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PHYTOCHEMICAL CONTENT OF *CANNABIS SATIVA* METHANOL EXTRACT AND IN VITRO ANTIOXIDANT ACTIVITIES OF ITS SOLVENT FRACTIONS

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ABSTRACT

Introduction: In this study, we estimated phytochemical content of *Cannabis sativa* methanol extract and the antioxidant potentials of the solvent fractions.

Methods: Methanol extract of the plant was screened qualitatively and quantitatively for bioactive phytochemicals. Fractions of the methanol extracts were assayed for antioxidant and lipid peroxidation activities using DPPH, FRAP, SRS and TBA.

Results: Methanol extract contained cardiac glycosides, saponins, flavonoids, alkaloids, tannins, and terpenoids. Methanol fraction (MF) was highly efficacious against ABTS radical (EC_{50}=94.83±0.02µg/mL) in a comparable manner with ascorbic acid (EC_{50}=96.02±0.03µg/mL). The order of efficacy of the fractions on DPPH radical scavenging activity was n-hexane fraction (HF, EC_{50}=78.75±0.08µg/mL) <MF (EC_{50}=120.30±0.04µg/mL) <chloroform fraction (CF, EC_{50}=123.00±0.02µg/mL). Iron chelation activity of CF (EC_{50}=100.00±0.03µg/mL) was close to that of EDTA used as standard (EC_{50}=80.41±0.02µg/mL), followed by MF (EC_{50}=130.7±0.02µg/mL). Close superoxide radical scavenging effect was observed in MF (EC_{50}=95.07±0.01µg/mL) and CF (EC_{50}=94.61±0.05µg/mL).**Conclusion:** Conclusively, ME of *Cannabis sativa* is a rich source of saponins, alkaloids and phenolic compounds. MF was highly effective against ABTS radical, thiobarbituric reactive species, with moderate efficacy on DPPH radical and iron chelation capacity while HF produced higher DPPH and CF has higher iron chelation property.

KEY WORDS: *C. sativa*, ROS, lipid-peroxidation, phytochemistry, antioxidant

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INTRODUCTION

The bioactive benefits of medicinal plants and their products have been attributed to the presence of phytochemical compounds with antioxidant properties such as phenols and alkaloids to mention a few [1, 2]. These plant-derived natural phytochemicals are capable of controlling oxidative stress derived from excessive production of free radicals. Oxidative stress which can cause damage to biological macromolecules may facilitate patho-progression of many human diseases including cancer, cardiovascular, and neurodegenerative diseases [3]. Many in glass assays have been frequently used to estimate the free radical scavenging abilities of plants and derivatives hence their antioxidant properties. Most of the free radical scavenging potential of plants results from the redox properties of their phenolic content. Antioxidant effects of polyphenols are exerted through different mechanisms. They are capable of reducing radical compounds or ions, scavenge free radicals and chelate metal ions [4]. The content of plant phenolic compounds varies in different plant species, based on environmental conditions, and different solvent extracts of the same plants [5]. Medicinal plants exhibiting good *in vitro* antioxidant activities have been showed to be good sources of natural antioxidants. *Cannabis sativa* is a popular plant of commercial, medical and sociological use around the world. The leaves and stem are very useful. The stem is used for the production of rope and pots. The leaves are medicinally consumed by various routes for medicinal purposes. The plant is used for management of several disorders and diseases like glaucoma, muscle spasm, neurological problems and cardiovascular diseases. Obtained from this plant are several cannabinoids which include tetrahydrocannabinol and cannabidiol with variety of clinical usage.

This plant and its extracts have been ratified for use as medicinal or recreational drugs in some countries. This feat is a recent development of an erstwhile criminalized use of this plant without prescription. To this end, scientifically based researches are being encouraged to further highlight the medicinal properties and uses of this plant. This is expected to further justify its decriminalization. Also, the phytochemicals of plants vary with environment and cultivation. In light of this, the present study aimed to estimate the phytochemical content of *Cannabis sativa* methanol extract and the antioxidant potentials of the solvent fractions as a basis for the medicinal value.

