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ORIGINAL ARTICLE

Phytochemical Study of *Aegle marmelos*: Chromatographic Elucidation of Polyphenolics and Assessment of Antioxidant and Cytotoxic Potential

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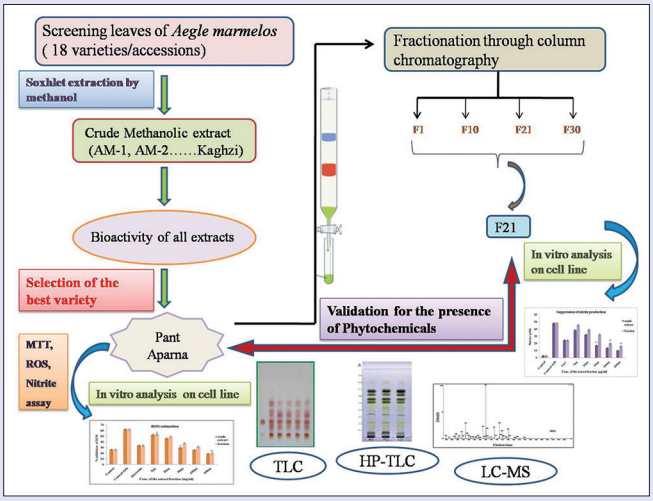
ABSTRACT

Background: The antioxidant potential of medicinal plants has been illustrated through many reports clearly depicting that plants are a rich source of antioxidants, making them a great resource of novel drugs and health-care products. **Objectives:** The current study is, therefore, focused toward the assessment of antioxidant properties along with the presence of phytochemicals in leaves of 18 varieties/accessions of *Aegle marmelos*. **Materials and Methods:** The antioxidant activities were initially measured using superoxide radical scavenging method, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and ferric-reducing ability of plasma assays. Further, thin-layer chromatography (TLC), high-performance TLC, and column chromatography were performed to isolate the potentially active fraction and anti-inflammatory activity of crude, and the isolated fraction was tested on J774 macrophage cell line. **Results:** The maximum inhibition of superoxide anions was shown by Pant Aparna. Additionally, Pant Aparna extract was most efficient, exhibiting 92.0% inhibition in scavenging the DPPH radicals. The content of total carotenoids was found to be higher in Pant Aparna among all the varieties/accessions. Furthermore, the crude extract and the fraction *A. marmelos* methanolic fraction 21 (AMMF21) were found to be nontoxic and significant reactive oxygen species, and NO inhibition was observed in a concentration-dependent manner. Moreover, the methanolic extract of variety Pant Aparna showed promising *in vitro* antioxidant activity, indicating its potency for therapeutic applications. **Conclusion:** In brief, this is the first ever report on Pant Aparna as the best variety in terms of phytocompounds and identification of potential antioxidant activity. In addition, the AMMF21 fraction of methanolic extract possessing best antioxidant activity on macrophage cells indicates its use as a novel phytotherapeutic agent.

Key words: *Aegle marmelos* (Bael), anti-inflammatory activity, antioxidant, chromatography, J774 macrophage cell line, phytochemicals

SUMMARY

- Our study identifies the best variety/accession of *Aegle marmelos* possessing the potential antioxidant and reactive oxygen species scavenging activity possessed by the methanolic crude extract of variety Pant Aparna along with the fraction *A. marmelos* methanolic fraction 21 isolated through column chromatography on J774 murine macrophage cell line
- The high-performance thin-layer chromatography fingerprinting profile obtained acts as a diagnostic tool to identify and determine the quality and purity of this extract and fraction in future studies
- On the basis of the results obtained, the above variety should be taken further to exploit its immense potential for other biological activities of medicinal importance
- Pant Aparna is an outstanding variety of *A. marmelos* and should be extensively studied for isolation of a novel and potential therapeutic agent.



Abbreviations used: AMMF21: *Aegle marmelos* methanolic fraction 21, DPPH: (2, 2-diphenyl-1-picrylhydrazyl), FRAP: Ferric-reducing ability of plasma, HP-TLC: High-performance-thin-layer chromatography, TLC: Thin-layer chromatography, TCA: Trichloroacetic acid, TPTZ: 2,4,6-Tripyridyl-s-triazine, DNPH: 2,4-dinitrophenyl hydrazine, NBT: Nitroblue tetrazolium, NADH: Nicotinamide adenine dinucleotide, PMS: Phenazine metho-sulfate, DMEM: Dulbecco's modified Eagle medium; MTT: (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide, DCFDA: 2',7'-dichlorofluorescein diacetate, LPS: Lipopolysaccharide, NED: N-(1-Naphthyl) ethylenediamine.

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INTRODUCTION

For thousands of years, plants served as a reservoir of various remedies. Even today, 80% of the population depend on traditional remedies such as herbs for their medicines as estimated by the World Health Organization.[1] The antioxidant potential of medicinal plants has been illustrated through many reports clearly depicting that plants are a rich source of antioxidants,[2-4] making them the major supplier of new pharmaceuticals and health-care products. Phytomedicine which

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comprises medicinal plant products, derived from various plant parts, produces a distinct physiological effect on the human body. The most essential natural bioactive components of plants are tannins, alkaloids, flavonoids, and phenolics. The antioxidant constituents of the medicinal plants are mainly responsible for disease prevention or control and are mostly linked with a wide array of amphipathic molecules referred to as polyphenolic compounds.[5] These compounds have manifold uses in pharmaceutical, cosmetic, and food industries[6] and usually present in both edible and inedible plants. Numerous studies on the isolation of natural antioxidants, the secondary metabolites of plants, have immensely increased during the last decade.[7] Natural antioxidants such as ascorbic acid, benzoic acids, flavonoids, carotenoids, cinnamic acids, folic acid, tocotrienols, and tocopherols are produced by the plant for their sustenance. Furthermore, these compounds act as metal chelators, reducing agents, singlet oxygen quenchers, or hydrogen donors, owing to their redox properties which depict their antioxidant capacity. Our study on quantitative phytochemical estimation has revealed that, among 18 accessions/varieties of Bael, the variety Pant Aparna contained the highest quantity of flavonoids and phenolics.[8] Flavonoids are well known for their valuable effects in many animal models and are extensively studied for their anti-inflammatory properties.[9] The antioxidant activities of plants are due to the presence of phenolics and flavonoids,[10,11] thus acting as anti-allergic, anti-inflammatory, anti-microbial, anti-thrombotic, cardioprotective, and vasodilatory agents.

The present study was, therefore, focused toward the evaluation of antioxidative potential in the leaves of 18 varieties/accessions of *Aegle marmelos* and identification of the phytocompound possessing free radical scavenging activity.

MATERIALS AND METHODS

Collection and identification of plant material

Fresh leaves of *A. marmelos* from 18 varieties/accessions were collected from the orchard of Narendra Deva University of Agriculture and Technology, Kumarganj, Faizabad, India. The taxonomy of the plant was authenticated. Finely powdered and air shade-dried leaves were taken for experiments.

Chemicals

2, 2-Diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), 2,4-dinitrophenyl hydrazine (DNPH), 2,4,6-Tripyridyl-s-triazine (TPTZ), ferric chloride ($FeCl_3$), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), phenazine metho-sulfate (PMS), Dulbecco's modified Eagle medium (DMEM), (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sulfanilamide, N-(1-Naphthyl) ethylenediamine (NED), 2',7'-dichlorofluorescein diacetate (DCFDA), lipopolysaccharide (LPS), and all other chemicals used were purchased from Merck, HiMedia, and Sigma-Aldrich. The high-performance-thin-layer chromatography (HP-TLC) plates Si $60F_{254}$ (20 * 10) were purchased from E. Merck (Darmstadt, Germany).

