International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2018; 6(2): 3509-3517

Received: 13-01-2018
Accepted: 14-02-2018

PN Timbadiya
Department of Biochemistry, College of Agriculture, Junagadh Agricultural University, Junagadh, Gujarat, India

MK Mandavia
Department of Biochemistry, College of Agriculture, Junagadh Agricultural University, Junagadh, Gujarat, India

BA Golakiya
Department of Biochemistry, College of Agriculture, Junagadh Agricultural University, Junagadh, Gujarat, India

Correspondence
PN Timbadiya
Department of Biochemistry, College of Agriculture, Junagadh Agricultural University, Junagadh, Gujarat, India

Phytochemical screening, Antioxidant, Anti-inflammatory and Anti microbial activities of *Aegle marmelos* leaf extracts

PN Timbadiya, MK Mandavia and BA Golakiya

Abstract
This study was performed to investigate the phytochemical screening, physicochemical activities, oxidative enzyme activities, anti microbial, antioxidant as well as anti-inflammatory properties from *Aegle marmelos* leaf different extracts. This investigation was achieved by extraction of medicinal plants in different solvents (Hexane, Chloroform, Methanol, water and 90% Methanol), preliminary phytochemical and physicochemical screening, oxidative enzyme activity, antimicrobial activity, antioxidant activity, antidiabetic activity, separation and identification of phytochemicals having antidiabetic activity from bioactive extracts. Results revels *Aegle marmelos* leaf all polar extracts had highest phytochemicals. The plant leaves rich in flavanoids 63.64±0.45 and phenol 53.45±0.39. The plant leaf powder contained the high level of calcium 51861.81 ppm, magnesium 1713.01 ppm and Iron 161.08. Different extracts of *Aegle maemelos* – leaf inhibited the growth of all four bacteria *Bacillus subtilis, Bacillus cereus, Bacillus megaterium* and *Escherichia coli* except hexane and chloroform extracts which had not showed activity against *Bacillus subtilis*. Antifungal activity was not found in plants extract against *Alterneria.* Highest activity was found against *Fussariumoxispora*, *Aspergillus parasiticus* followed by *Aspergillus flavus.* All five extracts of *Aegle maemelos* – leaf inhibited the growth of *Fussariumoxispora.* Only hexane, water and 90% methanol extracts inhibited the growth of *Aspergillus parasiticus,* while water and 90% methanol extractsinhibited the growth of *Aspergillus flavus.* It was observed that the extracts of *Aegle marmelos* (Beal) leaf had higher DPPH scavenging activity in methanol extract than that of the other extracts 79.52 ± 0.35%. The inhibition of albumin denaturation exerted by *Aegle marmelos* (Beal) leaf had higher activity in methanol extract than that of the other extracts 65.30±1.07%.

Keywords: DPPH, inflammation, anti inflammatory, oxidative enzymes, MP-AES

Introduction
Aegle marmelos (Linn) correa, commonly known as bael (or bel), belonging to the family Rutaceae, is a moderate-sized, slender and aromatic tree. The plant has been used in the Indian traditional medicines from time immemorial. It is associated with various important medicinal properties. Chemical investigation on the different parts of the plant has resulted in the isolation of a large number of novel and interesting metabolites. Some of the compounds have been screened for bioactivity. It is rich in alkalodis, among which aegline, marmesin, marmina and marmelosin are the major ones. Aqueous leaf extract should preventive effect myocardial diseases.

The World Health Organization (WHO) estimated that 80% of the earth's inhabitants rely on traditional medicines for their primary health care need, and most of this therapy involves the use of plant extracts or their active compounds. Secondary plant metabolites have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property. There are more than thousand known and many unknown phytochemicals in plants. It is well-known that plants produce these chemicals to protect themselves, but recent researches demonstrate that many phytochemicals can also protect human against diseases.

Secondary constituents are the remaining plant chemicals such as alkaloids, terpenes, flavonoids, lignans, plant steroids, curcumines, saponins, phenolics, flavonoids and glucosides.

Phytochemicals such as vitamins (A, C, E and K), carotenoids, terpenoids, flavonoids, polyphenols, alkaloids, tannins, saponins, pigments, enzymes and minerals which have antimicrobial and antioxidant activity (Madhuri and Pandey, 2009) [9].

Free radicals induce Oxidative damage to lipids, proteins and nucleic acids, which eventually cause atherosclerosis, ageing, cancer, diabetes, inflammation, AIDS and several degenerative diseases in humans are well documented (Maxwell *et al*., 1997) [10]. Antioxidants are compounds that show reducing activity.

Material and Method

Physicochemical Analysis

Determination of loss on drying

Two grams of crude powder of Medicinal plant was taken in an evaporating dish and then dried in an oven at 105 ºC till constant weight was obtained. The weight after drying was noted and loss on drying was calculated. The percentage was calculated on the basis of sample taken initially (WHO, 2002).

Determination of total ash

Total inorganic material in pulp and seed parts was estimated by AOAC method (AOAC, 1990). The Ash content was measured by gravimetrically.

pHcontent

Measurement of pH is one of the most common and useful analytical procedures in Biochemistry since the pH determines many important aspects of structure and activity of biological macromolecules. Many dyes such as phenol red, litmus and phenolphthalein are the indicators that change color at characteristic pH values.

