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STUDIES ON ISOLATION AND ANTIOXIDANT PROPERTIES OF BIOACTIVE PHYTOCHEMICALS FROM MANGO PEEL HARVESTED AT DIFFERENT DEVELOPMENTAL STAGES.

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ABSTRACT

The food and agricultural industries produce a large volume of wastes annually worldwide, causing a serious disposal problem. This is especially problematic in countries where the economy is largely based on agriculture and where the farming practice is very intensive. Mango is one of the most important tropical fruit and India ranks first in its world production. During the processing of mango, mainly for mango pulp and preparation of amchur powder, peel is a by-product. Currently, these agro-wastes are either allowed to decay naturally on the field subjected burnt. Hence, utilization of biological wastes is of great concern to the industry due to legislation and environmental reasons and therefore, the industry is forced to find an alternative use for its residual matter. One of the agro-wastes currently causing pollution problems is the peels of the mango (Mangiferaindica L.) fruit. Peel forms about 20% of the whole fruit and at present it is a waste product and its disposal has become a great problem. However, these wastes are rich in bioactive compounds, and other valuable compounds. Therefore, the present study attempts to examine the antioxidant activity and antiplatelet aggregation of mango peel extracts at various developmental stages. Further, the total polyphenol, anthocyanin, condensed tannins and flavonoid contents in acetone extract of peels were determined as well.

Highlights

- To assess the bioactive compounds present in various stages of Raspuri mango peels
- To determine the antioxidative potential and antiplatelet aggregation capacity by different maturity stages of mango peel acetone extracts

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Keywords: Mango Peel, Antiplatelet, Antioxidants, Bioactives and Anthocyanin

Introduction

Fruits and vegetables contain many antioxidant compounds, including phenolic compounds, carotenoids, anthocyanins, and tocopherols. Specially, fruit peels are rich in polyphenolic compounds, flavonoids, ascorbic acid, and many other biologically active components having positive influences on health (Leontowicz *et al.,* 2003, Ajila and Prasada Rao, 2008). Mango (*Mangiferaindica L.*), which belongs to the family Anacardiaceae, is one of the most popular tropical fruits, followed by banana, pineapple, papaya, and avocado. The mango plant has been the focus of attention of many researchers towards potent antioxidants. Parts of the mango, such as stem bark, leaves, and pulp are known for various biomedical applications, including antioxidative and free radical scavenging (Ajila CM *et al.,* 2007), anti-inflammatory (Hemandez *et al.,* 2007), and anticancer (Percival *et al.,* 2006) activities. As mango is a seasonal fruit, approximately 20% of fruits are processed for products, such as puree, nectar, pickles, and canned slices that are popular worldwide.

Peel is a major by-product of such processing and mango peels are not currently used commercially, but are discarded as waste and are becoming a source of pollution (Ling *et al.,* 2009). Peel contributes about 15–20% of the fruit (Beerh, Raghuramaiah, Krishnamurthy, & Giridhar, 1976).Peel has been found to be a good source of phytochemicals, such as polyphenols, carotenoids, vitamin E and vitamin C (Ajila CM *et al.,* 2007) and it exhibited good antioxidant properties. Recently, polyphenol profiles of mango by product including peel have been reported using HPLC-MS analysis (Barreto J *et al.,* 2008). Polyphenol content of peel was reported to be more than that of flesh. Ajila *et al.,* (2007) have shown that raw mango peel contains more polyphenols compared to ripe peels. Though considerable work has been done about antioxidants and polyphenols of mango pulp, very few reports are available about mango peel, especially peels from different maturity stages. Raw mangoes are used for pickle, chutney etc., even before they are fully matured. India is the major producer of mango, and peeled raw mangoes are processed for the preparation of amchur and ripe mangoes are processed for mango pulp and fruit bars.

Therefore, both raw and ripe peels are available in large quantities as a by-product in mango processing industry.Mango peel is a devoid of several worthful compounds such as polyphenols, anthocyanins, tannins and flavonoids. Therefore, the objective of the present study was to assess the bioactive compounds present in various stages of Raspuri mango peels. Further to determine the antioxidative potential and antiplatelet aggregation capacity by different maturity stages of mango peel acetone extracts.