EXPERIMENTAL

All chemical used for this study were of analytical grade.

Plant collection and processing

Cannabis sativa leaves were procured with the assistance of NDLEA (National Drug Law Enforcement Agency), Delta state, Nigeria; by releasing confiscated samples of the plant for the study. Authentication of the plant was done at the Botany Department, Delta State University, Nigeria (BDREFH/034/093). Four hundred grams of the pulverized dried leaves were soaked in 2000 mL methanol for 72 hours, then filtered with Whatman filter paper, the filtrate was then evaporated in a rotary evaporator to obtain the methanol extract. Eighty grams of the methanol extract was reconstituted with methanol and was partitioned

into n-hexane, chloroform and methanol fractions using an equal volume of solvents with a separatory funnel.

Phytochemical screening of methanol extract

The methanol extract of the plant was screened for cardiac glycosides, saponins, flavonoids, alkaloids, tannins, and terpenoids using the method described by Sofowora [6].

Quantitative phytochemical analyses of C. sativa methanol extract

The following phytochemicals were determined spectrophotometrically; carotenoids using a method described by Liaaen-Jensen & Jensen [7], lycopene using a method described by Maqsood et al [8], total chlorophyll using the method of Arnon [9], alkaloids using atropine as standard as described by Ghate et al. [9], tannins using tannic acid as standard [11], flavonoids using quercetin as standard [12], total phenolic content using gallic acid as standard [13] and saponin [14].

In vitro antioxidant profile of C. sativa solvent fractions

ABTS assay

Antioxidant activities of the fractions were also analyzed by investigating their ability to scavenge the $ABTS^{\bullet+}$ free radical using a method described by Ozgen et al. [15]. ABTS and the oxidant (potassium persulfate 0.00245 M) incubated in the dark for 15 h react to form a stable radical solution, dark blue-green in color. This resulting solution became the test reagent upon dilution to 0.7 ±0.01 at 734 nm absorbance. Reaction mixtures containing 10 µl (at varying concentrations) of samples and 1.5 ml of reagent were incubated at 30°C for 30 min in a water bath. The mixture turned colorless and the absorbance read at 734 nm.

DPPH scavenging activity

The antioxidant properties of each fraction were also investigated by determining the DPPH radical scavenging activity using the method by Brand-Williams et al. [16] 500 µL (sampleat varying concentrations) was mixed with 2700 µL of a methanol solution containing DPPH radicals (60 µM).The mixture was left to stand for 15min in the dark (until the absorbance stabilized). The ability to reduce DPPH radical was measured at 517 nm; RAS (Radical Scavenging Activity). Ascorbic acid was used as standard. The RSA was expressed as EC_{50}. The following equation was used to calculate RSA:

$$\% \text{ RSA} = ([A_{\text{DPPH}} - A_S]/A_{\text{DPPH}}) \times 100$$

A_S= solution absorbance when the fraction was added at different concentration

A_{DPPH} = DPPH solution absorbance.

Ferric reducing antioxidant power (FRAP)

This was done as described by Benzie and Strain [17]. The FRAP reagent was made up of 2500 µL of a 10 mmol/L (2,4,6-tripyridyl-s-triazine) TPTZ solution in 40 mmol/L HCl, 2500 µL of 20 mmol/L $FeCl_3 \cdot 6H_2O$ and 2500 µL of 300 mmol/L acetate buffer (pH 3.6). It was freshly prepared and warmed at 37^0C. The reaction mixture contained 900 µl FRAP reagent, 90 µl water and 30 µl of the fractions at different concentrations. Ascorbic acid was used as a control. The absorbance of the reaction mixture was read after 30 min at 593 nm after incubation at 37^0C.

Ferrous ion-chelating assay

The ferrous ion chelation activity was estimated by measuring the decrease in the absorbance (562 nm) of the iron (II) and ferrozine complex [18]. Each fraction at different concentration (2000 µL) was mixed with 3700 µL of methanol and 0.1 mL of 0.002 M $FeCl_2$. The reaction was initiated by addition of 0.2 mL of 0.005M ferrozine and control was prepared with-out plant fraction. After 10 min at room temperature, the absorbance (562 nm) was

measured. Methanol was used in the place of plant fraction in the control system. Methanol (200 µL) instead of ferrozine in sample blank.

