

Quantitative Phytochemical Analysis and *in vitro* Study of Antioxidant and Anti-inflammatory Activities of *Aegle marmelos* Fruit with Peel and without Peel: A Comparative Evaluation

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ABSTRACT

Background: *Aegle marmelos* is an important ethnomedicinal plant that is found commonly in India and other Asian countries. The peel of the fruit is hard and is often discarded as waste. However, a few studies have shown that even the peel has antioxidant and anti-inflammatory attributes. The aim of this research work was to perform a comparative evaluation of *Aegle marmelos* fruit extracts with the peel (whole fruit) and without the peel. Phytochemical investigations were carried out on the two extracts. **Materials and Methods:** The Folin-ciocalteu reagent method and the aluminum chloride colorimetric method were used to determine the total phenolic contents and total flavonoid contents, respectively. The marmelosin content was measured using the high-performance thin-layer chromatography technique. The antioxidant activity was determined *in-vitro* using the free-radical scavenging method, and the *in vitro* anti-inflammatory property was determined using the egg albumin denaturation method. The unpaired t-test (with Welch's correction) was used to determine *P* values. Any differences that were observed were considered

significant if $P < 0.05$. **Results:** The total flavonoid content of the extracts was found to be low, while the total phenolic content was high. There were no significant differences in the antioxidant activities of the extracts. The anti-inflammatory activities and marmelosin contents of the extracts were significantly different. **Conclusion:** The extracts displayed good antioxidant and anti-inflammatory activities. The anti-inflammatory activity of the extract of the fruit without the peel was found to be greater than that of the extract of the fruit with the peel.

Key words: Phenol, Flavonoid, Marmelosin, Antioxidant, Anti-inflammatory.

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INTRODUCTION

Herbal medicines have been used throughout the world for ages for curing and preventing various diseases. Today they are used in various countries in various medicinal systems such as Ayurveda (Indian), Unani medicine, Siddha medicine (Indian), traditional Chinese medicine, Korean Oriental Medicine, and Kampo medicine (Japanese).¹ These alternative and complementary medicinal systems are gaining popularity because they are perceived to have fewer side-effects and to be more effective in the treatment of various ailments, especially chronic ones. During the COVID-19 pandemic, the demand for herbal medicines surged globally, thus marking their international acceptance.^{2,3}

Aegle marmelos (L) Corr. is an Indian medicinal plant. It is an important plant in ethnomedicine and in ancient medicine. It has found uses in the treatment of diabetes, asthma, arthritis, inflammation, gastrointestinal disorders, and many other ailments.⁴ The fruit of the plant, although nutritive, is used more as a medicine than as a food. The unripe or partially ripe fruit is used as a digestive, demulcent, astringent, and stomachic. The ripe fruit is sweet, aromatic, and nutritive. The fruit is utilized in the management of chronic diarrhea and dysentery.⁵ It is also used to prepare a tonic for the brain and heart. The pulp is stimulant, antipyretic, and antiscorbutic. The pulp of the fruit is regularly used, while the peel and sometimes seeds are discarded. When only the pulp of the fruit is used, the waste index is higher.⁶ A few studies have shown that phytoactives are present in the peel of the fruit.⁷ Kushwah *et al.*⁸ have reported that an extract of the peel has antioxidant and antibacterial properties. They formulated silver nanoparticles using the peel extract.

The astringent property of the rind of the ripe fruit is also used in the treatment of acute dysentery.⁹

The present study aimed to carry out a quantitative phytochemical analysis and *in vitro* assessment of the antioxidant and anti-inflammatory activities of the fruit of *Aegle marmelos* with the peel and without the peel.

MATERIALS AND METHODS

Collection of Materials

Fruits of *A. marmelos* were collected during October–November 2020 from the Chitoda region, in Wardha District, Maharashtra, India. The samples were authenticated at Jannalal Bajaj College of Science, Wardha, India [herbarium sheet no. Bot Sp 04/2020–21]. The fruits were washed with water, wiped thoroughly and cut. The pieces of fruit were separated into two groups. The peel was removed from the fruit in one group. Both groups were dried in a tray dryer at a temperature below 50°C and the fruit pieces ground using a grinder.