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Material and Methods

Materials

Raspuri mango variety grown in CFTRI campus, Mysore, India was used in this study. This mango variety was harvested at different stages. Stage I mangoes represent fruits harvest after three months of flowering. Subsequently, Stage II mangoes were harvested after 18 days of first harvesting; Stage III mangoes were harvested after 49 days of second harvesting. Peel was removed from the fruit using anacute piercing knife and the underneath pulp removed by gently grating with its unsharpened edge. To obtain the ripe peel, some mangoes were kept to ripen at room temperature and the peel was removed as described earlier. The fresh peels thus obtained were used for analysis.

Gallic acid, catechol, Folin-Ciocalteu reagent, TCA, 2,4 dinitrophenylhydrazine,vanillic acid, ascorbic acid, adenosine diphosphate (ADP) were obtained from Sigma Fine Chemicals, St. Louis, USA. 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Himedia Laboratories Limited, Mumbai, India. All other chemicals and solvents were of analytical grade and obtained from Qualigens Chemicals (Mumbai, India).

Preparation of mango peel acetone extract and powder

Both raw and ripe mango peels were removed from the fruits. Peel was homogenized with acid washed sand in mortar and pestle. The homogenate was made up to 80% acetone by adding chilled acetone and subjected to stirring in a magnetic stirrer for 1 hour, then filtered the homogenate using Whatman filter paper 1 . The residue was washed with 80% chilled acetone, filtered and air dried (acetone powder). The filtrates (80% acetone extract) were combined and kept in 4°C for further studies. The 80% acetone extract was used for the estimation of total phenolic compounds, flavonoids, condensed tannins, DPPH, reducing power assay, anthocyanin and evaluated for antioxidant activity. The extract also subjected to lyophilisation for acetone extract powder (kothakota *et al.*, 2014) for the analysis of antiplatelet aggregation .

Estimation of phenolics

Determination of total phenolics content

The total Phenolic content (TPC) was determined by Folin-Ciocalteu assay (Singleton *et al.*, 1965) using the gallic acid as standard. The mixture of the sample solution (50µl), distilled water

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(3ml), 250 µl of Folin Ciocalteu's reagents solution, and 7% NaCO3 (750 µl) was vortexed and incubated for 8 min at room temperature. Then, a dose of 950 µl of distilled water was added. The mixture could stand for 2 h at room temperature. The absorbance measured at 765 nm.

Determination of total flavonoids

Total flavonoids content was determined using a colorimetric method previously (Heimler *et al.*, 2005). Briefly a dose of 0.25 ml of the acetone extract or (+)-catechin standard solution was mixed with 1.25 ml of distilled water in a test tube, followed by adding 75 µl of a 5% NaNO2 solution. After 6 min, 150µl of a 10% AlCl3.6H2O solution was added and allowed to stand for another 5 min before adding 0.5 ml of 1M NaOH. The mixture was brought to 2.5 ml, with distilled water and mixed well. The absorbance was measured immediately against the blank (the same mixture without the sample) 510 nm using uv-visible-spectrophotometer. The results were calculated and expressed as micrograms of (+)-catechin.

Anthocyanin assay by the pH-differential method

Prepare two dilutions of the sample, one with 0.025M potassium chloride buffer, pH 1.0, and the other with 0.4M sodium acetate buffer, pH 4.5, diluting each by the previously determined dilution factor (1:3 or 1:8). Let these dilutions equilibrate for 15min. Measure the absorbance of each dilution at the 700 nm and 515 nm against a blank with distilled water.

Calculate the absorbance of the diluted sample (A) as follows:

A = (A515 – A700) pH 1.0 – (A515 – A700) pH 4.5

Calculate the monomeric anthocyanin pigment concentration in the original sample using the following formula:

Monomeric anthocyanin pigment (mg/liter) = (A * MW *DF * 1000)/ (A* 1)
Where MW is the molecular weight, DF is the dilution factor, and A is the molar absorptivity.