Processing of plant samples

The leaves were washed and air dried at 35°C–40°C for 3 days. Finely powdered leaves were obtained and stored in air-tight containers at −20°C until use.

Ten grams of dried plant material was packed in a muslin cloth and subjected to Soxhlet apparatus using 180 ml of methanol as solvent. The extraction was carried out for 24 h and the extracts were dried in Petri plates. The dried extracts were weighed and stored in a refrigerator at 4°C.

Superoxide anion radical scavenging potential

The superoxide dismutase (SOD) scavenging potential of methanolic extract was performed as per the protocol mentioned by Nishikimi *et al.*, 1972, with slight modifications.[12] 0.1 ml of different concentrations of methanolic extract and reference compound (10, 25, 50, 75, and 100 µg) were taken and mixed with the reaction mixture and incubated at 25°C for 5 min. The absorbance was measured at 560 nm. Butylated hydroxyl toluene (BHT) was used as a reference compound.

Superoxide anion scavenging activity (%) = $([A_0-A_1]/A_0) \times 100$, where A_0 is the absorbance of the negative control and A_1 is the absorbance of reaction mixture or standard.

Determination of ferric-reducing/antioxidant efficiency

The total antioxidant potential of the sample was measured using the ferric-reducing ability of plasma (FRAP) assay as per Benzie and Strain.[13] Plant extract (150 µl) was reacted with 2.850 ml of the FRAP solution for 30 min in the dark condition for color development whose absorbance was taken at 593 nm. Linearity was observed by the standard curve between 10 and 100 µM. The results were expressed in terms of µmol Fe^{II}/g dry mass. Vitamin C was used as a standard. All the analyses were performed in triplicate.

Determination of 2, 2-diphenyl-1-picrylhydrazyl radical scavenging assay

2, 2 diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of plant extracts was evaluated against stable DPPH by method of Brand-Williams *et al.*, 1995, with slight modification.[14] Various concentrations (10–200 µg/ml) of plant extract solution were mixed with 1 ml of 0.1 mM DPPH solution in methanol. L-ascorbic acid (1–14 µg/ml) was used as a standard. The samples were incubated in the dark for 30 min and the decrease in absorbance was quantified at 517 nm. The IC_{50} value was calculated from the plot of percentage inhibition.

DPPH radical scavenging activity was calculated by the following formula.

% Inhibition = $([A_B-A_A]/A_B) \times 100$

Where A_B = absorption of blank sample (t = 0 min)

A_A = absorption of test extract solution (t = 30 min)

Estimation of total carotenoids

Total carotenoids were determined by following the protocol as described by Zakaria *et al.*, 1979.[15]

The experiment was performed in the dark for preventing the photolysis of carotenoids after saponification. Consequently, the homogenization and saponification of the sample (0.5 g) were done in a water bath containing 2.5 ml of 12% alcoholic potassium hydroxide at 60°C for 30 min. The extract obtained was taken in a separating funnel and mixed with 10–15 ml of petroleum ether. As a result, the upper layer of petroleum ether containing the carotenoids was collected while the lower aqueous layer was then transferred to another separating funnel. The extraction was carried out until the aqueous layer became colorless. The excess moisture was withdrawn from the petroleum ether extract by adding a small amount of anhydrous sodium sulfate. The final volume of the petroleum ether extract was noted and absorbance was measured at 450 nm using petroleum ether as blank.

The total carotenoids was estimated using the following formula,

$$\text{Amount of total carotenoids } (\mu g/g) = \frac{A_{450} \times \text{Volume of the sample} \times 10^4}{A^{1\%}_{1cm} \times \text{Weight of the sample}}$$

Thin-layer chromatography analysis of crude methanolic extract

The methanolic extract of Pant Aparna leaves was run one dimensionally in the mobile phase solvent (toluene:ethyl acetate:formic acid:glacial acetic acid in the ratio of 10:3:1:1) at room temperature (20°C–25°C). Identification of the separated phytocompounds in the extract was done under ultraviolet (UV) light of iodine. Rutin was used as reference compound. The retention factor (R_f) values of all the spots were determined by the following formula:

R_f = Distance traveled by the plant extract/distance traveled by the solvent system.

Isolation of flavonoid fractions by column chromatography

Briefly, 50 g of dried extracts was chromatographed over silica gel which was packed into a glass column (600 mm × 30 mm) and elution was done from nonpolar to polar solvents by gradient elution method. The elution of the column was performed with a solvent gradient of hexane:ethyl acetate (100:0–0:100 v/v) at a flow rate of 1 ml/min. Each fraction was weighed and stored at 25°C. The collected fractions were subjected to TLC analysis. TLC studies were carried out using toluene:ethyl acetate:formic acid:glacial acetic acid in the ratio of 10:3:1:1 and stained by iodine vapors. The flavonoid fractions based on the R_f values were pooled together. The fractions were labeled as *A. marmelos* methanolic fraction (AMMF1), the purified flavonoid fractions, namely, AMMF1, AMMF2., and AMMF30. All the fractions were dried and stored for further characterization in dark conditions at 4°C.

High–performance-thin-layer chromatography finger print profiles for crude extract and methanolic fraction of Pant Aparna

For HP-TLC analysis, 10–20 mg of methanolic crude extract of Pant Aparna and its fraction AMMF21 was prepared. A CAMAG Linomat V Automatic Sample Spotter (Camag Muttenz, Switzerland, USA) was used as the spotting device containing 100 µl Hamilton syringe, and CAMAG glass twin trough chamber (10 cm × 10 cm) as the developing chamber. The densitometer consisted of a CAMAG TLC scanner 3 attached to WINCATS software (CAMAG, Switzerland). Plates were developed using a mobile phase consisting of toluene:ethyl acetate:formic acid:glacial acetic acid in the ratio of 10:3:1:1. The developed plate was dried and kept in a photo-documentation chamber (CAMAG REPROSTAR 3) for scanning in CAMAG-TLC Scanner. The peaks were recorded at wavelengths of 254 nm and 366 nm.

Liquid column-mass spectrometry analysis

Liquid column-mass spectrometry (LC-MS) analyses of methanolic fraction of Pant Aparna (AMMF21) were performed on an ACCUCORE RP-MS system with chromatographic separation achieved on a column ODS-2 (100 × 3, 2.6 µm) operated at 30°C with gradient elution at 0.45 ml/min with electrospray ionization (ESI), MS mode. A 5 µl sample volume was injected using the system's autosampler. ESI parameters were as follows: nebulizer gas (N_2) temperature 350°C; flow 30 l/h; pressure 15000 psi; and capillary voltage 2000 V. The mobile phase consisted of four components: (a) ACN + WATER 5:95, (b) Acetonitrile, (c) Methanol, and (d) WATER + F. A at a flow rate of 0.45 ml/min in linear gradient mode as follows: until 0–1 min, 10% B and 90% D; at 6 min, 40% B and 60% D; at 8 min, 60% B and 40% D; at 12 min, 60% B and 40% D; at 14 min, hold 80% B and 20%; and at 17 min, hold 90% D. ESI was used as the interface and was conducted in positive selected ion monitoring (SIM) mode. The probe temperature was set at 20°C and the cone voltage was set at 30 V for all SIM scans with a span of 202–2000 amu/s for each SIM, acquisition range, 150–1000 m/z at 0.6 scan s^{-1}.

