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RESEARCH ARTICLE

ASSESSMENT OF ANTIOXIDANT ACTIVITY AND POLYPHENOLIC CONTENT OF COUROUPITA GUIANENSIS DURING FLOWER AND FRUIT MATURATION

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ABSTRACT

The present research work was carried out to assess the effect of maturation process on in vitro antioxidant activity of C. guianensis flower and fruit. The antioxidant activities of methanolic extracts of flower and fruit of young and matured stages were investigated spectrophotometrically against DPPH, ABTS, H2O2, NO, superoxide, hydroxyl radical and lipid per oxidation, along with ferric reducing power, metal chelating and β-carotene bleaching assay. Total phenols, flavonoids, ortho-dihydric phenols and anthocyanin were also determined. The methanolic extract obtained from young fruit was found to possess higher level of phenolic content, flavonoids as well as ortho-dihydric phenol content. The young stages were observed with highly potential free radical scavenging activity in comparison to that of matured one. The results demonstrated that the maturation process had profound impact on the antioxidant activity and polyphenol content of C. guianensis flower as well as fruit; also the young stages are suggested as the best harvest stage for medicinal purposes. A positive correlation was observed between polyphenolic contents and the antioxidant activity of the methanolic extracts. Further studies are required for the isolation of active principles responsible for its antioxidant activity.

INTRODUCTION

Free radicals, in the form of reactive oxygen species (ROS) and reactive nitrogen species (RNS), are an integral part of normal physiology (Cavas and Yurdakoc 2005). But over-production of these reactive species by any means brings out oxidative stress (Bramley, 2000). This free radical-mediated oxidative stress may lead to the development of various chronic and degenerative diseases such as cancer, atherosclerosis and also those related to the cardio- and cerebrovascular systems (Halliwell, 2000; Metodiewa and Koska, 2000; Young and Woodside, 2001; Heinecke, 2003). Antioxidant compounds are capable of mitigating the negative effects of oxidative stress as they are efficient scavengers of the free radicals. The trend of using natural products has increased and the active plant extracts are frequently screened for new drug discoveries for the presence of potential antioxidant components (Ramma *et al*., 2002). The fruits and vegetables are considered to be a good source of natural antioxidants and have been found to be beneficial for protection against the diseases related to oxidative stress (Chinnici *et al*., 2004).

Couroupita guianensis Aubl belongs to the family Lecythidaceae, commonly known as Naglingam or Cannon ball tree. It is a large deciduous evergreen tree growing to a height of 20 m. Leaves are alternate, oblong up to 20 cm long, entire to slightly serrate and hairy on the veins beneath. Inflorescence is racemose, arising from the trunk and other large branches. Flowers are reddish with a yellow tinge on the outside, fragrant, with stamens borne on an overarching and rophore. The tree produces globular brown woody, amphisarca (double fleshy) fruits of a considerably big size, each containing 200 to 300 seeds (Lester *et al.*, 1998). It is widely grown in tropical and subtropical region and established in botanical gardens as an ornamental plant (Kumar *et al.*, 2011).

Like other fruits and vegetables, *C. guianensis* is also a rich source of antioxidants and thus can help to prevent degenerative diseases. Different parts of this plant have been traditionally used in the folk medicine for healing various diseases. Umachigi *et al*., (2007) has provided a scientific rationale for the traditional use of *C. guianesis* in the management of skin diseases such as sores, boils and itching. The infusions prepared from the leaves, flowers, and barks of *C. guianensis* are used for the treatment of hypertension, tumours, pain, and inflammatory processes (Sanz- Biset *et al.*, 2009).

Secondary metabolites such as phenols and flavonols exist widely in plants and are considered as important dietary antioxidants, which are responsible for the prevention of consequences of oxidative stress in biological system (Hassimotto *et al.*, 2005; Andarwulan *et al.*, 2010). Similarly, Elumalai *et al*., (2012) have reported the presence of secondary metabolites in the leaves of *Couroupita guianesis* which might be responsible for its potential antioxidant, anti-arthritic and

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anti platelet activities. The fruit rind is found to possess significant antioxidant, antimicrobial and antifungal activities (Regina & Umaranjan, 2012).

