

VARIATION IN PHENOLICS, FLAVONOIDS AT DIFFERENT STAGES OF FRUIT DEVELOPMENT OF *POUTERIA CAMPECHIANA* (KUNTH) BAEHNI AND ITS ANTIOXIDANT ACTIVITY

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ABSTRACT

Objective: The work was aimed to study the changes in the level of phenols and flavonoids during fruit development and its antioxidant potential. The number of experiments conducted was twenty for each case. Polyphenols at different ontological stages may help producers and food technologists to identify which cultivar and/or maturity stage are most adequate for their need.

Methods: Egg fruits were harvested and classified into six developmental stages based on week after pollination (WAP): stage I (4WAP); stage II (8 WAP); stage III (12WAP); stage IV (16WAP); stage V (20 WAP) and stage VI (24 WAP). The total phenolics and flavonoids of egg fruits at different developmental stages were investigated. The antioxidant capacities of ethanolic and aqueous extracts were determined by different assays such as FRAP, DPPH, ABTS, superoxide anion, hydroxyl radical and H₂O₂ scavenging assays.

Results: The total phenolic contents varied from 30.35 to 2.26 mg chlorogenic acid equivalents/g dry weight (DW), and the total flavonoid contents ranged from 0.683 to 3.37 mg rutin equivalents/g DW. Total phenolics showed an initial increase and subsequently decreased during development. In contrast, flavonoids increased from stage I to VI. Antioxidant assays showed varied patterns of inhibition.

Conclusion: Significant correlations were observed between antioxidant capacities and total phenolic and flavonoid contents.

Keywords: Egg fruits, Phenolic, Flavonoid, Antioxidant, Development

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INTRODUCTION

Studies have indicated that phenolic compounds are a major source of natural antioxidants in foods of plant origin which in turn exhibit a wide spectrum of biochemical activities such as antimicrobial, antimutagenic, anticarcinogenic as well as the ability to modify the gene expression [1]. Numerous epidemiological studies confirm significant relationship between the high dietary intake of flavonoids and the reduction of cardiovascular as well as carcinogenic risks. Various factors (climatic, agronomic, genomic, pre- and post-harvest conditions and processing) may affect the chemical composition of plant foods and may have a significant role in determining the phenolic composition and the bioactivity of these compounds [2]. The ripening stage is another important factor that may influence the compositional quality of fruits and vegetables. Indeed, natural antioxidants warrant further scientific scrutiny, given their activity against free radicals, which contribute to chronic degenerative diseases. Medicinal plants play important roles in preventing various diseases, and have received much attention from many researchers over the last few decades. Studies on the antioxidant contents of fruits and vegetables are increasing because natural antioxidant consumption has been found to be related with decreased risk for cancer and heart diseases. Harvest time is essential to get a high quality fruit with storage potential. Some physiological properties can be affected by cultivar; agronomic conditions and maturity stage. Polyphenolics are present in vegetables and fruits functionally proven as antioxidants, anticarcinogenic, antimicrobial, antiallergic and also have antimutagenic and anti-inflammatory properties [3]. The anthocyanins, flavonoids and some non-flavonoids are also responsible of antioxidant properties [4]. Many of the modern diseases are caused by the oxidative stress which is the result of imbalance between formation and neutralization of reactive free radicals. These free radicals are continuously produced and neutralized in the body so as to maintain the constant redox state. These reactive free radicals are generated either by-products of respiration (in reducing molecular oxygen) or exogenous sources

like stress, UV radiation, rich polyunsaturated fatty acid diet, trace metals in diet or absence of exercise [5]. Reactive free radicals include reactive oxygen species (ROS) like superoxide anion radical (O₂⁻), hydroxyl free radical (OH), peroxy radical (ROO⁻) or reactive nitrogen species (RNS). The ROS or RNS cause oxidative damage of biological macromolecules which plays role in the pathogenesis of diverse degenerative disorders like diabetes, Alzheimer's, Parkinson's, cardiovascular diseases including programmed cell death i.e. aging [6]. All human cells can protect themselves through the antioxidant defense systems. The aim of this study was to investigate the level of potential antioxidant compounds and their *in vitro* antioxidant potentials of the aqueous and ethanolic fruit extracts from *Pouteria campechiana*. To the best of our knowledge, no scientific studies have been conducted so far related to the complete phytochemical profile of this fruit and hence the study was attempted. This is an underutilized fruit and no drugs have been reported.