Cell line maintenance

The murine macrophage cell line J774, purchased from NCCS, Pune, was maintained in DMEM (Dulbecco's modified Eagle medium) enriched with 10% fetal bovine serum, 100 u/ml penicillin, and 100 µg/ml streptomycin, under an atmosphere of 5% CO_2 at 37°C.

Cytotoxicity by (3-(4,5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay

The macrophage cell growth was quantified by the capacity of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide (MTT) to a blue formazan product.[16] Briefly, a 96-well culture plate was used for plating the cells at a concentration of 1 × 10^6 cells/ml. The various concentrations (5, 20, 50, 100, and 200 µg) of the filtered crude extract and fraction were added to the wells in triplicate. Finally, at the end of 24 and 48 h incubation, the medium in each well was replaced by MTT solution (5 mg/ml in phosphate-buffered solution). After 4 h incubation, MTT reagent was discarded and 100% dimethyl sulfoxide was used for dissolving the formazan crystals produced by viable cells and gently shaken. Viability was determined at 570 nm. The effect of the samples on the growth of macrophage was expressed as the % cell viability, using the following formula:

% cell viability = A570 of treated cells/A570 of control cells × 100%

Reactive oxygen species detection by 2′,7′-dichlorofluorescein diacetate

Murine macrophage cells were plated in 96-well culture plate at a concentration of 0.1 × 10^6 macrophage/well.[17] The cells were then treated with different concentrations of filtered crude extract and fraction (5, 20, 50, 100, and 200 µg) in DMEM (phenol red-free) and incubated for 24 h at 37°C in 5% CO_2. Lipopolysaccharide (1 µg/ml) was used as a mitogen. Each well was supplemented with 5 µM DCF-DA and incubated at 37°C in 5% CO_2 for 30 min. Cells were subjected to measurement by a fluorescent microscope (Labomed TCM400).

Nitrite assay

Griess reagent was used to detect nitrite as the amount of NO in the culture media and a major stable product.[18] The murine macrophage cell line J774 cells seeded at a concentration of 2 × 10^5 cells per well were let for adherence prior treating with the Vitamin C (289 µM) or various concentrations (5, 20, 50, 100, and 200 µg/ml) of crude extract and fraction AMMF21. The cells were stimulated with 1 µg/ml LPS following incubation of 1 h. Griess reagent comprising of 1% sulfanilamide, 0.1% NED, and 3% phosphoric acid was added after 24 h incubation. The incubation of the samples was performed in dark condition for 10 min and absorbance was measured at 540 nm using a Microplate Spectrophotometer System (Bio-Rad Laboratories, Inc., California, USA).

Statistical analysis

All the assays were performed in triplicate (n = 3) and results were indicated as a mean ± standard deviation.

RESULTS

Antioxidant capacity determination

Superoxide radical scavenging activity

To evaluate the antioxidant capacity of plants, several biochemical assays were performed and considered to be the most consistent and can be readily employed. Since the responses generated by a specific antioxidant are unsteady, it therefore becomes imperative to understand the mechanism of action of the bioactive principle involved via varied antioxidant assays.[19] Superoxide anions are produced by activated phagocytes such as monocytes, macrophages, eosinophils, and neutrophils, acting as a precursor to active free radicals that possess the potential of reacting with biological macromolecules, and thus responsible for tissue damage.[20] Superoxide anion radical scavenging activities of 18 varieties/accessions of Bael are shown in Table 1.

Superoxide anions serve as precursors of singlet oxygen and hydroxyl radicals causing indirect initiation of lipid oxidation.[21] The antioxidant properties of flavonoids are effective mostly through the scavenging of superoxide anion as studied by Robak and Gryglewski.[22]

The methanolic extract of 18 Bael accessions/varieties exhibited good scavenging activity on superoxide anions produced from PMS-NADH system at all the known concentrations. The superoxide anion inhibition ranged from 11.34% to 72.44% at 20–250 µg/ml concentrations.

The maximum inhibition was found to be 72.44% in Pant Aparna followed by Pant Sujata which showed 66.58%. An IC_{50} value of 50.50 ± 0.84 µg/ml was shown by the methanolic extract. Pant Aparna and Pant Sujata showed 50% inhibition even at a lower concentration of around 40–100 µg/ml. However, the other varieties/accessions depicted moderate inhibition of superoxide radicals in a dose-dependent manner [Table 1]. BHT used as a positive reference showed 52.23% inhibition at the maximum concentration of 100 µg/ml.

2, 2-diphenyl-1-picrylhydrazyl free radical scavenging activity

DPPH free radical scavenging potential of the methanolic extracts of *A. marmelos* was used as another parameter for corroboration of antioxidant activity. Table 2 summarizes data of methanolic extract of 18 Bael varieties/accessions. The data demonstrated that all the extracts of 18 varieties exhibited an inhibitory potential against DPPH radical.

The highest activity of 92.0% was obtained at a concentration of 80 µg/ml by Pant Aparna, clearly depicting its high antioxidant potential whereas other varieties/accessions such as AM-7, AM-8, Pant Sujata, and Pant Shivani showed 87.4%, 67.19%, 57.42%, and 57.27% inhibition, respectively. However, parallel results were obtained by AM-7 but at higher concentrations. The increase in DPPH radical scavenging ability of the extract might be due to the presence of high contents of phenolics and flavonoids.[23,24] Ascorbic acid was used as a positive control which demonstrated 95.6 ± 0.0018% inhibition at the concentration of 14 µg/ml.

From the above findings, it is evident that the methanolic extract of Pant Aparna possesses the ability of donating proton and could act as free radical inhibitors or scavengers, acting possibly as primary antioxidants. The lowest IC_{50} value of Pant Aparna (4.85 µg/ml) equivalent to that of ascorbic acid (7.29 µg/ml) adds to the value of the antioxidants in the extracts.

Ferric-reducing ability of plasma assay

FRAP assay is a versatile and novel method dependent on ferric to ferrous ion reduction at low pH in a dose-dependent manner, thereby forming a colored ferrous-tripyridyltriazine complex. The antioxidant capacity calculated on the potentiality to reduce ferric (III) ions to ferrous (II) ions was performed and the results obtained are presented in Table 3. The standard curve was prepared in the range of 10–100 µM/ml of ferrous sulfate and the results were expressed in terms of µmol ferrous ion equivalent per gram of sample dry weight ($y = 0.0117x + 0.099$, $r^2 = 0.998$).

As evident from the data obtained, the antioxidant power of these varieties/accessions varied from 2.82 µmol Fe^{2+}/g to 31.1 µmol Fe^{2+}/g dry mass. An 11-fold difference was noticed in the antioxidative potentiality of the extracts. The variety Pant Aparna revealed the maximum antioxidant capacity (31.1 µmol Fe^{2+}/g), followed by NB-17 (25.84 µmol Fe^{2+}/g), AM-7 (23.06 µmol Fe^{2+}/g), AM-1 (20.65 µmol Fe^{2+}/g), and AM-8 (15.1 µmol Fe^{2+}/g). NB-4 indicated the lowest antioxidant capacity (2.82 µmol Fe^{2+}/g) among these accessions. The ferric reducing ability of the standard ascorbic acid was found to be 38.56 µmol Fe^{2+}/g almost comparable to Pant Aparna.