Estimation of Moisture

Moisture content of plant parts were estimated by AOAC method (No. 930.15). 2 g of plant part were weighed separately in pre weighed glass Petri dishes and dried in hot air oven (80-95ºC) for 5 hours. The dishes were then transferred to desiccators, cooled to room temperature and recorded the weight. The drying was continued till the final weight become stable. The weight differences were noted and the moisture content was expressed in percentage weight of fresh plant parts.

Determination of extractive value in different solvent

Ten grams of dried powder of experimental material was extracted in soxhlet apparatus successively with hexane, ethyl acetate, acetone, methanol and water, respectively due to their nature of polarity.130ml solvent required per 10gm dried powder of experimental material.

Ten grams of dried powder of *Medicinal plant*s was taken in 100 ml of 80% Methanol in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 120 rpm for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105 °C till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially (WHO, 2008).

After extraction, the hexane, ethyl acetate, acetone, methanol, and water extracts were concentrated using rotary evaporator and dried in hot air oven at 50^0 C to get the solid mass and remained sample weighted and calculated % yield then stored yield after lyophilisation for further use.

Elemental analysis by MP-AES

Digestion Procedure of medicinal Plants: 0.5 gm of each sample was digested in nitic acid/Perchloric acid (3:1) using wet digestion method by heating slowly on a hot plate under fume hood chamber till a white residue was obtained. It was dissolved in 1 % Nitric acid and volume was made up to 50 ml. The digested sample were analyzed MP-AES Instrument**.**

Protein content

The protein content of each plant was measured to determine by Folin- Lowry method. True protein was estimated by the method, suggested by Lowry *et al*., (1951) [8]. The samples (0.1 ml) and standard BSA (0.5 -3ml from 0.1 mg/ ml BSA stock) were taken in a series of test tubes and the volume was made up to 3.0 ml with distilled water. Then added 5 ml solution –C, mixed well and incubated at room temperature for 10 mins. Then added 0.5 ml Folin-ciocalteau reagent, mixed immediately and incubated in dark for 30 mins. A reagent blank was prepared by taking only reagents and volume made up with distilled water. Light blue color was observed after incubation which measured at 600 nm in a spectrophotometer. The protein content was calculated by taking Bovine serum albumin as standard and value expressed as $mg.g^{-1}$

Oxidative enzyme activity

Peroxidase activity

Procedure:

Extraction of Samples

Take 0.15 gm of sample and crush with 1.5ml 50mM phosphate buffer (P^{H-} 7.0) in a chilled mortle and pestle. Then clearsupernatant is collected in 1.5ml eppendr of tube, centrifuged at the 15,000 RPM for 10-15 min at 4^0 C. [Note-Centrifuge the samples at the time of assay].

Blank: Take 3ml of the solution- D (i.e. without enzyme)

For samples

1) Take 3ml of solution-D
2) Add 10µl of aliquot (enzymes supernatant). Shake well by inverting it. Immediately keep in the spectrophotometer at 460nm, and record reading by 15sec interval for 3 min.

Catalase activity

Catalase assay is based on the absorbance of H2O2 at 240 nm in UV range. A decreased in absorbance is recorded over a time period as described by Aebi 1984.

Preparation for enzyme extract:

Take 0.2gm of leaves of leaves and crush in 1.5ml of 50mM phosphate buffer (pH - 7.0) in a chilled mortle and pestle. Then collect the sample in 2 ml eppendrof tube. Centrifuged the sample at the 15,000 RPM for 10-15 min at 4^0C. Collect the clear supernatant.

Procedure

The 3 ml reaction mixture consists of 50mM sodium phosphate buffer (pH 7.0), 10mM $H_2O_2$and 50 µl enzyme extract. The hydrogen peroxide dependent oxidation was estimated by measuring the decrease in the absorbance at 240nm.

Super oxide dismutase activity

The method employed at Worthington is essentially that of winter bourn *et al*. (1975) and is based on the ability of

superoxide dismutase to inhibit the reduction of nitro-blue tetrazolium by superoxide. One unit is defined as that amount of enzyme causing half the maximum inhibition of NBT reduction. The reaction velocity will depend largely on somewhat variable assay conditions such as light intensity and reaction temperature. Calibration of the method in individual laboratories is recommended.

Procedure
Prepare stock solution at one mg/ml.

Pipette into a series of tubes:

EDTA/Cyanide	0.2 ml
NBT	0.1 ml
Enzyme	*
Phosphate buffer	q.s. to 3.0 ml

Include several tubes with no enzyme as controls.
A series of samples ranging from 0.1-10 micrograms is recommended. A tube containing approximately 100 micrograms will generally produce maximum inhibition. Place the tubes in a light box providing uniform light intensity. (A foil-lined box approximately 4' long X 8" X 6" with an internally mounted 40 W fluorescent bulb has been used successfully). Incubate the tubes for 5-8 minutes to achieve a standard temperature. At zero time and at timed intervals add 0.05 ml riboflavin. Incubate all tubes in the light box for 12 minutes and at timed intervals read A560. Determine percent inhibition of NBT reduction. Plot percent inhibition versus amount of enzyme in test. Determine the amount of enzyme resulting in one half of maximum inhibition.

