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Phytochemical screening and in vitro antioxidant activities of the ethanolic extract of *Hibiscus rosa sinensis* L

Anusha Bhaskar*[1] Nithya, V[2] and Vidhya, V.G[3]

[1]Department of Biotechnology, PRIST University, Vallam, Thanjavur
[2]Department of Biotechnology, Srimad Andavan College of Arts and Science, Trichy
[3]Department of Biotechnology, Faculty of Science and Humanities, SRM University, Kattankulathur, Chennai

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ABSTRACT

The study was aimed at evaluating the antioxidant activity of the ethanolic extract of Hibiscus rosa sinensis flowers at varying concentration in vitro. The extract was found to contain large amounts of phenolic compounds and flavonoids. Additionally, the reducing power (capacity to reduce Fe^{3+} to Fe^{2+}), the capacity to scavenge hydrogen peroxide, superoxide radicals and nitric oxide were evaluated. The extract exhibited a concentration dependent scavenging activity. The GC-MS study carried out showed the presence of phytochemicals like hexadecanoinc acid (RT: 26.02) hexanedioic acid (RT: 33.57) and squalene (RT: 41.55).

Keywords: Reactive oxygen species, phenolic content, flavonoids, reducing potential, *Hibiscus rosa sinensis.*

INTRODUCTION

Living cells generate free radicals and other reactive oxygen species (ROS) by-products as a result of physiological and biochemical processes. The potentially reactive derivatives of oxygen are $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$, are continuously generated inside the human body as a consequence of exposure to a plethora of exogenous chemicals in our ambient environment and /or a number of exogenous metabolic processes involving redox enzymes and bioenergetics electron transfer. Under normal circumstances, the free radicals are detoxified by the antioxidants present in the body and there is equilibrium between the ROS generated and detoxified by the antioxidants present. However, overproduction of ROS and/or inadequate antioxidant defence can easily affect and persuade oxidative damage to lipids, proteins and DNA which may eventually lead to many chronic diseases, such as cancer, diabetes, aging and other degenerative diseases in humans (1).

In recent years, there has been an increasing interest in finding natural antioxidants, which can protect the human body from free radicals and retard the progress of many chronic diseases. Natural antioxidants such as α tocopherol and ascorbic acid are widely used because they are regarded as safer and causing fewer adverse reactions but their antioxidant activities are lower than the synthetic antioxidant butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT) which have been restricted by legislative rules because they are suspected to have some toxic effects and as possible carcinogens (2). Therefore, there is considerable interest in finding safer antioxidants from natural sources to replace the synthetic ones.

Hibiscus rosa sinensis L (Malvaceae) is an ornamental plant often planted as a fence or hedge plant. It is native to China and also occurs in India and Phillipines. The flowers have been reported to possess anti-implantation and antispermatogenic activities (3,4) traditionally the plant is attributed to antifertility activity in Ayurvedic literature (5). Leaves and flowers also possess hypoglycaemic activity (6,7). The extracts of *Hibiscus rosa sinensis* have also been shown a protective effect against the tumour promotion stage of cancer development (8). Therefore, our present study is to investigate the phytochemical composition, *in vitro* antioxidant and free radical scavenging potential of this plant and the chemical constituents were studied by GC-MS.

MATERIALS AND METHODS

Chemicals

Nitro blue tetrazolium (NBT), ethylene diamine tetra acetic acid (EDTA), sodium nitroprusside (SNP), tricholoro acetic acid (TCA), thio barbituric acid (TBA), potassium hexa cyno ferrate [$K_3Fe(CN)_6$] and L-ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd India. Gallic acid and BHA was purchased from Sigma Chemicals Co (St. Louis, MO, USA). All other chemicals solvents used were of analytical grade available commercially.

Plant materials: *Hibiscus rosa sinesis* flowers were collected from Trichy, Tamil Nadu. The plant was authenticated and molecular taxonomy of the plant was done by sequencing the 18S rDNA of the plant and the sequence submitted in the Genbank (Acc. No: FJ665614).

Preparation of the extract: The flowers were shade dried at room temperature and finely powdered with the help of a hand grinding mill. About 200 g of the powder was exhaustively extracted with ethanol. The extract was concentrated to a residue. The crude extract was used for further investigation for its phytochemical compounds and potential antioxidant properties.