Table 1: Superoxide free radical scavenging activity of methanolic extract of 18 *Aegle marmelos* varieties/accessions at different concentrations

Serial number	Bael varieties/accessions	Concentration (µg/ml) of methanolic plant extract				
		20	40	100	125	250
1	AM-1	0.927±0.25	2.57±0.31	4.74±0.56	7.73±0.41	14.94±0.43
2	AM-2	9.27±0.72	11.34±0.70	12.26±0.22	15.25±0.76	19.3±0.26
3	AM-3	5.62±0.15	11.25±0.41	13.92±0.18	16.51±0.44	19.55±0.23
4	AM-4	13.3±0.35	14.55±0.16	16.33±0.23	18.75±0.74	24.37±0.18
5	AM-6	20.42±0.75	29.87±0.82	40.85±0.71	50.91±0.36	61.28±0.47
6	AM-7	7.05±0.28	32.6±0.72	38.48±0.23	50.25±0.29	57.47±0.18
7	AM-8	15.64±0.54	18.09±0.34	24.65±0.53	36.72±0.46	42.34±0.73
8	NB-1	14.02±0.77	17.31±0.73	24.32±0.34	28.74±0.10	32.64±0.26
9	NB-4	5.76±0.18	11.52±0.41	16.82±0.78	20.5±0.44	25.8±0.52
10	NB-5	26.38±0.29	35.93±0.14	41.66±0.15	51.04±0.33	55.03±0.47
11	NB-7	21.61±0.52	34.50±0.60	42.05±0.71	49.74±0.83	54.68±0.56
12	NB-9	15.12±0.76	31.76±0.22	35.12±0.14	38.82±0.27	44.87±0.72
13	NB-16	2.34±0.70	6.25±0.25	10.15±0.32	16.79±0.23	20.7±0.27
14	NB-17	1.95±0.46	17.57±0.78	28.51±0.71	31.51±0.34	37.36±0.10
15	Pant Aparna	15.81±0.12	32.65±0.13	49.48±0.37	60.2±0.77	72.44±0.50
16	Pant Sujata	36.56±0.22	47.4±0.17	55.2±0.29	61.25±0.73	66.58±0.76
17	Pant Shivani	4.96±0.67	17.73±0.13	31.91±0.19	38.65±0.16	44.68±0.52
18	Kaghzi	8.67±0.75	12.5±0.24	21.93±0.93	29.84±0.36	38.26±0.31
Standard	BHT	18.58±0.26	31.52±0.40	41.88±0.45	46.35±0.30	52.23±0.80

Each value in the table was obtained by calculating the average of three experiments±SD. BHT: Butylated hydroxytoluene (The concentration used for the standard are 10, 25, 50, 75, 100 µg/ml); SD: Standard deviation; NB: Narendra Bael AM: *Aegle marmelos*

Table 2: Scavenging activity of different concentrations of methanolic extracts of 18 *Aegle marmelos* varieties/accessions on 2, 2-Diphenyl-1-picrylhydrazyl radical

Serial number	Bael varieties/ accessions	Percent inhibition									
		20 µg	40 µg	60 µg	80 µg	100 µg	120 µg	140 µg	160 µg	180 µg	200 µg
1	AM-1	16.74±0.59	26.87±0.74	37.88±0.79	49.33±0.38	61.89±0.41	72.24±0.70	80.83±0.70	81.27±0.53	81.49±0.45	81.93±0.54
2	AM-2	12.33±0.43	16.07±0.57	20.70±0.49	34.14±0.93	42.29±0.85	49.55±0.79	60.35±0.74	67.62±0.71	75.55±0.68	82.15±0.65
3	AM-3	15.65±0.41	21.95±0.70	30.0±0.46	37.82±0.28	44.34±0.21	51.95±0.39	57.82±0.70	64.34±0.28	70.0±0.70	71.73±0.26
4	AM-4	18.4±0.34	27.5±0.42	41.59±0.50	55.22±0.38	68.86±0.35	82.04±0.48	88.63±0.58	88.63±0.29	88.63±0.27	89.10±0.34
5	AM-6	4.87±0.98	12.86±0.84	21.06±0.21	29.49±0.91	37.91±0.82	44.78±0.84	53.65±0.42	57.42±0.38	65.18±0.70	67.2±0.56
6	AM-7	36.53±0.35	60.16±0.28	79.94±0.98	87.4±0.45	89.4±0.70	91.4±0.36	92.34±0.42	92.13±0.45	91.95±0.70	91.95±0.61
7	AM-8	18.2±0.70	37.07±0.98	53.7±0.84	67.19±0.42	82.92±0.84	88.98±0.49	89.08±0.56	89.21±0.70	89.21±0.21	89.66±0.35
8	NB-1	21.34±0.52	36.25±0.37	50.46±0.60	58.32±0.73	65.31±0.47	72.56±0.56	83.7±0.48	85.6±0.42	86.01±0.59	86.58±0.45
9	NB-4	9.95±0.77	23.8±0.21	34.2±0.40	46.0±0.28	55.7±0.25	65.2±0.23	74.5±0.31	82.1±0.28	88.8±0.39	89.34±0.46
10	NB-5	11.01±0.31	29.87±0.70	45.76±0.41	58.89±0.82	73.3±0.80	84.53±0.95	90.67±0.49	90.88±0.70	90.46±0.41	91.87±0.34
11	NB-7	12.80±0.41	25.61±0.42	31.61±0.60	38.42±0.53	53.30±0.43	63.22±0.37	72.10±0.33	80.99±0.31	90.46±0.29	93.67±0.43
12	NB-9	12.80±0.41	25.61±0.70	39.32±0.50	54.38±0.47	66.74±0.43	78.65±0.41	88.53±0.38	90.78±0.36	91.01±0.35	91.23±0.35
13	NB-16	5.86±0.36	11.73±0.45	17.15±0.78	23.02±0.43	30.02±0.28	35.21±0.68	40.40±0.44	46.04±0.75	53.04±0.44	55.04±0.45
14	NB-17	0.883±0.42	3.97±0.34	6.40±0.37	9.27±0.21	12.36±0.78	15.45±0.56	18.10±0.42	21.41±0.33	25.38±0.25	27.76±0.32
15	Pant Aparna	25.34±0.29	57.99±0.36	85.15±0.32	92.0±0.43	91.50±0.40	91.09±0.83	90.86±0.52	90.41±0.47	89.49±0.39	89.24±0.48
16	Pant Sujata	11.13±0.63	28.60±0.35	42.35±0.70	57.42±0.49	70.96±0.45	82.09±0.28	92.35±0.70	93.01±0.36	93.01±0.42	94.50±0.48
17	Pant Shivani	13.36±0.49	29.59±0.41	45.10±0.25	57.27±0.81	73.74±0.58	86.39±0.45	92.36±0.36	92.84±0.29	92.84±0.23	93.80±0.87
18	Kaghzi	7.57±0.29	15.36±0.35	22.07±0.25	28.13±0.31	34.84±0.48	40.25±0.37	46.75±0.26	52.59±0.42	59.30±0.47	62.12±0.24
Standard	Ascorbic acid (1 mg/ml)	11.5±0.07	22.4±0.015	38.8±0.059	56.17±0.052	70.7±0.047	84.1±0.044			95.6±0.063	

