Butwal Campus Journal, Vol. 7, No. 1: 86-98, August 2024
Research Management Cell, Butwal Multiple Campus, Tribhuvan University, Nepal
DOI: https://doi.org/10.3126/bcj.v7i1.71724

Antimicrobial and Phytochemical Analysis of Half-ripe Fruits of *Aegle marmelos* (l.) Correa Found in Butwal Area

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Abstract

In this work, the half-ripe fruit of *Aegle marmelos* found in the Butwal region was examined for antibacterial, phytochemical, and antioxidant activity as well as to quantify antioxidant activity, TPC, and TFC. Half-ripe *A. marmelos* fruits were collected from 12 to 24 April 2022 from Butwal area. Fruit pulp was shade-dried, crushed, and cold-macerated in methanol solvent to prepare methanolic extract. The crude extract was subjected to a preliminary phytochemical screening that found flavonoids, reducing sugar, polyphenols, alkaloids, steroids, tannins, terpenoids, coumarins, quinones, glycosides. The DPPH radical scavenging assay was used to evaluate the antioxidant activity, the agar well diffusion method was used to assess the antimicrobial activity, the Folin-Ciocalteu method was used to assess the total phenolic content, and the aluminum chloride colorimetric method was used to assess the flavonoid content. The DPPH radical scavenging activity in terms of IC_{50} of the fruit sample was 99.87±1.27µg/mL. The total phenolic content (TPC) was found 88.55±6.71 (mg of GAE/g), total flavonoid content (TFC) was found 10.98±0.134 (mg of QE/ g). Upon antimicrobial test, the extract was sensitive to both gram-positive bacteria *Staphylococcus aureus* (ZOI= 10.4±0.75 mm) and gram-negative bacteria to *Escherichia coli* (ZOI=11.22±0.18mm) almost equally.

Keywords: Antibacterial, Antioxidant, 2,2-Diphenyl -1-picrylhydrazyl, Flavonoid, content.

Introduction

Due to its fluctuating elevation ranging from 70 m to 8848 m (GC, et al., 2019) and an estimated 7000 species of flowering plants (Muhammad, et al., 2011), representing approximately 10% of medicinal flora, Nepal is characterized by diverse climatic conditions and geographical heterogeneity. Nevertheless, there exist limited empirical investigations pertaining to these therapeutic plants (Manandhar, et al., 2002). *Aegle marmelos*, commonly referred to as bel or bael in Nepali, constitutes a notable spiny aromatic tree distinguished by trifoliate leaves and greenish-white flowers. This species is a medium-sized arboreal entity exhibiting moderate growth, attaining heights of approximately 12 to 16 meters. It is characterized by its spreading, spiny branches, dense, soft bark that exhibits flaking, and a diminutive trunk. This tree is typically found at altitudes of up to 1200 meters in open, arid woodlands situated in both hilly and plain regions. The half-ripe bel fruit utilized for this study was sourced