Phytochemical analysis of plant extracts for active components

A small portion of the dry extract was used for the phytochemical tests for compounds which include tannins, flavonoids, alkaloids, saponins and steroids in accordance with the methods [9,10] with little modifications. Exactly 1.0 g of the plant extract was dissolved in 10ml of distilled water and filtered (using Whatman No.1 filter paper). A blue colouration resulting from the addition of ferric chloride reagent to the filtrate indicated the presence of tannins in the extract. Exactly 0.5 g of the plant extract was dissolved in 5 ml of 1% HCl on steam bath. About 1 ml of the filtrate was treated with few drops of Dragendorff's reagent. Turbidity or precipitation was taken as indicative of the presence of alkaloids. About 0.2g of the extract was dissolved in 2 ml of methanol and heated. A chip of magnesium metal was added to the mixture followed by the addition of a few drops of concentrated HCl. The occurrence of a red or orange colour was indicative of flavonoids. About 2ml of the extract was vigorously shaken in the test tube for 2 min. It was observed for frothing. To about 1 ml of the extract 5 drops of concentrated

H_2SO_4 was added in a test tube. Red coloration was observed which is indicative for the presence of steroids.

Determination of total phenols

The total phenol content in the ethanolic extract of *H. rosa sinensis* was determined with Folin Ciocalteau reagent by the method of [11]. To 2.5 m of 10% Folin Ciocalteu reagent and 2 ml of Na_2CO_3 (2% w/v) was added to 0.5 ml of each sample (3 replicates) of plant extract solution (1 mg/ml). The resulting mixture was incubated at 45° C with shaking for 15 min. The absorbance of the samples was measured at 765 nm using UV/visible light. Results were expressed as milligrams of Gallic acid (0-0.5 mg/ml) dissolved in water.

Estimation of flavonoids

Aluminium chloride colorimetric method was used for flavonoids determination. One millilitre (1 ml) of sample was mixed with 3 ml of methanol, 0.2 ml of 10% aluminium chloride, 0.2 ml of 1M potassium acetate and 5.6ml of distilled water and remains at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420 nm with UV visible spectrophotometer. The content was determined from using gallic acid as standard solution (0-0.8 mg/ml). The concentration of flavonoids was expressed in terms of mg/ml.

Determination of proanthocyanidins

Total proanthocyanidin was determined based on the procedure of Sun et al [12]. The mixture of 3 ml of vannilin-methanol (4% v/v), 1.5 ml of hydrochloric acid was added to 0.5 ml of extract dissolved and vortexed. The resulting mixture was allowed to stand for 15 min at room temperature followed by the measurement of the absorbance at 500 nm. Total proanthocyanidin content was expressed as gallic acid equivalent (mg/ml) from the standard curve.

Determination of antioxidant and antiradical assays

Total reduction capability

The reducing power of *H. rosa sinensis* was determined by the method of Oyaizu [13]. The mixture containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6.) and 2.5 ml of $K_3Fe(CN)_6$ (1% w/v) was added to 1.0 ml of extract dissolved in distilled water. The resulting mixture was incubated at 50 °C for 20 min, followed by the addition of 2.5 ml of TCA (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 ml), mixed with distilled water (2.5 ml) and 0.5 ml of $FeCl_3$ (0.1%, w/v). The absorbance was then measured at 700 nm against blank sample.

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay

One millilitre of 0.135 mM DPPH prepared in methanol was mixed with 1.0 ml of extract dissolved in distilled water ranging from 0.2-0.8 mg/ml. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm. The scavenging ability of the plant extract was calculated using this equation:

DPPH Scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$

Where $Abs_{control}$ is the absorbance of DPPH + methanol; Abs_{sample} is the absorbance of DPPH radical + sample (i.e the plant extract or standard)

Superoxide anion scavenging activity assay

The scavenging activity of superoxide anion was determined by the method of Yen and Chen [14]. The reaction mixture consists of 1 ml of plant extract (1 mg/ml), 1 ml of PMS (60 µM) prepared in phosphate buffer (0.1 M pH 7.4) and 1 ml of NADH (phosphate buffer) was incubated at 25 °C for 5 min, the absorbance was read at 560 nm against blank samples.

The superoxide anion scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0] \times 100,$$

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

Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by the plant extract was determined by the method of Ruch et al [15]. Plant extract (4 ml) prepared in distilled water at various concentrations was mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M, pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm against blank solution containing the plant extract without H_2O_2.

Nitric oxide scavenging activity

The method of Garratt [16] was adopted to determine the nitric oxide radical scavenging activity of extract of *H. rosa sinensis* reconstituted in distilled water. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generated nitric oxide which interacts with oxygen to produce nitrite ions determined by the use of Griess reagents. Two millilitre of 10 mM sodium nitroprusside dissolved in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract at various concentrations (0.2-0.8 mg/ml). The mixture was incubated at 25 °C after 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally 1.0 ml naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm was measured with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated.

