Journal of Pharmaceutical and Biomedical Analysis 192 (2021) 113678

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

HPLC-FRAP methodology and biological activities of different stem bark extracts of *Cajanus cajan* (L.) Millsp

Kouadio Ibrahime Sinan[a], Mohamad Fawzi Mahomoodally[b,*], Ozan Emre Eyupoglu[c], Ouattara Katinan Etienne[d], Nabeelah Bibi Sadeer[b], Gunes Ak[a], Tapan Behl[e], Gokhan Zengin[a,*]

[a] *Department of Biology, Science Faculty, Selcuk University, Campus, Konya, Turkey*
[b] *Department of Health Sciences, Faculty of Medicine and Health Sciences, University of Mauritius, 230 Réduit, Mauritius*
[c] *Department of Biochemistry, School of Pharmacy, Istanbul Medipol University, Turkey*
[d] *Laboratoire de Botanique, UFR Biosciences, Université Félix Houphouët-Boigny, Abidjan, Cote d'Ivoire*
[e] *Chitkara College of Pharmacy, Chitkara University, Punjab, India*

ARTICLE INFO

Article history:
Received 2 September 2020
Received in revised form
28 September 2020
Accepted 5 October 2020
Available online 12 October 2020

Keywords:
Antioxidants
Bioactive compounds
Pigeon pea
Enzyme inhibition
Natural products

ABSTRACT

Cajanus cajan. (L.) Millsp. (*C. cajan*) (Family: Fabaceae) also known as pigeon pea, is a famous food and cover/forage crop bearing a high amount of key amino acids (methionine, lysine and tryptophan). This study investigated into the total phenolic (TPC), flavonoid content (TFC), antioxidant [2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2 -azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity, total antioxidant capacity (TAC) (phosphomolybdenum) and metal chelating] activities and enzyme [α-amylase, α-glucosidase, tyrosinase, acetyl-(AChE), butyryl-(BChE) cholinesterase] inhibitory effects of four extracts (methanol, hexane, ethyl acetate, aqueous) prepared from *C. cajan* stem bark. Direct identification of antioxidants was also conducted using the high performance liquid chromatography-ferric reducing antioxidant power (HPLC-FRAP) system. The highest TPC and TFC were recorded with the methanolic (23.22 ± 0.17 mg GAE/g) and ethyl acetate extracts (19.43 ± 0.24 mg RE/g), respectively. The methanolic extract exhibited important antioxidant activity with DPPH (38.41 ± 0.05 mg Trolox equivalent (TE)/g), ABTS (70.49 ± 3.62 mg TE/g), CUPRAC (81.86 ± 2.40 mg TE/g), FRAP (42.96 ± 0.59 mg TE/g) and metal chelating (17.00 ± 1.26 mg ethylenediaminetetraacetic acid equivalent/g). *p*-coumaric and caffeic acid were the predominant antioxidants in the samples. Results from enzymatic assays showed the potential abilities of hexane extract in inhibiting the AChE, BChE, α-amylase and α-glucosidase enzymes. From the results obtained in this study, it can be concluded that *C. cajan* can be considered as a promising source of antioxidants and key enzyme inhibitors that can be exploited for future bioproduct development.

1. Introduction

Oxidative stress is one of the most burning topics among researchers around the globe as it is the underlying causative factor of numerous chronic diseases namely cancer, neurodegenerative disorders, cardiovascular diseases, dermatological problems, diabetes mellitus, among others. Continuously searching for antioxidants should be an ongoing challenge as it has been reported that antioxidants may hamper the oxidation process which consequently prevent or postpone oxidative stress related diseases [1]. Antioxidants from plant materials have garnered fair amount of attention from researchers as well as the public because of their demonstrated efficacy and safety. At present, most of the plant samples are screened in pursuit of novel and natural antioxidants with substantially high activity. For instance, in the year 2019, ScienceDirect recorded 12,901 articles related to the screening of plants in terms of their antioxidant activity. To be in line with the current trends, the present study aims to screen a nutritionally important plant, *Cajanus cajan* (L.) Millsp. (*C. cajan*) (Family: Fabaceae) also known as pigeon pea, for its antioxidative property using two different techniques namely spectrometry and chromatography. Since a major gap exists in the current *in vitro* antioxidant assays as well detailed in a recent review by Bibi Sadeer et al. [2], we

* Corresponding authors.
E-mail addresses: f.mahomoodally@uom.ac.mu (M.F. Mahomoodally), gokhanzengin@selcuk.edu.tr (G. Zengin).

https://doi.org/10.1016/j.jpba.2020.113678
0731-7085/

also assessed the antioxidants present in *C. cajan* using a recently developed system: high performance liquid chromatography-ferric reducing antioxidant power (HPLC-FRAP) for the direct identification of antioxidants in the complex matrices of plant samples.

The HPLC-FRAP system has been popularly used by numerous researchers eliminating tedious sample pre-treatment procedures and proved to be efficient and rapid [3]. According to previous reports, *in vitro* antioxidant assays suggested that *C. cajan* may be considered as an important natural antioxidant source [4,5]. However, to the best of our knowledge, there has been no report about the profiling of antioxidant compounds of *C. cajan* using HPLC-FRAP system. Thus, one of the aims of the present study is to identify antioxidants in the stem bark of *C. cajan* by such technique. Otherwise, this *Cajanus* species, being a plant food, is commonly used in traditional medicine. For instance, the leaf is used against diabetes, dysentery, hepatitis, measles and in China it is believed to treat blood-related diseases and alleviate pain [4]. In Trinidad, the leaf is used in food poisoning and to prevent constipation [6]. *Cajanus cajan* is a highly nutritious perennial legume and a good source of crude fibre, iron, sulphur, calcium, potassium, manganese and water-soluble vitamins [7]. Morphologically, it is a non-climbing shrub, erect, silky pubescent ribbed stems, spindly branches, leaves pinnately trifoliolate, leaflets in short stalks, flowers borne on corymbiform racemes, petals yellow, standard red outside and yellow inside, wings yellow, oblong linear pods constricted between the seeds [8].

Several pharmacological validations showed that pigeon pea possessed important biological properties. In a clinical evaluation, the extract of *C. cajan* was reported to reduce painful crises in sickle cell anaemia on the liver [9]. Compounds identified in the root and leaf extracts displayed moderate activity against the chloroquine-sensitive *Plasmodium falciparum* strain 3D7 [10]. Liu et al. [11] reported that the four stilbenes (cajaninstilbene acid, longistyline A, longistyline C, and cajanolactone A) isolated from the leaf of pigeon pea possessed neuroprotective effects. Another recent study showed that a natural stilbene (longistylin A) produced by *C. cajan* can significantly inhibit the growth of methicillin-resistant *Staphylococcus aureus* [12].

As evidenced by a number of publications, important pharmacological properties have been recorded from leaves and roots of *C. cajan*. In addition, the chemical composition has also been reported. However, no chemical and biological information's have reported from the stem bark of *C. cajan*. Therefore, to address this research gap in the corpus of scientific knowledge on this plant, the other aim of this study was to validate the use of this plant for its chemical profile and biological properties. Taken together, the presented results could fill the gap on *C. cajan* stem bark which subsequently could open new research avenues, particularly in development therapeutic bioproducts development.

2. Materials and methods

2.1. Plant material and extraction procedure

The stem bark of *C. cajan* was collected in the village of Tenikro (district of Yamoussoukro-Côte d'Ivoire, 6° 33′ 10″ N, 5° 15′ 11″ W, 293 m) in the year 2019 and it was authenticated by the botanist Ouattara Katinan Etienne (Université Félix Houphouet Boigny, Abidjan, Côte d'Ivoire). The plant materials (aerial parts) were dried in shade for about 10 days and then grounded using a laboratory mill.

Maceration was used for sample preparation with ethyl acetate, hexane and methanol as extraction solvents, separately. For this purpose, 5 g of the samples were macerated for 24 h at room temperature. After this, the extracts were filtered and then the solvents were removed *via* a rotary-evaporator. With respect to the water extracts, 5 g of the plant material were infused with 100 mL boiled water. Thereafter, the water was dried using freeze-drying. All extracts were stored at +4 °C until further studies.

