Research Article

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Free Radicals Scavenging Capability from Different Fractions of Cocoa Fresh Beans Aqueous Extract

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ABSTRACT

Cocoa fresh beans aqueous extract shows high free radicals scavenging capability from various studies based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Studies also showed that cocoa fresh beans aqueous extract contains few compounds which were believed contributing to the free radicals scavenging property. Nevertheless, report on which compounds or fractions from this cocoa fresh bean extract appear to be major sources in the free radicals scavenging capability is limited.

Cocoa fresh beans were extracted by water incubation at 80°C for 15 minutes. The extract was fractioned using preparative chromatography system fixed with C18 (21.2 x 150mm) column and diode array detector at 280nm wavelength. Each fractions were dried under nitrogen stream at 80°C and reconstituted with 2ml distilled water. Free radicals scavenging capability of the extract and its fractions were determined based on 0.06 mM DPPH solution.

Results showed that the cocoa fresh beans extract contains four prominent fractions as detected and isolated by Preparative liquid chromatography, namely fraction 1 to fraction 4. From this four fractions, only fraction 1 and fraction 2 showed positive result in free radicals scavenging capability. Fraction 1 and fraction 2 were identified as (+)-catechin and (-)-epicatechin respectively based on their retention time and mass spectrum.

Keywords

Cocoa fresh beans aqueous extract, DPPH, Free radicals scavenging.

Introduction

Theobroma cacao, is the scientific name of the cacao tree. The genus name, Theobroma, literally translates to "Food" (Theo) "of Gods" (broma) in Greek. Indeed, various research outcomes showed that cocoa is having many health-beneficial effects. For instance, studies had demonstrated that the consumption of cocoa or chocolate is able to reduce the risk of cardiovascular disease [1]. Extracts prepared from cocoa powder and cocoa beans gave positive results toward antihyperglycaemic effects on streptozotocin induced diabetic rats [2,3]. An ethanolic extract prepared from the Malaysian cocoa liquor was able to decrease the severity of hepatocarcinogenesis in rats [4].

Polyphenols have become an intense focus of research interest due to their physiological functions. Polyphenols are formed biogenetically from the shikimate and the acetic pathway [5]. Antioxidant, antimutagenic and antitumour is among the health-beneficial effects offered by phenolic compounds [6].

Cocoa beans are rich in polyphenols. The polyphenolic content of unfermented cocoa beans represent 12-18% of their dry weight [7] with 60% of the total phenolics in raw beans is flavanol monomers and procyanidin oligomer [8]. These compounds were reported to be a potential candidates to combat free radicals. Polyphenol content and composition and their final concentration depends on the variety of cocoa, processing and methods of extraction and these factors are significantly affected antioxidant capacity of the cocoa beans.

Antioxidant activity against free radicals based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay of the cocoa beans extract gave encouraging results. For instance, studies showed that ethanolic extract of Malaysia cocoa beans with concentration of 1.3mg/ml was sufficient to give EC50 value of scavenging activity based on DPPH assay [6]. Nevertheless, report on which compounds or fractions from cocoa fresh beans extract appears to be major sources in the free radicals scavenging capability is limited.

Materials and Methods

Fresh unfermented cocoa beans were collected from Malaysian Cocoa Board research centre located at Jengka, Pahang, Malaysia.

Preparation of Cocoa Fresh Beans Aqueous Extract

Unfermented cocoa beans were de-pulped. One gram of de-pulped cocoa fresh beans was added with 50ml of distilled water, ground with food processor in low speed for 3 seconds and incubated at 80°C for 15 minutes at 150 rpm stirring rate. The extract was then filtered with filter paper (Whatman no. 4).

Total Phenolic Content (TPC)

The extract was diluted six folds with distilled water. One millilitre of diluted extract was added with 2.5ml Folin-Ciocalteu reagent and 2.0ml 7.5% sodium bicarbonate. The mixture was vortexed and incubated at 45°C for 15 minutes prior to measure with UV-visible spectrophotometer at 750nm. A standard curve of gallic acid with a series concentration of 0 to 0.5 mg/ml was prepared. Total phenolic content, C in mg GAE/g sample, was calculated based on the equation below:

$$C\ (GAE) = k \times c \times V/M \qquad \{1\}$$

where

k = sample dilution factor

c = concentration determined from standard curve (mg/ml)

V = volume used during the assay (ml)

M = mass of the extract used during the assay (g)

Fractionation of the extract

The extract was fractioned using Agilent preparative liquid chromatography system fixed with C18 (21.2 x 150mm) column and diode array detector at 280nm wavelength. Each fractions were collected by in-line auto fraction collector.