Each value in the table was obtained by calculating the average of three experiments ± SD. (The concentration used for the standard are 2, 4, 6, 8, 10, 12, 14 µg/ml); SD: Standard deviation; NB: Narendra Bael; AM: *Aegle marmelos*

Table 3: Antioxidant capacity determination of 18 varieties/accessions of *Aegle marmelos* by ferric-reducing ability of plasma assay

Serial number	Different Bael varieties/ accessions	FRAP value (µmol Fe [II]/g dry mass)
1	AM-1	20.65±0.393
2	AM-2	4.57±0.456
3	AM-3	6.36±0.265
4	AM-4	4.61±0.237
5	AM-6	13.24±0.314
6	AM-7	23.06±0.445
7	AM-8	15.1±0.214
8	NB-1	6.64±0.251
9	NB-4	2.82±0.135
10	NB-5	12.58±0.204
11	NB-7	8.12±0.173
12	NB-9	11.05±0.198
13	NB-16	6.75±0.156
14	NB-17	25.84±0.261
15	Pant Aparna	31.1±0.256
16	Pant Sujata	9.81±0.293
17	Pant Shivani	10.74±0.327
18	Kaghzi	3.28±0.246
Standard	Ascorbic acid	38.56±0.417

Data represent mean±SD of three measurements. FRAP: Ferric-reducing ability of plasma; SD: Standard deviation; NB: Narendra Bael; AM: *Aegle marmelos*

Total carotenoid estimation

The carotenoid estimation of all the 18 varieties revealed its highest content in Pant Aparna (107.09 ± 0.84 µg/g) while AM-1, Kaghzi, and AM-7 had significantly lower values. Carotenoids are photoprotective in nature and help in reducing the risk of sunburns, photo-allergy, and even some types of skin cancer.[25] The present results obtained project Pant Aparna variety of *A. marmelos* as a good source of carotenoids and can be a favorable plant for designing the pharmacological products.

Chromatographic characterization

Thin-layer chromatography analysis

TLC analyses of the methanolic extract of the above-mentioned variety (Pant Aparna) showed that diverse types of phenolic compounds, flavonoids, and phenolic acids are present in the investigated extract as evident from Figure 1a and b under the visible and UV light. Moreover, the methanolic extract also denoted the presence of a large number of flavonoids such as rutin, quercetin, and some unidentified flavonoid glycosides. Similarly, phenolic acids such as chlorogenic acid and coumaric acids were also observed as characterized by their R_f-values.

Column chromatography

In addition, the separation and purification of the compounds, column chromatography, was performed by means of a gradient solvent system. The column was subjected to elution using a gradient solvent system [EtOAc-Hexane (1:99–100:0 v/v)] with the increasing polarity to provide thirty fractions. The yield of the fractions after evaporation was 42.8 mg to 107.5 mg consecutively. The fractions were analyzed for their antioxidant activities by DPPH, SOD, and FRAP assays. Moreover, among all the fractions isolated through column chromatography, the fraction number 21 possessed the significant ferric reducing ability, DPPH, and superoxide scavenging activity as indicated in Table 4.

The above fractions collected were subjected to TLC (toluene:ethyl acetate:formic acid:glacial acetic acid in the ratio of 10:3:1:1) at room temperature of 20°C –25°C. As shown in Figure 1c, the fraction number 21 appeared as a single band in contrast to others which consisted of more than one band.

High–performance-thin-layer chromatography profile

Furthermore, HP-TLC analysis exhibited the presence of many phytocompounds in the extract. The pattern obtained by HP-TLC fingerprint analysis demonstrated the presence of 17 polyvalent phytoconstituents in the crude extract of Pant Aparna [Figure 2a and b],

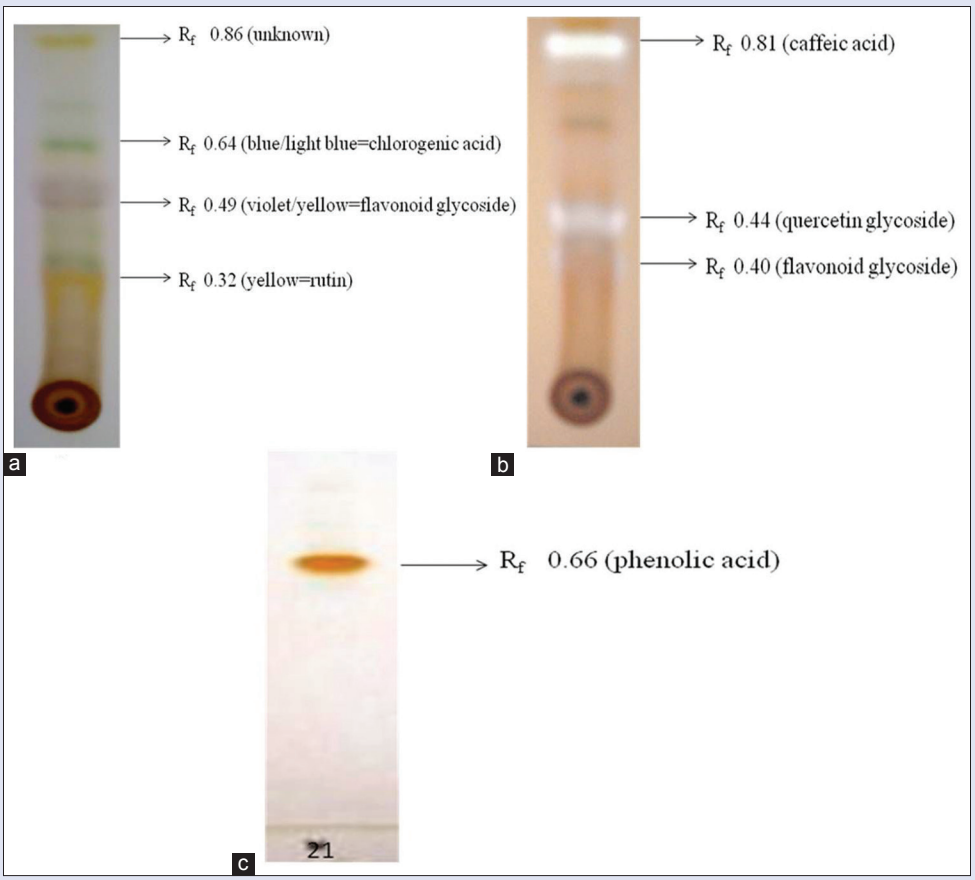

Figure 1: Thin-layer chromatography chromatogram with R_f values (a) Methanolic crude extract of variety Pant Aparna under visible light. (b) Methanolic extract under ultraviolet light. (c) Thin-layer chromatography chromatogram of methanolic fraction of Pant Aparna under visible light

Table 4: Antioxidant activity exhibited by few selected bioactive fractions isolated through column chromatography

Serial number	DPPH scavenging activity (percent inhibition)	FRAP assay (μmol Fe [II]/g dry mass)	Superoxide anion scavenging activity (percentage inhibition)
1	80.18±0.84 (AMMF21)	26.8±0.63 (AMMF21)	37.4±0.60 (AMMF21)
2	74.89±0.42 (AMMF28)	23.67±0.53 (AMMF19)	35.61±0.67 (AMMF20)
3	73.77±0.67 (AMMF27)	21.6±0.56 (AMMF27)	32.39±0.86 (AMMF19)
4 (standard)	Ascorbic acid (20 μg/ml) 86.96±0.86	Ascorbic acid (20 μg/ml) 32.64±0.65	BHT (100 μg/ml) 48.89±0.72

AMMF: *Aegle marmelos* methanolic fraction; BHT: Butylated hydroxytoluene; DPPH: 2, 2-Diphenyl-1-picrylhydrazyl; FRAP: Ferric-reducing ability of plasma

with R_f values ranging from 0.02 to 0.98. Two compounds exhibited the maximum percentage area of 15.97% and 12.58% whose corresponding R_f values were 0.02 and 0.61, respectively [Table 5a].

