ORIGINAL ARTICLE

Phytochemical analysis and *in vitro* antioxidant activity of *Uncaria gambir*

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The present research work was carried out to evaluate the antioxidant potential and antiradical property of methanolic extract of *Uncaria gambir.* Antioxidant and radical scavenging activity were determined by using different *in vitro* assays including reducing power assay, superoxide anion scavenging activity assay, hydroxyl radical scavenging activity assay, nitric oxide scavenging activity assay, DPPH free radical scavenging assay and hydrogen peroxide method. Preliminary phytochemical screening revealed that the extract of *U. gambir* possesses flavonoids, alkaloids and phenolic materials. In the present investigation, quantitative estimation of flavonoids content and phenolic content was also carried out by colorimetric methods, using aluminium chloride and Folin–Ciocalteu reagent method, respectively, to establish relationship between antioxidant activity and total phenolics and flavonoid contents. The total phenolic and flavonoid contents were found to be 18.37±2.79 mg gallic acid equivalents (GAE)/g dry weight and 5.82±2.23 mg rutin equivalents (RE)/g dry weight, respectively. The extract showed significant antioxidant activity in a dose-dependent manner in all the assays. The IC_{50} values of all parameters were determined. Ascorbic acid was used as standard. The results obtained in the present study indicated that *U. gambir* extract could be a potential source of natural antioxidant.

Key words: Antioxidant, ascorbic acid, DPPH, hydroxyl radical, *Uncaria gambir*

INTRODUCTION

Reactive oxygen species (ROS) such as singlet oxygen, superoxide anion, hydroxyl radical and hydrogen peroxide (H_2O_2) are often generated as by products of biological reactions or from exogenous factors.[1] These reactive species exert oxidative damaging effects by reacting with nearly every molecules found in living cells [2] including DNA, if excess ROS are not eliminated by the antioxidant system. They play important roles in aging and in the pathogenesis of age-related disorders such as cancer, hypertension, atherogenesis, Alzheimer's disease and Parkinson's disease.[3-5] Recent investigations have shown that the antioxidants with free-radical scavenging properties of plant origins could have great importance as therapeutic agents in ageing process and free radical-mediated diseases including neuro-degeneration.[6,7] Plant extracts[8] and plant products such as flavonoids and other polyphenolic constituents have been reported to be effective radical scavengers and inhibitors of lipid peroxidation.[9,10] Many synthetic antioxidant compounds have shown toxic and/or mutagenic effects, which have stimulated the interest of many investigators to search natural antioxidant.

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Uncaria gambir is known as safed kathha belongs to family Rubiaceae, a native Southeast Asian herbal plant, can mostly be found in countries such as Indonesia and Malaysia.[11] The earlier study have demonstrated potent anti-inflammatory activity and hypotensive effects.[12] Many general traditional medicinal uses of *U. gambir* include treatments for wounds and ulcers, fevers, headaches, gastrointestinal illnesses and bacterial/fungal infections.[13] It had been widely used as an astringent medicine for the treatment of spongy gums, tooth acne, diarrhoea and sore throat.[14] Besides being chewed, it is also imported in large quantities by the West for tanning, calico printing and dyeing purposes.[15] Previous studies showed that gambir consists mostly of the flavan monomer, which contains (+)-catechin, (+)-epicatechin and alkaloids.[16] Gambirdine and isogambirdine also isolated from *U. gambir.*[17]

Free radical-scavenging activity of *U. gambir* by the 2, 2-diphenyl-1-picryl hydrazyl (DPPH) method has been reported.[18] In the present investigation, phytochemical screening, quantitative estimation of total phenolic and total flavonoid have been carried out followed by antioxidant activity correlation between antioxidant activity and total phenolic and total flavonoid contents were also investigated in order to establish if there is a relationship between these groups of phytochemical and antioxidant activity. In view of its wide use and

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its chemical composition, the methanolic extract of *U. gambir* was evaluated for its *in vitro* anti oxidative activities. Antioxidant activity *U. gambir* was evaluated by the reducing power method, superoxide anion-scavenging method, hydroxyl radical-scavenging method, DPPH radical-scavenging method, nitric oxide-scavenging method, H_2O_2-scavenging method for the first time.

MATERIALS AND METHODS

Chemicals

Folin–Ciocalteu reagent, DPPH radical, nitro blue tetrazolium (NBT), phenazine methosulfate, nicotinamide adenine dinucleotide, sodium nitroprusside (SNP), trichloro acetic acid (TCA), thio barbituric acid (TBA), potassium hexa cyano ferrate [$K_3Fe(CN)_6$] and L-ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd, India. Catechin and rutin were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals and solvents used were of analytical grade available commercially.

Preparation of Extract

The leaf and shoot aqueous extract of *U. gambir* (100 g) was extracted exhaustively in a Soxhlet apparatus with methanol, (99%, 500 ml) for 6 h at 50ºC. The solvent was completely removed by rotary evaporator (Rotavapor® R-210, BUCHI Corporation) and obtained greenish gummy exudates. This crude extract was stored at low temperature in refrigerator and used for phytochemical analysis and evaluation of antioxidant activity.

Phytochemical Screening

The freshly prepared extract of *U. gambir* was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using standard procedures.[19,20]

Determination of Total Phenolic Content

Total phenols were determined by Folin–Ciocalteu reagent.[21] A dilute extract of plant extract (0.5 ml of 10 mg/ml) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by calorimetry at 765 nm (Schimadzu UV–Vis 1601). The standard curve was prepared using 25, 50, 100, 150, 200, 250, 300 µg/ml solutions of gallic acid in methanol. The concentration of total phenolic compounds in extract was expressed as milligram of GAE per g dry weight of extract.