Phytochemical Analysis
Qualitative phytochemical analysis
The extracts were screened for primary phytochemicals as described by Raja and Sama (2012) [13] & Reddy and Mishra (2012) with minor modifications. Methanol: water (9:1) extract of samples were analyzed for preliminary phytochemicals (Table 1),

Quantitative phytochemical analysis
Determination of total phenol content
Quantification of phenol was carried out using foline-ciocalteu method. Standard was prepared catechol was used as standard (10mg/10ml in methanol). (Jayasinghe *et al*., 2003) [7]. The extract (0.5 ml) and 0.1 ml of Folin-Ciocalteu's reagent (0.5 N) were mixed and the mixture was incubated at room temperature for 15 min. Then, 2.5 ml saturated sodium carbonate was added and further incubated for 30 minute at room temperature and the absorbance was measured at 760 nm using a digital spectrophotometer (Systronic, India), against a blank sample. The calibration curve was made by preparing gallic acid (10 to 100 µg/ml) solution in distilled water. Total phenolic content is expressed in terms of gallic acid equivalent (mg/g of extracted compounds).

Determination of flavonoid content
The amount of flavonoid content of *Medicinal plant* extract was determined by Aluminium chloride colorimetric method (Chang *et al.* 2002) [3]. The reaction mixture (3.0 ml) consisted of 1.0 ml sample (1 mg/ml), 1.0 ml methanol, 0.5 ml aluminium chloride (1.2 %) and 0.5 ml potassium acetate (120 mM) and was incubated at room temperature for 30 min. The absorbance was measured at 415 nm using a digital spectrophotometer (Systronic, India), against a blank sample. The calibration curve was made by preparing quercetin (5 to 60 µg/ml) solution in methanol. The flavonoid content is expressed in terms of quercetin equivalent (mg/g of extracted compounds).

Extraction of crude alkaloid content
Two gram of dried powder was taken in 100 ml flask and 40 ml 20 % glacial acetic acid in methanol was added to it and was allowed to stand for 4 h at room temperature. This was filtered and the extract was concentrated in a water bath to one quarter of the original volume. Concentrated ammonium hydroxide (25 %) was added drop wise to the extract until the precipitation was complete. Then it was taken in a separating funnel and an equal volume of chloroform was added. It was washed with distilled water three times to make the pH neutral. Sodium sulphate (Na2SO4) was added to remove moisture. It was filtered and dried. Crude alkaloid content was collected and weighed. Crude alkaloid content is expressed in mg/g of dried tissue powder (Djilani *et al*., 2006) [5].

Quantification of tannins
5g of the finely ground sample was weighed and transferred into 250 mL conical flask and 50 mL of distilled water added and shook vigorously for an hour. The resulting solution was filtered into a volumetric flask and 5 mL of the filtrate pipetted out into a test tube. 0.1g of tannic acid was dissolved in 100 mL of water to form tannic acid solution. 5 mL of the tannic acid solution was pipetted out into another 50ml volumetric flask. A blank sample was also prepared using 5ml of distilled water. The three samples were incubated for 1.5 hours at 20 - 300C and the sample was then filled with distilled water up to mark of 50 mL of the volumetric flask. The absorbance of the three samples was measured at 760 nm using spectronic 21D. The values generated were used to calculate the tannin content (Fecka *et al.*, 2015) [6].

Quantification of saponins
The total saponins content measured using double solvent extraction method (Obadoni and Ochuko, 2001) [12], by taking 20g of the finely ground sample was weighed out into a conical flask.100 mL of 20% aqueous ethanol was added into the sample, it was heated over a water bath for 4hrs with continuous stirring at about 55^0C. The mixture was filtered and the residue reextracted with another 100 mL of 20% ethanol. The combined extract was reduced to about 40 mL over a water bath at 90^0 c, and the concentrate transferred into 250 mL separating funnel and 40 mL of pet-ether was added and shook vigorously. Separation was by partition during which the ether layer was discarded and the aqueous layer reserved. The saponin was extracted with 60 mL of normal butanol. The combined extract were washed with 5% aqueous NaCl solution, and evaporated to dryness in a pre-weighed beaker (W1) and was dried at 60^0C in the oven and reweighed (W2). The saponin content was determined by difference and calculated as a percentage of the original sample.

Table 1: Procedures for the qualitative preliminary phytochemical screening

Sr.no.	Phytochemicals	Procedure	Nature of colourchange	Inference
1	Flavonoids	Substance+ 10% NaOH	Green brown	Present
2	Saponnin	Substance shake in water	Frothing present	Present
3	Steroids	0.5 ml of extract +1 ml conc. H_2SO_4	Wine red color	Present
4	Quinone	Sub. +conc. HCl	Green colour	Present
5	Cellulose	Extract + iodine followed by H_2SO_4	Brown colour	Present
6	Terpenoids	Substance + 2 ml chloroform +conc. H_2SO_4	Reddish brown colour at the interface	Present
7	Triterpenes	0.5 ml of extract + few drops of acetic anhydride + 1 ml conc. H_2SO_4 from the side of test tube	Red ring at the junction	Present
8	Cardiac glycosides	Substance + 2 ml glacial acetic acid+ 1 drop of $FeCl_3$ + 1 ml of conc.H_2SO_4 from the wall of test tube	Reddish brown ring at the junction of two solvents	Present
9	Phenol	Substance + alchohol + $FeCl_3$	Greenish yellow	Present
10	Tannin	0.5 g substance + 20 mal H_2O in boiled + 0.1% $FeCl_3$	Brownish green	Present
11	Alkaloids	2 ml test solution + 2 N HCl + Mayer's reagent	Yellowish orange precipitate	Present
12	Lignin	0.5 ml extract + shake + 2 ml of 2% (v/v) furfuraldehyde	Red colour	Present
13	Carbohydrates	Crude extract + shake + 2 ml conc. H_2SO_4 from the side of test tube	Violet ring at the junction	Present
14	Amino acid, and Protein	Crude extract boiled with 2 ml 0.2% ninhydrin	Violet colour	Present
15	Fat and Fixed oil	Substance + sudan III	Shiring orange colour	Present

Biological activity

Antimicrobial Activity

A modified agar well diffusion method (Bauer *et al*., 1966) was used to determine the antifungal activity.