Marmelosin was procured from Natural Remedies (Bangalore, India). Gallic acid and rutin trihydrate were purchased from Loba Chemie Pvt Ltd (Mumbai, India). Quercetin was purchased from Chemika Biochemika Reagents, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (free radical) was procured from Research-Lab Fine Chem. Industries (Mumbai, India). The other chemicals were procured from Loba Chemie Pvt Ltd. Distilled water obtained from a distillation apparatus was used in the study.

Preparation of Extract

Extracts were obtained from the powdered samples using the cold maceration method. The samples were macerated for 24 hr at room temperature. Aqueous ethanol (50% v/v) was used as the solvent with a solute-to-solvent ratio of 1:10. The extracts were then filtered and evaporated to dryness. The extract from the fruit with the peel was labelled AMPE and that without the peel was labelled AME. The extracts were kept in airtight glass containers.

Preliminary Qualitative Phytochemical Investigation

Preliminary qualitative phytochemical investigations of the two extracts were carried out using freshly prepared reagents and samples. The phytoconstituents of the extracts were determined using standard phytochemical tests. The extracts were analyzed to determine the presence of alkaloids, terpenoids, carbohydrates, proteins, tannins, and phenols. They were also subjected to the coumarin glycoside test and the resin test.

Quantitative analysis was performed to estimate the total phenolic content (TPC), total flavonoid content (TFC), and marker component (marmelosin) concentration of the extracts. The antioxidant and anti-inflammatory properties of the extracts were evaluated.

Analysis of Total Phenolic Content

The TPC of the extracts was determined using the modified spectrophotometric Folin-Ciocalteu method.¹⁰ A mixture with equal proportions of ethanol and water was utilized as the diluent. A standard calibration curve was obtained using gallic acid. For sample solutions, 25 mg of the extract was dissolved in 10 ml of the diluent. For treatment, 0.25 ml of the standard or sample solution was mixed with 10-fold diluted Folin-Ciocalteu reagent (5 ml) and 1 M sodium carbonate (4 ml). A blank solution was made similarly with the sample solution replaced with a diluent. The solutions were made to stand for 30 min at room temperature, and the analysis was performed at 765 nm. The analyses of the samples were performed in triplicates. The TPC, in milligrams of gallic acid equivalent to 1 g of extract (GAE), was calculated using the formula

$$TPC = C \times V/W, \quad (1)$$

where,

C = concentration of the standard, i.e., gallic acid, expressed in milligrams per milliliter,

V = volume of extract in milliliters and

W = weight of extract in grams.

Analysis of Total Flavonoid Content

The aluminum chloride colorimetric method with a few modifications was used to determine the TFC of the extracts. Quercetin was used as the standard.¹¹ A mixture with equal proportions of ethanol and water was used as the diluent. A standard calibration curve of quercetin was plotted using different concentrations. For sample solutions, 25 mg of extract was dissolved in 10 ml of the diluent. For color development, 1 mL the standard or sample solution was mixed with 0.1 ml 10% w/v aluminum chloride, 0.1 ml 1 M potassium acetate, 1.5 ml methanol and 2.8 ml water. The blank solution was made similarly, with the sample solution replaced with the diluent. All the solutions were made to stand at room temperature for 30 min, and the analysis was performed at 415 nm. The analyses of the samples were carried out in triplicate. The TFC, milligrams of quercetin equivalent to 1 g of extract (QE), was calculated using the formula

$$QE = C \times V/W, \quad (2)$$

where,

C = concentration of the standard, i.e., quercetin, expressed in milligrams per milliliter,

V = volume of extract in milliliters and

W = weight of extract in grams.

Comparative Thin-Layer Chromatography Profiling and Marmelosin Content

High-performance TLC profiling of the extracts was carried out on a precoated silica plate (F254, Merck). A CAMAG Linomat 5 with a 100 µl Hamilton syringe was used to apply the samples. A CAMAG TLC scanner 3 was used to scan the samples. The software used was WINCATS. n-Hexane and ethyl acetate in the ratio 12:4 was used as the mobile phase. Marmelosin was used as a standard marker compound for identification and quantification. A standard calibration curve of marmelosin was plotted. A sample of concentration 10 mg/ml was prepared in an equi-proportionate mixture of methanol and water. Ten microlitre of each sample was applied on the plate, and the plate was run in a CAMAG glass twin trough chamber that had been saturated with the mobile phase previously. Using 10% methanolic potassium hydroxide, the plate was later developed. Peaks were recorded at 366 nm.