(If the ε of the major pigment is not available, or if the sample composition is unknown, calculate pigment content as cyanidin-3-glucoside, where MW =449.2 and ε = 26,900)

Condensed tannin assay

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Analysis of condensed tannin content (CTC) was carried out according to the method of Broad Hurst and jones (Broadhurst *et al.,* 1978) and slightly modified in our laboratory. To 50 µl of the suitably diluted sample, 3 ml of a 4% methanol, vanillin solution and 1.5 ml of concentrated hydrochloric acid were added. The mixture stood for 15 min, and the absorption was measured at 500 min against methanol as a blank. The amount of condensed tannin was calculated and expressed as mg catechin equivalents (mg of GAE/g sample) using the calibration curve of (+) Catechin.

Ant oxidative assays

DPPH assay measurement of free radical scavenging activity

The effect of acetone extracts (polyphenolic) of mango peel and synthetic standard, BHA on DPPH radical was determined according to the method described by Blois (1958) with modification described by Brand–Williams et al (Cuvilier *et al.,* 1995). A 100 mM solution of DPPH in methanol was prepared and mango peel extract (200 ml) containing 1 to 5 mg GAE was mixed with 1 ml of DPPH solution. The mixture was shaken vigorously and left in the dark at room temperature for 20 min. The absorbance of the resulting solution was measured at 517 nm. The control contained all the reagents except peel extract/BHA. The capacity to scavenge DPPH radical was calculated by following equation.

Scavenging activity (%) = 1- (As/ A0) X100

Where A0 is the absorbance at 517 nm of the control and as is the absorbance in the presence of peel extract or BHA. The results were plotted as the % of scavenging activity against concentration of the sample. The half-inhibition concentration (IC50) was defined as the amount of GAE required for 50% of free radical scavenging activity. The IC50 value was calculated from the plots as the antioxidant concentration required for providing 50% free radical scavenging activity.

Reducing power assay

The reducing power of the mango peel phenolic extract and synthetic standard, BHA was determined according to the method of (Yen *et al.,* 1995). The mango peel extract containing 5 to 20 mg of gallic acid equivalent (GAE) was made up to 500 ml with 0.2 M phosphate buffer (pH 6.6) and mixed with 1 ml of potassium ferricyanide (0.1%) and the mixture was incubated at 50^0C for 20 min. Trichloroacetic acid (500 ml, 10%) was added to the reaction mixture and centrifuged at 3,000xg for 10 min. The supernatant obtained was mixed with equal volume of distilled water and 300 ml of 1% ferric chloride was added and the absorbance was measured at

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700 nm. Increased absorbance of the reaction mixture indicated the increased reducing power. The antioxidant activity of the extract was compared with BHA.

Isolation of rat platelets

Wistar rats (250-300 g) were anesthetized and blood was drawn by cardiac puncture and collected in plastic tubes containing sodium heparin (1000 units/mL) as anticoagulant at a ratio of 9:1 v/v. Platelet rich plasma (PRP) was obtained by centrifuging at 89g for 10 min at room temp. The platelet poor plasma (PPP) was separated by centrifuging the remaining residual blood at 559 g for 25 min.

Platelet aggregation

Aggregation of PRP was performed at 37°C using a computerized dual channel Chrono Log Aggregometer (Chrono-Log Corporation, Havertown, PA) as previously described (Anikisetty M *et.al.,* 2015). Typically, 0.45 ml platelet suspension (adjusted to1.5×108 platelets) was pre-incubated with antagonist along Mango peel (DMSO and Water) extract (100, 200, 400 and 800 µg/ml) for 5 min at 37ºC. Platelet aggregation was induced by adding agonist such as 40.0 µg/ml of ADP. Platelet aggregation was measured as the increase in light transmission for 5 min and data were expressed as percent inhibition of aggregation.