It is generally considered that different factors such as seasonal variation, genotype, maturation stages and environmental conditions influence the phytochemical composition as well as their bioactivity of fruits (Cordenunsi *et al.,* 2002). To our knowledge, there is no comprehensive data reported on the variation of antioxidants of *C. guianesis* flower and fruit during different maturation stages. Therefore, the present research work aimed at evaluating and quantifying the phytochemical components and related antioxidant activity of *C. guianesis* flower and fruit assayed at different stages of maturity.

MATERIAL AND METHODS

Plant material

The young and matured fresh flowers and fruits of *C. guianensis* were collected in the month of December 2010 from nearby Gate No. 2 of University of North Bengal. The specimen was identified and authenticated by Dr. A.P. Das, Professor, Taxonomy & Environmental Biology Laboratory, Department of Botany and University of North Bengal. A voucher specimen was deposited at laboratory herbarium, Taxonomy and Environmental Biology Laboratory, University of North Bengal and identified on 10.07.12 against the accession number: 09661 for the further references.

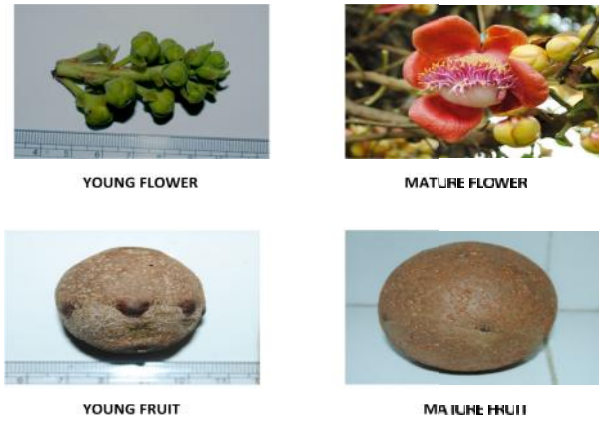

Figure 1 Young and Mature stages of flowers and fruits of *Couroupita guianensis*

Animal material

Goat liver, used for anti-lipid peroxidation assay, was collected from slaughter house immediately after slay and the experiment was conducted within an hour after collection.

Preparation of Extract

The collected flowers and fruits were crushed in a mixer grinder (Philips, HL 3294/C, India) and were packed into soxhlet apparatus (Borosil, 3840024, India) and extracted with methanol for 24 h. The refluxed samples were separated from the residues by filtering through Whatman No. 1 filter paper and the extract was concentrated to a definite concentration of 1g/ml using a vacuum rotary evaporator (Buchi, RC5100, India). The extracts thus obtained were stored in brown bottles and kept in refrigerator for further analysis.

DPPH based free radical scavenging activity

The radical scavenging activity of the extracts was measured by DPPH method (Bondet *et al.,* 1997). The reaction mixture contained 1.8 ml of 0.1mM DPPH and 0.2 ml of methanolic extracts. The reaction mixture was vortexed and kept in the dark at room temperature for 30 min. The absorbance was measured at 517 nm. A reaction mixture without test sample was considered as control.

Radical scavenging activity was expressed as percent inhibition from the given formula:

Percentage inhibition of DPPH radical = $[(A_0 – A_1)/A_0] \times 100$.

Where, A_0: absorbance of the control and A_1: absorbance of the extract. Then percentage inhibitions were plotted against concentration and from the graph, IC_{50} was calculated.

Hydroxyl radical scavenging activity

The $OH^{\cdot}$ radical scavenging activity of extracts was measured according to the standard method (Halliwell and Gutteridge, 1989). 200 µl of 2.8 mM 2-deoxyribose in potassium phosphate buffer (pH 7.4), 200 µl of 10 mM $FeCl_3$, 1 mM EDTA, 20 mM H_2O_2 , 200 µl of 1 mM L-ascorbic acid and 1ml methanolic extract were mixed to form a reaction mixture. The reaction mixture was incubated for 1 h at 37° C, and further heated in a boiling water-bath for 20 min after addition of 1 ml of 2.8% (w/v) trichloroacetic acid and 1 ml of 1% (w/w) 2-thiobarbituric acid. The colour development was measured at 532 nm against a blank containing phosphate buffer.