MATERIALS AND METHODS

Chemicals and reagents

NBT (Merck, India), 2-deoxyribose (Merck, India), tripyridyltriazine (Molbase, India), ABTS (Merck, India), EDTA solution (Merck, India), NADH (SRL, India), DPPH (Ottokemi, India), Ferric chloride (Alliance Global, India), Hydrogen peroxide (Arihant chemicals, India) Folin Ciocalteu's reagent (Merck, India), Aluminium chloride (Merck India) Potassium acetate (Merck India).

Plant material

Egg fruits at different stages (4WAP [early], 8WAP [young], 12WAP [green mature], 16WAP [green unripened], 20WAP [yellow unripened] and 24WAP [fully ripened]) were freshly collected from Palode region (8 ° 30' 0" N, 76 ° 55' 12" E) for estimating total phenol content and flavonoids. Fruits of fully ripened stage were also collected for analyzing the antioxidant capacity of the fruits. Voucher specimen (UCBD 13701) was deposited in the herbarium of the department of Botany, University College, Thiruvananthapuram.

Fully ripened fruits were thoroughly washed and wiped with clean tissue paper to avoid dust on the fruit surface. Then the fruits were cut into pieces and washed with water for further analysis.

Isolation and assay of phenols and antioxidants

Isolation of polyphenols was done with 70 % methanol with slight modifications using Folin-Ciocalteu reagent [7]. Absorbance was noted at 650 nm. Total flavonoids were determined by standard methodology using potassium acetate and aluminium chloride solution as reagents [8]. The absorbance was noted at 510 nm.

Antioxidant assays

Superoxide radical scavenging capacity of aqueous as well as ethanolic extract of fully ripened fruit was assessed [9]. Reaction mixture contained 1 ml of NBT, 1 ml NADH, 0.1 ml ascorbic acid and fruit samples at different concentrations. Hydroxyl ion scavenging assay was carried out in both extracts using 2-deoxyribose, ferrous sulphate, and H₂O₂ solution [10]. DPPH scavenging assay in aqueous and ethanolic extracts were also done using 3.9 ml 2, 2-diphenyl 1-picrylhydrazyl in sample at different dilutions [11].

FRAP assay of the fruit was also conducted and the reagent contained 0.1M phosphate buffer at pH 3.6, 10 mmol tripyridyltriazine solution, in 40 mmol HCl, and 20 mmol Ferric chloride [12]. Hydrogen peroxide scavenging assay of fully ripened

fruit in water and ethanolic extracts were assessed using H₂O₂ as reagent [13]. ABTS scavenging assay of fruits in both extract were measured as described by Re et al. [14]. The ABTS radical cation solution was pre generated by adding 7 mmol ABTS and 2.45 mmol potassium persulphate and incubated for 16 h in the darkness at room temperature.

The metal chelating activity of the two extracts was determined and reagent contains ferric chloride, ferrozine and sodium EDTA solution [15].

Statistical analysis

Experiments were carried out in 20 replicates. Data were presented as mean±standard deviation. Two way analysis of variance (ANOVA) was performed using The Origin 8.5 (Origin Lab Corporation, Northampton, USA) software to analyze the statistical significance. The $p < 0.05$ considered as significant.

RESULTS AND DISCUSSION

Total phenolics and flavonoids

The freshly-harvested egg fruits were classified into six groups according to their botanical characteristics: I (4WAP); stage II (8 WAP); stage III (12WAP); stage IV (16WAP); stage V (20 WAP) and stage VI (24 WAP) in table (1).