Analysis of free Radical-scavenging Antioxidant Property

The DPPH free radical scavenging assay with slight modifications was used to analyze the antioxidant property of the extracts.^{12,13} The antioxidant property was expressed as the half-maximal inhibitor concentration (IC_{50}). A mixture of ethanol and water in equal proportions was used as the diluent. A DPPH solution of concentration 0.5 mM was made by dissolving 19.7 mg of DPPH in ethanol (100 ml). Rutin trihydrate was used as the standard and for plotting a calibration curve. The stock solution of the standard was diluted using the diluent to prepare solutions of concentration 4, 8, 12, 16, and 20 µg/ml. The stock solutions (1000 µg/ml) of the extracts were prepared using the diluent and were further diluted to get solutions of different concentrations (20, 40, 60, 80, and 100 µg/ml). One milliliter of the 0.5 mM DPPH solution was added to the standard or sample solution (3 ml), and the mixture was made to stand for 30 min in the dark. The reading was taken at 525 nm. A control sample was made in the same way, with the 3 ml of the standard/sample solution replaced with the diluent. The diluent was used as the blank. The percent inhibition (% INH) values of the standard and samples were calculated for each concentration using the following formula:

$$\% INH = [(A1 - A2)/A1] \times 100, \quad (3)$$

where,

A1 = absorbance of the control sample and

A2 = absorbance of the standard/sample.

The IC_{50} values of the standards and extract samples were determined using a graph of the percent inhibition (% INH) against the standard/sample concentration. The samples and standards were prepared in triplicate, and the results were written as the mean ± SD.

In vitro Anti-inflammatory Bioassay

Fresh chicken eggs were used in the experiment. A phosphate buffered saline solution was prepared by dissolving 8 g (0.137 M) of sodium chloride, 0.2 g (0.0027 M) of potassium chloride, 1.44 g (0.01 M) of sodium phosphate dibasic anhydrous, and 0.245 g of potassium dihydrogen orthophosphate anhydrous in distilled water (800 ml). The pH was adjusted to 6.4 with hydrochloric acid, and the volume was made up to 1000 ml using distilled water. Ethanol and water in the ratio 1:1 was used as the diluent for preparing the standard and extract solutions. Standard solutions of diclofenac sodium (1000 µg/ml) were prepared

using the diluent and were further diluted to get solutions of different concentrations (50, 100, and 200 µg/ml). Similarly, stock solutions of the AME and AMPE extracts were made separately using the diluent and were diluted to make solutions of concentrations 50, 100, and 200 µg/ml. Five milliliters of the reaction mixture was made by mixing egg albumin (0.2 ml), phosphate buffered saline (2.8 ml, pH 6.4) and the standard/sample of each concentration (2 ml). For the controlled sample, the sample solution was replaced with 2 ml of the diluent. The reaction mixtures were incubated for 15 min at $37 \pm 2^\circ\text{C}$ in a biological oxygen demand incubator and then maintained at 70°C for 5 min. The samples were then cooled at room temperature and centrifuged at 5000 rpm for 10 min. The readings of clear solutions were taken at 600 nm utilizing the diluent as the blank.^{14,15} The egg albumin protein denaturation % INH was calculated using the formula

$$\% \text{INH} = [(A1 - A2)/A1] \times 100, \quad (4)$$

where,

A1 = absorbance of the control sample and

A2 = absorbance of the standard/ sample

Samples and standards were prepared in triplicate, and the results were written as the mean \pm SD.

RESULTS

Preliminary Qualitative Phytochemical Investigation

The results of the preliminary phytochemical evaluation of the AME and AMPE extracts are shown in Table 1. The presence of phenols, flavonoids, tannins, terpenoids, coumarin glycoside, alkaloids, carbohydrates and proteins was detected.