from the Sukkhanagar-8 Butwal of Nepal between April 12 and April 24, 2022. Butwal City is located in the Rupandehi district of Nepal's Lumbini Province, at an elevation of 150 meters above sea level. Various plant parts, including leaves, roots, and fruits, have been extensively employed in numerous indigenous medicinal practices, notably in the treatment of dysentery, diabetes, and appetite loss, as endorsed by Indian Ayurveda and allopathic medicine. This species is indigenous to Southeast Asia and the Indian subcontinent, being prevalently distributed across India, Nepal, Bangladesh, Pakistan, Malaysia, Sri Lanka, and Thailand. *Aegle marmelos* has been demonstrated to possess anti-inflammatory, antiviral, antimicrobial, radioprotective, anticancer, antipyretic, anti-fertility, antiulcer, and antidiarrheal properties, as evidenced by multiple experimental and clinical studies (Rahman, et al., 2014).The leaves and young shoots of bael are utilized in the preparation of salads, while the fruit pulp is employed in the creation of summer beverages, murabba, puddings, confectionery, squash, toffee, and pulp powder, among other products. The juice extracted from its leaves, when combined with honey, can be utilized to alleviate fever and treat conditions such as tuberculosis and dysentery, whereas its oil extract serves as a remedy for respiratory ailments. The bark of *Aegle marmelos* is abundant in antioxidants and facilitates insulin secretion, thereby exerting beneficial effects on diabetes. The fruit extract of *A. marmelos* encompasses phytochemicals such as polyphenols, carotenoids, pectin, alkaloids, flavonoids, coumarins, terpenoids, tannins, and phenolic acids, representing a vital area of research due to its diverse pharmacological properties (Sharma, et al., 2022). Baliga, et al. (2010) in their publication documented the presence of phytochemicals such as alkaloids, flavonoids, lignins, coumarins, phenols, terpenoids, and glycosides in the raw fruit extract. Furthermore, bael fruits have been employed in various traditional remedies as a laxative and for the treatment of respiratory conditions, chronic diarrhea, dysentery, and peptic ulcers. Reports indicate that the fruit possesses a multitude of medicinal properties, including free radical scavenging, antioxidant, antibacterial, antiviral, anti-diarrheal, gastroprotective, anti-ulcerative colitis, antidiabetic, cardioprotective, and radioprotective effects. Scientific investigations have corroborated numerous ethnomedicinal applications. Many medicinal qualities and classes of compounds have been identified from different anatomical areas of *A. marmelos*; these include coumarins (Marmelosin, marmesin, imperatorin), alkaloids (Aeglin, aegelenine), tannins (skimmianine), carotenoids, and seed oils (Dhankhar, et al., 2011).

Oxidative stress within the organism is the cause of chronic pathologies like diabetes, as parkinson's disease, alzheimer's disease, cardiovascular illnesses, and neoplasms (Rezaeizadeh, et al., 2011). In conditions characterized by oxidative stress, the equilibrium between the body's antioxidant levels and reactive oxygen species (ROS) and reactive nitrogen species (RNS) are disrupted, leading to the degradation of proteins, lipids, and nucleic acids within cellular structures, ultimately culminating in cellular apoptosis. ROS and RNS are predominant contributors to free radical generation, which are implicated in critical disorders (Willcox, et al., 2004). Antioxidants are defined as compounds that inhibit oxidative processes, thereby attenuating or postponing oxidative stress, as articulated by Shyur, et al. (2005). An

elevation in antioxidant intake can mitigate health hazards and stave off diseases. Synthetic antioxidants such as BHA, BHT, TBHQ, and gallic acid esters are commonly employed. However, these agents exhibit particular adverse effects. Within plant extracts, polyphenols, and in particular flavonoids, are recognized as potent antioxidants (Bernard, et al., 2010). Flavonoids possess the capability to effectively scavenge the majority of oxidizing agents. The pulp of *A. marmelos* fruit has been documented as a remedy for gastrointestinal ailments in humans, as reported by Rajan, et al., (2011) due to elevated antioxidant properties of the fruit pulp extracts. The aqueous extract revealed the presence of flavonoids, lignin, tannins, terpenoids, saponins, and steroids. Conversely, the alcoholic extract exhibited the presence of alkaloids, while being devoid of saponins. Aqueous extracts demonstrated significant antioxidant efficacy, with IC_{50} values ranging from 37.11±3.50 to 158.99±59.46 µg/mL, whereas the alcoholic extract exhibited antioxidant potential with IC_{50} values spanning from 35.02±8.10 to 283.06 ± 135.80 µg/mL (Rajan, et al., 2011). As per the findings of Pandey, et al., (2011), methanolic and water extracts of the plant exhibited the least antibacterial efficacy in comparison to ethanolic and ethyl acetate extracts. The minimum inhibitory concentration (MIC) values were determined to be 1.98 mg/mL in fruit extracts containing ethanol and ethyl acetate against *S. aureus*, and 11.90 mg/mL in methanol extract against *Pseudomonas aeruginosa*. The principal antibacterial compounds identified in *A. marmelos* comprised tannins, saponins, terpenoids, alkaloids, and polyphenols (Pandey, et al., 2011). Devi, et al., (2020) noted that the methanolic extract of *A. marmelos* exhibited the most pronounced inhibitory effect against *B. cereus*, and followed against *P. aeruginosa* and *Escherichia coli*. An analysis of the phytochemical composition of the methanolic extract revealed the presence of steroids, glycosides, alkaloids, tannins, and saponins. According to Bhera, et al., (2014), the crude fruit pulp extract from *Aegle marmelos* (Linn.) Correa was found to contain reducing sugars, tannins, saponins, phenols, and flavonoids. The antibacterial efficacy was observed against *Staphylococcus aureus* at various concentrations. The ethanolic extracts of the plant was potent against *S. aureus* ATCC 29213 at concentrations ranging from 50 to 100 µg/mL. Furthermore, he observed, both petroleum ether extracts and aqueous extracts effectively suppressed the growth of the specified strains. In conclusion the finding of this study revealed that methanolic extract of raw fruit of *Aegle marmelos* rich in antioxidant, antibacterial activities with measurable TPC and TFC contents.