Superoxide radical scavenging (SRS)

The superoxide anion radical scavenging potentials of the fractions were determined by the method of Salah et al. [19]. Plant fractions at different concentrations (100 µL) were mixed with 1000 µL of nitro blue tetrazolium (NBT) in a buffer (16 mM, pH 8) Tris-HCl and 1 mL of NADH solution in Tris-HCl buffer. The reaction was initiated by adding a solution of phenazine methosulfate (500 µL) to the mixture. The absorbance (560 nm) of this resultant overall mixture was read against the control samples (DMSO, 1 mL, replaced test samples in the mixture). The result equated as the scavenging activities of the fractions. Ascorbic acid was used as the standard agent in this experiment. SOS scavenging effect (%) = $[(A_1 - A_0)/ A_0]$ X100 Where A_0 = control absorbance, A_1 = sample/standard absorbance.

TBA radical scavenging property

In vitro lipid peroxidation inhibition was estimated using a modified method described by Garcia-Alonso et al. [20]. A volume of 40 µL each of the fractions (at varying concentrations) was added to a volume of 230 µL KCl (150 mM). The first control (1) was empty of the test sample. While the second control (2) contained all the reagents except the oxidized substrates. Lipid peroxidation was induced by adding 40 µL $FeCl_3$ (0.02 M) plus 40 µL ascorbate (0.05M). The reaction that was initiated by incubating the mixtures for 60 min at 37°C for 60 min, was halted by the addition of 0.01 ml of BHT, thiobarbituric acid (0.4% in HCl 0.25 N) and trichloroacetic acid (0.8 mL of 20% solution). This was then heated (15 min), cooled and centrifuged. The absorbance of the second control (used as zero) was measured (532) against that of the supernatant. The lipid peroxidation inhibiting ability was obtained as follows: Peroxidation inhibition (%) = (Control1 OD – sample OD/Control OD) × 100.

Data analysis

Results were presented as mean ± S.E.M of triplicate determination. EC_{50} were estimated with EC_{50} plot using GraphPadPrism 6.0

RESULTS

Phytochemical screening revealed the presence of cardiac glycoside, saponin, flavonoid, alkaloid, tannins, and terpenoids. Quantitative phytochemical estimation revealed high content of saponins, alkaloids, phenolic and flavonoids (Table 1).

Legend: AE: Atropine equivalent, TAE: Tannic acid equivalent, QE: Quercetin equivalent, GAE: Gallic acid equivalent.

In vitro antioxidant analyses of the fractions showed that methanol fraction exhibited comparable ABTS radical scavenging activity with ascorbic acid (Figure 1A). DPPH radical scavenging activities of the fraction were in order HF (n-hexane fraction) < MF (methanol fraction) < CF (chloroform fraction) (Figure 1B). Hexane fraction produced higher ferric acid reducing property (Figure 1C). Iron chelation capacity was highest in CF followed by methanol with hexane having the least chelation effect (Figure 1D).