Statistical analysis

All measured quality attributes of mango peel samples were determined in three replications by using analysis of variance (ANOVA) and a Duncan's multiple range test in the statistical analysis

Results and Discussion

Estimation of total polyphenol, flavonoids, anthocyanins and condensed tannins

Raspuri mango fruits were harvested at different stages of maturity. Peels from these fruits were removed and various physical parameters were determined as shown in Table 1. The peel content was found to be around 10% of the fruit weight. However, there was slight less value in matured mangoes (stage II compared to stage I mangoes as well as and ripened mangoes. However, the dry powder content increased with development of fruit.

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As acetone extracts were reported to have significant levels of polyphenols (Ajila *et al.,* 2007), mango peels of Raspuri variety mango fruits were extracted with 80% (v/v) acetone separately and the total phenolic content in the extracts were determined. Polyphenol contents in acetone extracts of various stages in the dried mango peels varied from 35 to 80mg GAE/g peel (Table 1). The polyphenol content was found to be significantly higher in early stages. Earlier (Ajila *et al.,* 2007) reported that raw peel contains more extractable polyphenols compared to ripe fruit peel. The content of total polyphenol was higher in the peel than the pulp at any stage of mango fruit development (Subramanyam *et al.,* 1970).The acetone extracts were used for further studies such tannins, anthocyanins and antioxidant properties.

Flavonoid content in the extracts was determined. Flavonoid contents in acetone extracts of various stages of mango peels was found to be significantly higher in initial stages and decreased in the later stages of fruit development (Table 2).Earlier, presence of the flavonoids like Rutin, quercetin, isoquercetin (quercetin glucoside) were reported (Ajila CM and Prasadr Rao, 2013)

Anthocyanins are a group of phenolic compounds in the plant kingdom and they exhibit good antioxidant properties. The anthocyanin content in the mango peel extract ranged from 7 to 19 mg/g and was more in early developmental stage. Anthocyanin content was found to be significantly higher in 1st and 3rd stages when compared to 2nd stage of matured fruit **(**Table 2).

Tannins are polyphenols, which bind with protein, basic compounds such as alkaloids or heavy metallic ions in a solution and making them insoluble and inducing precipitation (kothakota *et al*., 2015 and 2016). Condensed tannins are polymers of polyhydroxyflavan-3-ol monomers. In the present study, condensed tannins in mango peels observed and ranged from 2.53 to 6.9 mg/g peel. The tannin content was found to be more in ripe fruit compared to that of matured fruit (Table 2).

Determination of antioxidant properties of mango peel acetone extract at various developmental stages

Fruits and vegetables contain phenolic compounds, carotenoids, anthocyanins and tocopherols and these compounds exhibit antioxidant properties. Many studies have shown that free radicals in the living organisms cause oxidative damage to different molecules such as lipids, proteins, nucleic acids and these are involved in the interaction phases of many degenerative diseases. Antioxidants are substances that delay or prevent the oxidation of cellular oxidable substrates. They exert their effect byscavenging reactive oxygen species (ROS) and reactive nitrogen

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species (RNS) or preventing the generation of ROS/RNS (Halliwell *et al.,* 1992). So, it is imperative to assess the antioxidative properties of mango peel obtained from various developmental stages.

Reducing power of mango peel extract

The reducing powers of a compound are related to its electron transfer ability and therefore, serve as a significant indicator of its antioxidant activity. Figure.1 shows the reducing power of the Raspuri mango peel extracts of various stages of mango. The reducing power increases with the concentration of peel extracts. The reducing power of initial stages was more compared to final stages of fruit development. Phenolics, carotenoids and anthocyanins present in the peel are good electron donors and could reduce Fe^{3+}ferricyanide complex to ferrous form, which indicates the antioxidant activity (Chen *et al.,* 1995).