Table 1: Physical parameters for the identification of different stages of egg fruit

parameters	Stage 1 (4 WAP)	Stage 2 (8WAP)	Stage 3 (12 WAP)	Stage 4 (16 WAP)	Stage 5 (20 WAP)	Stage 6 (24 WAP)
Peel color	green	green	green	green	Light yellow	Yellowish orange
Pulp color and nature	White, hard, gummy	White, hard, gummy	Cream, hard, gummy	Yellow, hard, gummy	Yellow-orange, hard, gummy	Yellowish orange, soft (edible)
Seed coat nature and color	Not prominent	Membraneous, white	Thick membrane, white	Hard, thick, brown	Hard, thick, black	Hard thick, black
Peel weight (g)	0.23±0.02	0.55±0.09	1.34±0.082	2.50±0.06	3.43±0.32	3.63±0.08
Pulp weight(g)	2.38±0.09	4.85±0.05	11.69±0.03	33.65±1.2	59.73±0.82	64.79±2
Seed weight(g)	0.37±0.07	0.220±0.01	2.64±0.1	5.56±0.07	8.15±0.52	9.13±0.5
Fruit circumference (cm)	5.8±0.04	7.80±0.026	12.05±0.5	17.92±0.04	21.05±0.76	23.03±0.45

mean±SD n=20 $p < 0.05$

Total phenols and flavonoids

The total phenolic and flavonoid contents of egg fruits at different developmental stages were measured by using a Folin-Ciocalteu assay and NaNO₂-Al (NO₃)₃-NaOH test system.

As shown in the table (2), the total phenolic contents of egg fruits increased during development i.e., stage I to III and then declined i.e., at stage VI, total phenols was 2.26 mg/g. Meanwhile, the flavonoids increased from stage (I) to stage (VI) from 0.683 and 3.37 mg/g fresh weight (FW) in table 2.

Many researchers have observed the decrease of total phenolic and/or flavonoid contents of other plant tissues during development. For example, variations in metabolites throughout plant development in *Artemisia annua* was reported [16]. Chemical changes and antioxidant activity in pomegranate arils during fruit development was also noticed [17]. Changes of antioxidative activity

during florescence and flower senescence of Peonies petal were correlated [18]. Variations of polyphenols during fruit development in apples were also analyzed [19].

This may be due to the rapid growth of plant tissues in the early period, leading to a dilution effect of polyphenolic compounds. In the late period, the plant tissue grows slowly, while the oxidation and transformation of phenolic compounds may be accelerated due to the senescence of plant tissue.

A negative correlation between polyphenol content and peach blossoms during development was noted, which suggested that the increase in weight during the early periods may be one of the main reasons for the decline of phenolic content during fruit development [20]. Similarly, decline of some active components reported in the leaves of pigeon pea during vegetative growth has been attributed to the rapid growth of plant during that period [21].

Table 2: Estimation of total phenol content and flavonoids at different developmental stages of egg fruit

Stages	Total phenol (mg/g)	Flavonoid (mg/g)
Stage 1(4WAP)	30.35±0.3	0.683±0.07
Stage 2(8WAP)	41.59±0.4	0.774±0.02
Stage 3(12WAP)	46.59±0.3	0.818±0.02
Stage 4(16WAP)	26.46±0.2	1.26±0.02
Stage 5(20WAP)	17.52±0.3	2.6±0.02
Stage 6 (24WAP)	2.26±0.08	3.37±0.03

mean±SD n=20, $p < 0.05$

Estimation of antioxidant potentiality (AOX)**Superoxide anion radical (O₂⁻) scavenging capacity**

The superoxide anion radical (O₂⁻) scavenging capacities of aqueous and ethanolic fruit extracts from *Pouteria campechiana* were evaluated. As shown in table 3, the fruits of *P. campechiana* exhibited remarkable scavenging capacities towards superoxide

anion radicals in ethanolic extract than aqueous. The superoxide anion radical scavenging capacity showed an ascending pattern with an increase of 71.9% (700 µg/ml) for ethanolic antioxidant capacity and 60% for aqueous extract antioxidant capacity (1000 µg/ml). The difference in value indicated that the content of antioxidant components in different solvent extracts greatly vary depending on their polarity.

Table 3: Superoxide radical scavenging assay of stage VI fruit (24WAP)

Concentration (µg/ml)	% inhibition Ethanol extract (%)	Concentration (µg/ml)	% inhibition Aqueous extract (%)
100	19.2±1.0		
200	25.3±0.82	200	12.5±0.08
300	32±0.71	400	21.3±0.14
400	42.03±0.16	600	28.03±0.1
600	60.1±0.42	800	49.08±0.35
700	71.9±0.31	1000	60.01±0.1

The number of experiment n=20. The data were given in mean±SD.