Extract sample was filtered with 2.0µm nylon syringe filter. An injection volume of 2.0ml was injected into preparative liquid chromatography system. The eluent A was water and eluent B was acetonitrile. The mobile phase flow-rate was 2.0ml/min, whilst gradient elution program was applied as follow: initial 15% B; after 4 minutes, percentage of B ramped to 20%; after 5 minutes, eluent B ramped to 30% and 50% after 7 minutes. After 8 minutes, eluent B was decreased to 10% and the run time ended at the tenth minutes.

Free Radicals Scavenging Capability

Each fractions collected from the fraction collector were dried under nitrogen stream at 80°C and reconstituted with 2ml distilled water. Free radicals scavenging capability of the extract and its fractions were determined by drawing 0.5ml sample added with 5.0ml 0.06 mM DPPH solution, mixed well and incubated in dark for 30 minutes prior to measure with UV-Visible spectrophotometer at 520nm. Percentage of free radicals scavenging capability was calculated as the equation below:

$$RSC\ (\%) = [(Abs(c) - Abs(S)/Abs(c)] \times 100 \qquad \{2\}$$

where:

RCS = DPPH radical scavenging capability

Abs(c) = Abs for control

Abs(S) = Abs for sample

Qualification of each fraction

Fractions with positive result on DPPH assay were determined by UHPLC-MS/MS system of ABSciex TripleToF 4600. Electrospray ionization (ESI) source was operated in negative mode. Instrument setting were Declustering Potential at -80V; Collision Energy was -5V for ToF MS scan and -30V for product ion; desolvation temperature 400°C. The data were acquired and processed using Analyst 1.6 software. The analytical column was Luna Omega 1.6µm C18 (50mm x 2.1mm i.d.) and kept at constant temperature of 30°C during chromatographic separation. Injection volume was 2.0µl and the eluent A was water with 0.01% formic acid and eluent B was mixture of acetonitrile: methanol at 75:25 respectively. The mobile phase flow rate was 150µl/min. The gradient elution program were 10% B at initial; after 1 min, B was ramped to 30% and kept isocratic at 2 min for 30 seconds and decrease to 10%. The total run time was 4 min.

Results and Discussion

The total phenolic content (TPC) of the aqueous extracted cocoa fresh beans was estimated as 49.02 mg GAE/g in average of five replicates. It represents 4.9% total phenolic contents of the fresh beans weight.

Four prominent peaks were detected by the preparative liquid chromatography system and therefore, 4 fractions were collected by the in-line fraction collector (Figure 1) from the cocoa fresh beans aqueous extract.

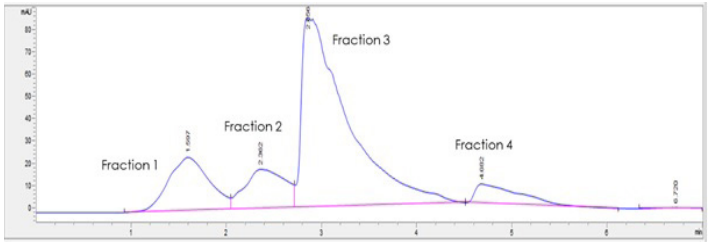

Figure 1: Four compounds isolated by the preparative liquid chromatography fixed with C18 column and diode array detector at 280nm wavelength.

Free radicals scavenging test using DPPH assay showed that cocoa fresh beans aqueous extract was able to inhibit 79% of free radicals in 0.06mM of DPPH reagent. From the four fractions collected, only fraction 1 and fraction 2 showed positive result towards DPPH assay, with the capability to scavenge 31% and 49% of free

radicals available in 0.06mM of DPPH assay respectively.

Fraction 1 and fraction 2 were determined as (+)-catechin and (-)-epicatechin respectively based on their mass spectrum and retention time with reference to standard injections as in table 1.

Fraction	Retention time (min)	ToF MS scan, [M-H]-	MS2	Compound
1	1.25	289.0581	289.0709, 245.0709, 205.0415, 203.0623	(+)-catechin
2	1.43	289.0585	289.0587, 245.0708, 205.0413, 203.0623	(-)-epicatechin

Table 1: Retention time, ToF MS scan ([M-H]-), and product ions (MS2) for fraction 1 and fraction 2 isolated by preparative liquid chromatography system.

Both (+)-catechin and (-)-epicatechin shared similar mass spectrum because they are stereoisomer.

Conclusion

Four prominent compounds were isolated by the preparative liquid chromatography system fixed with C18 column and diode array detector at 280nm wavelength. Two out of this four isolated compounds showed positive results towards DPPH assay and were determined as (+)-catechin and (-)-epicatechin with the fraction of (-)-epicatechin in cocoa fresh bean aqueous extract showed higher free radicals scavenging capability.

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