



A comparative study on antioxidant activity and biochemical profile of exotic cocoa (*Theobroma cacao* L.) clones

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Plants are natural source of polyphenols and related secondary metabolites which possess medicinal value and are widely used in traditional and modern medicine. Reactive oxygen species (ROS) has been found to have a significant role in the initiation and/or progression of various diseases such as atherosclerosis, inflammatory injury, cancer and cardiovascular disease (Halliwell, 1997). Excess ROS in the body can lead to cumulative damage in proteins, lipids, DNA *etc.*, resulting in oxidative stress. Studies have explored the possibility of plant polyphenols as antioxidants against various diseases induced by free radicals (Hou *et al.*, 2003).

Theobroma cacao L. is an important tropical rain forest species, grown for its oil- rich beans, to produce cocoa and cocoa butter. Cocoa is rich in polyphenols, oligomers of polyphenol molecules (procyanidins), fat and theobromine. Studies have revealed that cocoa beans have health beneficial effects due to their polyphenol contents which exhibits high antioxidant activity *in vivo* (Kondo *et al.*, 1996) as well as *in vitro* (Baba *et al.*, 2000) conditions and offering protection against chronic diseases. Cocoa, cocoa extracts, purified cocoa flavanols and procyanidins exert strong antioxidant effects (Rosmawati and Azriena, 2013). The antioxidant properties of flavanols are based in part on their structural characteristics, including the hydroxylation of the basic flavan ring. Polyphenols in cocoa have established variety of effects associated with blood pressure (Buijsse *et al.*, 2006), diabetes-induced cataract formation (Osakabe *et*

al., 1998), platelet activation (Rein *et al.*, 2000) and regulation of nitric oxide (Duffy and Vita, 2003). Total phenols and antioxidant activities of unfermented beans are being utilized for cosmetic use in Malaysia (Norliza *et al.*, 2013).

Characterization of germplasm using biochemical techniques has received attention because of the increasing recognition of genetic resources in crop improvement and in selection of desirable genotypes to be used in breeding programs (Ghafoor and Ahmad, 2005). Cocoa germplasm with high polyphenols, fat content and superior antioxidant activities may have great attention to industrial use, in which cocoa is being utilized for making chocolates, cocoa powder and cocoa drink. There is little information about polyphenols, procyanidins or condensed tannins, fat and antioxidant activities of cocoa beans cultivated in India. Hence, the present study was carried out to determine the above biochemical parameters present in different cocoa clones and their correlation among the clones.

Cocoa germplasm collections of exotic origin conserved at field gene banks of ICAR-Central Plantation Crops Research Institute (ICAR-CPCRI), Regional Station, Vittal, Karnataka were included in the study. Fermented and dried beans of twenty one cocoa clones comprised of collections from Ecuador, Malaysia, Nigeria, Peru and Trinidad were used for biochemical estimation.

Biochemical profile of cocoa clones

Total phenols: Total phenolic content was determined by the Folin–Ciocalteu method (Swain

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and Hillis, 1959). One hundred and fifty microliters of cocoa bean extract fraction of water and 0.25 N Folin–Ciocalteu reagents were combined and then mixed well. The mixture was allowed to react for 3 min followed by addition of 1N Na₂CO₃ solution mixed well. The solution was incubated at room temperature in the dark for 2 hrs. The absorbance was measured at 725 nm and the results were expressed in milligram of catechin equivalents (GAE) per gram of extract/ fractions.

Condensed tannins (proanthocyanidins): Proanthocyanidins were determined by a botanical-HCl assay (Porter *et al.*, 1986). After addition of butanol-HCl reagent (95:5 butanol-HCl, 3.0 mL) and 2% ferric reagent (2% ferric ammonium sulfate in 2.0 M HCl, 0.1 mL) to the extract (0.5 mL) test tubes were vortexed and placed in a boiling water-bath for 60 min. After cooling, absorbance was recorded at 550 nm. Proanthocyanidins were expressed as g leucoanthocyanidin per kg dry bean, assuming that the specific absorbance of leucoanthocyanidin was 460 nm.

Fat estimation of cocoa beans: The cocoa fat content was determined using the Soxhlet extraction according to the official method (AOAC, 1960). From each cocoa clone 50 g of beans were ground and then extracted with petroleum ether in a Soxhlet apparatus for 6 h. After extraction, the samples were ground again, more finely, and extracted for 6 hrs (second extraction). Petroleum ether was evaporated under reduced pressure using a rotavapor. Lipid content was expressed as g per 100 g of bean fresh weight.