GC – MS analysis

Preparation of the extract

Flowers of *Hibiscus rosa sinensis* were shade dried. 20 g of the powdered flowers were soaked in 95% ethanol for 12 h. The extracts were then filtered through Whatmann filter paper No. 41 along with 2 g sodium sulphate to remove the sediments and traces of water in the filtrate. Before filtering, the filter paper along with sodium sulphate was wetted with 95% ethanol. The filtrate was then concentrated by bubbling nitrogen gas into the solution. The extract contained both polar and non-polar phytocomponents of the plant material used. 2µl of these solutions was employed for GC/MS analysis [17].

GC analysis

GC-MS analysis was carried out on a GC clarus 500 Perlin Elmer system comprising a AOC-20i autosampler and gas chromatograph interfaced to a mass spectrophotometer (GC – MS) instrument employing the following conditions: column Elite – 1 fused silica capillary column (30 x 0.25 mm ID x 1 EM df, composed of 100% Dimethyl polysiloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1 ml/min

and an injection volume of 0.5 EI was employed (split ratio of 10:1 injector temperature 250 C; ion-source temperature 280 C. The oven temperature was programmed from 110 C (isothermal for 2 min). with an increase of 10 C/min, to 200 C then 5 C/min to 280 C, ending with a 9 min isothermal at 280 C. Mass spectra were taken at 70 eV; a scan interval of 0.5s and fragments from 40 to 550 Da.

Identification of components

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

Statistical analysis

All values are expressed as mean ± S.D. Statistical analysis were performed by Student's *t*-test. The values of p lower than 0.05 were considered significant (p is probability)

RESULTS AND DISCUSSION

Table 1 shows the results of phytochemical analysis of the flower extract of *H. rosa sinensis*. Tannins, saponins, alkaloids, steroids, flavonoids were found in the extract. Total phenolics present in the ethanolic extract was 259±6.5 mg/g and flavonoids and proanthocyanidin were found to be 347±7.4 and 55 ± 0.5 respectively, all of which are expressed as gallic acid equivalent. Since phenolic compounds and flavonoids are responsible for the antioxidant activity, the amounts present in the extract are high indicating good antioxidant activity. The scavenging ability of the phenolics and flavonoids are mainly due to the presence of hydroxyl groups. The presence of phenolic compounds in the plant contributed to its antioxidant activity and thus usefulness of the plant as a medicament. Flavonoids have been shown to exhibit the actions through effects on membrane permeability, and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2 [18].

The reducing power of the compound may serve as a significant indicator of its potential antioxidant activity. Reducing power assay is used to evaluate the ability of natural antioxidant to donate electron [19]. Fig 1 shows the reducing power of the ethanolic extract of *H. rosa sinensis* compared to BHT as standard. The reducing power of the extract was found to be significant and dose dependent.

The capacity to scavenge DPPH, $O_2^{\bullet -}$, H_2O_2 and NO by the ethanolic flower extract of *H. rosa sinensis* is presented in Table 2. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. A freshly prepared DPPH solution exhibited a deep purple colour with a maximum absorption at 517 nm. The purple colour disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals and convert them to a colourless product, resulting in a decrease in absorbance at 517 nm. In this quantitative assay the extract exhibited a notable dose dependent inhibition of the DPPH activity. At a concentration of 250 µg/ml, *H. rosa sinensis* flower extract scavenged 95% of DPPH radicals and had an IC_{50} value of 45 µg/ml.

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated [20]. Although O2 is the primary free radical in the biological system it by itself is quite un-reactive. However, the system converts it into more reactive species viz., H_2O_2 and OH radicals. The scavenging activity of the flower extract compared favourably with the standard BHA suggesting that the plant is also a potent scavenger of superoxide radical.

Hydrogen peroxide though a weak oxidizing agent is important because of its ability to penetrate biological membranes, once inside the cell it can probably react with Fe^{2+} and Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects [21]. The extract also scavenged H_2O_2, however compared to O_2, H_2O_2 was scavenged weakly by the extract.

The toxicity and damage caused by NO and O2 is multiplied as they react to produce reactive peroxynitrile (ONOO-), which leads to serious toxic reactions with biomolecules. In the present study the crude ethanol extract of *H. rosa sinensis* showed a remarkable dose dependent inhibitory effect of nitric oxide radical scavenging activity (Table 2). A maximum inhibitory effect was seen at 50 µg/ml of the extract. The plant may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Nitric oxide is implicated in inflammation, cancer and other pathological conditions [22].