Test organisms

Four Bacteria and Four fungi were used to test antimicrobial activity of extrcts. Microorganisms were provided by Department of Biotechnology, Junagadh Agricultural University, Junagadh. Microorganisms were stored at 4°C on Nutrient agar slant and potato dextrose agar slant before use.

List of Bacteria used in antimicrobial activity

Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Escherichia coli

List of Fungi used in antimicrobial activity

Aspergillus paracitica, Fusarium oxispora, Aspergillus flavus, Alterneria

Procedure

The Nutrient agar (for bacteria) and Potato dextrose agar (for fungi) was inoculated with microbial cell suspension (100 µl) and poured into sterile Petri dishes. Agar well diffuse of 8 mm diameter were impregnated with 60 µl extract solution equivalent to 1500 µg of the each dried extract in 100% DMSO (dimethylsulphoxide) and air dried.

Chloramphenicol and streptomycin were used as two controls for antibacterial activity, while ketoconazole and Fluconazole (5 mg/ml) were used as two controls for antifungal activity. Well filled with 60 µl of DMSO served as negative control.

The plates were incubated at 28ºC for 24h, for all tested bacteria and 48 h for all the tested fungi. The experiment was done three times to minimize error. After incubation period the antibacterial and antifungal activity was evaluated by measuring the inhibition zones. An inhibition zone of 8 mm or greater (including diameter of the well) was considered as high antimicrobial activity. Activity index was calculated by Zone of inhibition of treated / Zone of inhibition of control

***In Vitro* Antioxidant Assays**

The antioxidant activity of the extracts was evaluated by DPPH free radical.

Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH•) free radical scavenging activity

Procedure:

The DPPH free radical scavenging activity was measured by the modified method of McCune and Johns (2002). The reaction mixture (3.0 ml) consisted of 1.0 ml DPPH in methanol (0.3 mM), 1.0 ml methanol and 1.0 ml different concentrations of the methanolic extract were incubated in dark for 10 min, after which the absorbance was measured at 517 nm against blank. For control, 1.0 ml of methanol was used in place of extract. Ascorbic acid was used as positive control. Percentage of inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = (A0 - A1 / A0) \times 100$$

Where, A0 is the absorbance of control and A1 is the absorbance of sample.

In order to calculate IC50 value, plant extract solution in methanol was further diluted and tested for DPPH assay to find out 50% inhibition. IC50 value was calculàted by graph method.

Anti inflammatory activity

Procedure

The anti inflammatory activity was measured by method of (standard protocol, Plant Research Scheme).Take the 1 ml of all suitable concentrations of test/ standard solutions. Add 1 ml of 25 mM TRIS-HCl buffer. & 1 ml 0.2% BSA solution. Allow to keep it for 20 min at 37oC. After incubation, heat all the solutions at 70oC for 4-5 min. till turbidity comes. Allow to cool solutions at lab temperature. Take absorbance at 660 nm in spectrophotometer & record absorbance. Prepare one blank solution which contains the 1 ml of 25 mM TRIS-HCl buffer. & 1 ml 0.2% BSA solution with 1 ml distilled water. All the experiment should carried out in triplicate for suitable statistical calculations

Result and Discussion

Physicochemical and phytochemical screening

Physicochemical Analysis

Extract Yield (%)

The percentage yield of leaf extracts of *Aegle marmelos* in different solvents are 2.73±0.16, 5.30±0.07, 17.43±0.37,

22.83±0.04 and 6.53±0.25 % for Hexane, Chloroform, methanol, Water and 90% Methanol respectively. Extraction efficiency was high in *Aegle marmelos* (water) with the highest extraction yield (%) 22.83±0.04 % and (MeOH) (17.43±0.37%).

Moisture Content
Moisture content of the freshly harvested plant samples was found to be 44.02±1.58in *Aegle marmelos* (Beal) leaf.

Total ash Content
The range of total ash content found in all four medicinal plants was 7.09±0.06 in *Aegle marmelos* (Beal) leaf.

Total Fibre Content
The fibre is the fraction of carbohydrate that remains after treatment with acid and alkali. The total fibre content found in all selected medicinal plants was.04±0.01 *in Aegle marmelos* (Beal) leaf.

pH content
The range of pH content found in all five medicinal plants was 6.18±0.29 in *Aegle marmelos* (Beal) leaf.

Total Protein Content
Protein content was found (14.03±0.85) in *Aegle marmelos* (Beal) leaf.

Total Phenol Content
Aegle marmelos (Beal) leaf (53.45±0.39 mg/g)

Total Flavonoid Content
Aegle marmelos (Beal) leaf had theflavonoid content (63.64±0.45 mg/g)

Total Alkaloid Content
The alkaloid content was obtained from the *Aegle marmelos* (Beal) leaf (36.51±0.45 mg/g).

Total Tannin Content
Aegle marmelos (Beal) leaf (8.07±0.95 mg/g)

Total Saponin Content
Aegle marmelos (Beal) leaf (11.66±0.57mg/g).