Analysis of Total Phenolic Content

The TPC of the extracts was expressed as the GAE (mg/g of extract). The equation of gallic acid obtained was $y = 0.002x + 0.006$, and the correlation coefficient was $R^2 = 0.996$. The TPC of the AME (181.13 mg/g GAE) and AMPE (180.80 mg/g GAE) were found to be nearly same (Table 2).

Analysis of Total Flavonoid Content

The TFC was written as QE (mg/ g of extract). The linear equation and correlation coefficient obtained for quercetin were $y = 0.010x + 0.024$

Table 2: TPC, TFC, and marmelosin content in extracts.

Extract	TPC (GAE)* (mg/g)	TFC (QE)* (mg/g)	Marmelosin content (%)
AME	181.13 \pm 1.68	7.30 \pm 0.43	0.368
AMPE	180.80 \pm 1.84	5.71 \pm 0.25	0.163

*Mean \pm standard deviation ($n = 3$)

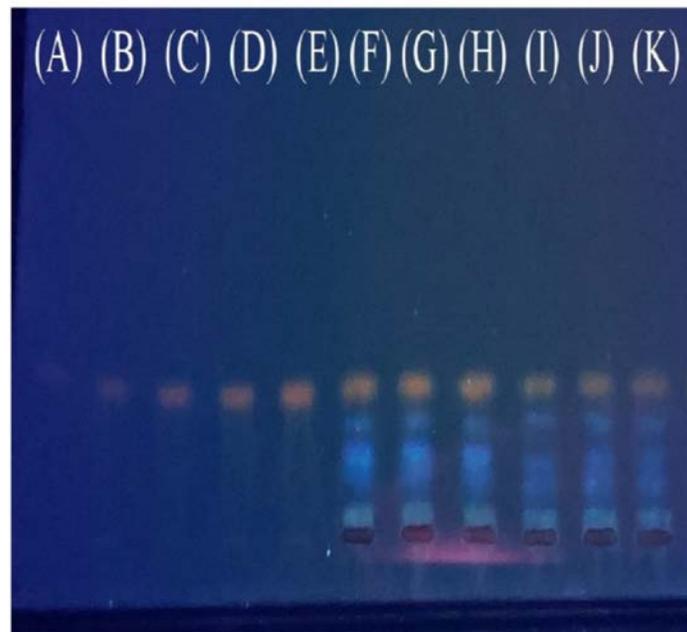


Figure 1: HPTLC Analysis for quantitative estimation of marker (marmelosin) in the *A. marmelos* extract without and with rind (A, B, C, D, E: marmelosin standard; F, G, H: AME samples; I, J, K: AMPE samples).

and $R^2 = 0.999$, respectively. The TFC was found to be low in both the extracts (Table 2). The TFC of the AMPE was 5.71 mg/g QE, whereas that of AME was 7.30 mg/g QE.

Comparative Thin-Layer Chromatography Profiling and Marmelosin Content

The HPTLC scanning revealed the presence of numerous phytoconstituents in both the extracts (Figure 1). Figure 2 shows the HPTLC profiles of the AME, AMPE, and standard. The R_f of marmelosin was found to be 0.28. A straight line was obtained when the standard calibration graph of marmelosin was plotted. Its equation was $y = 11.63x + 431.5$, and the correlation coefficient $R^2 = 0.999$. The concentrations of the samples were determined using the equation. The marmelosin concentration in the AME was found to be 0.368% w/w, and that of the AMPE was 0.163% w/w (Table 2).

Analysis of free Radical Scavenging Antioxidant Property

The antioxidant properties of the extracts with and without the peel are graphically shown in Figure 3. The IC_{50} values of the AMPE and AME were found to be 60.55 µg/ml and 65.28 µg/ml. The IC_{50} value of the standard was found to be 9.84 µg/ml.

In vitro Anti-inflammatory Bioassay

The % INH of egg albumin denaturation is shown in Figure 4. The egg albumin denaturation was inhibited most by the samples of

Table 1: Preliminary qualitative phytochemical investigation.