The fraction AMMF21, however, appeared as a single band in TLC [Figure 2c], but the HP-TLC fingerprinting analysis resulted into 12 polyvalent phytocompounds as shown in Figure 2c and d. The R_f values ranged from 0.03 to 0.98 in which the maximum concentration of the phytocompound was found to be 28.24% whose corresponding R_f value at 0.56 was recorded [Table 5b]. This was followed by peak recorded at number 9 with a high percentage area of about 24.62% and R_f value 0.67.

From the HP-TLC studies, it has been found that methanolic extract contain not a single compound but a mixture of compounds and so it can be concluded that the activities shown by the extract are due to the combined reaction of all the compounds.

Liquid column-mass spectrometry analysis

In addition, LC-MS (ESI) performed in a positive mode in the range of 200–450 nm under the optimized conditions of the Pant Aparna fraction AMMF21 demonstrated the presence of three prominent compounds having molecular weight of 300, 276, and 248, respectively, which were identified as Kaempferide, Ebselon, and Vanillic acid 4-sulfate by spectrum analysis, respectively [Figure 3]. Kaempferide O-methylated flavonol is well reported for peroxynitrite free radical scavenging and antioxidant activities.[26] Ebselon (2-phenyl-1,2-benzisoselenazol-3 (2H)-one) having molecular formula $C_{13}H_9NOSe$ is a lipoxygenase inhibitor whereas vanillic acid 4-sulfate (3-methoxy-4-(sulfooxy) benzoic acid) having molecular formula $C_8H_8O_7S$ belongs to the class of phenolic acids (hydroxybenzoic acid) and are well-known for their broad array of biological activities, namely, antioxidative, anticarcinogenic, and anti-inflammatory.[27,28]

Anti-inflammatory activity of crude extract and selected fraction

(3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide assay

To rule out the cytotoxicity of the crude extract of *A. marmelos* (var. Pant Aparna) and its fraction, the formazon assay (MTT) was

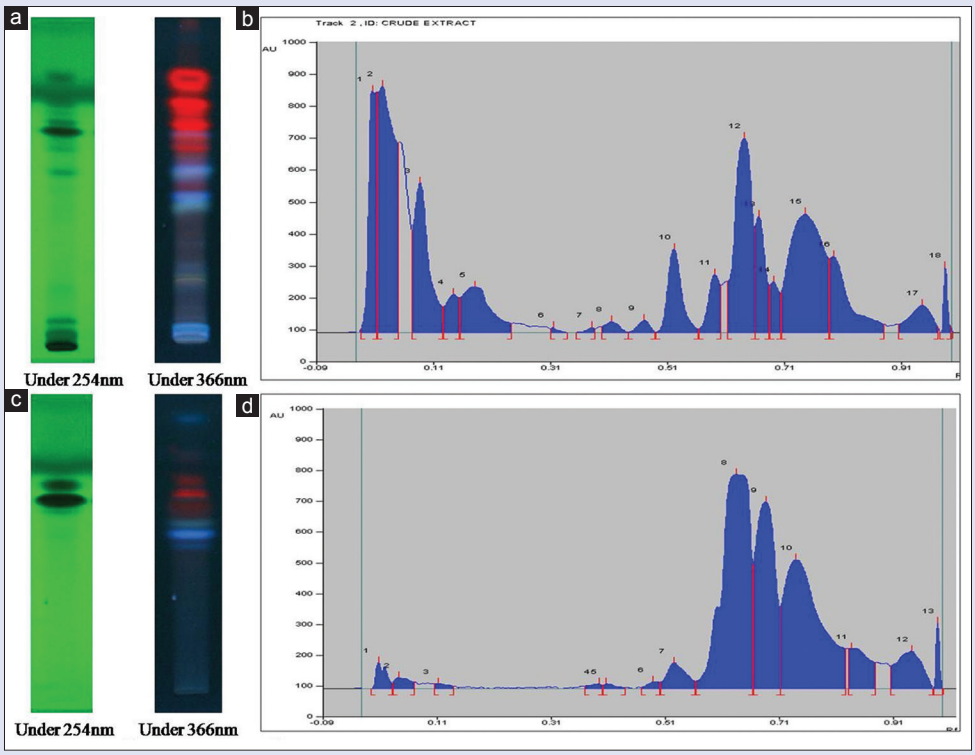

Figure 2: (a) High-performance-thin-layer chromatography fingerprint profiling of methanolic extract of leaves of *Aegle marmelos* variety. Pant Aparna under 254 nm and 366 nm (b) High-performance-thin-layer chromatography chromatogram of a methanolic extract of *Aegle marmelos* (c) High-performance-thin-layer chromatography fingerprinting profile of methanolic fraction (d) High-performance-thin-layer chromatography chromatogram of methanolic fraction

performed. The results obtained revealed that neither the crude nor the fractions adversely affected the cell viability even at higher concentrations at both time periods of 24 h and 48 h of incubation.

Assessment of intracellular reactive oxygen species scavenging activity

The reactive oxygen species (ROS) scavenging analysis by DCFDA revealed a significant reduction by the crude extract at a dose of 50 µg/ml as compared to the positive control, quercetin (10 µM). This was further enhanced with increasing concentration as presented in Figure 4a and b. The potential of fraction AMMF21 was not as influential as the crude extract. It could exert a significant effect only at a higher concentration of 200 µg/ml [Figure 4a and c].

Nitrite assay

The nitric oxide (NO) production from LPS-stimulated macrophage cells was measured by Griess reagent. Antioxidant Vitamin C was used as the standard for samples of crude extract and fraction AMMF21. As clearly depicted by Figure 4d, the production of NO was increased to about 48.72 µM in LPS-stimulated control cells. The unstimulated cells secreted basal levels of NO around 3.41 µM.

Dose-dependent suppression of NO was noticed after treating macrophage cells with crude extract and fraction. Statistically, a significant reduction was observed at the 50 µg/ml concentration of crude extract which further enhanced at 200 µg/ml. However, the fraction was able to exert this effect at a higher concentration of 100 µg/ml. Notably, the crude extract was a better reducer of nitrite production than fraction.