The GC-MS study of *Hibiscus rosa sinensis* flowers has shown many phytochemicals which contribute to the medicinal activity of the plant (Table 3). The major components present in the flowers are 2,3-hexanediol (RT:17.59), n-Hexadecanoic acid (RT:26.02), 1,2-Benzenedicarboxylic acid (RT: 35.91) and squalene (RT: 41.55).

Table 1: The phytochemical components of *H. rosa sinensis* based on the preliminary crude flower extract screening

Phytochemical Compounds	Presence	Extract equivalent to Gallic (mg/g)
Tannins	++	ND
Flavonoids	+++	ND
Steroids	++	ND
Alkaloids	++	ND
Saponins	+	ND
Total phenols	+++	259 ± 6.5
Total flavonoids	+++	347 ±7.4
Total proanthocyanidin	+	55 ± 0.5

+++ = appreciable amount (positive within 5 min); ++ = moderate amount (positive after 5 min but within 10 min); + = trace amount (positive after 10 min but within 15 min)

Table 2 Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) by *H. rosa sinensis* flower extract

Group	DPPH (% of control)	$O_2^{\bullet-}$ (% of control)	H_2O_2 (% of control)	NO (% of control)
Control	100 ± 3.5	100 ± 3.2	100 ± 3.7	100 ± 3.0
H. rosa sinensis extract (µg/ml)				
10	23.4 ± 4.0	31.7 ± 6.0	47.4 ± 3.2	32.9 ± 2.6
20	35.7 ± 5.4	46.9 ± 5.4	59.3 ± 3.5	41.7 ± 3.8
50	49.7 ± 4.5	85.4 ± 4.9	95.2 ± 4.0	83.0 ± 3.2
100	38.5 ± 3.3	77.8 ± 5.9	90.5 ± 3.6	80.7 ± 2.8
250	4.0 ± 0.34	62.4 ± 5.0	82.7 ± 4.9	65.4 ± 2.6
500	0	54.2 ± 3.8	61.6 ± 4.0	54.2 ± 0.9
BHA (µM)				

20	54.8 ± 4.5	-	-	-
200	-	33.2 ± 2.3	31.2 ± 2.0	62.4 ± 3.4

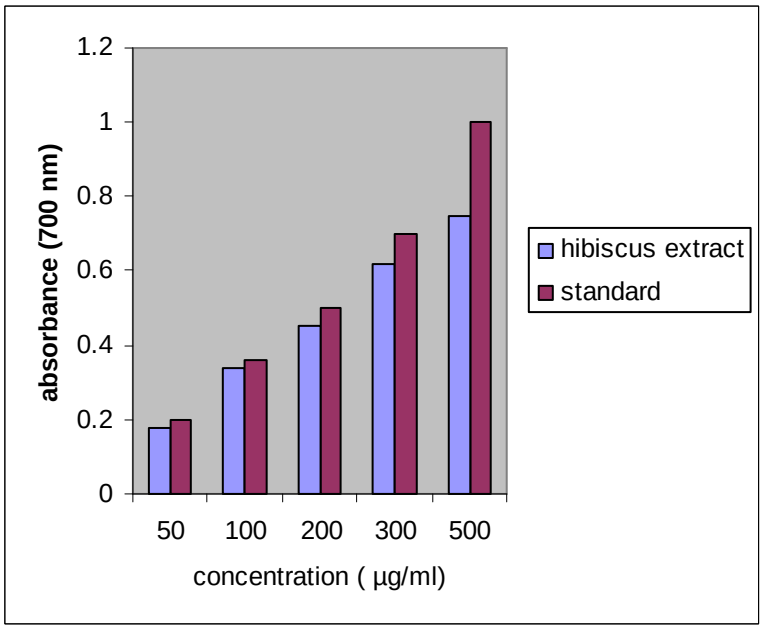

Fig.1. Reducing power of *Hibiscus rosa sinensis* and butylated hydroxyl toluene (BHT). Reducing power was measured spectrophotometrically as Fe3+ to Fe2+ transition. Each value is mean of 5 replicates.