There were variations in antioxidant potentiality among baobab, kiwi, orange, bilberry, and strawberry i.e., 1.2-386 (depending on the plant part), 0.73, 17.0, 1.95, and 1.72 µmol ascorbate/g respectively [22]. So the present antioxidant potential of egg fruits was more significant and may play an important role in preventing human health from oxidative stress. It has been reported that, the increasing order in the superoxide anion scavenging activity was due to the presence of hydroxyl group of phenolics.

Hydroxyl radicals (OH) scavenging power

Hydroxyl radical (OH) scavenging effects showed a dose-dependent pattern for ethanolic extracts. Moderate inhibition of OH radicals against concentrations was displayed by alcoholic extract i.e., in ethanolic extract the inhibition ranged from 8.9 to 56% with 100 to 1000 µg/ml concentrations while no results were noticed with aqueous extract in table 4.

Table 4: Hydroxyl radical scavenging assay of ethanolic extract of 24 WAP fruits

Concentration (µg/g)	% inhibition Ethanol extract (%)
200	8.9±0.02
400	15.4±0.11
600	20.81±0.1
800	45.8±0.17
1000	56.0±0.1

The number of experiments n=20. The data were given in mean±SD.

Scavenging capacity of DPPH radicals

Significant DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging capacities were noticed with ethanolic and aqueous extracts and the results were superior to synthetic antioxidants such as quercetin and ascorbate (table 5). At concentration of 700 µg/ml, the scavenging percentage was higher by 94.01%, while it was only 64.6% at concentration of 1400 µg/ml. Moreover, the DPPH radical scavenging effects also showed a dose-dependent pattern and increased with

concentrations. As compared with hydroxyl radical assay, the scavenging capacity towards DPPH radicals was higher. The antioxidant capacity of pomegranate flower petal was determined and an IC₅₀ value of 344.35 and 107.78 mg/l for DPPH and hydroxyl radical assay was obtained respectively, indicating that the flower petal extract could scavenge hydroxyl radical more efficiently [23]. This difference may be ascribed to the difference of composition of phytochemicals in the test samples, and thereby the reaction process as well as the mechanism involved may also differ from each other.

Table 5: DPPH scavenging assay using ethanol and water extract of fully ripened fruit

Concentration (µg/ml)	% of inhibition Ethanol extract (%)	Concentration (µg/ml)	% of inhibition Aqueous extract (%)
100	23.3±0.1	200	21.31±0.12
200	44.0±0.17	400	37.63±0.2
300	60.5±0.2	600	52.63±0.16
400	71.5±0.31	800	59.09±0.2
500	80±0.14	1000	64.8±0.1
600	87.0±0.12	1200	64.7±0.1
700	94.01±0.24	1400	64.6±0.26

The number of experiments n=20. The data were given in mean±SD.

FRAP assay

Table 6 showed the FRAP of ethanolic and aqueous extracts of egg fruits. Ethanolic extract significantly affected ($p<0.05$) the

FRAP content at different concentrations, the highest values were recorded in 400 µg/ml (81.5 mmol Fe²⁺/g) and the lowest in 100 µg/ml (41 mmol Fe²⁺/g). It is important to note that the entire antioxidant capacity of the sample was derived by

measuring their capacity to reduce Fe³⁺ to Fe²⁺ using FRAP test. The differences in the results obtained from different tropical fruits may be due to the increase in FRAP ability because of hydroxyls present in their lead molecules (106±28 mg TE/100g FW in papaya [24], 26-1393 mg TE/100 g FW in banana [25], 160-180 mg TE/100 g FW in mango [24]). Analysis of

antioxidant activity in different fractions of tomatoes has been conducted [26]. In mango fruit, the fibre content was associated with antioxidant capacity that points its importance as bakery product ingredient [26]. Analysis of the activity and contents of polyphenolic antioxidants in the whole fruit, flesh and peel of three apple cultivars was performed [27].