2.2. Profile of bioactive compounds

The total phenolic (TPC), and flavonoid (TFC) contents of the extracts were measured and detailed methods were described in our previous paper [13]. Standards, namely gallic acid (GAE) for phenolics, and rutin (RE) for flavonoids, were used to express the results.

2.3. HPLC-Method

The Shimadzu LC-2010C-HT HPLC compact system (Japan) used for the analysis included thermostable column unit, the autosampler unit, degasser, gradient manager pump and UV- Diode array detectors combined with a second supported on-line reagent programmable single channel syringe pump (IPS 12-RS model, Inovenso laboratory devices, Turkey). The experiments were conducted using a Purospher star RP-18 encapped, C18 column with guard column (4.6 × 250 mm, 5 μm) (Merck, Germany). Data procurement (peak area, retention time) was executed by utilizing Shimadzu ChemStation Program. The injection volume of all samples was 50 μL. Analysis time was 30 min. The mobile phase consisted of four solvents: solvent A was methanol, solvent B was a mixture of acetic acid/water/acetonitrile (0.5/49.75/49.75, v/v/v), solvent C was a mixture of 0.2 % acetic acid: water (v/v) and solvent D was ultra-pure grade acetonitrile [14].

HPLC-FRAP methodology, one of the methods that allows simultaneous application of HPLC separation and antioxidant activity determination, was used. DAD signal was set at 280 nm (maximum absorption for phenolic contents) and UV on-line antioxidant activity detection signal was set at 595 nm (maximum absorption after the post column FRAP reaction). Mobile phase flow rate was set at 1.2 mL/min. The flow rate of the syringe pump was set at 0.25 mL/min (optimized value). Column temperature was set at room temperature, 25–30 °C. The reaction coil, which made of polytetrafluoroethylene (PTFE) tubing (0.25 mm i.d.), was adjusted as 2.5 m length (optimized value) [14]. The FRAP reagent was prepared such as in the study of Benzie and Strain [15] and adapted to post-column assay. For HPLC-FRAP assay, FRAP reagent was freshly prepared, drawn into the syringe, covered with aluminium foil and put into the syringe pump. HPLC-FRAP system reacts with the opening of the splitter switch system connected to the manifold in the 1.5 cm reaction coil of the pigeon pea plant sample extracts separated according to the polarity difference with the solvent flow from the main pump, and this reaction peaks with extra peak detection in the UV detector besides the DAD separation were analysed (Fig. 1).

Lyophilized and powdered (5 mg) forms of *C. cajan* stem barks for four different extracts (ethyl acetate, hexane, methanol, and infusion) were solubilized with HPLC grade high purity methanol (10 mL). Firstly, they were filtered through a 0.45 μm filter prior to HPLC analysis. Their final concentrations were adjusted to 0.5 mg / mL before HPLC analysis. Ethyl acetate, hexane, methanol, and infusion extracts of *C. cajan* stem barks was run with HPLC-FRAP system at least three parallel each other. Limit of detection (LOD) and limit of quantitation (LOQ) are the analytical processing arguments of an analyte that can be accurately measured by the analytical procedure. For this study, validation parameters about LOD and LOQ were determined according to the International Conference on Harmonization guidelines. While at the end of the running of four different extracts (ethyl acetate, hexane, methanol, infusion) from *C. cajan* stem barks, chromatograms with positive FRAP peaks were obtained at UV 595 nm simultaneous with DAD 280 nm. The detec-

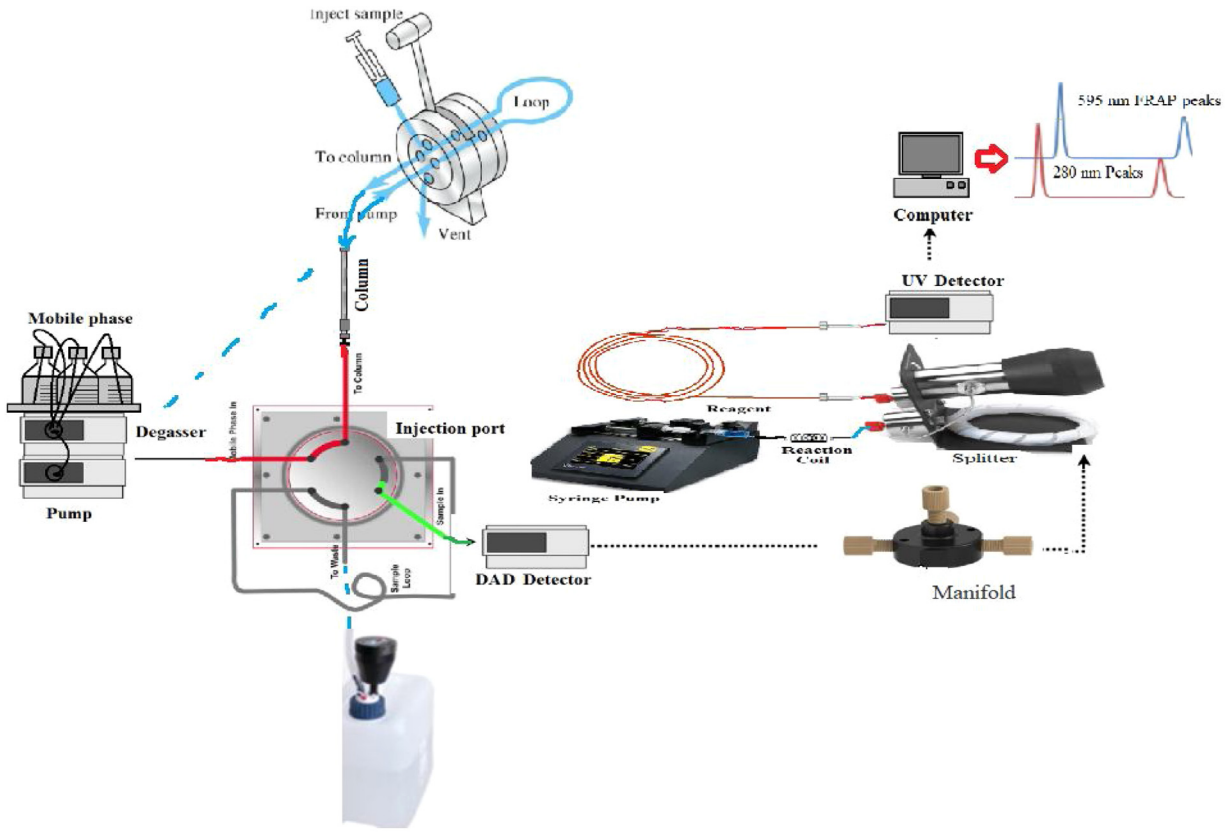

Fig. 1. HPLC-FRAP antioxidant analysis system.

tion limits of HPLC-FRAP method about phenolic acid contents of *C. cajan* stem barks extracts were determined as 3 times and 10 times of the average standard deviation of noise. Linearity of the method was tested in the range of 0.4–9 ppm for detected 12 phenolic acids (*p*–OH benzoic acid, chlorogenic acid, caffeic acid, syringic acid, ferulic acid, *p*-coumaric acid, vanillin, vanillic acid, rosmarinic acid, gentisic acid, syringaldehyde, protocatechuic acid). The LOD and LOQ data obtained for 280 nm and 595 nm was calculated. All statistical ChemStation detection limit analysis data were reported significantly ($p < 0.05$) with standard deviation. There are no FRAP peak equivalents of p–OH benzoic acid, p-coumaric acid, vanillin and vanillic acid

2.4. Determination of antioxidant and enzyme inhibitory effects

To detect antioxidant properties, we used several chemical assays with different mechanisms namely, radical scavenging, reducing power and metal chelating. Trolox (TE) and ethylenediaminetetraacetic acid (EDTA) were used as standard antioxidant compounds. Obtained results were expressed as equivalents of these compounds Grochowski et al. [16]. To detect inhibitory effects on enzymes, we used colorimetric enzyme inhibition assays and these assays included tyrosinase, α-glucosidase, α-amylase and cholinesterases. Some standard inhibitors (galantamine, kojic acid and acarbose) were used as positive controls.