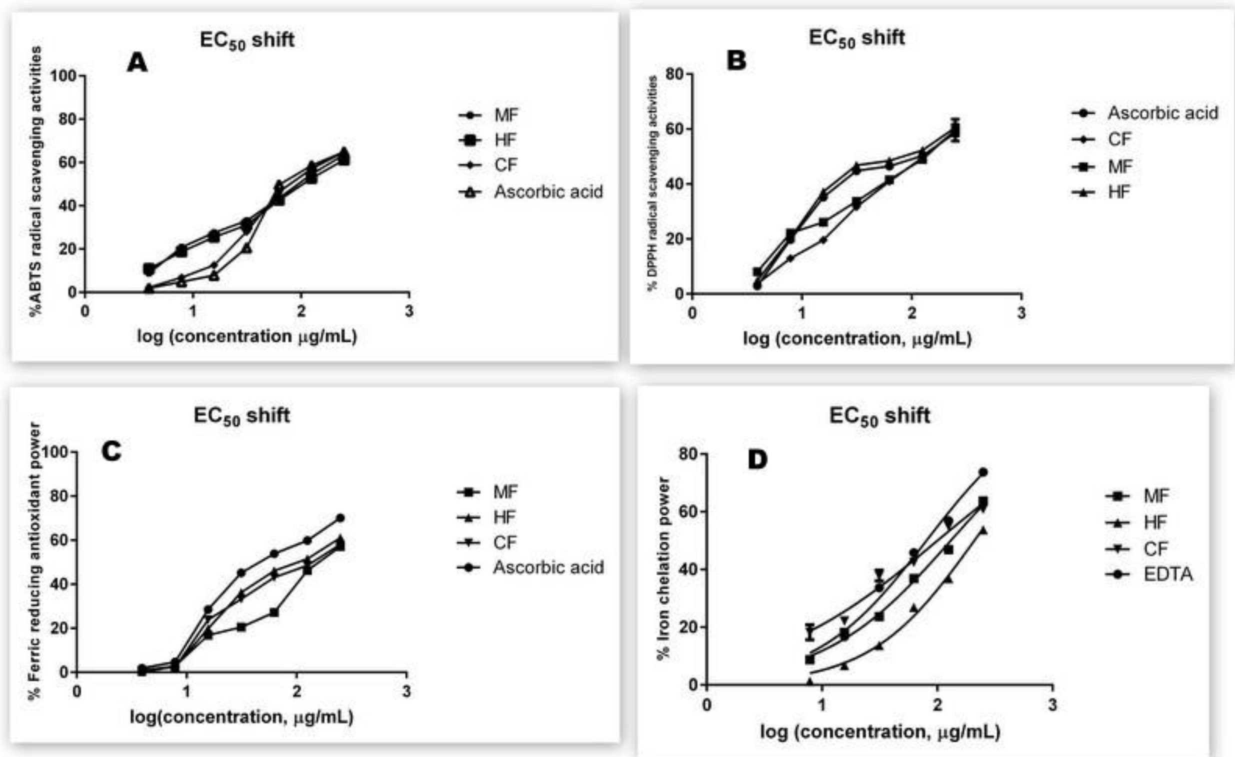

Figure 1: EC_{50} shift of the *In vitro* effects of *Cannabis sativa* fractions on (a) ABTS radical (b) DPPH radical (c) Iron reduction (d) Iron chelation

Legend: MF- methanol fraction, HF- n-hexane fraction, CF- chloroform fraction

Closely ranged superoxide radical scavenging property was observed in methanol and chloroform fractions with n-hexane fraction having the least effect (Figure 2A). Thiobarbituric species scavenging property was highest in n-hexane fraction followed by chloroform while methanol produced the least efficacy (Figure 2B).

