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Antiplatelet aggregation and antimicrobial activities of bael (*Aegle marmelos* Linn.) extracts

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Bael (*Aegle marmelos* Linn.) has been used in the Indian traditional systems for treating various ailments. In the recent past there have been numerous studies indicating various bioactivities with different parts of the plant. A study has been undertaken to assess the antiplatelet aggregation activities and antimicrobial activities of the Bael. Five solvent extracts from different parts of the plant were analysed for the antibacterial activity against seven bacteria. It was found that methanol extract of pulp, rind and seed has shown the inhibitory zone of 4mm against *Klebsiella pneumoniae* and *Staphylococcus aureus* and the hexane extract of rind and seed has shown inhibitory zone for *Pseudomonas aerogenosa.*The methanol extract was analysed for antiplatelet aggregation assay. It was found that highest per cent activity was found in freeze-dried pulp (32.50%), rind (28.33%) and seed (10.13%). The bael plant part extracts shown to be potential antibacterial and anti-platelet aggregation activities.

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INTRODUCTION

Bael has been extensively used for various purposes since ancient times in India. Recently there has been a surge in the literature with regard to potential bioactivities for various parts of the plant. A recent study examined the antidiabetic potential of Bael bark in a diabetic rat model. Treatment with *Aegle marmelos* significantly increased insulin level, resulted in the regenerative effect on the β-cells and also increased insulin-immunoreactive β-cells of diabetic rats Gandhi *et al.* (2012). A study evaluated the immunomodulatory potential of methanol extract of *Aegle marmelos* in an experimental animal model. The methanol extract of *Aegle marmelos*showed immunomodulatory potential by stimulating cellular and humoral immune mechanisms. Low dose of methanol extract of *Aegle marmelos* was more effective for augmenting cellular immunity, whereas, high dose was more inclined towards humoral immunity Govinda and Asdaq (2011). Leaves of *Aegle marmelos* were tested for its β-amylase inhibitory activity to establish antidiabetic potential.The plant extracts of aqueous, 50 per cent methanol and 100 per cent methanol were subjected to an *in vitro* amylase inhibitory assay using starch as a substrate and pancreatic amylase as the enzyme.The results show that *Aegle marmelos*has shown inhibitory activity and, therefore, might be effective in lowering postprandial hyperglycemia Saha and Verma (2012).

A study has been investigated to study the effect of *Aegle marmelos* leaf extract on early stage DCM in alloxan-induced diabetic rats.Aegle marmelos extract (AME) was found to decrease the levels of FBG, total cholesterol, TBARS, LDH and CK, and increase the levels of GSH, CAT and SOD dose dependently as compared to diabetic control groups.The investigations revelaed that treatment with the extract attenuates the severity and improves the myocardium in the early stages of alloxan-induced DCM at a dose of 200 mg kg

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Bhati *et al.* (2011).

It was studied to evaluate and compare the anti-inflammatory activity of the aqueous root bark extract of *Aegle marmelos* in experimental acute and chronic inflammatory animal models. The percentage inhibition with indomethacin and Bilwa in carrageenan induced paw edema were 52.7 per cent and 46 per cent and in cotton pellet induced granuloma were 24.7 per cent and 9.2 per cent, respectively. Indomethacin showed highly significant anti-inflammatory activity in both the models. However, Bilwa showed highly significant activity in acute model and but a trend of anti-inflammatory activity in chronic model studied Benni *et al.* (2011). A study reported the *in vitro* antimicrobial activity of serial petroleum ether, chloroform and methanol extracts from leaves of *Aegle marmelos* against bacterial and fungal species. It was observed that all the extracts exhibited broad spectrum antimicrobial activity with zones of inhibition against bacteria: *Staphylococcus aureus*, beta *Streptococcus haemolyticus* group A, *Proteus mimrabilis*, *Klebsiella pneumoniae*, *Pseudomonas aenrginosa*, *Escherichia coli, Salmonella typhi*, fungi: *Candida albicans, Candida tropicalis* and *Aspergillusflavus* Kothari *et al.* (2011). However, the study did not use various parts of bael for assessing their antimicrobial activities. Also, there are no studies in the literature indicating the beneficial cardiovascular activities of bael. Current study also attempts to know the antiplatelet aggregation activities of bael plant parts.

METHODOLOGY

Statistical analysis:

The experiments was carried out in triplicates and significant differences ($P < 0.05$) were determined by Duncan's Multiple Range Test (DMRT). Duncan. (1955).