2.5. Data analysis

All experiments were performed in triplicate and results were aggregated and gave as mean ± SD standard deviation. Data were statistically analysed by using One-way analysis of variance, principal component and hierarchical clustered analysis respectively. R v 3.6.2 software was used for the analysis.

Table 1
Total bioactive components of *C. cajan* stem bark.

Extracts	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg RE/g extract)
Hexane	5.55 ± 0.08^{d}	1.25 ± 0.03^{c}
EA	21.69 ± 0.30^{b}	19.43 ± 0.24^{a}
MeOH	23.22 ± 0.17^{a}	11.49 ± 0.06^{b}
Infusion	19.14 ± 0.18^{c}	1.51 ± 0.23^{c}

* Values expressed are means ± S.D. of three parallel measurements. GAE: Gallic acid equivalent; RE: Rutin equivalent; EA: Ethyl acetate; MeOH: Methanol. Different superscripts indicate significant differences in the extracts ($p < 0.05$).

3. Results and discussion

3.1. Total bioactive compounds and in vitro antioxidant properties

Phenolic compounds are widely distributed in plant foods. In addition to fruits, coloured vegetables and spices, legumes are equally rich in phenolic compounds. As a matter of fact, *C. cajan* is expected to be abounded by polyphenols as it is a highly nutritious legume and this has indeed been confirmed several times by previous publications indicating that the seed contained high quantity of phenolic, flavonoid, and tannin contents [7]. Since little is known on the phytochemical profile of stem bark of the plant of interest, the present study attempted to quantify the total phenolic (TPC) and total flavonoid content (TFC) of different extracts of stem bark. Results are shown in Table 1. In contrast to seeds whereby some studies revealed low TPC ranging from 1.054 to 18.3 mg GAE/g [17,18], the data presented herein showed relatively higher quantity of phenolic and flavonoid contents in stem bark. For instance, the highest TPC and TFC recorded was from the methanolic extract

Table 2
Antioxidant activities of *C. cajan* stem bark.

Extracts	DPPH (mg TE/g extract)	ABTS (mg TE/g extract)	CUPRAC (mg TE/g extract)	FRAP (mg TE/g extract)	Phosphomolybdenum (mmol TE/g)	Metal chelating ability (mg EDTAE/g)
Hexane	na	4.75 ± 0.32^{d}	13.48 ± 0.30^{d}	8.54 ± 0.19^{d}	0.31 ± 0.02^{d}	6.52 ± 0.29^{c}
EA	12.39 ± 0.09^{c}	22.45 ± 0.70^{c}	72.09 ± 1.40^{b}	25.91 ± 0.44^{c}	1.68 ± 0.09^{a}	10.24 ± 0.03^{b}
MeOH	38.41 ± 0.05^{a}	70.49 ± 3.62^{a}	81.86 ± 2.40^{a}	42.96 ± 0.59^{a}	1.32 ± 0.06^{b}	17.00 ± 1.26^{a}
Infusion	25.84 ± 0.64^{b}	64.40 ± 1.09^{b}	53.51 ± 0.34^{c}	36.43 ± 0.08^{b}	1.00 ± 0.05^{c}	10.16 ± 0.69^{b}

* Values expressed are means ± S.D. of three parallel measurements. TE: Trolox equivalent; EDTAE: EDTA equivalent; EA: Ethyl acetate; MeOH: Methanol; na: not active. Different superscripts indicate significant differences in the extracts ($p < 0.05$).

(23.22 ± 0.17 mg GAE/g) and ethyl acetate extract (19.43 ± 0.24 mg RE/g), respectively. However, the lowest yield for both TPC and TFC was obtained from the hexane extracts (5.55 ± 0.08 mg GAE/g and 1.25 ± 0.03 mg RE/g, respectively).

Ever since epidemiological studies correlate diets rich in natural antioxidants with a decreased risk of oxidative stress-related diseases, phenolic compounds and their antioxidant activities have been of great concern to both consumers and researchers since the past decades [19]. Due to this keen interest, assessment of antioxidants present in the extracts of *C. cajan* were conducted herein. Since there is still no universal method to determine antioxidative power *in vitro*, six different tests involving different mechanism of antioxidant defence system were conducted to evaluate the antioxidant activity of pigeon pea. For instance, DPPH and ABTS were performed to evaluate the free radical scavenging capacity of *C. cajan*, FRAP and CUPRAC to investigate into the reducing potential, phosphomolybdenum to determine total antioxidant capacity and ferrous ion chelating assay to measure the chelating ability of the extracts. Results are shown in Table 2.

Overall, it can be observed that a good correlation existed between the different assays. Additionally, extracts with high antioxidant activity were associated with high phenolic contents. For instance, methanolic extract yielding the highest TPC, exhibited the highest activity in five assays namely DPPH (38.41 ± 0.05 mg TE/g), ABTS (70.49 ± 3.62 mg TE/g), FRAP (42.96 ± 0.59 mg TE/g), CUPRAC (81.86 ± 2.40 mg TE/g) and metal chelating (17.00 ± 1.26 mg EDTAE/g). Scientists have claimed that extracts obtained from highly polar solvents, especially methanol, have high antioxidant effects [20], hence supporting our results. Such response is common among plant extracts and was observed with several other studies [21,22]. On the other hand, hexane extract possessing the lowest TPC, displayed the lowest antioxidative power with all six methods (DPPH: inactive; ABTS: 4.75 ± 0.32 mg TE/g; FRAP: 8.54 ± 0.19 mg TE/g; CUPRAC: 13.48 ± 0.30 mg TE/g; phosphomolybdenum: 0.31 ± 0.02 mmol TE/g and metal chelating: 6.52 ± 0.29 mg EDTAE/g).

3.2. -HPLC-FRAP analysis

To further scrutinize our extracts in terms of antioxidants and bearing in mind that the current *in vitro* antioxidant assays do not always give realistic results [2], we also screened our samples using a more extensive technique such as HPLC-FRAP.

Antioxidant activity determination, corresponding to their ferric reducing identity, was based on the ability of antioxidant compounds to reduce Fe^{3+} to Fe^{2+}. Therefore, the research group of Shi from China suggested that HPLC–FRAP assay could be designed by detecting the blue coloured Fe^{2+}-TPTZ complex at 593 nm [3]. On the HPLC-FRAP system, more phenolic peaks were detected as positive peaks at 280 nm compared at 595 nm. For instance, for sample 1 (ethyl acetate) seven phenolic peaks were detected at 280 nm while four at 595 nm, for sample 2 (hexane) eight phenolic peaks were detected at 280 nm while four at 595 nm, for sample 3 (methanol) five peaks were confirmed whereas two were detected as positive at 595 nm and for sample 4 (infusion) four peaks were detected at 280 nm but only two were confirmed at 595 nm. Identification of a total of 12 different antioxidants (such as p-coumaric acid, p−OH benzoic acid, syringic acid, ferulic acid, vanillin, caffeic acid, chlorogenic acid, syringaldehyde, protocatechuic acid, vanillic acid, rosmarinic acid and gentisic acid) was achieved at 280 nm by systematic analysis of their retention time (Tables 3–6) from the four samples of *C. cajan*. The chemical structures of the identified antioxidants are illustrated in Fig. 2. The HPLC chromatograms are given in Figs. 3–6.

In sample 1, the seven antioxidants detected at 280 nm were p-coumaric acid (or 4-hydroxycinnamic acid), p−OH benzoic acid (or 4-hydroxybenzoic acid), syringic acid, ferulic acid, vanillin, caffeic acid and chlorogenic acid. At 280 nm, p-coumaric acid was the predominant antioxidant with a peak area of 247.3. At 595 nm, the peaks of p−OH benzoic and p-coumaric acid were absent and caffeic acid was most predominant (210.3). Interestingly, the same predominant antioxidant compounds were found in abundance in sample 3 whereby the peak area of p-coumaric acid was 213.5 at 280 nm and that of caffeic acid was 227.6 at 595 nm. Despite this similarity in the main antioxidant compounds, the *in vitro* revealed different antioxidant activities. In addition to the fact that *in vitro* assays do not always give realistic results, this difference can also be linked to the chemical structures of the phenolic antioxidants.