Total Glycoside Content
Aegle marmelos (Beal) was also contained of glycoisde (31.99±1.01mg/g).

Table 2: Physico-chemical analysis of medicinal plants

Parameters	*Aegle marmelos* leaf	*Gymnemasylvestre*leaf	*Pterocarpus marsupium* stem	*Tinospora cordifolia* stem	*Trigonella FoenumGraecum*Seeds
MoistureContent(mg%)	44.02±1.58	7.06±0.72	2.44±0.66	34.44±0.07	4.21±0.27
Total ash (mg%)	7.09±0.06	9.46±0.11	2.15±0.11	11.43±0.18	3.23±0.03
pH content	6.18±0.29	7.00±0.09	4.52±0.31	7.02±0.11	7.03±0.03
Total fibre(mg %)	10.04±0.01	11.53±0.30	15.18±0.52	14.65±0.36	6.39±0.14
TotalProtein (mg%)	14.03±0.85	10.75±0.26	2.28±0.26	1.53±0.31	23.40±0.32
Total phenol(mg/g)	53.45±0.39	62.50±0.12	28.77±0.71	156.58±0.38	17.51±0.31
Total tannin(mg/g)	8.07±0.95	2.11±0.03	1.51±0.32	13.12±0.04	111.31±7.6
Totalalkaloid(mg/g)	36.51±0.45	10.09±0.08	2.57±0.08	16.04±0.11	4.35±0.15
Totalflavanoid(mg/g)	63.64±0.45	18.01±0.88	36.88±1.48	6.64±0.28	38.34±0.20
Totalsaponin(mg/g)	11.66±0.57	22.46±0.50	7.37±0.19	19.22±0.02	74.09±3.60
Totalglycoside(mg/g)	31.99±1.01	21.35±0.682	5.39±0.22	1.99±0.05	122.26±1.8
Extract yield (%)					
Hexane	2.73±0.16	0.89±0.07	1.89±0.04	5.88±0.08	2.35±0.09
Chloroform	5.30±0.07	0.61±0.02	0.50±.007	4.67±0.03	1.35±0.03
Methanol	17.43±0.37	4.70±0.22	7.13±0.08	9.25±0.29	6.66±0.16
Water	22.83±0.04	5.73±0.06	0.97±0.06	8.34±0.11	2.57±0.08
Methanol:Water	6.53±0.25	4.57±0.06	5.69±0.18	6.31±0.09	4.68±0.07
(90:10)					

Elemental analysis of medicinal plants
The elemental analysis of all five plants found 3 macro (Ca, Mg and Fe), 3 micro (Zn, Cu and Mn) and 2 other elements (Ni and Cd).

Aegle marmelos (Beal) leaf contained highest amount of Ca (51861.81 ppm) followed by Mg (1713.01 ppm), Fe (161.08 ppm), Zn (51.24 ppm),Mn (46.04 ppm). While Cu, Cd, and Ni were absent

Table 3: Elemental analysis of medicinal plants

Parameters	*Aegle Marmelos*
Macro elements (ppm)	
Calcium	51861.81
Magnesium	1713.01
Iron	161.08
Microelements (ppm)	
Zinc	51.24
Copper	Nil
Manganese	46.04
Other elements (ppm)	
Nickel	Nil
Cadmium	Nil

Phytochemical analysis

The presence of various phytochemicals was observed in the leaf of *Aegle marmelos* Phytochemicals such as alkaloid, saponin, terpenoids, glycosides, tannin, steroid, flavonoid andphenol were found to be present (Table 4.). Triterpenoids werepresent in chloroform extract. Chloroform extract did not contain steroids**.** 90% methanol and methanol extracts was found rich in alkaloids, however moderate amount of flavonoids, saponins, steroid, glycosides, and protein was also observed. Among all extracts 90% Methanol and methanol extract showed the presence of more secondary metabolites followed by water, chloroform and hexane extract respectively.

Table 4: Preliminary Phytochemical Screening of *Aegle marmelous* Leaf Extract

Test	Hexane extract	Chloroform extract	Methanol extract	Water extract	90% Methanol extract
Flavonoids	+	+	++	+	++
Saponins	+	+	++	++	++
Steroids	-	-	++	+	++
Quinones	+	+	+	--	+
Cellulose	+	+	+	+	+
Terpenoids	-	+	-	-	-
Triterpenoids	-	+	-	-	-
Glycosides	+	+	++	++	++
Phenol	+	+	+	+	+
Tannins	+	+	+	-	+
Alkaloids	+	+	+++	++	+++
Carbohydrates	+	+	+	+	--
Proteins	-	+	++	-	++

(+++, High concentration; ++, moderate concentration; +, low concentration; −, absence)

Oxidative enzyme activities

Superoxide dismutase (SOD)

The activity of super oxide dismutase specific ($U.mg^{-1}$ $protein.min^{-1}$) of *Aegle marmelos* (Beal) leaf were 1.68.

Catalase (CAT)

The specific activity of Catalase ($U.mg^{-1}$ $protein.min^{-1}$) *Aegle marmelos* (Beal) leaf were 0.11.

Peroxidase

The specific activity of peroxidase ($U.mg^{-1}$ $protein.min^{-1}$) *Aegle marmelos* (Beal) leaf were 0.48.