Superoxide anion radical scavenging activity

The superoxide radical scavenging activity was measured by the method described by Wei *et al.* (2010) with slight modification. All solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed using fluorescent lamps (Philips, FTG 14/111, India). The reaction mixture contained 1 ml of NBT solution (312 µM prepared in phosphate buffer, pH- 7.4), 1 ml of NADH solution (936 µM prepared in phosphate buffer, pH-7.4), and 1ml of methanolic extract of different concentrations. After 5 min incubation, 100 µl of PMS (120 µM prepared in phosphate buffer, pH-7.4) was added to the reaction mixture. The reactant was illuminated at 25°C for 30 min and the absorbance was measured at 560 nm against methanol as control. The inhibition percentage of superoxide anion generation was calculated by using the following formula:

Superoxide radical scavenging effect (%) = [(Abs. of control – Abs. of sample) / Abs. of control] x 100

Metal chelating activity

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method described by Dinis *et al.* (1994) with slight modification. To 0.4 ml of methanol extract, 1.6 ml of methanol was diluted and mixed with 0.04 ml of $FeCl_2$ (2 mM). After 30sec, 0.8 ml ferrozine (5 mM) was added. After 10 min at room temperature, the absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated as

$$\text{Chelating rate (\%)} = (A_0 - A_1) / A_0 \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in presence of the extract.

Nitric oxide scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction (Marcocci *et al.,* 1994). 320

µL methanol extract, 360 µL (5 mM) sodium nitroprusside-PBS solutions, 216 µL Greiss reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride) was mixed and incubated at 25^oC for one hour. Lastly 2 ml water was added and absorbance was taken at 546 nm.

Radical scavenging activity was expressed as percent inhibition from the given formula:

% inhibition = [(Abs. of control – Abs. of sample) / Abs. of control] x 100.

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch *et al.* (1989) with some modifications. At first, a solution of H_2O_2 (43 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). 0.4 ml of methanolic extract was diluted in 3.4 ml phosphate buffer and 0.6 ml of H_2O_2 solution was added to the mixture. After incubation of 10 mins, the absorbance value of the reaction mixture was recorded at 230 nm. Blank solution contained sodium phosphate buffer without H_2O_2.

The H_2O_2 scavenging activity was expressed as percent inhibition from the given formula:

H_2O_2 scavenging (%) = $[(A_0 – A_1)/A_0] \times 100$.

Where, A_0: absorbance of the control and A_1: absorbance of the extract. Then percentage inhibitions were plotted against concentration and from the graph, IC_{50} was calculated.

$ABTS^+$ radical cation(s) decolourization assay

The spectrophotometric analysis of $ABTS^+$ radical cation(s) scavenging activity was determined according to Re *et al.* (1999) method with some modifications. This method is based on the ability of antioxidants to quench the $ABTS^+$ radical cation, a blue/green chromophore with characteristic absorption at 734 nm. The $ABTS^+$ was obtained by reacting 7 mM $ABTS^+$ radical cation(s) in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for 6 hrs. Then, 2 ml of $ABTS^+$ solution was added to 1 ml of the methanolic extract. After 30 min, percentage inhibition at 734 nm was calculated for each concentration, relative to a blank absorbance. Solvent blanks were run in each assay.

The $ABTS^+$ scavenging was calculated using the following formula:

$ABTS^+$ scavenging effect (%) = $[(Ac – As)/Ac] \times 100$

Where A_C is the initial concentration of the $ABTS^+$ radical cation(s) and A_S is absorbance of the remaining concentration of $ABTS^+$ radical cation(s) in presence of the extract.

Ferric reducing antioxidant power (FRAP) Assay

The reducing antioxidant power of the extracts was determined by the standard method (Oyaizu, 1986). Different concentrations of 1 ml of extracts were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50^oC for 20 min. Then, 2.5 ml of trichloroacetic acid (10%) was added to mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank using UV-VIS Spectrophotometer (Systronics, 2201, India). Increased absorbance of the reaction mixture indicated increase in reducing power.

