

Original Research Article

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

ABSTRACT

Phytochemicals are responsible constituents for the therapeutic potential in medicinal plants. In this study, *Aegle marmelos* leaf powder was extracted through cold percolation and fractionation process. Analysis of the leaf chloroform extract through phytochemical screening confirmed the presence of alkaloids, glycosides, phenolics, flavonoids, 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 Germacrene-B, 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* with an inhibition zone of 7 mm. The IC₅₀ of the chloroform extract against DPPH radical was found to be 308.21 µg/mL. The extract demonstrated cytotoxic against brine shrimp larva, with a lethal concentration 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 of 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, Pakistan, 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 [3]. *A. marmelos* has numerous ethnomedicinal uses in conventional and folk medicine [4]. 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, diarrhea, gastric problems, constipation, inflammation, febrile delirium, acute bronchitis, snakebite and laxative [5,6]. 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 environment-friendly creation of gold and silver nanoparticles [7]. Bioactive compounds found in plants, such as alkaloids, coumarins, carotenoids,

phenolics, flavonoids, and 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 sugar, fiber, fat, minerals potassium, calcium, phosphate, iron, thiamine, vitamin B1, ascorbic acid, nicotinic acid, riboflavin, are other nutrients found in Bael [10].

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 an altitude of 1.2 KM above sea level. The identification of the plant was conducted with the help of the Department of Botany at Amrit Science Campus, Kathmandu. The leaves were cleaned with distilled water, shaded-dried, and ground into powder using an herbal medicine disintegrator.

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 about ten minutes. The heavy aqueous layer at the bottom of the funnel was collected in a beaker and a lighter 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

The chloroform extract was subjected to phytochemical screening. Several tests were carried out using standard methods [11].

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 [12].

2.5. Total Phenolic Content (TPC)

The TPC was evaluated by using the Folin-Ciocalteu reagent method and the same methodology was followed as mentioned by Kumar et al. 2013 [13]. The absorbance was measured at 765 nm using methanol as the blank.

2.6. Total Flavonoid Content (TFC)

TFC in the plant extract was estimated by the aluminum chloride colorimetric technique and protocol was used as done by Alighiri et al. (2020) [14]. At 415 nm, the absorbance of the reaction mixture was measured. methanol was taken as blank and quercetin was used as the standard.

2.7. Antibacterial Activity

The agar well diffusion method was used to evaluate the antibacterial potential of chloroform extracts and was carried out as done by Athanassiadis et al. 2009 [15]. The plant extract (15 μ L of 25 mg/mL) as a working solution was then added to each well using a micropipette (Microlit company). To ascertain the antibacterial effectiveness, the ZOI was measured using a ruler, and the mean value was noted [16]. The microbial species *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus leutus*, *Pseudomonas aeruginosa*, *Enterobacter cloacae* subsp. *Disolvens*, and *Klebsiella pneumonia* were taken to evaluate the 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. 2023 [17]. The absorbance of the reaction mixture was measured at 517 nm. The IC₅₀ (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 and the protocol was followed as mentioned by Sarah et al. 2017 [18].

The mortality for 50% of the test subjects after a specific exposure duration. Relation (1) was used to calculate the percentage mortality of the nauplii.

$$\% \text{ Mortality} = \frac{\text{Number of dead nauplii}}{\text{Total number of nauplii}} \times 100\% \dots \dots \dots (1)$$

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. Table 1 contains detailed information about the tests carried out.

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 ($y = 0.0083x - 0.0014$, $R^2 = 0.996$) 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 (milligram gallic acid equivalent per gram) 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 chloroform extract was found to have 142.29 mg QC/g (milligram quercetin equivalent per gram) flavonoid content. 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 [19]. Phenolic substances exhibit a broad range of physiological properties, including anti-inflammatory, antioxidant, anticarcinogenic, cardioprotective, and gene expression modifying capabilities [20-23]. The health advantages associated with consuming abundant fruits and vegetables have been linked to the presence of phenolic compounds [24]. They also provide protection against harmful radiation 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 in human welfare [25]. Flavonoids demonstrate pharmacological effects such as inhibiting histamine release, preventing blood platelet adhesion, and reducing the action of lens aldose reductase. 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.