Chemicals:

All the organic solvents used for extraction (Hexane, Chloroform, Ethyl acetate, Acetone and Methanol) were of AR grade from E.Merk (Mumbai, India). Nutrient Agar and Nutrient Broth were purchased from HiMedia lab, Pvt. Ltd., Bombay, India. Collagen was purchased from Sigma chemical Co. (St. Louis, MI, USA). Trisodium citrate and other chemicals were of extra pure analytical grade were purchased locally.

Preparation of Bael powder:

Freshly harvested matured and ripened fruits were obtained from the orchard. Fruits were washed and pulp, rind and seeds were separated and they are subjected to hot air oven drying at 40-48^{0}C and also freeze-drying at – 28^{0}C. The dried fruit parts was powdered by apex grinder of 60-120 mesh size.

Solvent extraction of powder:

Dry powder of plant material was considered for extraction to avoid interference of water. For isolating bioactive molecules from a plant material, the samples were extracted with a variety of solvents, sequentially starting from low polarity to high polarity. Most preferred solvent sequence used for extracting the plant material with unknown composition is as follows, which is based on the polarity of solvents. Hexane < Chloroform < Ethyl acetate < Acetone < Methanol. The extract was subjected to distillation by flash evaporation and it is lyophilized to get concentrated powder.

Antibacterial assay:

*Bacterial Strains and culture Conditions:*Clinical isolates *viz.*, *Bacillus subtilis*, *Enterobacter aerogens*, *Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeroginosa*, *Salmonellatyphi,Staphylococcus aureus* were used from the stock cultures. The bacterial stock cultures were maintained on nutrient agar slants at 37$\pm$1^{0}C.

In-vitro screening of antibacterial activity by agar well diffusion assay: Antibacterial activities of Bael extracts with different solvents were assayed separately using agar well diffusion method modified from Perez *et al. (*1990). About 10^6 cfu/ml bacteria were seeded in to the molten nutrient agar (42-43^{0}C) and plated immediately into sterilized petriplates, allowed to set for 30 min. About 7mm diameter cuplets were bored in the plate at equidistance using a sterile cork borer. To each cuplet 100 µl of fest sample (2mg / ml in Dimethyl sulfoxide) (DMSO) was added. Absolute DMSO was used as control. After 3 hours of chilling treatment at 4^{0}C in upright position, petriplates were inverted and transferred to bacterial incubator maintained at 37$^0\pm$ 1^{0}C and incubated for 24 hours. After 24 hours of incubation, plates were examined for inhibitory zones formed around the well, which were measured and expressed as zone inhibition in mm. Three replicates were maintained for each treatment.

Platelet aggregation inhibitory activity:

Platelet preparation:

Blood samples were taken from healthy volunteers who assured not to have taken any drugs during last 2 weeks prior to the blood sampling. Blood was collected in to buffered sodium citrate (3.8% w/v) of pH. 6.5 as the anticoagulant at a ratio of 9:1v/v and used within 3 hours of collection. Platelet Rich Plasma (PRP) was obtained by centrifugation of the citrated blood at 1100 rpm for 20 min. The residual blood was again centrifuged at 2500 rpm for 20 min to obtain the homologous Platelet Poor Plasma (PPP)(8).

Platelet aggregation:

Aggregation was measured turbidometrically at 37^{0}C with constant stirring at 1000 rpm in a Chronolog Dual Channel Aggregometer. About 0.45 ml of PRP was kept stirred at 1200 rpm at 37^{0}C, and aggregation was induced by collagen (10

Table A. Antibacterial activity of different solvent extracts of oven dried and freeze dried bael fruit parts by agar well diffusion method

Bacteria	Hexane			Chloroform			Ethyl acetate			Acetone			Methanol		
	Pulp	Rind	Seed	Pulp	Rind	Seed	Pulp	Rind	Seed	Pulp	Rind	Seed	Pulp	Rind	Seed
Bacillus subtilis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Enterobacter aerogenes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Klebsiella pneumoniae	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Pseudomonas aeroginosa	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella typhii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+

Sample concentration: 2mg/ml "+" = 4mm Inhibition zone was observed "-" = No inhibition zone was observed

µM). The change in turbidity was recorded with reference to PPP using an Omniscribe recorder for at least 2-3 min. The slope was calculated for collagen (agonist) and it is used as control.