Table 5: Oxidative enzyme specific activity in (U.Mg-1 Protein.Min-1) from Different Antidiabetic Medicinal Plants

Medicinal Plant	specific activity ($U.mg^{-1}$ $protein.min^{-1}$)		
	Peroxidase (POD)	Catalase (CAT)	Super oxide Dismutase (SOD)
A. marmelous	0.48	0.11	1.68

Biological activities of medicinal plant extracts

Antimicrobial activity by well diffusion method

The antimicrobial potential of all the experimental plants was evaluated according to their zone of inhibition against various pathogens and the results (zone of inhibition) were compared with the activity of the antibacterial standards (viz., Chloramphenicol 1.0 mg/ml, Streptomycin 1.0 mg/ml) and antifungal standards (viz., Ketoconazole 1.0 mg/ml, Fluconazole 1.0 mg/ml).

Different extracts of *Aegle maemelos* – leaf inhibited the growth of all four bacteria *Bacillus subtilis, Bacillus cereus, Bacillus megaterium* and *Escherichia coli* except hexane and chloroform extracts which had not showed activity against *Bacillus subtilis.*

Antifungal activity was not found in plants extracts against *Alterneria.* Highest activity was found against *Fussariumoxispora*, *Aspergillus parasiticus* followed by *Aspergillus flavus.* All five extracts of *Aegle maemelos* – leaf inhibited the growth of Fussarium*oxispora.* Only hexane, water and 90% methanol extracts inhibited the growth of *Aspergillus parasiticus,* while water and 90% methanol extractsinhibited the growth of *Aspergillus flavus.*

Table 6: Qualitative antibacterial activity and Activity index of Different Medicinal plant extract

Medicinal plant	Extract	*Bacillus cereus*			*Bacillus megaterium*			*Bacillus subtilis*			*Escherichia coli*		
		ZI	AI (CHL)	AI (STR)	ZI	AI (CHL)	AI (STR)	ZI	AI (CHL)	AI (STR)	ZI	AI (CHL)	AI (STR)
Aegle marmelos- leaf	Hexane	17	1.54	1.13	11	0.78	0.57	-	-	-	13	0.81	0.65
	Chloroform	14	1.27	0.93	10	0.71	0.52	-	-	-	14	0.88	0.7
	Methnol	15	1.36	1	11	0.78	0.57	12	1.33	1.33	12	0.75	0.6
	Water	14	1.27	0.93	10	0.71	0.52	10	1.11	1.11	13	0.81	0.65
	90% methanol	16	1.45	1.06	12	0.85	0.63	15	1.67	1.67	12	0.75	0.6

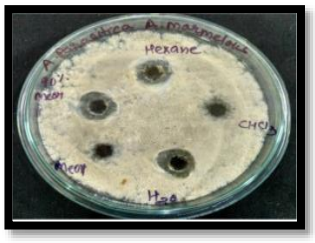
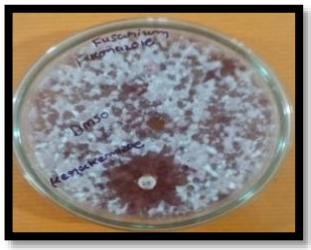
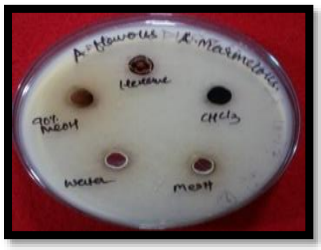
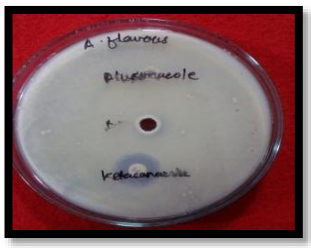
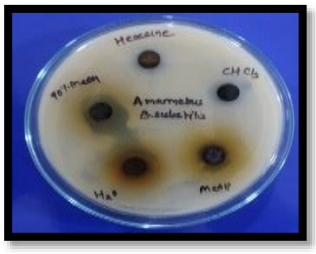
ZI= Zone of inhibion, AI= Antibacterial index of plant extracts, CHL= Chloramphenicol (antibiotic control), STR= Streptomycin (antibiotic control)

Table 7: Qualitative antifungal activity and Activity index of Different Medicinal plant extract

Medicinal plant	Extract	*Aspergillus parasiticus*			*Fussariumoxisporium*			*Aspergillus flavus*			*Alterneria*		
		ZI	AI (KT)	AI (FLC)	ZI	AI (KT)	AI (FLC)	ZI	AI (KT)	AI (FLC)	ZI	AI (KT)	AI (FLC)
Aegle marmelos- leaf	Hexane	9	0.4	-	16	0.69	-	-	-	-	-	-	-
	Chloroform	-	-	-	8	0.34	-	-	-	-	-	-	-
	Methnol	-	-	-	9	0.39	-	8	0.61	-	-	-	-
	Water	10	0.45	-	9	0.39	-	8	0.61	-	-	-	-
	90% methanol	10	0.45	-	11	0.47	-			-	-	-	-

ZI= Zone of inhibion, AI= Antifungal index of plant extracts, ketoconazole (KT) (antibiotic control) amd fluconazole (FLC) (antibiotic control)

Table 8: Qualitative antimicrobial activity plates Different Medicinal plant extract

Fungi	**Plant extracts**	**Control**
A.parasiticus		
F. oxysporum		
A.flavus		
Bacteria	**Plant extracts**	**Control**
B.cereus		
B. subtilis		