Many studies have associated the chemical structures of phenolic compounds with high antioxidant power. Basically, phenolic compounds contain one or more hydroxyl groups (−OH) attached to a benzene ring [23]. The antioxidant activity centre of phenolic acids is phenolic hydroxyl (−OH), thus the number and position of phenolic hydroxyls have a direct link with the antioxidant activity of phenolic acids [24]. Furthermore, methoxy ($-OCH_3$) and carboxylic acid (−COOH) groups also have significant effects on the antioxidant property of phenolic acids [25]. In this study, eight typical phenolic acids have been identified and they are p−OH benzoic acid, vanillic acid, protocatechuic acid, caffeic acid, ferulic acid, p-coumaric acid, syringic acid and gentisic acid. Natella et al. [26] reported that hydroxycinnamic acid (−CH = CHCOOH) has stronger antioxidant activity than hydroxybenzoic acid (−COOH) when the other substituents of the benzene ring remain the same [26]. These results can be attributed to the electron-donating ability of carboxylic acid groups. The conjugation effect and induction effect together determine that −COOH is a strong electron-withdrawing group and −CH = CHCOOH is a weak electron-withdrawing group. An electron-donating group can increase the electron cloud density of a benzene ring, decreasing the dissociation energy of the phenolic hydroxyl bond which consequently promote its free scavenging ability. Therefore, it is speculated that carboxylic acid groups affect the antioxidant activity of phenolic acids based on their electron-donating ability (−CH = CHCOOH > −COOH) [27]. Nonetheless, these facts do not corroborate with the *in vitro* results of sample 2 (hexane). For instance, the sample showed low or no activity with ABTS and DPPH assays despite five phenolic acids were identified in it.

In addition to carboxylic acid groups, methoxy groups can also improve the antioxidant activity of phenolic acids. In point of fact,

Table 3
Detection and quantitation limits of the peaks defined at 280 nm and 595 nm for hexane extract from *C. cajan* stem bark.

Peak Numbers	Component Name	Retention Time (RT) (min.)	Peak Area (280 nm) (mAU x min.)	Concentration (for 280 nm) (ppm)	Peak Area (595 nm) (mAU x min.)	Concentration (for 595 nm) (ppm)	Limit of Detection (LOD, ppm, 280 nm)	Limit of Quantitation (LOQ, ppm, 280 nm)	Limit of Detection (LOD, ppm, 595 nm)	Limit of Quantitation (LOQ, ppm, 595 nm)
1	Protocatechuic acid	8.1	115.5	12.2	92.8	15.4	1.2 ± 0.03	4.0 ± 0.04	1.0 ± 0.03	3.3 ± 0.04
2	Gentisic acid	11.9	68.8	7.3	77.6	12.9	0.4 ± 0.02	1.3 ± 0.02	0.6 ± 0.02	2.0 ± 0.03
3	Chlorogenic acid	13.9	158.3	16.7	230.3	38.3	1.6 ± 0.03	5.3 ± 0.04	2.8 ± 0.03	9.2 ± 0.04
4	*P*-OH benzoic acid	15.3	280.7	29.6	*	*	1.8 ± 0.04	6.0 ± 0.04	*	*
5	Vanillic acid	17.5	83.5	8.8	*	*	0.8 ± 0.02	2.6 ± 0.03	*	*
6	Vanillin	20.3	78.6	8.3	*	*	0.6 ± 0.02	2.0 ± 0.03	*	*
7	Syringaldehyde	23.2	86.6	9.1	200.4	33.3	0.9 ± 0.02	3.0 ± 0.04	2.5 ± 0.03	8.2 ± 0.04
8	*p*-Coumaric acid	24.9	75.2	7.9	*	*	0.5 ± 0.01	1.7 ± 0.02	*	*

±SD: Average Standard Deviation, 95 % confidence interval, critical ratio: $p < 0.05$, *: non-detected, ppm: parts per million.

Table 4
Detection and quantitation limits of the peaks defined at 280 nm and 595 nm for ethyl acetate extract from *C. cajan* stem bark.

Peak Numbers	Component Name	Retention Time (RT) (min.)	Peak Area (280 nm) (mAU x min.)	Concentration (for 280 nm) (ppm)	Peak Area (595 nm) (mAU x min.)	Concentration (for 595 nm) (ppm)	Limit of Detection (LOD, ppm, 280 nm)	Limit of Quantitation (LOQ, ppm, 280 nm)	Limit of Detection (LOD, ppm, 595 nm)	Limit of Quantitation (LOQ, ppm, 595 nm)
1	*p*-OH benzoic acid	9.2	31.5	3.8	*	*	0.2 ± 0.01	0.7 ± 0.02	*	*
2	Chlorogenic acid	10.4	130.8	15.6	87.6	15.6	0.8 ± 0.02	2.6 ± 0.04	0.6 ± 0.02	2.0 ± 0.03
3	Caffeic acid	12.6	178.3	21.3	210.3	37.4	1.2 ± 0.03	4.0 ± 0.04	1.4 ± 0.03	4.6 ± 0.04
4	Syringic acid	16.6	180.7	21.6	205.6	36.6	1.3 ± 0.03	4.3 ± 0.04	1.5 ± 0.03	5.0 ± 0.04
5	Ferulic acid	21.3	46.2	5.5	58.3	10.4	0.4 ± 0.01	1.3 ± 0.03	0.5 ± 0.01	1.7 ± 0.03
6	*p*-Coumaric acid	25.0	247.3	29.5	*	*	1.6 ± 0.03	5.3 ± 0.04	*	*
7	Vanillin	29.1	21.6	2.6	*	*	0.1 ± 0.01	0.3 ± 0.01	*	*

±SD: Average Standard Deviation, 95 % confidence interval, critical ratio: $p < 0.05$, *: non-detected, ppm: parts per million.

Table 5
Detection and quantitation limits of the peaks defined at 280 nm and 595 nm for methanol extract from *C. cajan* stem bark.

Peak Numbers	Component Name	Retention Time (RT) (min.)	Peak Area (280 nm) (mAU x min.)	Concentration (for 280 nm) (ppm)	Peak Area (595 nm) (mAU x min.)	Concentration (for 595 nm) (ppm)	Limit of Detection (LOD, ppm, 280 nm)	Limit of Quantitation (LOQ, ppm, 280 nm)	Limit of Detection (LOD, ppm, 595 nm)	Limit of Quantitation (LOQ, ppm, 595 nm)
1	*p*-OH benzoic acid	11.8	71.5	10.3	*	*	0.7 ± 0.02	2.3 ± 0.03	*	*
2	Caffeic acid	13.9	168.8	24.4	227.6	70.4	1.9 ± 0.03	6.3 ± 0.04	2.4 ± 0.03	7.9 ± 0.04
3	Vanillic acid	15.1	198.3	28.6	*	*	2.1 ± 0.03	6.9 ± 0.04	*	*
4	Syringic acid	19.5	40.7	5.9	95.6	29.5	0.4 ± 0.01	1.3 ± 0.03	0.8 ± 0.02	2.6 ± 0.03
5	*p*-Coumaric acid	25.7	213.5	30.8	*	*	2.2 ± 0.03	7.3 ± 0.04	*	*

±SD: Average Standard Deviation, 95 % confidence interval, critical ratio: $p < 0.05$, *: non-detected, ppm: parts per million.

according to a recent study, the higher the number of methoxy groups present, the higher is the antioxidant activity of phenolic acids [27]. It is worth mentioning that the difference between the chemical structure of vanillic acid (identified as major antioxidant in sample 4) and p−OH benzoic acid (identified as major antioxidant in sample 2) is the presence of one methoxy ($-OCH_3$) group. Since, $-OCH_3$ increases antioxidant power, sample 4 can be said to be stronger than sample 2 in terms of antioxidant activity. This finding is consistent with the *in vitro* results which also revealed sample 4 to be stronger than sample 2.