Anti-lipid peroxidation (ALP) assay

The anti-lipid peroxidation activity of the methanolic extracts was determined by the standard method (Bauchet *et al.,* 1998), followed by slight modifications with the goat liver homogenate. 2.8 ml of 10% goat liver homogenate, 0.1 ml of 50 mM $FeSO_4$ and 0.1 ml extract was mixed. The reaction mixture was incubated for 30 minutes at 37^oC. 1 ml of reaction mixture was added with 2 ml 10% TCA-0.67% TBA in acetic acid (50%) for terminating the reaction. Mixture was boiled for 1 h at 100^oC and centrifuged at 10,000 rpm using centrifuge (Remi, R24, India) for 5 min. Supernatant was taken for absorbance at 535 nm.

ALP % was calculated using the following formula:

ALP percent = $[(A_F – A_S)/ (A_F – A_C)] \times 100$

Where, A_F: absorbance of Fe^{2+} induced peroxidation; A_S : absorbance of the extract and A_C : absorbance of the control. Then percentage inhibitions were plotted against concentration and from the graph, IC_{50} was calculated.

β-Carotene–linoleate bleaching (BCB) assay

The antioxidant activity was assayed based on the β-carotene bleaching method (Velioglu *et al.,* 1998). β-carotene (0.2 mg in 1 ml chloroform), linoleic acid (0.02 ml) and Tween 20 (0.2 ml) were transferred into a round bottomed flask. Chloroform was removed at room temperature under vacuum at reduced pressure using a rotary evaporator. After evaporation, 50 ml of distilled water was added to the mixture, and then shaken vigorously to form an emulsion. 2 ml of emulsion was then added to 0.2 ml of methanolic extract or methanol (as control) into test tubes and immediately placed in a water bath at 50 °C. The absorbance was read at 30 min intervals for 2 h at 470 nm. Degradation rate (DR) was calculated according to first order kinetics, using the following equation: (Al-Saikhan *et al.*, 1995)

Ln a/b x 1/t = DR_{sample} or $DR_{standard}$

where Ln is natural log, *a* is the initial absorbance (470 nm) at time 0, *b* is the absorbance (470 nm) at 30, 60, 90 or 120 min and t is the incubation time after which the absorbance was taken.

Antioxidant activity (AA) was expressed as percent of inhibition relative to the control, using the following formula:

AA = $[(DR_{control} - DR_{sample}) / DR_{control}] \times 100$

Where, $DR_{control}$: Degradation rate in absence of extract and DR_{sample}: Degradation rate in presence of extract.

Total phenolic content (TPC)

Total phenolic contents of the extracts were determined according to the standard protocol (Chandler and Dodds, 1993). 1 ml of the various methanolic extracts was mixed in a test tube containing 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of 50% Folin-ciocalteau reagent. The resultant mixture was allowed to react for 5 min and 1 ml of 5% Na_2CO_3 was added. It was mixed thoroughly and placed in dark for 1h. Finally the absorbance of coloured reaction product was measured at 765nm against the reagent blank. The

total phenolic content was expressed as mg of gallic acid equivalent (GAE) per gram fresh weight (fw).

Total flavonoid content (TFC)

The TFC were measured following a standard spectrophotometric method (Sultana *et al.,* 2009). 1 ml of methanolic extracts was diluted with water (4 ml) in a 10 ml volumetric flask. Initially, 5% $NaNO_2$ solution (0.3ml) was added to each volumetric flask; at 5 min, 10% $AlCl_3$ (0.3 ml) was added; and then after 6 min, 1M NaOH (2 ml) was added. Next water (2.4 ml) was added to the reaction flask and mixed well. Absorbance of the reaction mixture was read at 510 nm. The total flavonoid content in different extracts was calculated as quercetin equivalent (QE) per gm fresh weight.