Table 6: FRAP assay of ethanolic and water extract from fruit (24WAP)

Concentration (µg/ml)	Ferric reducing ability Ethanolic extract (mmol Fe ²⁺ /g)	Concentration (µg/ml)	Ferric reducing ability Aqueous extract (mmol Fe ²⁺ /g)
100	41±0.1	500	30.1±0.18
150	56±0.11	750	38.9±0.31
200	63±0.21	1000	44.5±0.26
250	71±0.3	1250	49.0±0.42
300	79±0.21	1500	57.3±0.2
350	80±0.2	1750	62.01±0.1
400	81.5±0.7	2000	62.0±0.1.3

The number of experiments n=20. The data were given in mean±SD.

Hydrogen peroxide (H₂O₂) radical scavenging activity

The scavenging effect of different solvent extracts of fruit on hydrogen peroxide (H₂O₂) was concentration-dependent (10-800 µg/ml) as shown in table 7. Ethanolic extract displayed strong H₂O₂ scavenging activity (31.3 to 68 % with 10 to 70 µg/ml)

whereas that of the standard, ascorbic acid exhibited 89% of inhibition at 50 µg/ml. The scavenging activity of aqueous extract was less (24.6 to 65.94 % with 200 to 800 µg/ml) in table (7). EC₅₀ values of the fractions in scavenging hydrogen peroxide were significantly different (*p*<0.05) from the EC₅₀ values obtained for ascorbic acid.

Table 7: Hydrogen peroxide scavenging assay of ethanolic and aqueous extract (24WAP)

Concentration (µg/ml)	% inhibition Ethanolic extract (%)	Concentration (µg/ml)	% inhibition Aqueous extract (%)
10	31.3±0.1	200	24.6±0.39
20	44.7±0.22	300	29±0.65
30	49.0±0.23	400	33.1±0.1
40	56.9±0.17	500	39.1±0.41
50	62.1±0.3	600	53.0±0.33
60	68.01±0.19	700	62.51±0.08
70	68±0.1	800	65.94±0.06

The number of experiments n=20. The data were given in mean±SD.

ABTS radical scavenging activity

Both the extracts scavenged ABTS radical in a concentration-dependent way (100-500 µg/ml alcoholic while in aqueous 100-1000 µg/ml) in table 8. Present results showed that the ABTS radical scavenging ability of samples can be ranked as ethanolic followed by aqueous.

Metal chelating activity

Table 9 shows the dose response curves for metal chelating power of ethanolic and aqueous extract (100-170 µg/ml). Most ROSs were generated as by-products during mitochondrial electron transport and other metabolic reactions.

Table 8: ABTS scavenging assay of ethanolic and water extract from fruit (24WAP)

Concentration (µg/ml)	% inhibition Ethanolic extract (%)	Concentration (µg/ml)	% of inhibition Aqueous extract (%)
100	23.02±0.19	200	21.2±0.11
150	32.55±0.13	300	29.03±0.16
200	45.05±0.1	400	34.7±0.16
250	53.0±0.23	500	41.24±0.1
300	60.39±0.2	600	56.02±0.3
350	69.71±0.25	700	61.01±0.13
400	69.41±0.2	800	61.39±0.2
450	69.3±0.15	900	61.30±0.18
500	69.2±0.19	1000	61.3±3.3

The number of experiments n=20. The data were given in mean±SD.

In addition, ROS were formed as necessary intermediates of metal catalyzed oxidation reactions. The transition metal ion Fe²⁺ possessed the ability to perpetuate the formation of free radicals

by gain or loss of electrons. Therefore, the reduction of the formation of reactive oxygen species can be achieved by the chelation of metal ions with chelating agents. Chelation power assay

was carried out to assess the chelation capacity of the crude extracts which illustrated that both the crude aqueous and ethanolic extracts possessed remarkable chelation power from 100 to 170 µg/ml (73.6 and 68.4 % respectively). Excess of metal ions could lead to various anomalies in the body. The iron (II) chelating activity of plant extracts is of great significance, because it has been proposed that the transition metal ions contribute to the oxidative damage in neurodegenerative disorders like

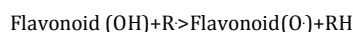
Alzheimer's and Parkinson's diseases. Also, chelation therapy is a common practice of neutralising iron overload in the body especially in cases of treatment of Thalassemia and other anemias. The current scenario suggests that the chelation therapy makes use of synthetic compounds which have certain side effects as well [29, 30]. Therefore, chelation of metal ions by natural phytochemicals from egg fruits can prove to be of therapeutic importance.

