-cycloundecatriene,1,5,9,9, tetramethyl	14.271	$C_{15}H_{24}$	204	5.50
7	GermacreneD	14.831	$C_{15}H_{24}$	204	6.79
8	Cubenol	15.538	$C_{15}H_{26}O$	222	4.48
9	GermacreneB	16.396	$C_{15}H_{24}$	204	20.65



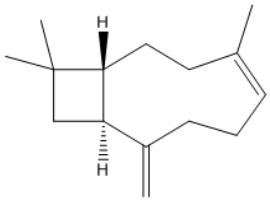
GermacreneB



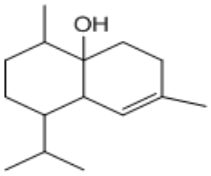
Limonene dioxide



GermacreneD



Cis-Caryophyllene



Cubenol

Fig. 4. Chemical structures of some l compounds obtained from GC-MS

3.4. Antibacterial Activity

The antibacterial test of the chloroform extract was assessed against six different bacterial strains. The chloroform extract was able to show 7 mm zone of inhibition on *B. subtilis* culture and all other bacterial cultures were found to be resistant to the extract. Ampicillin (1 mg/mL) was taken as a positive control, and DMSO was used as a negative control. The chloroform extract was found to be effective against bacillus bacterial culture.

Table 4. The diameter (mm) of the ZOI of the chloroform extract

Bacterial culture used	Zone of inhibition
S. aureus KCTC 1916	No activity
B. subtilis KACC 17047	7mm
M. leutus KACC 13377	No activity
P. aeruginosa KACC 10232	No activity
E. cloaceae subsp. disolvens KACC 13002	No activity
K. pneumonia KCTC 2242	No activity

3.5. Antioxidant Activity Evaluation

The antioxidant potential exhibits an inverse relationship with the IC_{50} value, which can be calculated through linear regression analysis of the percentage inhibition against concentration. A lower IC_{50} value signifies its greater antioxidant potential.

Fig. 5.(??)

The chloroform extract revealed an IC_{50} of 308.21 µg/mL (Figure 5) against the DPPH radical and also showed significant antibacterial activity against *B. subtilis.* Free radicals, specifically reactive oxygen species (ROS) and reactive nitrogen species (RNS) contribute to age-related damage to lipids, proteins, enzymes, and nucleic acids, leading to cellular or tissue injury and oxidative stress. Presence of these free radicals are associated with degenerative conditions including inflammation, cancer, atherosclerosis, diabetes, liver damage, Alzheimer's disease, Parkinson's disease, and coronary heart disease [22]. Any constituent with high antioxidant potential could be applicable as the therapeutic to reduce such stress and support to defence against diseases. The inhibitory concentration value of the chloroform extract towards DPPH free radical suggested the mild antioxidizing ability of the extract.

3.6. Brine Shrimp Toxicity Assay

Fig. 6. Plot of concentration of chloroform extract versus % mortality

This study showed that the LC_{50} value of the chloroform extract was found to be 157.49 µg/mL (Figure 6). Brine Shrimp Lethality Assay, serves as a preliminary toxicity screening method for plant extracts, fungal toxins, heavy metals, cyanobacterial toxins, pesticides, and cytotoxicity of dental substances [23]. A lethal concentration of less than 200 µg/mL could be considered as pharmaceutically potential and mild toxic substance [24]. The LC_{50} of the chloroform extract showed the moderate toxic nature of the extract towards brine shrimp.

4. Conclusion

In this study, the potential bioactivity test and chemical composition demonstration of the extract through GCMS was carried out. GC-MS analysis of chloroform extract showed the presence of nine major compounds. Chloroform extract showed antibacterial activity against *Bacillus subtilis* at 7 mm zone of inhibition. The extract showed mild oxidizing phenomena towards DPPH free radical and was found less toxic to the brine shrimp larva. All the results showed the therapeutic potential of *A. marmelos* in this study. The isolation of the phytochemicals and evaluation of their therapeutic aptitude to the various diseases *invitro* and *in vivo* approach would help to expose the pharmaceutical significance of this plant further.

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