Total ortho-dihydric phenol content (TOPC)

The determination of ortho-dihydric phenol was based on Arnow's reagent method (Kim *et al.,* 2003). Firstly, 0.2 ml of methanolic extracts was diluted up to 1 ml volume. Then the diluted extract was added to a mixture containing 1 ml of 0.05N HCl, 1 ml of Arnow's reagent, 10 ml of water and 2 ml of 1N NaOH. Finally, after 5 min the absorbance was recorded at 515 nm. The total ortho-dihydric phenol content in different extracts was calculated as catechol equivalent (CE) per gm fresh weight.

Total anthocyanin content

Total anthocyanin content was measured using a spectrophotometric method (Fuleki and Francis, 1968a; 1968b), with some modifications. Two buffer of pH 1.0 (potassium chloride) and pH 4.5 (sodium acetate) were prepared. 1ml of each extracts were properly diluted with in test tubes containing 4.0 ml of pH 1.0 and pH 4.5 buffers and they were thoroughly mixed by vortex for 10 sec. After incubating at room temperature for 20 min, the absorbance of each solution was measured at 520nm and 700nm against blanks of pH 1.0 and 4.5 buffers. Total anthocyanin calculation was calculated by

Total anthocyanin (mg/L) = $(A \times MW \times DF \times 1000) / (\varepsilon \times 1)$

Where: A is adjusted absorbance = $(A_{520}\text{-}A_{700})$ buffer 1.0 – $(A_{520}\text{-}A_{700})$ buffer 4.5, 1000 = molar to ppm conversion, DF = dilution factor, MW is molecular weight of cyanidin-3-glucoside = 449.2 and ε is the molar absorptivity of cyanidin-3-glucoside = 26,900.

Statistical analysis

The software package, MS Excel 2007 (Microsoft, Redmond, WA, USA) was used for comparing the antioxidant attributes of the *C. guianensis* fruits and flowers. The different group means were compared by Duncan's Multiple Range Test (DMRT) through DSAASTAT software (version 1.002; DSAASTAT, Peruglia, Italy); $P<0.05$ was considered significant in all cases. The software package Statistica (Statsoft Inc., Tulsa, OK, USA) was used for analysis of other data. Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA and USA) was used for determining the IC_{50} values of antioxidants and their standard error of estimates (SEE). Principal component analysis (PCA) was conducted using SPSS (Version 12.00, SPSS Inc., Chicago, IL, USA) to indentify the patterns between antioxidant activity and phytochemical attributes.

RESULTS AND DISCUSSION

Total phenolic and flavonoid contents

The phenolic and flavonoid contents of fruits are influenced by several factors such as species, variety, cultivation, topography, climatic conditions, and environment etc (Wang and Lin, 2000; Iqbal and Bhanger 2006). Among these the environmental factors are considered to play a major role on polyphenol composition of fruits and vegetables. These factors may include agronomic (biological culture, greenhouses, hydroponic culture *etc*.) or climatic (sun exposure, soil type, rainfall) (Javaria *et al.,* 2012). Besides these, the polyphenol content is also influenced by the extent of fruit maturation process (Kondakova *et al.,* 2009). The total phenol, flavonol and ortho-dihydric phenol contents of both the young and matured fruits are presented in Table 1. The results of total phenolic contents observed in our work is also supported by the findings reported in medlar fruit (Rop *et al.,* 2011) and *Cassia fistula* (Siddhuraju *et al.,* 2002) which showed a gradual decreasing trend in the phenolic contents during fruit maturation process. It can be suggested that the decrease in the phenol contents during maturation process might be associated with an amplified polyphenol oxidase activity (Parr *et al.,* 2000). Such trend was also reported in case of developing floral stages of chrysanthemum (Stickland, 1972).

Total anthocyanin content

The results demonstrated that the anthocyanin pigment was absent in young stage but good amount of anthocyanin pigment was present in the extract obtained from matured flower (0.584 mg/g fresh weight) and fruit (3.924 mg/g fresh weight). Similar findings were reported by Wang and Lin (2000) who demonstrated that total anthocyanin content increased with maturity in blackberry, raspberry and strawberry fruits.