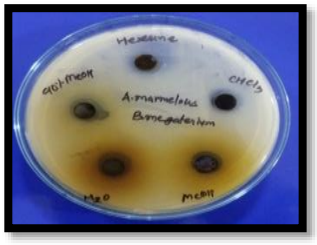
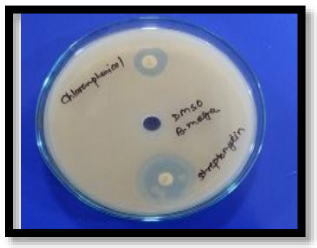
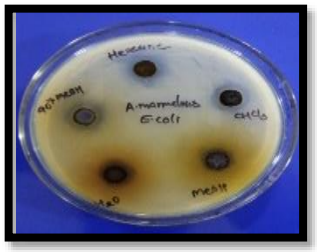
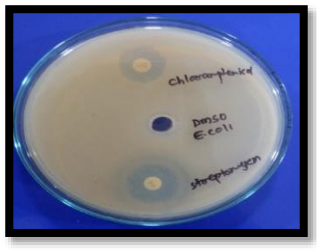
B.mega		
E.coli		

Table 9: Antioxidant Activity by DPPH Method

Medicinal plant	Extract	Concentration (µg/ml)	DPPH scavenging activities	IC50 Values (µg/ml)
Aegle marmelos - leaf	Hexane	100 µg/ml	33.68 ± 0.61[a]	50.84
	Chloroform	100 µg/ml	16.14 ± 0.35[ab]	65.93
	Methanol	100 µg/ml	79.52 ± 0.35[ab]	21.25
	Water	100 µg/ml	41.52 ± 0.91[b]	37.09
	90% methanol	100 µg/ml	52.58 ± 0.73[b]	34.01

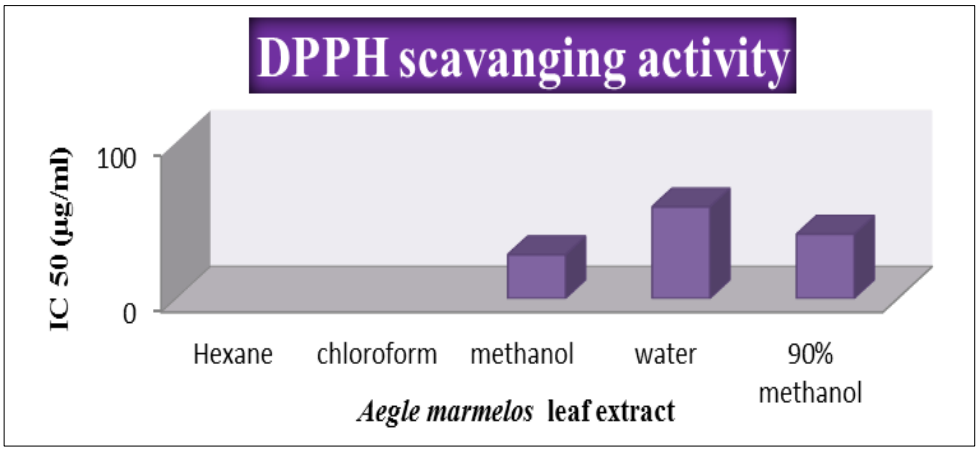


Fig 1: IC 50 (µg/ml) DPPH Scavanging activities of *Aegle marmelos*leaf extracts

It was observed that the extracts of *Aegle marmelos* (Beal) leaf had higher activity in methanol extract than that of the other extracts (Table 9). At a concentration of 100 µg/mL, the scavenging activity of the Hexane, Chloroform, Methanol, Water and 90% Methanol extracts reached 33.68, 16.14, 79.52, 41.52 and 52.58% respectively. The result showed that Hexane, Chloroform, Methanol, Water and 90% Methanol extracts are good scavangers, and the IC50 of that extracts were 50.84, 65.93, 21.25, 37.09 and 34.01µg/ml, respectively (Table 9).

Table 10: Anti inflammatory activity by inhibition of albumin denaturation method

Medicinalplant	Extract	Concentration(µg/ml)	Inhibition of albumin denaturationActivities	IC50 Values (µg/ml)
Aegle marmelos - leaf	Hexane	1000 µg/ml	22.22± 1.41[b]	-
	Chloroform	1000 µg/ml	24.42±0.43[b]	-
	Methanol	100µg/ml	65.30±1.07[a]	43.90
	Water	100 µg/ml	46.06±1.13[ab]	61.58
	90% methanol	100µg/ml	52.42±0.15[ab]	51.27

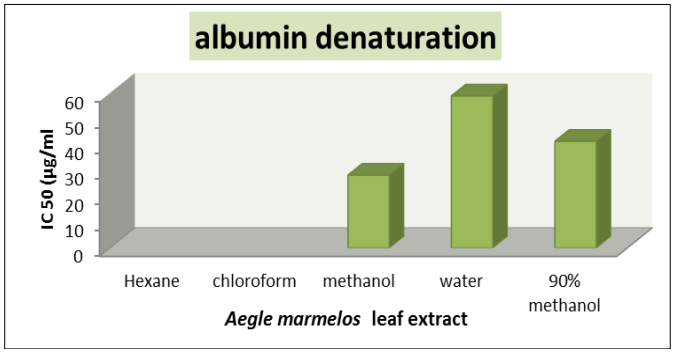


Fig 2: IC 50 (µg/ml) albumin denaturation activities of *Aegle marmelos*leaf extracts

It was observed that the extracts of *Aegle marmelos* (Beal) leaf had higher activity in methanol extract than that of the other extracts (Table 10). At a concentration of 1000 µg/mL, the anti-inflammatory activity of the Hexane and Chloroform and 100 µg/mL in Methanol, Water and 90% Methanol extracts reached up to 22.22, 24.42, 70.24, 46.06, and 52.42% respectively. The result showed that Methanol, Water and 90% Methanol extracts are good inhibitors, and the IC50 of that extracts were 43.90, 61.58 and 51.27 µg/ml, respectively (Table 10).