Since −CH=CHCOOH > −COOH in terms of electron-donating ability, both samples 1 and 3 are thus considered to be stronger than sample 2 in terms of antioxidant activity due to the predominant presence of p-coumaric acid in samples 1 and 3 which contains the −CH = CHCOOH group attached to the phenol group while p−OH benzoic acid, present in majority in sample 2, does not possess the −CH = CHCOOH group but instead the −COOH group. However, this result is not in line with the *in vitro* findings. Instead, *in vitro* assays classified only sample 3 as the strongest antioxidant. It is important to also bear in mind that the interactions among other antioxi-

Table 6
Detection and quantitation limits of the peaks defined at 280 nm and 595 nm for infusion from *C. cajan* stem bark.

Peak Numbers	Component Name	Retention Time (RT) (min.)	Peak Area (280 nm) (mAU x min.)	Concentration (for 280 nm)(ppm)	Peak Area (595 nm) (mAU x min.)	Concentration (for 595 nm) (ppm)	Limit of Detection (LOD, ppm, 280 nm)	Limit of Quantitation (LOQ, ppm, 280 nm)	Limit of Detection (LOD, ppm, 595 nm)	Limit of Quantitation (LOQ, ppm, 595 nm)
1	*p*-OH benzoic acid	11.8	75.5	16.8	*	*	0.7 ± 0.02	2.3 ± 0.03	*	*
2	Caffeic acid	16.7	68.8	15.3	207.6	54.9	0.5 ± 0.02	1.7 ± 0.03	1.4 ± 0.03	4.6 ± 0.04
3	Vanillic acid	19.5	218.3	48.7	*	*	1.5 ± 0.03	5.0 ± 0.04	*	*
4	Rosmarinic acid	28.9	85.7	19.1	170.6	45.1	0.9 ± 0.02	3.0 ± 0.04	1.2 ± 0.03	4.0 ± 0.04

±SD: Average Standard Deviation, 95 % confidence interval, critical ratio: $p < 0.05$, *: non-detected, ppm: parts per million.

dants present in the samples can be synergistic, thus explaining such *in vitro* response.

3.3. Enzyme inhibition effects

Inhibiting digestive enzymes such as α-amylase and α-glucosidase can be considered as an effective way to monitor blood glucose level. Type 2 diabetes is global and chronic condition affecting about 422 million people around the globe (WHO). Diabetes is associated with microvascular and macrovascular complications damaging vital organs including kidney, heart, eyes, brain, and also cause cutaneous manifestations [28]. It is reported that there is a 30 % risk that people with diabetes develop cutaneous manifestations due to the damage caused to the vascular and nerve structures by high blood sugar levels [29]. Nowadays, agents which are based on natural resources are preferred to manage diseases since side effects are minimal and the therapies are well-tolerated compared to synthetic oral drugs currently available on the market [30]. The present study was thus designed to evaluate the extracts of *C. cajan* for their antidiabetic activity (anti-amylase and anti-glucosidase), cutaneous manifestations (anti-tyrosinase) and Alzheimer's disease (anti-cholinesterase). Results are shown in Table 7.

Overall, the hexane extract exhibited the highest activity with most enzymes whereas the aqueous extract was least successful in depressing most enzymatic activities. In the amylase assay, the ethyl acetate extract exerted the highest amylase inhibitory activity with an acarbose equivalent of 0.74 mmol/g. However, the same extract exhibited lower glucosidase inhibition (0.68 ± 0.01 mmol ACAE/g). Instead, the hexane extract showed the highest glucosidase inhibition (0.81 ± 0.01 mmol ACAE/g) but low anti-amylase effect (0.42 ± 0.01 mmol ACAE/g), nearly two times lower than glucosidase inhibitory activity. α-Amylase breakdown polysaccharides to form disaccharides which are further broken down by glucosidase to produce monosaccharides. The inhibition of α-amylase is sometimes linked with gastrointestinal disturbance due to digestion of carbohydrates [31]. Thus, antidiabetic drugs exhibiting moderate α-amylase but higher α-glucosidase inhibitory potential is preferred.

The highest tyrosinase activity was recorded with methanolic extract (55.90 ± 1.20 mg KAE/g) followed by the ethyl acetate extract (47.31 ± 1.22 mg KAE/g), hexane (38.02 ± 4.04 mg KAE/g) and aqueous (no activity was reported). These results may be attributed to the presence of high phenolic content in the methanolic extract. Previous studies have also reported the potent inhibitory activity of plant extracts on tyrosinase is attributed to their phenolic profiles [32], and are thus in agreement with our findings. Hexane extract was the best cholinesterase inhibitor with both AChE (2.61 ± 0.06 mg GALAE/g) and BChE (4.87 ± 0.25 mg GALAE/g) enzymes. Although flavonoids are reported to be efficient cholinesterase inhibitors, results from this study do not correlate with the observed activities. For instance, hexane extract which contained the least amount of flavonoid demonstrated the highest anti-AChE and anti-BChE activity and the ethyl acetate extract yielding the highest TFC, was least successful in depressing AChE enzyme activity (1.25 ± 0.14 mg GALAE/g). Therefore, it can be said that the level of flavonoid present in extracts may not always be linked with cholinesterase activity. In addition to this approach, several studies [6,33] reported that *C. cajan* contains other classis of phytochemicals including saponins, terpenoids and stilbenes (cajanin *etc.*) and they could be responsible for the mentioned differences in enzyme inhibition assays.

3.4. Multivariate analysis

Firstly, principal component analysis (PCA) was achieved in order to reduce the dimensionality of the data whilst preserving as far as possible most of the relevant information in the data set. It accomplishes this reduction by generating a few numbers of dimensions along which the variability in the dataset is maximal. Through the factorial plan erected by a few dimensions, it is possible to visually assess differences and similarities between samples. The PCA results are shown as Fig. 7, before starting the interpretation of the obtained results, the optimal number of dimensions that must be considered were identified. Thus, we followed two rules suggested by Solanas et al. [34]. Accordingly, three dimensions were found to satisfy the above-mentioned rule; they had an eigenvalue above 1 and encompassed together 99.7 % of the information in the original data (Fig. 7A). The contribution of the original variables on the retained dimensions was determined by referring to the circle of correlation (Fig. 7B). the first dimension which captured the maximum quantity of information, was positively correlated with ABTS, DPPH, FRAP and negatively linked to BChE and glucosidase. This would suggest that the first dimension separated the samples predominantly in relation to their ability to scavenge ABTS and DPPH radical, reduce Fe^{3+} ion and inhibit the activity of BChE and glucosidase enzymes. Dimension 2 optimized 31.3 % of the variance and was positively determined by CUPRAC, PPBD, MCA, amylase and tyrosinase. As a result, it distinguished the samples in terms of said biological activities. Dimension 3 which represented the leftover information was highly positive loaded for AChE and negative loaded for PPBD and amylase. Accordingly, it discriminated the samples according to their total antioxidant, anti-AChE and anti-amylase activities. After the reduction of the dimensionality of the data, hierarchical clustered analysis was subjected to the results of PCA in order to bring out the different clusters. The obtained factor map revealed four clusters (Fig. 7C), however ethyl acetate and methanol extracts were close together relatively to hexane and infusion extracts. This implies that among the four extraction solvents, ethyl acetate and methanol presented some similarities concerning their biological activities. A large number of publica-