Table 1 Different phytochemical content of methanolic extract of young and mature fruits of *C. guianensis.*

Phytochemical contents	Young Flower	Mature Flower	Young Fruit	Mature Fruit
Total Phenol Content (mg GAE/g FW)	11.47±0. 5b	3.5±0.22c	12.61±0.52a	0.7 ± 0.09d
Total Flavonol Content (mg QE/g FW)	0.87±0.051b	0.53±0.03c	0.97±0.03a	0.06±0.02d
Ortho-dihydric Phenol Content (mg CE/g FW)	0.64±0.04a	0.11±0.01c	0.57±0.025b	0.02±0.008d

Results are represented as mean ± SEM, n=3. Values with different letters (a, b, c & d) are significantly (P<0.05) different from each other by Duncan's multiple range test (DMRT).

Free radical scavenging activity (FRSA)

The evaluation of the antioxidant activity of fruits and vegetables is an important parameter for assessing their nutritional value (Rice- Evans *et al.,* 1996; Pellegrini *et al.,* 1999; Scalfi *et al.,* 2000). The mode of action of antioxidant components, which helps in defence mechanisms in all stages of plant, depends on plant's growth and development (Howard *et al.,* 1994; Deepa *et al.,* 2007). The significant variation in

Table 2 Free radical scavenging activities of methanolic extracts of young and mature flowers and fruits of *C. guianensis*.

Antioxidant activity	Young Flower	Mature Flower	Young Fruit	Mature Fruit
DPPH IC_{50} (mg/ml)	0.021±0.005a	0.28±0.012a	0.022±0.005a	7.67 ± 0.89b
ABTS IC_{50} (mg/ml)	0.164±0.015a	0.55±0.056b	0.141±0.020a	1.58±0.069c
Super Oxide IC_{50} (mg/ml)	2.91±0.5a	6.17±0.8a	2.86±0.90a	37.18±4.20b
Nitric Oxide IC_{50} (mg/ml)	37.15±1.8b	47.02±3.67c	25.04±1.99a	169.03±4.01d
Hydroxyl radical IC_{50} (mg/ml)	30.84±2.89a	56.88±7.79a	49.21±5.99a	224.45±12.39b
Hydrogen peroxide IC_{50} (mg/ml)	2.06±0.32a	33.74±1.44b	32.62±1.44b	60.67±2.11c

Results are represented as mean± SEM, n=3. Values with different letters (a, b, c & d) are significantly (P<0.05) different from each other by Duncan's multiple range test (DMRT).

the free radical scavenging activities among young and matured stages of both flower and fruit of *C. guianensis* indicates that the potential efficacy of antioxidants varies considerably during maturation stages. In this study, we used free radical-generating systems such as DPPH, superoxide, nitric oxide, $ABTS^{+}$, H_2O_2 and hydroxyl to evaluate changes of antioxidant activity. The results of FRSA of methanolic extracts of both the stages of fruits and flower are presented in Table 2, where it was observed that the young stage possesses significantly higher free radical-scavenging activities in comparison to that of matured fruit as well as flower. The present decreasing trend of free radical scavenging activity of *C. guianensis* fruit is in agreement with the investigation performed by (Kulkarni and Aradhya, 2005) who claimed decrease in the antioxidant activity of pomegranate arils during fruit development. Hence it can be suggested that significant decrease in scavenging rate on each free radical mentioned above occurred during maturation process.

Reducing antioxidant power (FRAP) assay

The reducing capacity of a biological compound acts as a significant indicator of its antioxidant potential (Moein *et al.*, 2008). The reducing ability of the extracts was determined with ascorbic acid equivalent (AAE). Higher ascorbic acid equivalent value indicates higher reducing capacity of samples, thus greater antioxidant potential. The result of FRAP assay revealed that the methanolic extract of young fruit and flower had the highest antioxidant activity (Table 2) which too indicated decrease in antioxidant activity towards maturation. Since the polyphenolic compounds are considered as very efficient reducing agents therefore, it can be suggested that such decline in reducing potential might be associated with the trends of the phenolics contents of the fruit extracts as the maturation process progresses.