Purpose of the Study

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The specific objectives of this research study are as follows:

a) To perform a phytochemical analysis on methanolic extract of *A. marmelos's* half-ripe fruit.

b) To carry out an antibacterial activity test

c) To ascertain the antioxidant potential, total flavonoid content, and total phenolic content in the methanolic extract.

Materials and Methods

Tools Employed

The equipment included mechanical grinders, micropipettes, conical flasks, funnels condensers, beakers, rotary evaporators, water baths, test tubes, reagent bottles, biosafety cabinets, vials, digital balances, cuvettes, incubators, autoclaves, UV-visible spectrophotometers (Aczel, Model: AUV-8S SR NO: 20210702206), petri plates etc.

Required Chemicals and Reagents

All along, analytical-grade chemicals were used. Folin-Ciocalteu reagent, DMSO, 2,2-diphenyl-1-Pycryl Hydrazyl, (Qualigenes Pharma Pvt. Ltd. India), mueller-hinton agar, nutrient agar, tetracycline (Himedia Pvt. Ltd. India), quercetin, methanol, gallic acid, NH_4SCN, $FeSO_4$, NH_4OH, distilled water, $NaNO_2$, $AlCl_3$, Ascorbic acid, etc. (Fischer India Pvt. Ltd.). Reagents including Meyer's, Molish's, and Dragendorff's were prepared in the laboratory.

Collection and Identification of Plant

Between April 12 and April 24, 2022, fresh, half-ripe fruits of *Aegle marmelos* (L.) Correa were collected from Sukkhangar-8, Butwal Sub-Metropolitan City, Lumbini province. It lies 150 m above from sea level and coordinates is 27.70°N to 83.466ºE. The plant was identified by botanist Dr. Anant Gopal Singh.

Drying and Preparation of Extract

Following a tap water wash, distilled water was used to clean the harvested fruits. Then the fruit pulp was extracted and allowed to dry in the shade for two weeks before being ground using a machine grinder. The pulverized specimen was gathered in a sterile plastic bag. To extract the methanolic fruit pulp extract, cold maceration was used. 500 mL of methanol were used to soak 50 grams of powdered dry material, which was shaken constantly for 72 hours. Following that, the mixture was filtered using Whatman No. 1 filter paper. A rotary evaporator operating at 37°C was used to concentrate the filtrate. In vials, the dehydrated methanolic extract was stored at 4°C for further analysis.

Analysis of secondary metabolites

The protocol from Wankhar, et al., (2015) was applied for phytochemical screening. The colour reaction was used in the study together with a number of specialized reagents (Shrestha, et al., 2022). The symbols (+) and (-) in the qualitative data denote the presence and absence of phytochemicals, respectively. Tests for alkaloids, flavonoids, terpenoids, coumarins, quinones, polyphenols, reducing sugar, glycosides, tannins, saponins, and steroids were conducted as part of the phytochemical screening process.