DISCUSSION

It is widely recognized that disease aggravation is because of the excessive oxidative stress that occurs due to an instability between ROS generation and its neutralization.[29] Most of the naturally occurring substances have been known to have antioxidant abilities and various *in vitro* methods have been used to evaluate their free radical scavenging and antioxidant activities. In the present study, the scavenging activities of 18 accessions/varieties of *A. marmelos* were assessed at different concentrations. The extracts exhibited a significant superoxide scavenging activity in a concentration-dependent manner and reached a plateau beyond which no further activity was observed. The highest activity was possessed by Pant Aparna which showed 72.44% inhibition with an IC_{50} value of 50.50 ± 0.84 µg/ml. This potential of Pant Aparna may be due to the presence of the highest amount of flavonoids in comparison to all the other varieties/accessions studied.[8]

Parallel results were achieved from the FRAP assay depicting reduction of Fe^{3+} ferricyanide complex to Fe^{2+} in a dose-dependent manner and the highest reductive property was possessed by Pant Aparna. Earlier reports on the presence of reductones in plant extracts reveal their reducing ability.[30]

Furthermore, the DPPH assay was also performed to further approve its antioxidant potential. The results corroborated with the above findings by exhibiting higher percentage inhibition of free radicals. As lower IC_{50} value indicates higher scavenging activity, it was seen that the methanolic extract of Pant Aparna exhibited utmost scavenging potential of 92.0% at the concentration of 80 µg/ml followed by AM-7. Nevertheless, the IC_{50} value of variety Pant Aparna was 4.85 µg/ml, almost comparable to that of ascorbic acid (7.29 µg/ml).

Table 5: Peak table with Rf values, height, and area of unknown compounds

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area%	Assigned substance
(a) Track 2, Id: Crude extract										
1	-0.01 Rf	10.7 AU	0.01 Rf	754.9 AU	15.64%	0.01 Rf	50.3 AU	10148.1 AU	7.90%	unknown*
2	0.02 Rf	752.0 AU	0.02 Rf	771.2 AU	15.97%	0.05 Rf	93.5 AU	22057.2 AU	17.16%	unknown*
3	0.07 Rf	321.5 AU	0.09 Rf	466.6 AU	9.66%	0.13 Rf	80.8 AU	12543.7 AU	9.76%	unknown*
4	0.13 Rf	81.0 AU	0.15 Rf	120.0 AU	2.49%	0.16 Rf	08.6 AU	2742.9 AU	2.13%	unknown*
5	0.16 Rf	109.0 AU	0.18 Rf	142.3 AU	2.95%	0.24 Rf	29.1 AU	7216.1 AU	5.61%	unknown*
6	0.31 Rf	11.6 AU	0.32 Rf	16.4 AU	0.34%	0.34 Rf	0.2 AU	189.0 AU	0.15%	unknown*
7	0.35 Rf	0.6 AU	0.38 Rf	15.5 AU	0.32%	0.39 Rf	11.7 AU	211.8 AU	0.16%	unknown*
8	0.40 Rf	19.0 AU	0.41 Rf	33.9 AU	0.70%	0.44 Rf	1.1 AU	884.6 AU	0.69%	unknown*
9	0.44 Rf	1.1 AU	0.47 Rf	38.0 AU	0.79%	0.49 Rf	0.8 AU	789.6 AU	0.61%	unknown*
10	0.49 Rf	1.1 AU	0.52 Rf	261.0 AU	5.40%	0.56 Rf	11.3 AU	6350.6 AU	4.94%	unknown*
11	0.56 Rf	11.5 AU	0.59 Rf	181.8 AU	3.77%	0.60 Rf	47.4 AU	3695.0 AU	2.88%	unknown*
12	0.61 Rf	159.9 AU	0.64 Rf	607.2 AU	12.58%	0.66 Rf	25.6 AU	18214.9 AU	14.17%	unknown*
13	0.66 Rf	329.5 AU	0.67 Rf	362.9 AU	7.52%	0.68 Rf	47.7 AU	6064.4 AU	4.72%	unknown*
14	0.69 Rf	147.8 AU	0.69 Rf	158.4 AU	3.28%	0.70 Rf	23.9 AU	2603.4 AU	2.03%	unknown*
15	0.71 Rf	125.6 AU	0.75 Rf	371.5 AU	7.69%	0.79 Rf	31.2 AU	21856.0 AU	17.01%	unknown*
16	0.79 Rf	231.3 AU	0.79 Rf	237.2 AU	4.91%	0.88 Rf	26.7 AU	8346.8 AU	6.49%	unknown*
17	0.91 Rf	26.6 AU	0.95 Rf	85.4 AU	1.77%	0.97 Rf	18.7 AU	3332.9 AU	2.59%	unknown*
18	0.98 Rf	4.2 AU	0.99 Rf	204.1 AU	4.23%	0.99 Rf	19.8 AU	1269.3 AU	0.99%	unknown*
(b) Methanolic fraction										
1	-0.01 Rf	0.4 AU	0.01 Rf	84.7 AU	3.44%	0.03 Rf	17.2 AU	1510.5 AU	1.45%	unknown*
2	0.03 Rf	18.5 AU	0.04 Rf	36.2 AU	1.47%	0.07 Rf	22.0 AU	1004.5 AU	0.97%	unknown*
3	0.11 Rf	15.7 AU	0.11 Rf	17.2 AU	0.70%	0.14 Rf	7.5 AU	411.2 AU	0.40%	unknown*
4	0.37 Rf	8.4 AU	0.39 Rf	15.4 AU	0.63%	0.40 Rf	11.9 AU	341.7 AU	0.33%	unknown*
5	0.40 Rf	12.1 AU	0.41 Rf	15.5 AU	0.63%	0.44 Rf	3.7 AU	352.8 AU	0.34%	unknown*
6	0.47 Rf	4.6 AU	0.49 Rf	23.2 AU	0.94%	0.50 Rf	21.0 AU	490.4 AU	0.47%	unknown*
7	0.50 Rf	21.2 AU	0.53 Rf	84.2 AU	3.42%	0.56 Rf	24.4 AU	2894.2 AU	2.78%	unknown*
8	0.56 Rf	24.6 AU	0.64 Rf	694.6 AU	28.24%	0.66 Rf	02.7 AU	35964.5 AU	34.57%	unknown*
9	0.67 Rf	406.0 AU	0.69 Rf	605.7 AU	24.62%	0.71 Rf	63.9 AU	21217.8 AU	20.39%	unknown*
10	0.71 Rf	266.5 AU	0.74 Rf	417.7 AU	16.98%	0.83 Rf	30.4 AU	28052.4 AU	26.96%	unknown*
11	0.83 Rf	130.1 AU	0.84 Rf	131.1 AU	5.33%	0.88 Rf	34.8 AU	4859.0 AU	4.67%	unknown*
12	0.91 Rf	73.5 AU	0.94 Rf	121.8 AU	4.95%	0.98 Rf	0.1 AU	5711.0 AU	5.49%	unknown*
13	0.98 Rf	0.0 AU	0.99 Rf	212.8 AU	8.65%	1.00 Rf	1.7 AU	1227.6 AU	1.18%	unknown*

Polyphenolics are an important group of secondary metabolites possessing the antioxidant potential in addition to numerous other beneficial effects. Our earlier findings have revealed the presence of polyphenolics in an appreciable amount in variety Pant Aparna among all the varieties and accessions of *A. marmelos.* Thus, it can be inferred that Pant Aparna is an outstanding variety of *A. marmelos* and should be extensively studied for isolation of a novel and potential therapeutic agent.

The TLC analysis detected the occurrence of phenolic compounds such as flavonoids and phenolic acids which have earlier been reported[31-34] as principal contributors to the scavenging effect of the investigated extract. The scavenging ability of quercetin, rutin, flavonoid glycosides, and phenolic acids (chlorogenic, caffeic, coumaric, and vanillic acids), present in the extract, is ascribed to their hydrogen-donating ability and scavenging effect.