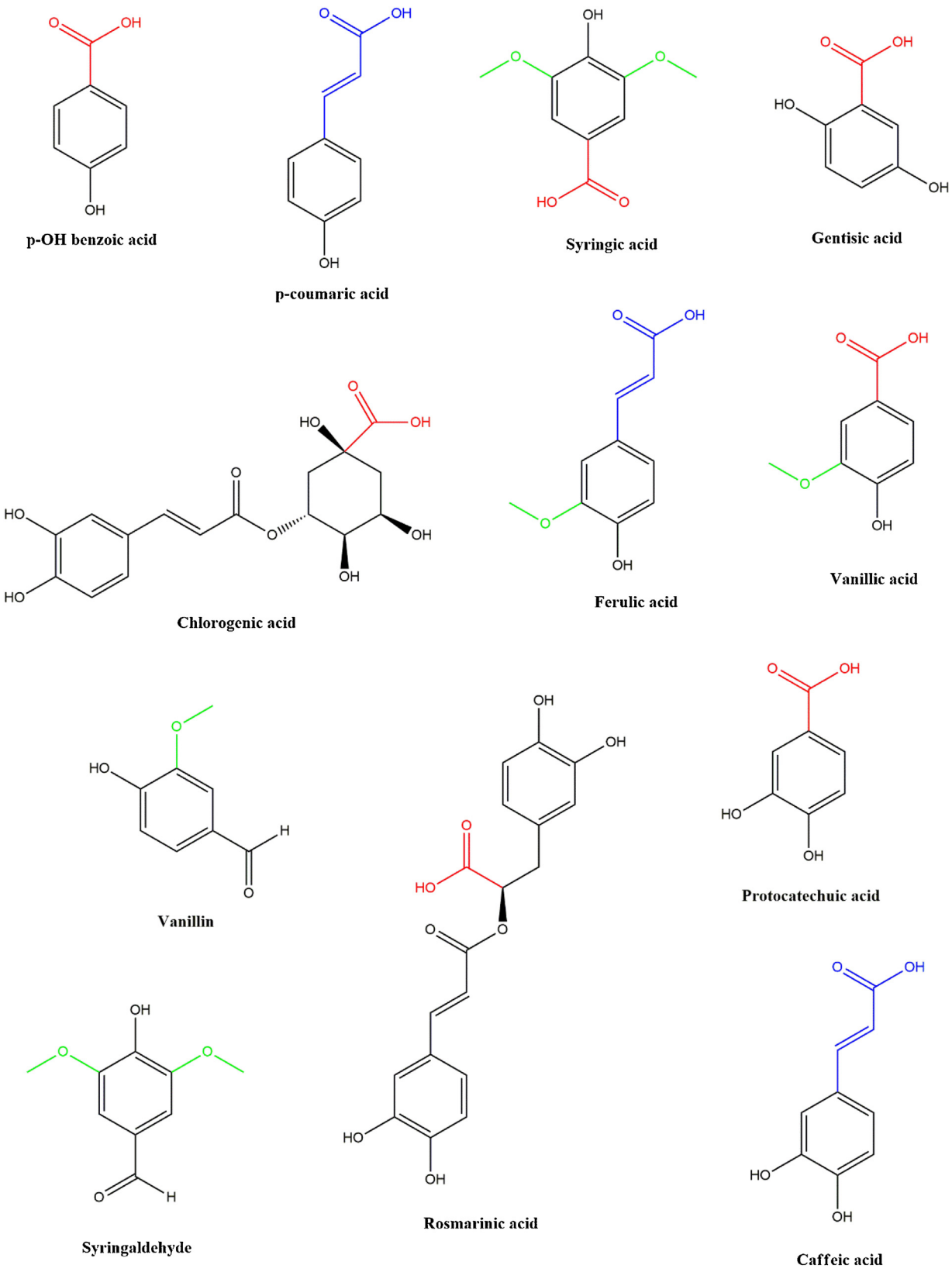

Fig. 2. Chemical structures of 12 different phenolic antioxidants identified by HPLC-FRAP from sample 1 (ethyl acetate), 2 (hexane), 3 (methanol) and 4 (infusion).

tions can be found on the influenced of the extraction solvent on the recovery of biopharmaceutical molecules from plants. This influence focuses on the quality and number of secondary metabolites extracted from the raw materials. Methanol and ethyl acetate provided overall excellent biological activities; they exhibited high phenolic content, strong antioxidant activities and relatively good enzyme inhibitory properties. However, from the perspective of food and pharmaceutical security, it is preferable to employ sol-

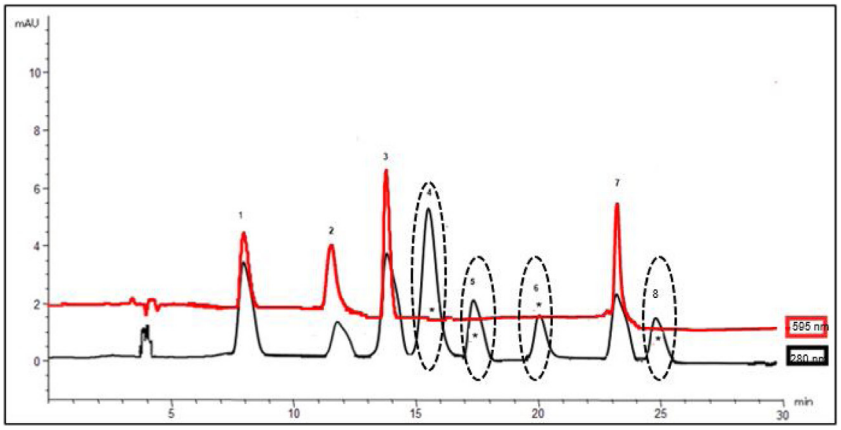

Fig. 3. Overlapped chromatograms with HPLC-FRAP 595 nm and DAD 280 nm detections of hexane extract from *C. cajan* stem bark.

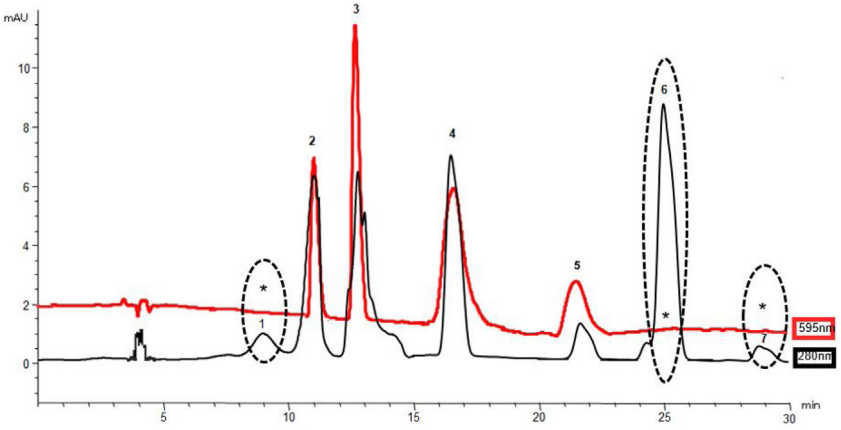

Fig. 4. Overlapped chromatograms with HPLC-FRAP 595 nm and DAD 280 nm detections of ethyl acetate extract from *C. cajan* stem bark.

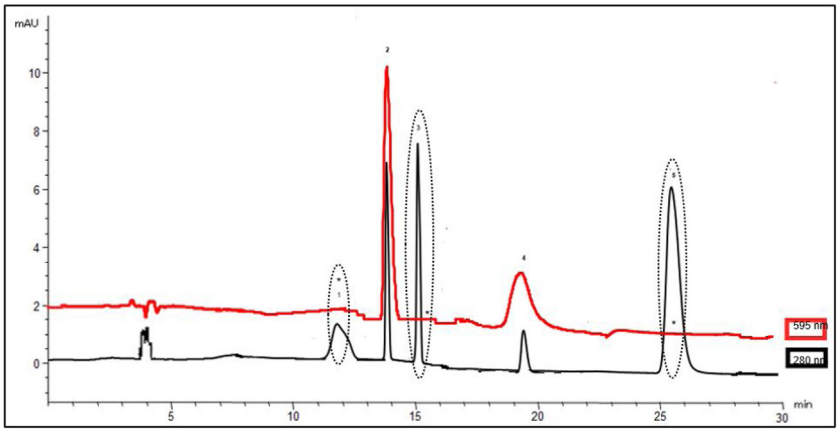

Fig. 5. Overlapped chromatograms with HPLC-FRAP 595 nm and DAD 280 nm detections of methanol extract from *C. cajan* stem bark.

Table 7
Enzyme inhibitory properties of *C. cajan* stem bark.