Free radical scavenging activity using DPPH

Scavenging the stable DPPH radical model is another widely-used method to evaluate antioxidant activity. DPPH is a stable free radical with characteristic absorbance at 517nm and antioxidant reacts with DPPH and converts it to 2, 2- diphenyl-1-picrylhydrazine. The degree of discoloration indicates the antioxidant extract, which is due to the hydrogen donating ability (Vengadow *et al.,* 1997). The IC50 values ranged from 2.4 to 6.1 µg of GAE (Table 2). The mango peel extracts showed a concentration dependent scavenging of DPPH radical (Figure 2), which may attribute to its hydrogen donation ability. Initial stages of Raspuri mango peel extracts showed low IC50 values (2.4µg and 2.1µg of GAE) compared to that of final stages of peel (ripe) extracts (6.1µg of GAE).

Antiplatelet Aggregation

Rat platelet aggregation was induced by using ADP (40µg/ml) as agonist and its inhibition was studied by using mango peel extracts at various stages of mango maturity. Inhibition of platelet aggregation by water and DMSO extracts of mango peel at various stages of maturity is shown in (Figure 3). Inhibition of platelet aggregation induced was lower by mango peel water extract compared to that of DMSO. The percentage of inhibition by ripened mango peel DMSO extract at concentration of 800 µg/ml is 70% showed highest among all other extracts. From the figure 3. it can be found that the ripened mango peels (Stage 3) have given better inhibition of platelet aggregation. The inhibition of platelet aggregation can be linked with the antioxidant phytochemicals in ripened mango peel extract which subsequently helps in preventing various

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cardiovascular diseases such as arthrosclerosis, coronary syndrome and stroke. (Zhang *et al.* 2015)

Conclusion

The present study showed that mango peel extract extracted from the three different developmental stages (Stage I, II and III) were rich in number of valuable phytoceuticls and nutraceutical components such as polyphenols, flavonoids and antioxidants. The acetone extract of mango peel showed excellent antioxidant properties and antiplatelet aggregation with good inhibitionat different stages of maturity. The results indicated that the mango peel extracts exhibited good antioxidative potentials especially at the initial levels of fruit development and the ripe fruit peel extract showed better platelet aggregation inhibitory properties. The difference in the antioxidant activity can be attributed to the synergetic effect of various bioactive components present in the mango peels at various stages of development. Therefore, there is possibility for isolation of bioactive components and their utilization as an ingredient in various healthy food formulations and products. In addition, the utilization of mango peel can reduce the waste in mango processing industries while cutting off the cost of main product.

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Table 1 Physical parameters of mangoes harvested at different stages of maturity

Harvesting stage	Average mango weight (g)	Average weight of mango peel (g)	Average weight of dry powder (g)
1	85±14.14[a]	9±2.32[a]	0.73±0.34[a]
2	190±4.21[b]	14.5±3.23[b]	0.95±0.12[b]
3	228±46.66[c]	24±5.34[c]	1.6±0.45[c]

All data are the mean ± SD of ten replicates. Mean value followed by different letters in the same column differs significantly (P≤0.05).

Table 2 Bioactive components and reducing power of mango peel extract from different stages of maturity.

Mango peel	Polyphenol content (mg/g)	Flavonoid content(mg/g)	Anthocyanin content (mg/g)	Condensed tannins content(mg/g)	IC50(µg of GAE)
Stage I	79.8±0.86[c]	1.8±0.12[a]	7±0.41[a]	2.5±0.12[a]	4.1±0.08[c]
Stage II	34.5±0.22[a]	3.8±0.11[b]	12±0.43[b]	5.6±0.32[b]	3.4±0.02[b]
Stage III	48.6±1.13[b]	9±0.36[c]	19±0.21[c]	6.9±0.03[c]	3.1±0.04[a]

All data are the mean ± SD of three replicates. Mean followed by different letters in the same column differs significantly (P≤0.05)