Furthermore, the compounds identified through LC-MS analysis further authenticated the scavenging ability of the fraction AMMF21. Various phenolics such as chlorogenic acid (136.8 µg/g), ellagic acid (248.5 µg/g), ferulic acid (98.3 µg/g), gallic acid (873.6 µg/g), protocatechuic acid (47.9 µg/g), and quercetin (56.9 µg/g) have been characterized through LC-MS and LC-MS/MS scans and HPLC studies in Bael fruit by Dhan *et al.*[35]

Nevertheless, its antioxidant potential was verified by *in vitro* assays using mouse macrophage cells J774. The results corroborated with the antioxidant assays by exhibiting intracellular ROS inhibition and reduction of NO in a dose-dependent manner from LPS-stimulated macrophages. However, the fraction AMMF21 demonstrated significant antioxidant potential but at higher doses. Whereas the crude extract was able to exert similar efficacy at a lower concentration. The strong antioxidant activity manifested by the crude extract as compared to the compounds separated may be due to the synergistic action of the collective biologically active compounds.

CONCLUSION

The findings of this study support the view that medicinal plants are promising sources of potential antioxidants that may be valuable for therapy of human diseases.

This is a first ever report on the best antioxidant and ROS scavenging potential of methanolic crude extract and its fraction AMMF21 of variety Pant Aparna of *A. marmelos.* The chromatographic analysis revealed the presence of potential phytocompounds which in combination can be used as a promising phytotherapeutic agent for its varied applications against free radical-mediated ailments. Therefore, we strongly recommend that the above variety should be taken further to exploit its immense potential for other biological activities of medicinal importance.

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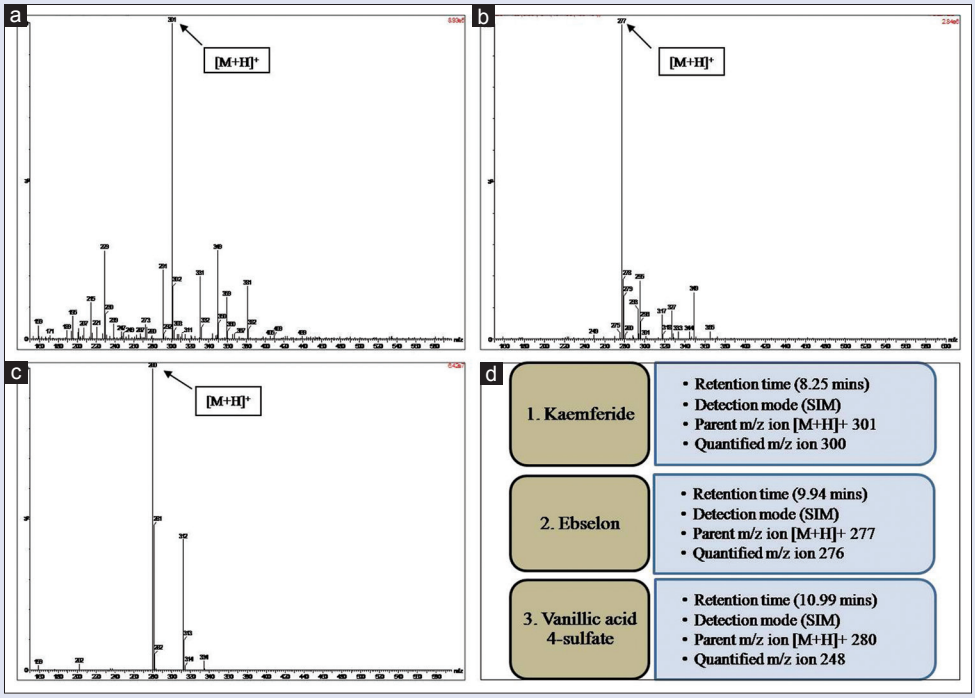

Figure 3: Chromatograms-(liquid column-mass spectrometry-electrospray ionization) analysis of *Aegle marmelos* methanolic fraction 21 of Bael variety Pant Aparna. Identification of peaks: (a) Kaemferide. (b) Ebselon. (c) Vanillic acid 4-sulfate. (d) Main signals exhibited in the liquid column-mass spectrometry spectra of *Aegle marmelos* methanolic fraction 21 (variety Pant Aparna of Bael)

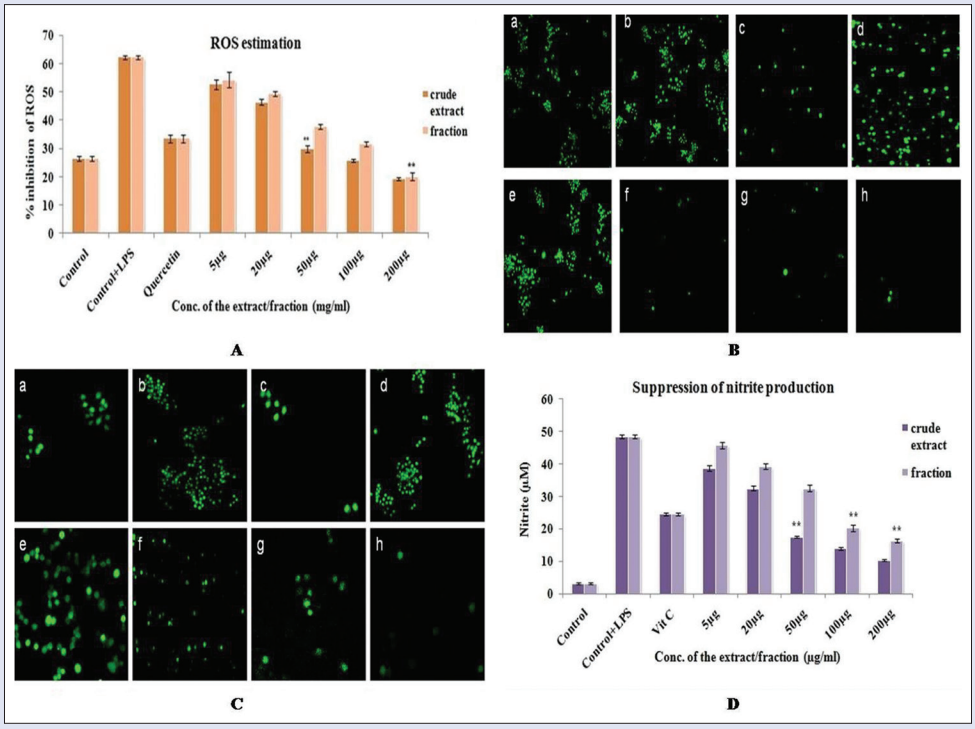

Figure 4: (A) Cellular radical scavenging activity in lipopolysaccharide-activated J774 cells by the DCFH-DA (B) reactive oxygen species detection by DCFH-DA assay: (a) Control (b) Control + lipopolysaccharide (c) quercetin (positive control) (d) 5 µg extract (e) 20 µg extract (f) 50 µg extract (g) 100 µg extract (h) 200 µg extract (C) reactive oxygen species detection by DCFH-DA assay in fraction (D) The crude extract and fraction suppressed lipopolysaccharide-induced nitrite production in J774 macrophage cell line. The values are means ± standard deviation ($n = 3$). The significance of the test was determined using one-way ANOVA by Dunnett's test. * $P < 0.05$, ** $P < 0.01$

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Conflicts of interest

There are no conflicts of interest.

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