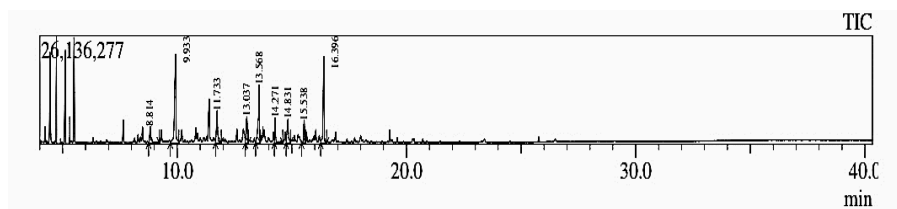


Fig. 3. GC-MS chromatogram of chloroform extract of *A. marmelos*

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

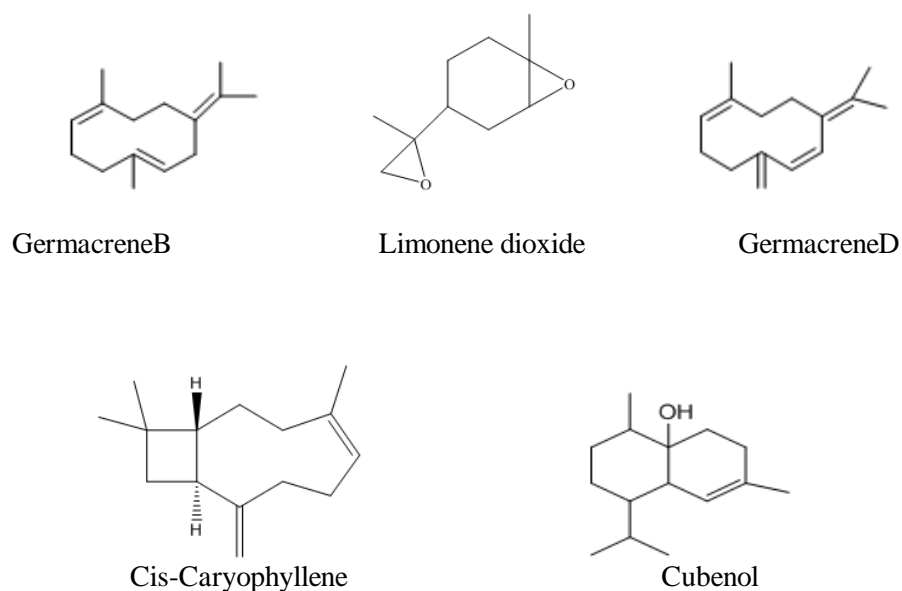


Fig. 4. Chemical structures of some of the compounds obtained from GC-MS

3.4. Antibacterial Activity

The antibacterial test of the chloroform extract was assessed against six different bacterial strains. It 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
<i>S. aureus</i> KCTC 1916	No activity
<i>B. subtilis</i> KACC 17047	7mm
<i>M. leutus</i> KACC 13377	No activity
<i>P. aeruginosa</i> KACC 10232	No activity
<i>E. cloaceae</i> subsp. <i>disolvens</i> KACC 13002	No activity
<i>K. pneumoniae</i> KCTC 2242	No activity

3.5. Antioxidant Activity Evaluation

The antioxidant potential exhibits an inverse relationship with the IC₅₀ value, which can be calculated through linear regression analysis of the percentage inhibition against concentration. A lower IC₅₀ value signifies its greater antioxidant potential.

Fig. 5. DPPH scavenging activity of chloroform extract

The chloroform extract revealed an IC₅₀ of 308.21 µg/mL (Figure 5) against the DPPH radical and also showed significant antibacterial activity against *B. subtilis*. Free radicals including reactive nitrogen species (RNS) and reactive oxygen species (ROS) are responsible for age-related damage, tissue injury, and oxidative stress. The presence of these free radicals is associated with degenerative conditions in vital organs in the human body including the pancreas, intestine, lungs, heart, liver, and nervous system [26]. Any constituents with high antioxidant potential could be applicable as therapeutic to reduce such stress and support defense 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. Graph plot of concentration of chloroform extract versus mortality percentage

This study showed that the lethal concentration of chloroform extract against *Artemia*

salina larvae was found to be 157.49 µg/mL (Figure 6). Brine Shrimp Lethality Assay is a technique of preliminary screening of toxicity [27]. A lethal concentration of less than 200 µg/mL could be considered as a pharmaceutically potential and mild toxic substance [28]. The LC₅₀ of the chloroform extract showed the moderate toxic nature of the extract towards brine shrimp.

4. Conclusion

In this study, the *in vitro* bioactivity and chemical composition demonstration of the extract through GCMS was tested. 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 *in vitro* and *in vivo* approach would help to bolster evidence for the pharmaceutical significance of this plant further.

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