Table 1: Phytochemical screening of methanolic extract of fruit

SN	Test	Results
1	Alkaloids	+
2	Terpenoids	+
3	Coumarins	+
4	Flavonoids	+
5	Quinones	+
6	Polyphenols	+
7	Reducing sugars	+
8	Glycosides	+
9	Tannins	+
10	Saponins	+
11	Steroids	+

Note: Where, (+) for present and (-) for absent of phytochemicals

Evaluating Antioxidant Inhibition

Antioxidant activity was measured using the Paulsamy, et al., (2012) method of scavenging radicals 2,2-Diphenyl-1-picrylhydrazyl (DPPH). A solution of 500 µg/mL of different concentrations of extract and a solution of 2 mL (0.1mM) DPPH were combined. To ensure a full reaction, the mixture was kept in the dark for thirty minutes. Finally, each plant sample's absorbance at 517 nm was determined using a UV spectrophotometer. Using the following formula, the sample's radical scavenging activity was determined:

Percent Radical Scavenging (%) = $[(A_0 - A_1 / A_0) \times 100\%]$

where A_0 denotes the absorbance of the control and A_1 the sample extract's absorbance. Control was the test solution without sample. Ascorbic acid was the standard dosage. The technique employed a concentration-based ascorbic acid solution (10–100 µg/mL) as a standard. The fruit sample's antioxidant activity was determined using the 50% inhibition coefficient (IC_{50}), which is the dose required to prevent the production of DPPH radicals. Fifty percent inhibitory concentration (IC_{50}) is the effective concentration of the sample required to scavenge fifty percent of the DPPH free radicals. The inhibition curve was used to get the IC_{50} values by plotting the extract concentration against the corresponding scavenging effect.

Calculating the Phenolic Content in Total

The total phenolic content was ascertained by following the procedure provided by Khan, et al., (2018). The FCR method was used to determine the extract's total phenolic content using gallic acid as a standard. 1 mL of fruit extract containing 500 ug/mL was mixed with 5 mL of 10% FCR. After five minutes of standing, 4mL of 7% (w/v) sodium carbonate was mixed and stirred on its own. The mixture's absorbance at 765 nm was measured after 30 minutes of incubation. Every experiment was

conducted with three different sample concentrations in triplicate. To create the calibration curve, several quantities of gallic acid (10, 20, 30, 40, 50, 60, 70, and 80 µg/mL) in methanol were utilized as the standard.
The calibration curve was used to calculate the total phenolic content, and the findings were represented as mg of GAE/gm dry weight of extract.
The formula is TPC= C×V/M where M = weight of plant extract in gram, V = volume of extract in mL, and C = concentration of gallic acid as determined by the calibration curve in mg/mL. The linear correlation coefficient (R^2) value and regression equation were obtained using the gallic acid calibration curve. The TPC value of extract was determined using the regression equation.

Calculating the Total Flavonoid Concentration

The flavonoid content of the samples was determined by using the aluminum chloride colorimetric technique according to the protocols provided by Bag, et al. (2015). After combining 1 mL of an extract with a concentration of 500 µg/mL with 4 mL of pure water, 0.3 mL of 5% sodium nitrite was promptly added. Followed by adding 0.3 cc of 10% aluminum chloride after five minutes and wait for an additional six minutes. The absorbance was measured at 415 nm after thoroughly shaking the mixture and adding 2 mL of 1 M sodium hydroxide and 2.4 mL of distilled water to create the volume 10 mL. The calibration curve was made using quercetin as the standard at different concentrations (10, 20, 30, 40, 50, 60, 70, and 80 µg/mL). The total flavonoid concentration was determined using the calibration curve, and the results were expressed as mg QE per gram of the extract's dry extract weight. The formula is TFC= C×V/M, where M = weight of plant extract in g, V = volume of extract in mL, and C = concentration of quercetin as determined by the calibration curve in mg/mL.

Statistical Analysis

For every concentration, three observations were made. Statical analysis were performed using one-way ANOVA. The mean and standard deviation (SD) of the data were used to express them. Microsoft Excel 2019 was used to perform additional statistical analysis and get the IC_{50} values.