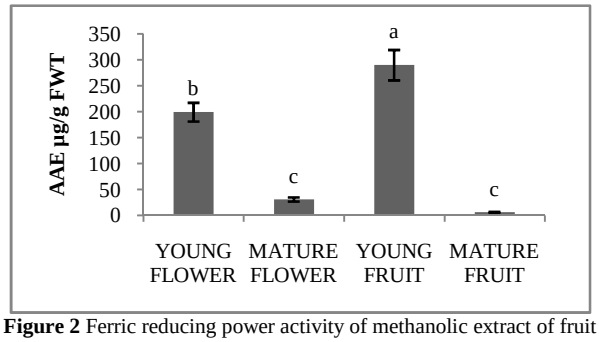

Figure 2 Ferric reducing power activity of methanolic extract of fruit and flower *C. guianensis* at young and mature stage

Results are represented as mean± SEM, n=3. Values with different letters (a, b & c) are significantly (P<0.05) different from each other by Duncan's multiple range test (DMRT).

Metal Chelating Activity

Ferrous ion (Fe^{2+}) is known to be involved in generation of free radicals through Fenton and Haber–Weiss reaction (Halliwell, 1996). The secondary antioxidant compounds are efficient metal chelating agents and also prevent free radical generation (Gordon, 1990). These chelating agents are found to reduce the redox potential by stabilizing the oxidized form of the metal ion through formation of σ-bonds with these metal ions (Kumar *et al.*, 2008). During metal chelation assay, ferrozine, a chelating agent reacts with ferrous chloride (Fe^{2+}) and forms purple coloured complex which gives maximum absorbance at 562 nm. Table 2 shows the chelating activity of the methanolic extracts indicating higher chelating potential of young fruit as well as flower.

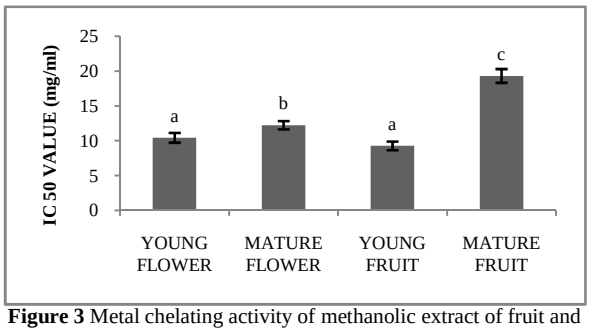

Figure 3 Metal chelating activity of methanolic extract of fruit and flower *C. guianensis* at young and mature stage

Results are represented as mean± SEM, n=3. Values with different letters (a, b & c) are significantly (P<0.05) different from each other by Duncan's multiple range test (DMRT).

β-Carotene linoleate bleaching (BCB) assay

The mode of action of beta-carotene bleaching is a free radical-mediated phenomenon resulting from oxidation of linoleic acid. The linoleic acid free radical, formed upon the release of a hydrogen atom from its diallylic methylene groups, targets the highly unsaturated beta-carotene molecules for degradation. As beta-carotene molecules get oxidized in this model system, the compound loses its chromophore and characteristic orange colour, in the absence of antioxidant compound, which is spectrophotometrically measured (Wang *et al.*, 2009).

From BCB assay it was established that the methanolic extract of young fruit (inhibition percentage – 77.85%) had the highest antioxidant activity (Table 2). It was observed that the antioxidant activity gradually decreased towards maturation.

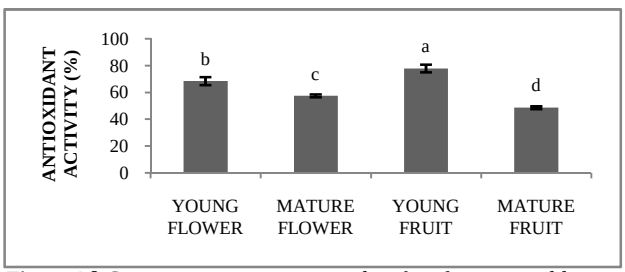

Figure 4 β-Carotene protective activity of methanolic extract of fruit and flower *C. guianensis* at young and mature stage

Results are represented as mean± SEM, n=3. Values with different letters (a, b, c & d) are significantly (P<0.05) different from each other by Duncan's multiple range test (DMRT).