Antioxidant potential

DPPH scavenging activity: The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of cocoa beans ethanol extracts was measured as described by Kolevla *et al.* (2002). One mL of cocoa bean extract at a fixed concentration was prepared separately using respective solvent (40 µg mL⁻¹) mixed with 1 mL of DPPH ethanol solution (0.1 mM). An equal amount of methanol and DPPH (0.1 mM) served as control. After incubation in the dark for 30 min., absorbance was measured at 517 nm using a UV spectrometer. The percentage inhibition was calculated by comparing the absorbance values of control and samples. The capability to scavenge the DPPH radical was

calculated using the following equation: DPPH· scavenging effect (%) = [Absorbance of control – Absorbance of test sample/ Absorbance of control] x 100.

The experiment was arranged in a random block design with four replications. The data were subjected to analysis of variance (ANOVA). Mean comparisons were conducted using the Fisher's least significant difference test. Simple correlation analysis was performed to understand relation among biochemical traits in cocoa beans. Principal component analysis was also done to identify important biochemical factors of cocoa genotypes.

Comparison between clones

Biochemical characteristics of different cocoa clones with respect to polyphenols, procyanidins, fat content and antioxidant activities are presented in Table 1. Flavonoids and phenolic acids are known to possess antioxidant potential due to the

Table 1. Polyphenols, procyanidins, fat and antioxidant activities of different cocoa clones

Cocoa clones	Polyphenol (mg g ⁻¹)	Procyanidin (mg g ⁻¹)	Fat (%)	Antioxidant activity (%)
EET-272	136.2	52.5	47.7	98.8
SIAL -93	98.2	51.3	35.7	83.6
NA-33	81.4	48.3	45.7	77.2
NA-242	93.3	55.2	24.7	93.8
ICS-95	113.3	59.2	35.3	82.1
ICS-1	142.3	62.3	39.3	97.5
ICS-6	100.2	64.3	28.0	81.2
V-1	117.1	52.8	52.3	87.3
III-35	110.2	55.7	54.3	83.3
I-14	93.2	49.6	40.3	86.6
IV-20	82.4	48.9	53.7	92.9
I-56	105.2	59.2	40.0	84.6
I-21	113.3	63.5	53.7	93.2
II-67	134.9	60.2	48.7	98.1
V-7	95.2	51.1	49.0	92.6
SCA-12	100.2	51.2	39.7	89.5
III-105	102.3	42.6	30.3	66.6
II-52	98.6	52.5	32.0	82.5
I-56	103.1	51.2	49.0	92.1
NC-46	97.5	52.2	54.3	87.3
NC-67	115.5	48.6	54.3	82.2
CD	0.7	0.8	1.2	—

presence of hydroxyl groups in their structures and thus contribute to defense system against the oxidative damage. Here, the clone EET-272 showed highest polyphenol content of 136 mg g⁻¹ whereas least polyphenol content of 82.4 mg g⁻¹ was found in IV-20. Condensed tannins or procyanidins recorded in ICS-6 was the highest with 64.3 mg g⁻¹ and the least content of 48.6 mg g⁻¹ was found in NC-67. Fat content also varied from the highest of 54.3 per cent in III-35 to the lowest 24 per cent in NA-242, among the clones studied.

DPPH radical is one of the few stable organic nitrogen free radicals, which has been widely used to determine the free radical scavenging ability of the various samples. The antioxidant activity of cocoa clones using DPPH assay showed significant difference among the clones, widely ranging between a high of 98 per cent in EET-272 and the lowest of 77 per cent in NA-33. Higher percentages indicating higher antioxidant properties were seen in clones ICS-1, I-21, II-67 except, SCA-12, ICS-95, III-105 and NC-67. The reason might be that these ethanolic extracts of clones attributed genotypic variation in reaction kinetics with ethanolic DPPH free radicals.

In the present study, the biochemical contents exhibited significant positive correlation between polyphenols and antioxidant activity ($p < 0.05$, $r = 0.439$) and between polyphenols and procyanidins ($p < 0.05$, $r = 0.473$). The procyanidins also tend to show positive significant correlation with antioxidant activity ($r = 0.418$). Beans of cocoa clones had significant variation in polyphenols, procyanidins and antioxidant activities. High contents of polyphenols and procyanidins are related with high antioxidant activities. This information may be targeted for close screening of cocoa clones further for food, health and nutritional purposes.

Correlation between assays

The associations between biochemical contents estimated for correlation are presented in Table 2. The correlation coefficient was higher between the antioxidant capacity and anthocyanins ($r = 0.93$) compared to the antioxidant capacity and total phenolic content ($r = 0.89$). Significant correlations between the total polyphenol content and antioxidant activity of whole wheat or milling

Table 2. Correlation of biochemical contents in different cocoa clones

Content	Polyphenols	Procyanidins	Fat	Antioxidant
Polyphenol	1.000	0.473 *	0.166	0.439 *
Procyanidin		1.000	-0.101	0.418
Fat			1.000	0.330
Antioxidant				1.000

*Correlation is significant at the 0.05 level (2-tailed)

fractions of wheat have also been reported (Zhou et al., 2004). Our results are in agreement with Scibisz and Mitek (2007 correlating the antioxidant capacity of highbush blueberries (*Vaccinium corymbosum* L.) with total anthocyanins and total phenolics.