Test for Antimicrobial Activity

Antibacterial activity was evaluated by the agar well diffusion method following protocols Sharma, et al., (2023). The diameter of the zone of inhibition (ZOI) was measured in order to assess the antibacterial activity of plant extracts. The antimicrobial assay was conducted at BMLT Department of Crimson College of Technology (CCT) Butwal, Rupandehi.

Collection of Test Organisms, Antibiotics and Preparation of Plant Extract:

The positive control antibiotic drug tetracycline (1mg/mL) was purchased from local market of Butwal and prepared in autoclaved double distilled water. Two bacterial strains *Staphylococcus aureus* and *Escherichia coli* were were acquired from the BMLT Department of Crimson College of Technology, 50% DMSO was prepared as negative control. Exactly, 50 mg of plant extract was dissolved in 1000 µL 50% DMSO in sterile vials as test sample.

Mueller Hinton Agar (MHA) Media Preparation

In a 1000 mL sterile conical flask, capped with aluminum foil, 500 mL autoclaved distilled water was used to dissolve precisely 19 g of MHA. Subsequently, the conical flask underwent 15 minutes of autoclaving at 121°C and 15 Ibs of pressure for sterilization. In a biosafety cabinet, the heated conical flask medium was allowed to cool between 40 and 50°C. Next, each petri dish was filled with 25 mL of this media, which was then allowed to set and stored in the refrigerator.

Antibacterial Activity Assessment:

Mueller Hinton Broth (MHB) was used for the growth of test microorganisms *Staphylococcus aureus* and *Escherichia coli* and it was incubated at 37ºC for 24 h and its turbidity was maintained by using 0.5 McFarland. The MHA plates were lawn cultured with the microbial inoculum. Then, 50 mg/mL concentration of methanolic extract was prepared in 50 % DMSO solvent. 6 mm well were bored in the lawn cultured media with the help of cork borer. Each well was filled with 50 µL of prepared samples with positive control (tetracycline 1 mg/mL) and negative control (50% DMSO) and the petri dishes were left for 15 minutes for diffusion and incubated for 18-24 h at 37°C. After, incubation of zone of inhibition (ZOI) was measured with the help of scale in mm. Bioassays were performed in triplicate.

Results and Discussion

Extraction Yield Value:

Half-ripe fruit powder of *Aegle marmelos* was found to have a 13.56% (w/w) yield of methanolic extract.

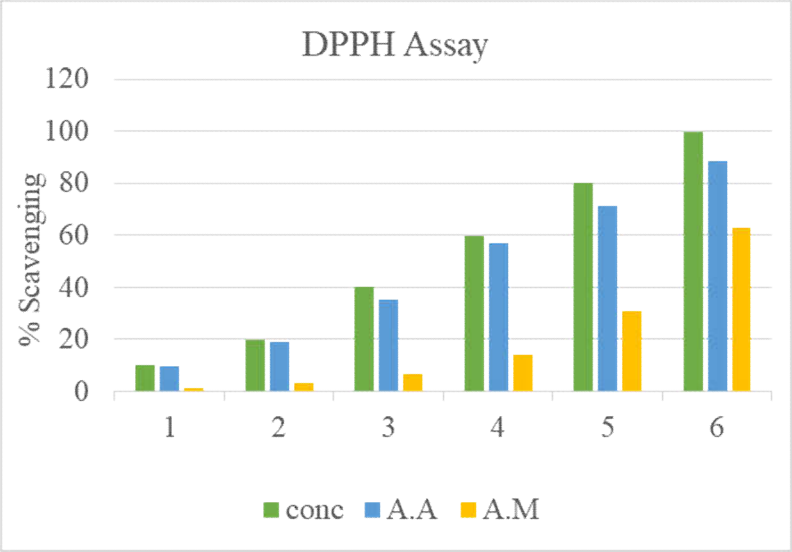
Phytochemical Screening

Following phytochemical investigation, the samples contained flavonoids, alkaloids, steroids, polyphenols, terpenoids, coumarins, quinones, tannins, glycosides and reducing sugar. These compounds are responsible for a number of pharmacological effects.