Anti-lipid peroxidation (ALP) assay

Lipid peroxidation is the oxidative degradation of lipids through peroxide formation which induces severe damage to adjacent bio-molecules in cells causing cell death. Thus, the inhibition of such destructive processes is very important for the protection of the living system (Yang *et al.*, 2008). Interestingly, it was observed (Fig. 11) that anti-lipid peroxidation activity was absent in flower but methanolic extracts of both young and matured fruit exhibited almost same anti-lipid peroxidation effect against goat liver, IC_{50} values of 2.77 and 2.70 mg/ml for young and matured fruit respectively.
The overall results indicated that there was decrease in FRSA, accompanied by a significant reduction in phenolics and flavonoid concentration towards maturity. This clearly specifies that the increase in FRSA and subsequent accumulation of phenolics were positively correlated and were inversely related to the fruit maturation process. In agreement with the previous study (Castrejon *et al.*, 2008), our results suggested decrease in antioxidant activity on progress of fruit maturation. This phenomenon was also reported in case of fruit maturation of guava (Javaria *et al.*, 2012).

Principal component analysis

Principal component factor loadings plot was used to determine the importance of antioxidant assays and the contribution of different secondary metabolites in this aspect. The loadings of first (PC1) and second (PC2) principal components accounted for 82.04% and 13.86% (fig 5) of the total data variance, respectively. Interestingly almost all antioxidant attributes (IC_{50} values for scavenging different free-radical species) were clustered together with positive loadings on PC1, whereas the reducing potential and phenolics components exhibited strong negative loadings on the same component.

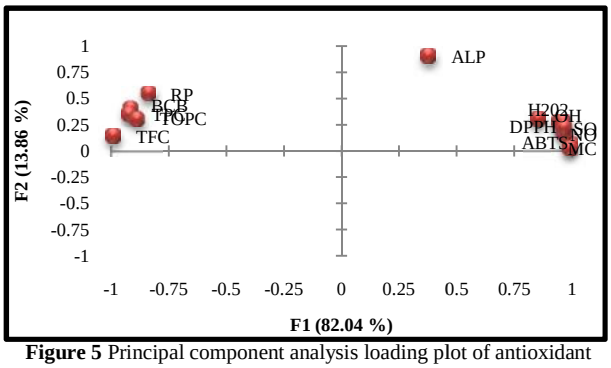

Figure 5 Principal component analysis loading plot of antioxidant activity and phytochemical attributes

This indicates that the polar secondary metabolites generated through phenyl propanoid pathway mainly contributed for scavenging of all these free-radicals. Previously similar observations were recorded from Tunisian pomegranate (*Punica granatum*) cultivars, where antioxidant activity and phenolic compounds along with anthocyanin pigments were loaded on PC1 at opposite co-ordinates (Zaonay *et al*., 2012). Anti-lipid peroxidation activity (ALP) was mainly emphasised on PC2 indicating that the accumulation of phenolics was not necessarily translated into protection of membrane lipids from oxidation. Similar relevant findings were also obtained during analysis of 'Bihi' fruits (*Solanum incanum* and *S. anguivi*) available in Eastern Himalaya (Ghosal *et al*., 2012). Principal component analysis was also employed earlier to highlight the discriminant characters of antioxidants and phytochemicals among different stages of development of flowers of *Couroupita* (Gupta *et al*., 2014).

CONCLUSION

Based on the results obtained, it may be concluded that the maturation process had profound effects on antioxidant activity and polyphenol contents of *C. guianensis* flower and fruit. Hence the methanolic extract of young stage exhibited strong antioxidant activity as well as high phenolics content when compared to matured stage. Moreover, we might speculate that the *in vitro* antioxidant activity of *C. guianensis* could be related with the high concentration of flavonoids and phenolics. Therefore the immature stage of both flower and fruit may have great relevance in the prevention and treatment of diseases in which oxidants or free radicals are implicated. The observed results claim further analyses to confirm its prophylactic effect in the treatment of free radical-mediated diseases and isolation of active principles responsible for its antioxidant activity.

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