Extracts	AChE (mg GALAE/g extract)	BChE (mg GALAE/g extract)	Tyrosinase (mg KAE/g extract)	α-amylase (mmol ACAE/g extract)	α-glucosidase (mmol ACAE/g extract)
Hexane	2.61 ± 0.06^{a}	4.87 ± 0.25^{a}	38.02 ± 4.04^{c}	0.42 ± 0.01^{b}	0.81 ± 0.01^{a}
EA	1.25 ± 0.14^{c}	4.66 ± 0.07^{a}	47.31 ± 1.22^{b}	0.74 ± 0.03^{a}	0.68 ± 0.01^{b}
MeOH	2.01 ± 0.01^{b}	2.49 ± 0.12^{b}	55.90 ± 1.20^{a}	0.42 ± 0.01^{b}	0.39 ± 0.02^{c}
Infusion	na	na	na	0.12 ± 0.01^{c}	na

* Values expressed are means ± S.D. of three parallel measurements. GALAE: Galantamine equivalent; KAE: Kojic acid equivalent; ACAE: Acarbose equivalent; EA: Ethyl acetate; MeOH: Methanol. na: not active. Different superscripts indicate significant differences in the extracts ($p < 0.05$).

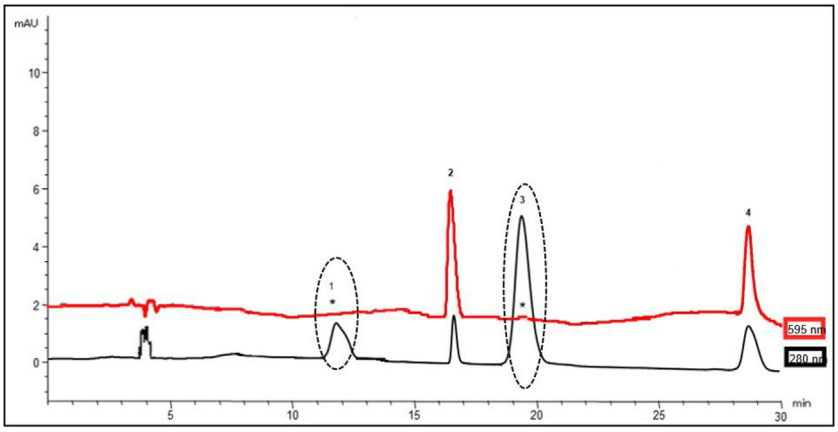

Fig. 6. Overlapped chromatograms with HPLC-FRAP 595 nm and DAD 280 nm detections of infusion from *C. cajan* stem bark.

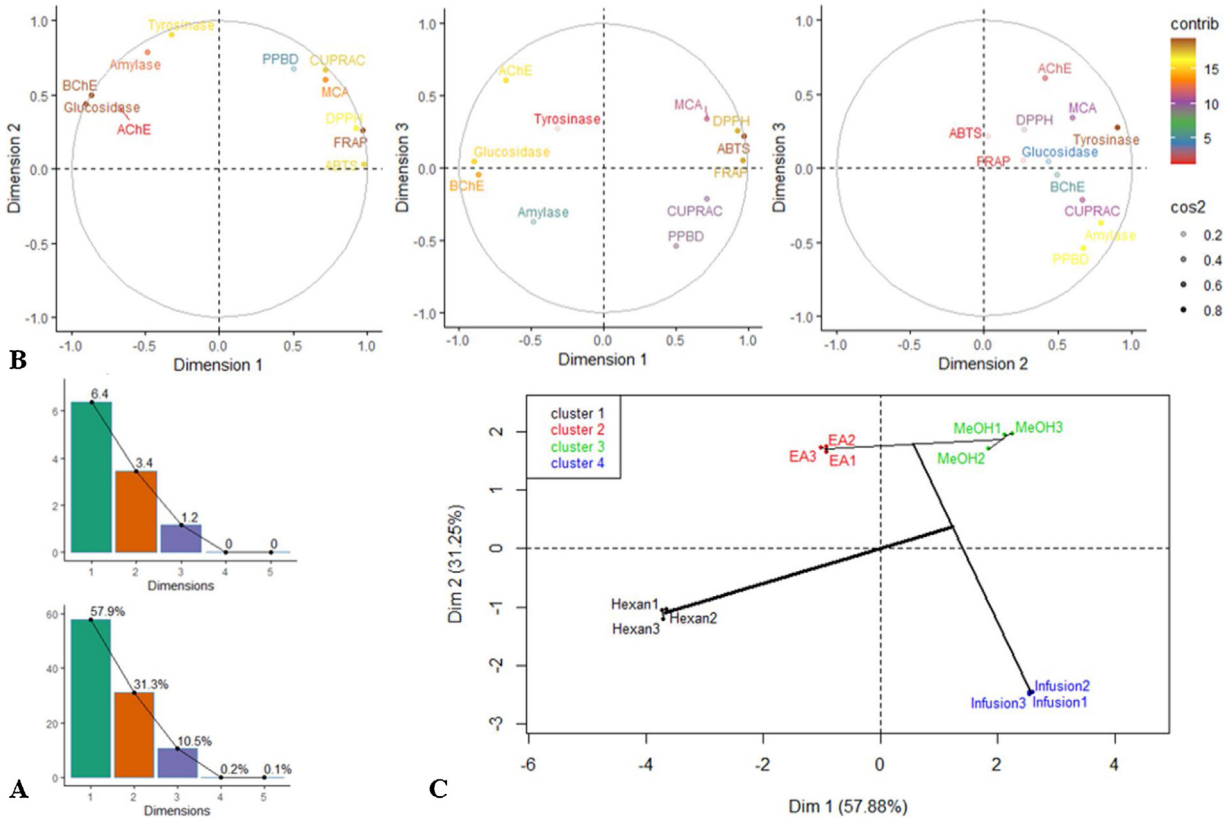

Fig. 7. Principal component and hierarchical clustered analyses applied to biological activities of *C. cajan* stem bark. A. eigenvalue and percentage of explained variances of dimensions of principal component analysis. B. Appreciation of the biological contribution on the first three dimensions of principal component analysis through the circle of correlation. C. Factor map of Hierarchical clustering analysis.

vent such ethanol or ethanol-water since they are in compliance with safely manufacturing practice.

4. Conclusion

The present study, for the first time, has employed the HPLC-FRAP system to identify a total of 12 phenolic antioxidants in the different extracts of *C. cajan*. Plants with high level of phenolics are considered as a good source of antioxidants and therefore it can be said that *C. cajan* might be considered as a future source of antioxidants for development of bioproducts. However, cytotoxicity test should be conducted on the plant food to determine its safety. Contrary to other studies [35], in this present work, the observed biological activities (antioxidant and enzymatic activities) could not be fully attributed to the polyphenolic profile of the extracts as the activities varied; whereby certain extracts possessing high bioactive compounds demonstrated low biological activity and vice versa. As a future work, the hexane extract, depressing most studied enzymes, can be subjected to kinetic studies to determine the type of inhibition involved.

CRediT authorship contribution statement

Kouadio Ibrahime Sinan: Methodology, Formal analysis, Investigation. **Mohamad Fawzi Mahomoodally:** Investigation, Writing - original draft, Writing - review & editing. **Ozan Emre Eyu-**

poglu: Methodology, Formal analysis. **Ouattara Katinan Etienne:** Methodology. **Nabeelah Bibi Sadeer:** Investigation, Writing - original draft, Writing - review & editing. **Gunes Ak:** Investigation, Writing - original draft, Writing - review & editing. **Tapan Behl:** Writing - review & editing. **Gokhan Zengin:** Investigation, Writing - original draft, Writing - review & editing, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] A. Rahal, A. Kumar, V. Singh, B. Yadav, R. Tiwari, S. Chakraborty, K. Dhama, Oxidative stress, prooxidants, and antioxidants: the interplay, Biomed Res. Int. 2014 (2014).

[2] N. Bibi Sadeer, D. Montesano, S. Albrizio, G. Zengin, M.F. Mahomoodally, The versatility of antioxidant assays in food science and safety—chemistry, applications, strengths, and limitations, Antioxidants 9 (8) (2020) 709.

[3] S. Shi, K. Guo, R. Tong, Y. Liu, C. Tong, M. Peng, Online extraction–HPLC–FRAP system for direct identification of antioxidants from solid Du-zhong brick tea, Food Chem. 288 (2019) 215–220.

[4] Y.-S. Lai, W.-H. Hsu, J.-J. Huang, S.-C. Wu, Antioxidant and anti-inflammatory effects of pigeon pea (*Cajanus cajan* L.) extracts on hydrogen peroxide-and lipopolysaccharide-treated RAW264. 7 macrophages, Food Funct. 3 (12) (2012) 1294–1301.