Antioxidant Activity Estimation using DPPH Assay

Figure 1 illustrates the percentage of fruit samples' DPPH radical scavenging activity at various methanol concentrations. The DPPH radical scavenging activity of the fruit sample was 99.872±1.27µg/mL, as determined by the IC_{50}. The ascorbic acid standard IC_{50} for DPPH radical scavenging activity was 55.40±0.89µg/mL. Antioxidants found in plants have the ability to visibly change the stable, purple-coloured DPPH radical into a yellow hue.

Figure 1: The percentage results of the scavenging of DPPH free radical by methnolic



extract of raw fruit in comparison to ascorbic acid, with the mean ± and standard deviation (n=3) at concentration of 10 to 100 ppm (A. A=Ascorbic acid, A.M=*Aegle marmelos*)

Finding the Total Phenolic Content (TPC)

A concentrated gallic acid solution with a regression coefficient (R^2) of 0.9923 at 765 nm confirmed the validity of Beer's rule (Figure 2). The average phenolic concentration in raw fruit extract was 88.55 mg GAE/g dry extract weight.

Determination of Total Flavonoid Content (TFC)

The aluminum chloride colorimetric technique was used to quantify the total flavonoid concentration in methanolic extracts, with quercetin serving as a reference. The quercetin solution with a concentration of 10-80 µg/mL at 415 nm and a regression coefficient (R^2) of 0.9903 (Figure 3) demonstrated the validity of Beer's rule. The average total flavonoid content was 10.98±0.134 mg QE/g.

Table2: Absorbance of gallic acid at different concentrations

The concentration of Gallic acid (ppm)	Absorbance (Y)
10	0.4326
20	0.877
30	1.2923
40	1.6976
50	2.047
60	2.367
70	2.526
80	2.71
90	2.673

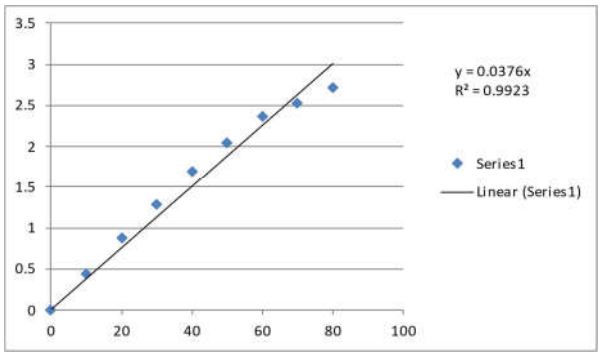


Figure 2: Calibration curve for standard gallic acid

Table 3: Calculation of Total Phenolic Content (TPC) of fruit extract

Conc.of extract(ppm)	Absorbance (Y)	G.A.E. conc.(x) (µg/mL)	TPC = C×V/M (mg of GAE/g)	TPC Ave (mg of GAE/g)
500ppm	1.645	44.4594	88.91	88.558±6.175
500 ppm	1.646	44.4864	88.92	
500ppm	1.624	43.8918	87.783	

Table 4: Absorbance of Quercetin at different concentrations

Conc. of standard Quercetin (ppm)	Absorbance (Y) (mean)
10	0.1213
20	0.525
30	0.896
40	1.1466
50	1.15586
60	1.7685
70	2.12
80	2.4425