Sr. no.	Phytochemical compounds	Method	Observation	Inference
1	Alkaloid	Mayer's test	Cream color precipitate at bottom	Present
2	Terpenoids test	Salkowski reaction	Reddish brown coloration at interface	Present
3	Carbohydrates	Molish test	Violet ring at junction	Present
4	Proteins	Biuret test	Violet color	Present
5	Tannins	Ferric chloride test	Dark green coloration	Present
6	Phenol	Lead acetate test	White precipitate	Present
7	Coumarin glycoside	Alkali test (ammonia test)	Blue-green fluorescence	Present
8	Resin	HCl test	Pink color developed	Present

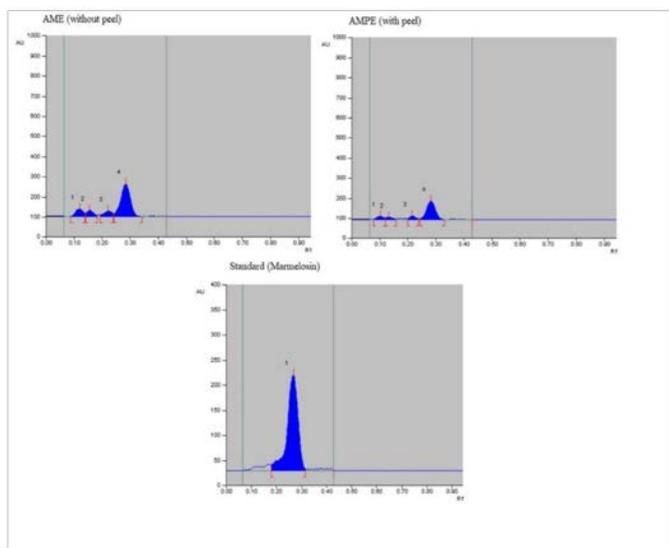


Figure 2: HPTLC graph of AME, AMPE, and standard (marmelosin).

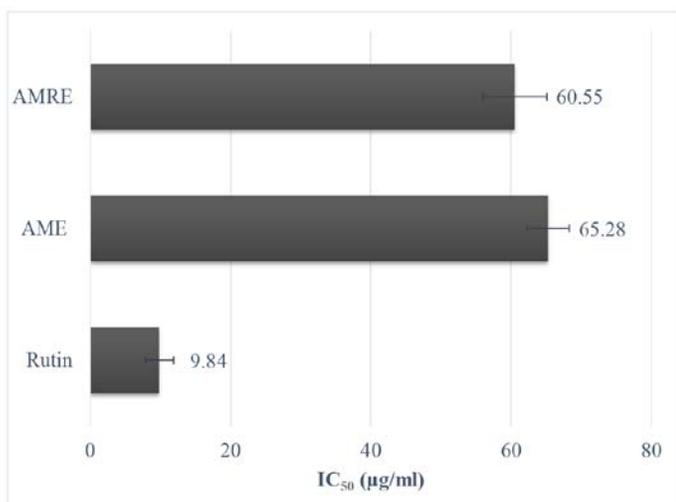


Figure 3: IC₅₀ values of standards and extracts.

concentration 200 µg/ml. The egg albumin denaturation was inhibited most by diclofenac sodium (91.83%), followed by the AME (89.54%). It was inhibited least by the AMPE (77.79%).

DISCUSSION

The fruit of *A. marmelos* is popularly known as *bael*, *bilwa*, *sriphala*, golden apple, wood apple, stone apple and Bengal quince. It has been used in Ayurveda and folk medicine to treat various ailments. The *A. marmelos* tree has a unique importance in Ayurveda. The use of the *bael* fruit in *pravahika* (dysentery), *agnimandya* (dyspepsia) and *grahaniroga* (chronic diarrhea with malabsorption) is mentioned.¹⁶ Ethnobotanical studies have found that the *bael* fruit is used for fever, diarrhea, abdominal colic and diseases of the digestive system.¹⁷ The fruit is reported to have antibacterial, antiviral, antioxidant, anti-inflammatory, antiarthritic and radioprotective activities.