Summary and Conclusion

The result of physicochemical analysis showed 44.02, 7.09 and 10.04 % moisture content, total ash and total fiber percent respectively. The extractive value of crude powder was maximum in water (22.83 %), followed by methanol (17.43 %). The qualitative phytochemical analysis of different extracts revealed the presence of secondary metabolites like glycosides, alkaloids, saponins, tannin, steroid, flavonoid and phenol. The crude powder was rich in flavonoids 63.64 mg/g, quantitatively estimated total alkaloid content was higher (36.51 mg/g) than that of glycosides content (31.99mg/g). Maximum alkaloid content was in methanol and 90% methanol extract. The elemental analysis revealed the presence of 8 elements; in which (Ca, Fe, Mg, Zn and Mn) were present. While (Cu, Ni and Cd) were absent. Oxidative enzyme results revealed that highest specific activity was found in peroxidase enzyme (1.31U.mg^{-1} protein.min^{-1}) than catalase and superoxide dismutase enzymes.

Result of Antimicrobial activity revealed that all extracts show good inhibitory activity against three bacteria and one fungus. *In vitro* antioxidant activity of the extracts was carried out by DPPH free radical scavenging activity. It was found that methanol extract had higher DPPH activity (79.52%) followed by 90 % methanol extract (52.58%), The IC_{50} value of both the extracts was 21.25 and 34.01µg/ml in DPPH free radical scavenging activity.

In vitro anti-inflammatory activity of the extracts was carried out by albumin denaturation activity. It was found that methanol extract had higher inhibitory activity (65.30%) followed by 90 % methanol extract (52.42%), The IC_{50} value of both the extracts was 43.90 and 51.27µg/ml in albumin denaturation activity.

References

1. Aebi H. Catalase in vitro. Methods Enzymol. 1984; 105:121-126.
2. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology. 1966; 45:493-496.
3. Chang C, Yang M, Wen H, Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Analaysis. 2002; 10:178-182.
4. De Benoist B, McLean E, Egli I, Cogswell M. World Health Organization, Centers for Disease Control and Prevention. Worldwide prevalence of anaemia 1993-2005, 2008.
5. Djilani A, Legseir B, Soulimani R, Dickob A, Younos C. New Extraction Technique for Alkaloids. JBraz. Chem. Soc. 2006; 17(3):518-520.
6. Fecka I, Kucharska AZ, Kowalczyk A. Quantification of tannins and related polyphenols in commercial products of tormentil (Potentillatormentilla).Phytochem Anal. 2015; 26(5):353-66.
7. Jayasinghe C, Gotoh N, Aoki T, Wada S. Phenolics composition and antioxidant activity of sweet basil (Ocimum basilicum L.)J Agric Food Chem.2003; 51(15):4442-9.
8. Lowry 0H, Rosebrough NJ, Farr AL, Randall RJ. JBiol. Chem. 1951; 193:265-275.
9. MadhuriS, PandeyG. Some medicinal plants as natural anticancer agents. Pharmecognocy Review. 2009; 3:259-63.
10. Maxwell SR, Thomason H, Sandler D, Leguen C, Baxter MA, Thorpe GH, *et al*. Antioxidant status in patients with uncomplicated insulin-dependent and non-insulin-dependent diabetes mellitus. Eur. JClin. Investigation. 1997; 27:484-490.
11. McCune LM, Johns T. Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the indigenous peoples of the North American boreal forest. Journal Ethnopharmacology, 2002; 82:197-205.
12. Obadoni BO, Ochuko PO. Phytochemical studies and comparative efficacy of the crude extract of some homeostatic plants in Edo and Delta states of Nigeria. Global JPure Appl. Sci. 2001; 8:203-208.
13. Raja AV, Sama K. Preliminary phytochemical screening of root bark of delonix regia. International research journal of pharmacy. 2012; 2(10):42-43.
14. Reddy MN, Mishra GJ. Preliminary phytochemical screening and antibacterial analysis of the leaf extracts of launaea procumbens roxb. International Journal of Phytopharmacology. 2012; 3(2):147-151.
15. Standard protocols. Plan Research Scheme. Evaluation of Pharmacological activity of Indigenous Medicinal Plants of Surashtra region at junagadh. In-vitro anti-inflammatory activity Dept. of Veterinary Pharmacology & Toxicology. Junagadh Agricultural University.16:19-23.
16. Standard protocols. Plan Research Scheme. Evaluation of Pharmacological activity of Indigenous Medicinal Plants of Surashtra region at junagadh. In-vitro anti-diabetic activity Dept. of Veterinary Pharmacology & Toxicology. Junagadh Agricultural University. 23:27.
17. Winterbourn CC, Carrell RW. Studies of hemoglobin denaturation and Heinz body formation in the unstable hemoglobins. J Clin. Invest. 1974; 54:678-689.