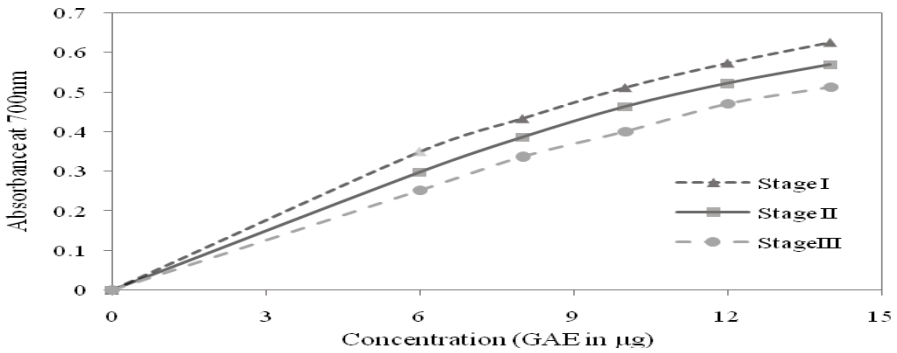

Figure 1 Reducing power of mango peel extractat various stages of maturity

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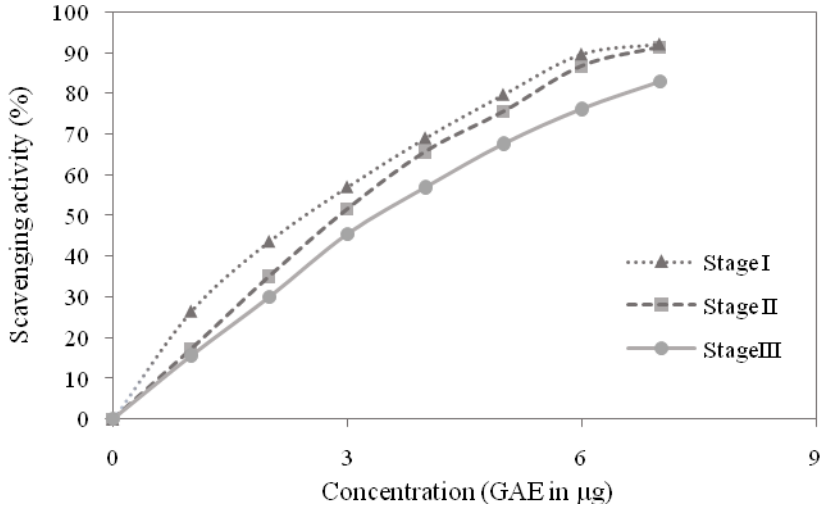

Figure 2 DPPH radical scavenging activity of mangopeel extractat various stages of maturity

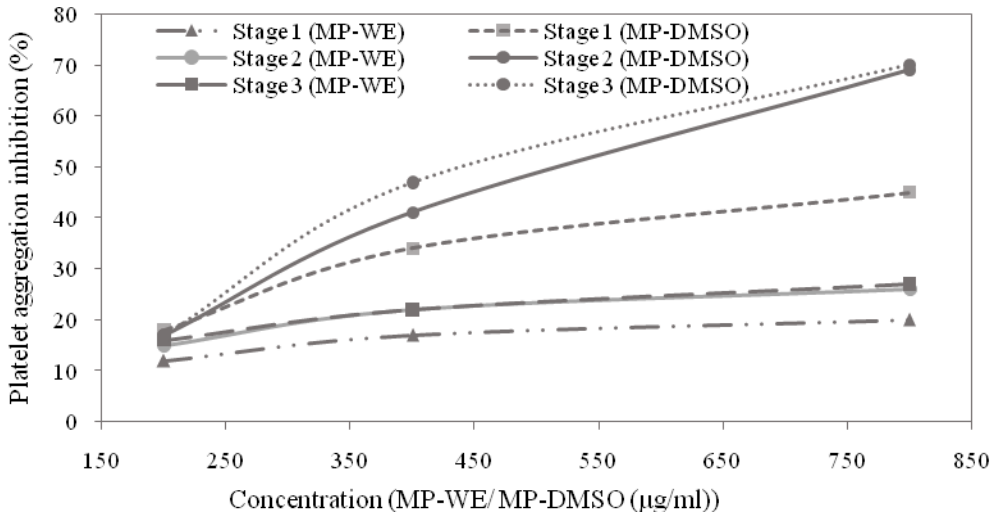

Figure 3 Inhibition of platelet aggregation by water and DMSO extracts of mango peel extract at various stages of maturity