Table: 9 Metal chelating activity of ethanolic and aqueous extract from fully ripened fruit

Concentration (µg/ml)	% inhibition Ethanolic extract (%)	Concentration (µg/ml)	% inhibition Aqueous extract (%)
100	23.33±0.17	100	43.02±0.13
110	31.0±0.1	110	51.7±0.1
120	40.11±0.24	120	59.0±0.2
130	52.0±0.29	130	65.11±0.2
140	57.6±0.3	140	70.01±0.31
150	63.2±0.4	150	73.33±0.22
160	68.1±0.001	160	73.30±0.01
170	68.4±0.21	170	73.6±0.24

The number of experiments n=20. The data were given in mean±SD.

Mechanism of flavonoids as antioxidants for protecting the body against reactive oxygen species may be via the additive effect to the endogenous scavenging compounds. Flavonoids can interfere with ≥3 different free radical-producing systems, which are described below, but they can also increase the function of the endogenous antioxidants. Flavonoids can prevent injury caused by free radicals in various ways. One way is the direct scavenging of free radicals. Flavonoids are oxidized by radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilize the reactive oxygen species by reacting with the reactive compound of the radical. Because of the high reactivity of the hydroxyl group of the flavonoids, radicals are made inactive, according to the following equation [31].



Where R· is a free radical and O· is an oxygen free radical. Selected flavonoids can directly scavenge superoxides, whereas other flavonoids can scavenge the highly reactive oxygen-derived radical called peroxynitrite. Epicatechin and rutin are also powerful radical scavengers. The scavenging ability of rutin may be due to its inhibitory activity on the enzyme xanthine oxidase. By scavenging radicals, flavonoids can inhibit LDL oxidation *in vitro*. This action protects the LDL particles and, theoretically, flavonoids may have preventive action against atherosclerosis.

The antioxidant compounds and total antioxidant activity in fruits of acerola Cv. Flor Branca, Florida Sweet and Brs 3661 were assessed [32]. In apples, antioxidant activity at different maturity stages and fruit parts was compared [33]. Correlation of total phenolics, flavonoid content and antioxidant profile of different date varieties were also done [34]. In *Eugenia jambolana*, fruit maturity, phenolic content and antioxidant activity were evaluated [35]. Metal ion chelating activity and hydrogen peroxide scavenging activity of *Kalanchoe pinnata* were analyzed [36]. In tomato fruits, the evolution of antioxidant activity and bioactive compounds during growth and ripening was also reported [37]. The fruit of *Phyllanthus emblica* showed antioxidant property due to certain phytochemical compounds such as tannins, flavonoids, terpenoids and alkaloids [38]. The extract of *Myxopyrum smilacifolium* showed higher antioxidant property than control but less than ascorbic acid [39]. All these support the present antioxidant power of egg fruits.

CONCLUSION

The assessment of the chemical composition and antioxidant activity of egg fruits may allow increased utilization of the fruit as a functional food. In this study, total phenols, flavonoids and antioxidant activity were commendable, compared with other common fruits. Total phenol and flavonoids of egg fruit examined in this study was comparable with that of cranberry, red grape or

strawberry, peach, apple, orange, banana, pear, pineapple, some blueberry cultivars and grapefruit. Correlations between polyphenols and antioxidant activity suggested that phytochemical components of egg fruit have an important effect on antioxidant capacity, as reported for other fruits and vegetables. The bioactivities of the underutilized species points to a new source of natural health promoting compounds.

AUTHORS CONTRIBUTION

Both Sunila and Murugan contributed in the analysis of variation shown by the phytochemical constituents along with antioxidant activity related to the fruits of *Pouteria campechiana* at different stages.

CONFLICT OF INTERESTS

Declared none

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