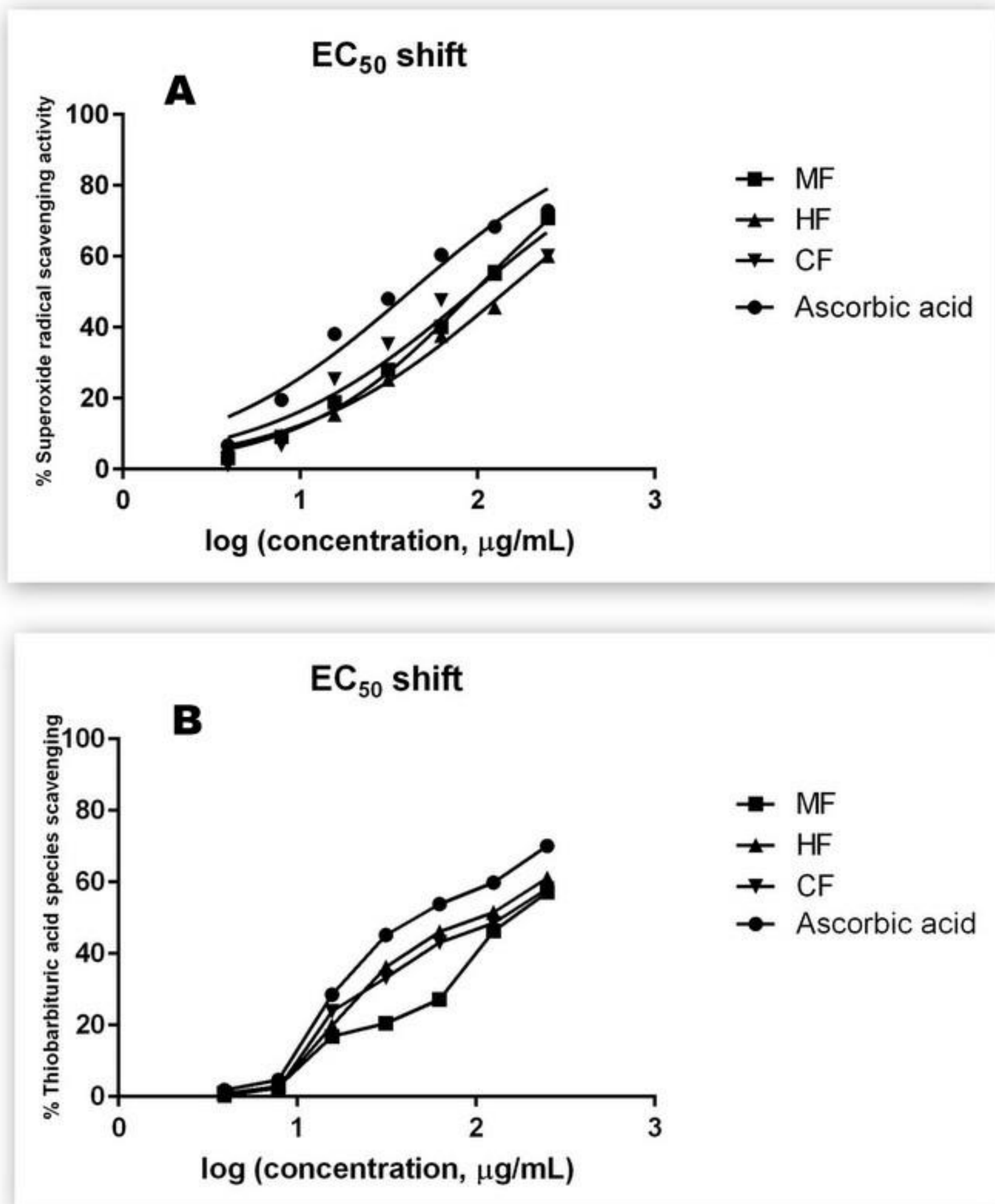

Figure 2: EC_{50} shift of the *In vitro* effects of *Cannabis sativa* on (a) Superoxide radicals (b) thiobarbituric reactive species.

Legend: MF- methanol fraction, HF- n-hexane fraction, CF- chloroform fraction

DISCUSSION

Phytochemical evaluation of *Cannabis sativa* revealed the presence of alkaloids, saponins, tannins, phenolic and flavonoids in varying quantities. The listed secondary metabolites possessed by this plant are often attributed to different therapeutic activities exhibited by some medicinal plants. Major natural alkaloids and their derivatives are medicinal agents used as anticancer, analgesic and antibacterial [21, 22]. Flavonoids in small quantities are free radical scavengers, which prevent oxidative cell damage. They have been known to produce anti-allergic, anti-inflammatory, antimicrobial, and anticancer activities [23].

As a result of the complex nature of phytochemicals in medicinal plants, a single method cannot be used to evaluate their antioxidant activity. There are several mechanisms through which antioxidants exhibit their effects i.e. by chelating metal ions, donating hydrogen to radicals, quenching singlet oxygen and radical scavenging to mention few. Hence there is a need for studying multiple antioxidant methods to attain conclusion since each antioxidant testing only measure one mechanism.