[5] B. Mahitha, P. Archana, M.H. Ebrahimzadeh, K. Srikanth, M. Rajinikanth, N. Ramaswamy, In vitro antioxidant and pharmacognostic studies of leaf extracts of *Cajanus cajan* (L.) millsp, Indian J. Pharm. Sci. 77 (2) (2015) 170.

[6] D. Pal, P. Mishra, N. Sachan, A.K. Ghosh, Biological activities and medicinal properties of *Cajanus cajan* (L) Millsp, J. Adv. Pharm. Technol. Res. 2 (4) (2011) 207–214.

[7] J. Sekhon, S.K. Grewal, I. Singh, J. Kaur, Evaluation of nutritional quality and antioxidant potential of pigeonpea genotypes, J. Food Sci. Technol. 54 (11) (2017) 3598–3611.

[8] U. Quattrocchi, CRC World Dictionary of Medicinal and Poisonous Plants: Common Names, Scientific Names, Eponyms, Synonyms, and Etymology (5 Volume Set), CRC press, 2012.

[9] A.O. Akinsulie, E.O. Temiye, A.S. Akanmu, F.E.A. Lesi, C.O. Whyte, Clinical evaluation of extract of *Cajanus cajan* (ciklavit®) in sickle cell anaemia, J. Trop. Pediatr. 51 (4) (2005) 200–205.

[10] G. Duker-Eshun, J.W. Jaroszewski, W.A. Asomaning, F. Oppong-Boachie, S. Brøgger Christensen, Antiplasmodial constituents of *Cajanus cajan*, Phytother. Res. 18 (2) (2004) 128–130.

[11] Y.-m. Liu, S.-n. Shen, F.-b. Xia, Q. Chang, X.-m. Liu, R.-l. Pan, Neuroprotection of stilbenes from leaves of *Cajanus cajan* against oxidative damage induced by corticosterone and glutamate in differentiated PC12 Cells, Chin. Herb. Med. 7 (3) (2015) 238–246.

[12] J. Wu, B. Li, W. Xiao, J. Hu, J. Xie, J. Yuan, L. Wang, A. Longistylin, A natural stilbene isolated from the leaves of *Cajanus cajan*, exhibits significant anti-MRSA activity, Int. J. Antimicrob. Agents 55 (1) (2020), 105821.

[13] G. Zengin, A. Aktumsek, Investigation of antioxidant potentials of solvent extracts from different anatomical parts of *Asphodeline anatolica* E. Tuzlaci: an endemic plant to Turkey, Afr. J. Tradit. Complement. Altern. Med. 11 (2) (2014) 481–488.

[14] N.A. Burnaz, M. Küçük, Z. Akar, An on-line HPLC system for detection of antioxidant compounds in some plant extracts by comparing three different methods, J. Chrom. B 1052 (2017) 66–72.

[15] I.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of ïantioxidant power: the FRAP assay, Anal. Biochem. 239 (1) (1996) 70–76.

[16] D.M. Grochowski, S. Uysal, A. Aktumsek, S. Granica, G. Zengin, R. Ceylan, M. Locatelli, M. Tomczyk, In vitro enzyme inhibitory properties, antioxidant activities, and phytochemical profile of *Potentilla thuringiaca*, Phytochem. Lett. 20 (2017) 365–372.

[17] K. Saxena, R. Kumar, C. Gowda, Vegetable pigeonpea–a review, J. Food Legu. 23 (2) (2010) 91–98.

[18] S.A. Marathe, V. Rajalakshmi, S.N. Jamdar, A. Sharma, Comparative study on antioxidant activity of different varieties of commonly consumed legumes in India, Food Chem. Toxicol. 49 (9) (2011) 2005–2012.

[19] N. Pourreza, Phenolic compounds as potential antioxidant, Jundishapur J. Nat. Pharm. Prod. 8 (4) (2013) 149–150.

[20] A. Altemimi, N. Lakhssassi, A. Baharlouei, D.G. Watson, D.A. Lightfoot, Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts, Plants 6 (4) (2017) 42.

[21] N.B. Sadeer, G. Rocchetti, B. Senizza, D. Montesano, G. Zengin, A. Uysal, R. Jeewon, L. Lucini, M.F. Mahomoodally, Untargeted metabolomic profiling, multivariate analysis and biological evaluation of the true mangrove (*Rhizophora mucronata* Lam.), Antioxidants 8 (10) (2019) 489.

[22] N.B. Sadeer, K.I. Sinan, Z. Cziáky, J. Jekő, G. Zengin, R. Jeewon, H.H. Abdallah, K.R.R. Rengasamy, M.F. Mahomoodally, Assessment of the pharmacological properties and phytochemical profile of *Bruguiera gymnorhiza* (L.) lam using in vitro studies, in silico docking, and multivariate analysis, Biomolecules 10 (5) (2020) 731.

[23] R. San Miguel-Chávez, Phenolic antioxidant capacity: a review of the state of the art, Phenolic Compounds-Biological Activity (2017).

[24] P. Rodríguez-Bonilla, F. Gandía-Herrero, A. Matencio, F. García-Carmona, J.M. López-Nicolás, Comparative study of the antioxidant capacity of four stilbenes using ORAC, ABTS+, and FRAP techniques, Food Anal. Method. 10 (9) (2017) 2994–3000.

[25] S. Johnny, M. Asnaashari, N. Molaahmadibahraseman, A. Sharif, Structure–antioxidant activity relationships of o-hydroxyl, o-methoxy, and alkyl ester derivatives of p-hydroxybenzoic acid, Food Chem. 194 (2016) 128–134.

[26] F. Natella, M. Nardini, M. Di Felice, C. Scaccini, Benzoic and cinnamic acid derivatives as antioxidants: structure−activity telation, J.Agri. Food Chem. 47 (4) (1999) 1453–1459.

[27] J. Chen, J. Yang, L. Ma, J. Li, N. Shahzad, C.K. Kim, Structure-antioxidant activity relationship of methoxy, phenolic hydroxyl, and carboxylic acid groups of phenolic acids, Sci. Rep. 10 (1) (2020) 2611.

[28] W.T. Cade, Diabetes-related microvascular and macrovascular diseases in the physical therapy setting, Phys. Ther. 88 (11) (2008) 1322–1335.

[29] E. Baselga Torres, M. Torres-Pradilla, Cutaneous manifestations in children with diabetes mellitus and obesity, Actas Dermosifiliogr. 105 (6) (2014) 546–557.

[30] V. Gulati, I.H. Harding, E.A. Palombo, Enzyme inhibitory and antioxidant activities of traditional medicinal plants: potential application in the management of hyperglycemia, BMC Complement, Altern. Med. 12 (1) (2012) 77.

[31] S.A. Tucci, E.J. Boyland, J.C. Halford, The role of lipid and carbohydrate digestive enzyme inhibitors in the management of obesity: a review of current and emerging therapeutic agents, Diabetes Metab, Syndr. Obes. 3 (2010) 125–143.

[32] S. Zolghadri, A. Bahrami, M.T. Hassan Khan, J. Munoz-Munoz, F. Garcia-Molina, F. Garcia-Canovas, A.A. Saboury, A comprehensive review on tyrosinase inhibitors, J. Enzyme Inhib. Med. Chem. 34 (1) (2019) 279–309.

[33] Q.-F. Luo, L. Sun, J.-Y. Si, D.-H. Chen, Hypocholesterolemic effect of stilbenes containing extract-fraction from *Cajanus cajan* L. on diet-induced hypercholesterolemia in mice, Phytomedicine 15 (11) (2008) 932–939.

[34] A. Solanas, R. Manolov, D. Leiva, Retaining principal components for discrete variables, Anu. Psicol. 41 (1–3) (2011) 33–50.

[35] G. Zengin, A. Aktumsek, A. Mocan, K.R.R. Rengasamy, C.M.N. Picot, M.F. Mahomoodally, *Asphodeline cilicica* Tuzlaci: from the plant to its most active part extract and its broad bioactive properties, S. Afr. J. Bot. 120 (2019) 186–190.