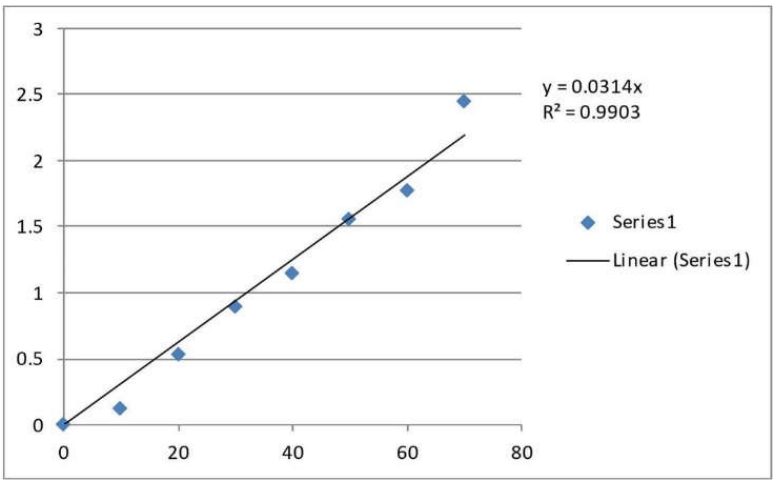


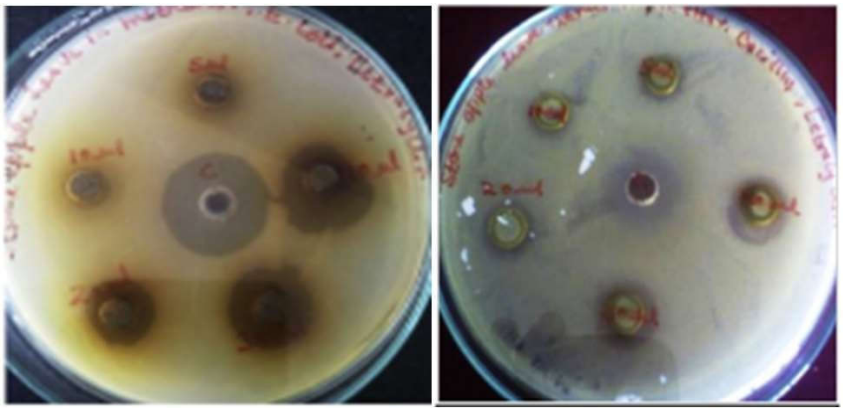
Figure3: Calibration curve for standard Quercetin

Table5: Calculation of Total Flavonoid Content (TFC) of fruit extract

Concentration of extract (ppm)	Absorbance (Y)	Q.E conc (x) (µg/mL)	TFC = C×V/M (mg of QE/g)	TFC Ave (mg QE/g)
500ppm	0.172	5.548	11.0968	10.98±0.134
500 ppm	0.171	5.516	11.0323	
500ppm	0.168	5.419	10.8387	

Antibacterial Test Results

Two bacterial strains were tested for their susceptibility to the methanolic extract. Tetracycline, and dimethyl sulphoxide (DMSO) were used as positive and negative control respectively. The methanolic fruit extract was reported to be significant almost equally against pathogens *S. aureus* (ZOI=10.4±0.75 mm) and *Escherichia coli* (11.22±0.18 mm bacteria).



(A) (B)

(A) Inhibition zone of *S. aureus* (B) Inhibition zone of *E. coli*

Figure 4: Antibacterial activity of methanolic fruit extract of *A. marmelos*

Table 6: Antimicrobial activity of methanolic fruit extract of *A. marmelos*

Samples	Zone of Inhibition (mm)	
	E.coli	*S. aureus*
Methanolic extract from fruit	11.22±0.18 mm	10.4±0.75 mm
Tetracycline (+ve control)	18±0.55 mm	19±0.34 mm
DMSO (-ve control)	-	-

Note: (-) indicate inactive in the evaluated concentration.

Conclusion

According to the study, the methanolic extract of the *A. marmelos* half-ripe fruit has strong antioxidant and antibacterial properties. The extract was effective against *S. aureus* and *E. coli* bacteria. The extracts displayed strong antioxidant with effective IC_{50} value. The presence of bioactive components like polyphenol, flavonoids, alkaloids, steroids, tannins, terpenoids etc. may be what gives the substance its increased antioxidant and antibacterial property. Additionally, a significant amount of TPC and TFC were discovered in fruit extract. As a result, *A. marmelos'* raw fruit could be a powerful source of natural medicines. More thorough phytochemical and pharmacological research must be conducted.

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