The phytochemical analysis of the AME and AMPE extracts suggested that many bioactive and phytochemical compounds are present. The

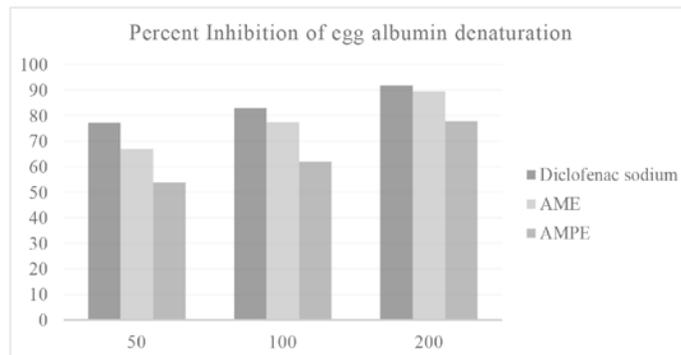


Figure 4: Comparative percent inhibition of egg albumin denaturation of diclofenac sodium, AME, and AMPE at different concentrations.

beneficial and protective roles of the *bael* fruit may be because of the presence of phenolics, flavonoids, tannins, and coumarin glycosides in the fruit. Phenolic compounds have been reported to have beneficial roles as antioxidant, anti-inflammatory, anti-aging and anti-proliferative agents.^{18,19} Phenolic compounds in plants can reduce the hazard of oxidative stress by improving the endogenous antioxidative system and the oxidant-antioxidant balance.²⁰ High levels of phenolic compounds were found in both the extracts. The difference between the TPCs of the AME and AMPE was not significant ($P = 0.8307$).

Flavonoids are very diverse natural compounds. They possess a wide spectrum of biological activities. The flavonoid contents of both the extracts were low. The TFC of the AMPE was found to be significantly less than that of the AME ($P = 0.0117$).

Marmelosin is a biomarker furanocoumarin found in the fruit of *A. marmelos*. Hasitha and Dharmesh²¹ reported that marmelosin exhibits antioxidant, anti-inflammatory, and anti-proliferative activities. The content of the marker in the AME was found to be higher. The difference in the marmelosin content was very significant ($P = 0.0040$). The peel of the fruit does not contain marmelosin, and hence the concentration of the marmelosin was less in the AMPE.

The antioxidant activities of the extracts were studied using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay and were expressed as IC₅₀ values. Rutin trihydrate was used as the standard. The DPPH method depends on the reduction of DPPH in the presence of an H-donating antioxidant, resulting in the discoloration of DPPH. As the IC₅₀ value is inversely proportional to the antioxidant activity, the smaller the IC₅₀ value is, the higher is the antioxidant activity. Both the extracts exhibited good antioxidant activity. The antioxidant activities of the extracts may be attributed mainly to their high phenolic content. The antioxidant activities of the AME and AMPE are not significantly different ($P = 0.2316$).

Denaturation of tissue proteins causes inflammation. Hence, compounds that can protect proteins from denaturation would be important in the development of anti-inflammatory agents. Hence the potential anti-inflammatory activities of the extracts were evaluated. The albumin protein denaturation bioassay was used. The extracts and the reference standard (diclofenac) inhibited heat-induced egg albumin protein denaturation that had been dose-dependent. The protein stabilization could be estimated by comparing the % INH of the protein denaturation of the samples and that of the control. At 200 µg/ml, the albumin protein denaturation of the AME was found to be comparable with that of the standard (diclofenac sodium). It was found to be lower in the AMPE.

CONCLUSION

The *A. marmelos* tree bears fruits only once a year. When the peel of the fruit is removed, the phytoactives in it are lost and the waste index is higher. Hence, comparative phytochemical studies of the whole fruit and the peeled fruit were carried out. The study reveals that the extracts of the *A. marmelos* fruit, both with the peel and without the peel, have high total phenolic contents and exhibit good antioxidant activity. Thus, the whole fruit can be utilized as a natural source of antioxidants to prevent the progress of diseases caused by oxidative stress. The anti-inflammatory activity of the extract without the peel was found to be of a higher level, which may be attributed to its higher marmelosin content. Further detailed investigations are needed to explore the mechanisms and constituents accountable for the antioxidant and anti-inflammatory activities of the extracts. Also, stability studies of both the extracts need to be carried out.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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