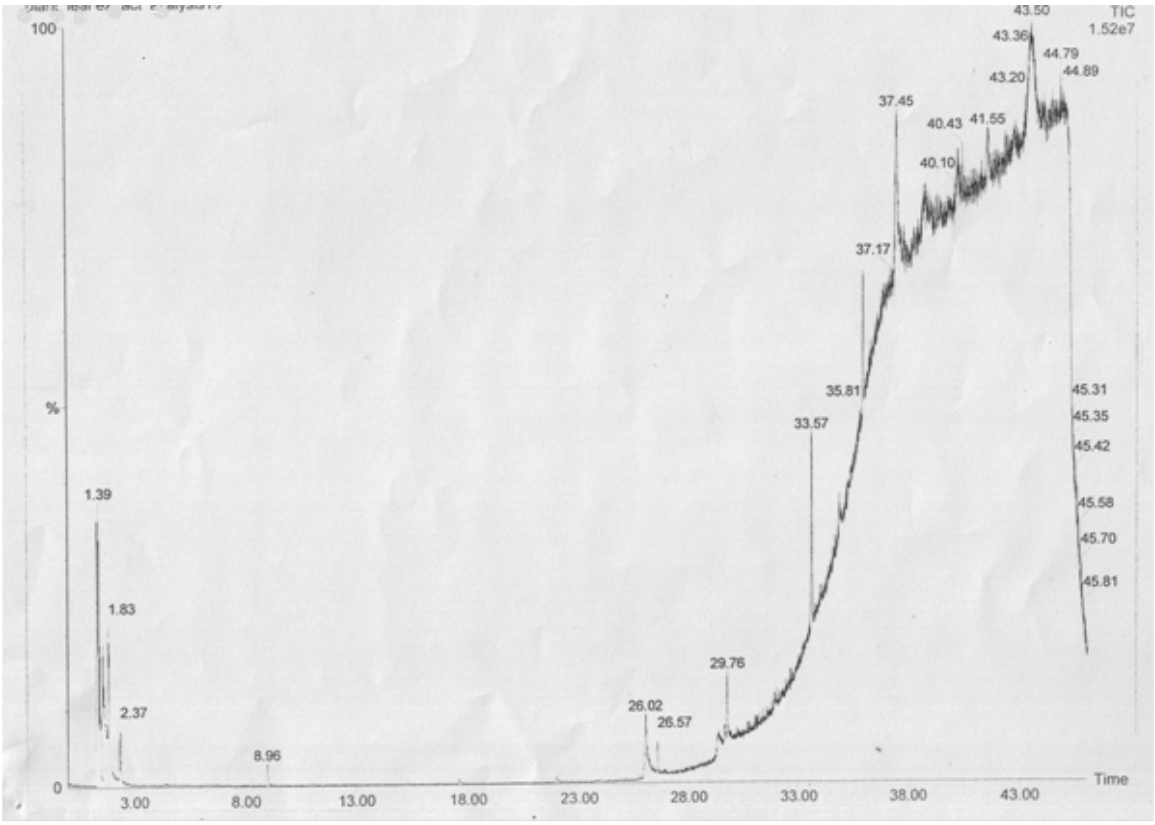

Fig 2: Chromatogram of *H. rosa sinensis* by GC-MS

Table 3 Phytocompounds identified in the ethanolic extract of the flowers of *H. rosa sinensis* by GC-MS

RT	Name of the compound	Peak area (%)
8.96	Propanol,3,3'-dithiobis(2,2-dimethyl-	1.39
17.59	SS)- or (RR)-2,3-hexanediol	0.45
21.98	2-Hydroxy-2-methylbutyric acid	0.32
26.02	n-Hexadecanoic acid	8.78
26.57	Heptanoic acid, 2-ethyl-	1.42
29.65	Trans-(2-Ethylcyclopentlyl)methanol	1.83
29.76	3-N-Hexylthiolane, SS-dioxide	7.24
33.57	Hexanedioic acid, bis(2-ethylexyl) ester	12.88
35.91	1,2-Benzenedicarboxylic acid, diisoocytl ester	7.66
37.45	1,3-Benzodioxole, 5.5'- (tetrahydro-1H,3H-furo {3,4-c)furan-1,4-diyl)bis-, (1S-(1α,3a α,4β,6a α))-	23.49
41.55	Squalene	5.25
43.50	2R-Acetoxymethyl-1,3,3-trimethyl-4t-(3-methyk-2-buten-1-yl)-1c-cyclohexanol	29.28

CONCLUSION

This study affirms the in vitro antioxidant potential of crude extract of the flower of *H. rosa sinensis*, with comparable results to that of the standard BHA. The phytochemicals in the flower makes it a pharmacologically effect antioxidant. However, we are now focussing on the active principle in the extract which contributes to such activity. The findings suggest that *H. rosa sinensis* could be a potential source of natural antioxidant that could have great importance as therapeutic agent.

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