$ABTS^+$ radical cation requires electron to neutralize the positive charge, hence the scavenging of this radical is an electron transfer process. *Cannabis sativa* fractions, especially the methanol and chloroform fractions may possess bioactive compounds which are capable of producing or donating an electron, thereby enabling the formation of stable ABTS molecule.

DPPH method measures the ability of antioxidants to scavenge stable free radicals [24]. The n-hexane fraction showed higher free radical scavenging compared to chloroform and methanol fraction in the present study. This suggested that fractions of *Cannabis sativa* especially the hexane fraction may contain compounds that can donate hydrogen to free radicals to remove odd electrons.

The reducing ability of antioxidants in medicinal plants against reactive species is measurable by FRAP assay. This measurement is achieved by the ability of an antioxidant to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II). In the current study, n-hexane and chloroform fractions of *Cannabis sativa* possess greater ferric radical reduction compared to methanol fraction.

The chelating capacity of plant bioactive components is one of the mechanisms through which they exert their antioxidant effects. Since it involves reducing the concentration of catalytic transition metals, they may trigger lipid peroxidation [25]. Of the three samples, chloroform exhibit higher iron chelation effect.

Chloroform and methanol fractions produce higher superoxide radical scavenging activities compared to hexane fraction. Superoxide radical, an oxygen centred anion with selective reactivity is produced by cellular enzyme via auto-oxidation, or by non-enzymatic electron transfer [26]. The mechanism of scavenging of the radical by plant fractions may be through inhibition of the generation of the radical in the reaction mixture.

There are pointers to the medicinal benefits of cannabis shown in clinical trials (chronic pain, inflammation and Crohn’s disease), cancer (murine and cell line research models) and anecdotal claims (Alzheimer’s disease and multiple sclerosis) [27, 28]. Oxidative stress as a result of reactive oxygen species (ROS) has been shown to be responsible for these conditions. It may be inferred, therefore, that the mechanism of the medicinal effects could be the radical scavenging activities of the bioactive compounds; shown in the present study.

The crude extract of cannabis has been documented to contain a number of active metabolites, up to 500, including terpenes, flavonoids and cannabinoids [29]. Scientific evidence showed the synergistic effects of these metabolites which is responsible for the beneficial effects [30].

CONCLUSION

The extract of *Cannabis sativa* assayed in the present study showed a high content of saponins, alkaloids, phenolic and flavonoids. The fractions also showed high activities in scavenging free radicals in in-vitro studies. The present study showed varied antioxidant effects of the fractions using different methods. By inference, different bioactive molecules may be isolated and 'trapped' in different solvent-fractions. Further studies will seek to elucidate the isolated bioactive compounds with a view to studying the medicinal value.

Table 1: Phytochemical content of *Cannabis sativa* methanol extract

Phytochemicals	Content (mg/g)
Carotenoids	2.41±0.04
Lycopene	1.88±0.03
Total chlorophyll	4.36±0.11
Alkaloids (AE)	24.44±1.40
Tannins (TAE)	9.44±0.87
Flavonoids (QE)	12.66±2.31
Total phenol (GAE)	14.19±2.17
Saponin	33.98±1.95

Results were mean ± S.E.M

Table 2: EC_{50} of *in vitro* antioxidant assays of *Cannabis sativa* fractions

Fractions / Assays	MF	HF	CF	Standard	Standard used
ABTS scavenging	94.83±0.02	108.60±0.01	96.02±0.03	96.21±0.04	Ascorbic acid
DPPH scavenging	120.30±0.04	78.75±0.08	123.00±0.02	92.74±0.09	Ascorbic acid
Ferric reducing	167.70±0.03	103.40±0.06	122.00±0.06	62.86±0.06	Ascorbic acid
Iron chelation	130.7±0.02	209.10±0.02	100.00±0.03	80.41±0.02	EDTA
Superoxide radical scavenging	95.07±0.01	143.50±0.02	94.61±0.05	41.57±0.04	Ascorbic acid
Thiobarbituric acid reactive species scavenging	167.7±0.03	103.4±0.06	122.00±0.06	62.86±0.06	Ascorbic acid

Legend: MF- methanol fraction, HF- n-hexane fraction, CF- chloroform fraction

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