Similarly 15-50 µl of the methanol extracts of bael fruit pulp, rind and seed were added separately to PRP and incubated for 1min. after which agonist *i.e.*, collagen was added. Platelet aggregation was recorded using an Omniscribe recorder for 2-3 min. The slope was calculated. The difference in the slope between the control and the treated was expressed as per cent inhibition of platelet aggregation by bael fruit extract of different parts. Three replicates were maintained for each treatment.

Antibacterial Assay (Agar well diffusion assay):

Methanol extract of pulp, rind and seed has shown antibacterial activity against *Klebsiella pneumoniae*, *Staphylococcus aureus*. Hexane extract of rind and seed has also showed antibacterial activity against *Pseudomonasaerogenosa*. The inhibitory zone in both methanol extract and also hexane extract was around 4mm diameter (Table A). The antibacterial activity of methanol extract may be due to the presence of phenolics in it Gerrard (1982).

Table B. Antiplatelet aggregation activity of methanol extract of Bael fruit parts

Sample preparation	Activity (%)
Oven dried	
Pulp	32.41c
Rind	28.27b
Seed	9.16a
Freeze dried	
Pulp	32.50c
Rind	28.33b
Seed	10.13a

Sample concentration: 2mg/ml

Each value represents mean of three different observations. Mean values in rows with different subscripts (a,b,c…) differ significantly at $P< 0.05$.

Anti platelet aggregation activity of methanol extract:

As the methanol extract has shown higher antioxidant activity compared to other solvent extracts. Methanol extracts were subjected to antiplatelet aggregation assay. The highest per cent of inhibition was observed in freeze-dried pulp (32.50%), rind (28.33%) and seed (10.13%) when compared to oven dried pulp (32.41%), rind (28.27%) and seed (9.16%) (Table B). However, there was no significant difference in antiplatelet aggregation activity between the freeze-dried and oven dried extracts.

OBSERVATIONS AND ASSESSMENT

Bael is a perennial fruit crop of Rutaceae family. It has been widely used as a fruit crop since time immemorial. Its medicinal uses in traditional system of medicine are well documented in the ancient literature. Almost all the parts like pulp, root, bark and leaves have shown Nutraceutical properties, which includes hypoglycemic, regeneration of damaged pancreas, fertility control, anticancer, antioxidant etc. Thus bael is emerging as a food / fruit with myriad applications and biological activities, besides it's traditional functions as fruit and also medicinal uses. In the present study the bioactivity of the different solvent extracts of bael fruit pulp, rind and seeds were examined.

The ripened fruits were freshly harvested and they are manually separated in to pulp, rind, and seed. The pulp was taken for the estimation of the physico-chemical properties and it was found that pulp is a rich source of ascorbic acid (66.65mg/ 100g) and phenols (1.76g/100 g). The different parts of bael fruits (pulp rind and seed) after manual separation they are subjected to drying by hot air oven (at 48-50^{0}C) and freeze drying (at–20^{0}C). The dried parts are subjected to powdering by the apex grinder of mesh size 60. After powdering the freeze-dried and oven dried pulp, rind and seed were examined for the percent powder yield, which showed high yield in freeze dried pulp, rind and seed compared to oven dried.

The freeze-dried and oven dried pulp, rind and seed were subjected to sequential solvent extractions separately by Hexane < Chloroform < Ethyl acetate < Acetone < Methanol based on the polarity *i.e.*, from non polar solvent to polar solvent. The obtained extracts from each solvent was subjected to concentration by flash evaporation followed by lyophillization to obtain crude extract.

All the five extracts of solvents were analysed for the antibacterial activity against seven bacteria. It was found that methanol extract of pulp, rind and seed has shown the inhibitory zone (antibacterial activity) of 4mm against *Klebsiella pneumoniae* and *Staphylococcus aureus* and the hexane extract of rind and seed has also shown inhibitory zone for *Pseudomonas aerogenosa.*

As methanol extract has shown highest antioxidant activity, it was analysed for antiplatelet aggregation assay. It was found that highest percent activity was found in freeze-dried pulp (32.50%), rind (28.33%) and seed (10.13%) with respect to their oven dried counterpart. Assay of different parts of bael fruit indicated highest activity in pulp, a edible portion of the fruit, compared to rind and seed. The bioactivity of sequential solvent extraction of bael fruit indicated that, polar solvents like methanol, acetone and ethyl acetate, has shown higher bioactivity compared to chloroform and hexane.

Conclusions:

From the study studies it is clear that various parts of bael are very potent against several microorganisms and shown to potentially inhibit platelet aggregation. Hence, bael can be considered for further exploration for its nutraceutical properties.

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