Principal component analysis

The distribution of data for PC-1 and PC-2 are shown in Figure 1. This plot describes 89.95 per cent of the sample set variation. Polyphenols, procyanidins, fat and antioxidant activities are present in PC-1. A classical representation of the scores on PC-1 and PC-2 has been shown in Fig. 2. Principal component analysis showed that first two components contributed to explain (PC-1=48.24% and PC-2=27.82%) a total of 89.95 per cent variation in biochemical traits of cocoa. Polyphenols and procyanidins of cocoa beans were the major contributors of total variation of antioxidant activities in 48.24 per cent. PC-2 is the contributor of variation in 27.82 per cent. There is a right grouping of all products within the PC-1.

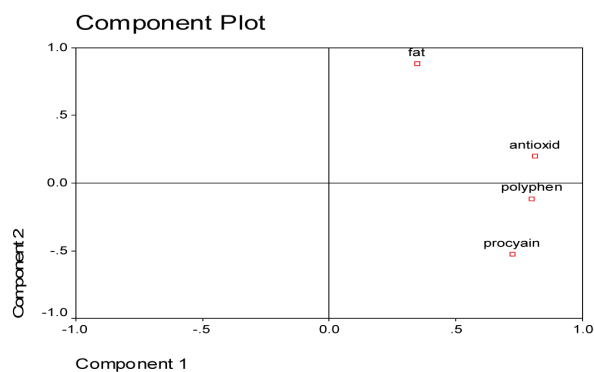


Fig. 1. Principal Component plot of PC-1 and PC-2 showing the distribution of cocoa polyphenols, procyanidins, fat and antioxidant activities. The PC-1 axis is correlated at the top with the level of fat content in the product

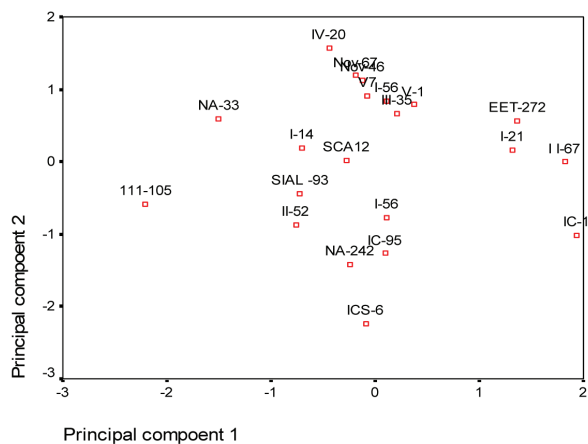


Fig. 2. Scatter diagram of the first two principal components for total polyphenols, procyanidins, fat and antioxidants from different cocoa clones

In PC-1 the attributes strongly associated with the X-axis are total polyphenols, procyanidins and antioxidant activities of cocoa beans. The level of fat is associated with nearly at the top of the Y-axis. These results corroborated with other studies on the commercially available cocoa-containing products in the United States, where, in PC-1, the attributes non-fat cocoa solids oxygen radical absorbance capacity, total polyphenols and procyanidins were strongly associated with the X-axis. The level of fat was associated with PC-2 at the top of the Y-axis (Miller *et al.*, 2006).

Scatter diagram in Figure 2 represents the first two principal components, for total polyphenols, procyanidins, fat and antioxidants from different cocoa clones. Bean of clone EET-272 contained a large amount of polyphenols and procyanidins, fat and antioxidant activities and PC-1 evidently split it from the other samples. Examining the two-dimensional scores plot in the space defined by PC-1 and PC-2 showed that distribution of the samples followed a pattern. The separation between groups was related to the polyphenolic content of beans.

The first group of six cocoa clones (EET-272, NA-33, ICS-1, II-67, III-105, 1-56) containing high amounts of Eigen value of equations in the first principal component analysis because these genotypes had high contents of polyphenols, procyanidins, fat and antioxidant activities. The second group (NA-242, ICS-95, ICS-6, IV-20, V-7, NC-46, and NC-67) contained high amounts of

Eigen value of equations in second principal component analysis group had average contents of polyphenols, procyanidins, fat and antioxidant activities. The scatter diagram can thus explain other cocoa genotypes in the principal components based on the biochemical contents.

The significant difference among cocoa clones with respect to total phenolics, procyanidins, fat and antioxidant activity is probably largely due to the genotypic effect, though they were grown under the same environmental conditions, as has been reported in other plant species (Scalzo *et al.*, 2005).

In short, polyphenols and procyanidins showed significant positive correlation with antioxidant activity. Cocoa clones also showed a wide range of difference in polyphenols, procyanidins, fat and antioxidant activities. High content of polyphenols and procyanidins contribute for phytochemical improvement and will play an important role in the development of superior cocoa clones for better nutritional and health benefits.

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