Determination of Total Flavonoid Content

The aluminium chloride colorimetric method was used for flavonoids determination.[21] Plant extract (0.5 ml of 10mg/ml) in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm (Schimadzu UV-Vis 1601). The calibration curve was prepared by preparing rutin solutions at concentrations 10 to 100 µg/ml in methanol. The concentration of total flavonoids compounds in extract was expressed as mg of rutin equivalents per gram dry weight of extract.

In vitro Antioxidant Activity

Reducing power assay

The reducing power of *U. gambir* was determined according to the method described.[22] Different concentrations of *U. gambir* extract (10–50 µg/ml) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml. 0.1%) and the absorbance was measured at 700 nm (Schimadzu UV–Vis 1601). Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a reference standard. Phosphate buffer (pH 6.6) was used as blank solution.

Superoxide anion scavenging activity assay

The scavenging activity of the *U. gambir* towards superoxide anion radicals was measured.[23] Superoxide anions were generated in a non-enzymatidc phenazine methosulfate nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments, the superoxide anion was generated in 3 ml of phosphate buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 µM) solution, 0.75 ml of NADH (936 µM) solution and 0.3 ml of different concentrations (10 µg/ml – 50 µg/ml) of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in a spectrophotometer (Schimadzu UV–Vis 1601). The super oxide 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 (without extract) and A_1 was the absorbance of the extract or standard.

Hydroxyl radical scavenging activity assay

The scavenging activity for hydroxyl radicals was measured with Fenton reaction.[24] Reaction mixture contained 60 µl

of 1.0 mM $FeCl_2$, 90 µl of 1mM 1,10-phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M H_2O_2 and 1.5 ml of extract at various concentrations. Adding H_2O_2 started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560 nm was measured with a spectrophotometer (Schimadzu UV–Vis 1601). The hydroxyl radicals scavenging activity was calculated.

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

Where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance of the extract or standard.

Nitric oxide scavenging activity assay

Nitric oxide radical activity was determined according to the method.[25] Sodium nitroprusside in an aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. Two millilitre of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of *U. gambir* extract at various concentrations and the mixture was incubated at 25ºC for 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 naphthyl ethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm.

% 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 of the extract or standard.

Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical

The free radical-scavenging capacity of the extracts was determined using DPPH.[26] A DPPH solution (0.004% w/v) was prepared in 95% methanol. Methanolic extract of *U. gambir* was mixed with 95% methanol to prepare the stock solution (1 mg/ml). The freshly prepared DPPH solution (0.004% w/v) was taken in test tubes then *U. gambir* extract was added followed by serial dilutions (10 to 50 µg/ml) to every test tube so that the final volume was 3 ml and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (Schimadzu UV–Vis 1601). Ascorbic acid was used as a reference standard and dissolved in double distilled water to make the stock solution with the same concentration (1 mg/ml) followed by serial dilutions (10 to 50 µg/ml). Control sample was prepared containing the same volume without any extract and reference ascorbic acid.

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 of the extract or standard.

Scavenging of Hydrogen Peroxide

The ability of *U. gambir* to scavenge H_2O_2 was determined according to the method.[27] A solution of H_2O_2 (2 mM) was prepared in phosphate buffer (pH 7.4) and concentration was determined spectrophotometrically at 230 nm (Schimadzu UV–Vis 1601). *U. gambir* extract (5–25 µg/ml) in distilled water was added to a H_2O_2 solution (0.6 ml, 2 mM) and the absorbance of H_2O_2 at 230 nm was determined after 20 min against a blank solution in phosphate buffer without H_2O_2. The percentage of scavenging of H_2O_2 of *U. gambir* and standard compounds was calculated by using the equation.

% 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 of the extract or standard.

RESULTS

Phytochemical Screening

Preliminary phytochemical screening of the extract of *U. gambir* revealed the presence of various bioactive components of which flavonoids and tannins were the most prominent and the result of phytochemical test are summarized in Table 1.

Total Phenolic Content

The total phenolic content of *U. gambir* extract was measured by using the Folin–Ciocalteu colorimetric method. In *U. gambir* extract, the phenolic content was found to be 18.37±2.79 mg gallic acid equivalents (GAE)/g dry weight [Table 2].

Total Flavonoid Content

Therefore, in the present study, total flavonoid content present in extract was estimated using the aluminium chloride colorimetric method. In *U. gambir* extract, the

Table 1: Phytochemical screening of methanolic extract of *Uncaria gambir*

Alkaloid	+
Sterol	+
Carbohydrate	+
Phenolic compound	+
Flavonoid	+
Proteins and amino acids	+
Lipid/fat	–
Mucilage	–
Resin	+

flavonoid content was found to be 5.82±2.23 mg rutin equivalents (RE)/g dry weight [Table 2].

In vitro Antioxidant Activity

Reducing power assay

The reducing potential of the *U. gambir* extract and ascorbic acid was very potent and the power of the extract was increased with quantity of sample [Figure 1]. It was determined by using a modified iron (III) to iron (II) reduction assay.

Superoxide anion scavenging activity assay

The superoxide anion radical scavenging activity of *U. gambir* extract assayed by the PMS–NADH system is shown in Figure 2. The superoxide scavenging activity of *U. gambir* extract was increased markedly with the increase in concentrations. Thus, higher inhibitory effects of the rhizomes extracts on superoxide anion formation noted herein possibly renders them as a promising antioxidants. The half inhibition concentration (IC_{50}) of *U. gambir* extract was 35.95µg/ml while IC_{50} value for ascorbic acid was 16.15µg/ml. These results suggested that *U. gambir* extract has a potent superoxide radical scavenging effects.

Hydroxyl radical scavenging activity assay

Activity of the rhizomes extract on hydroxyl radical has been shown in Figure 3. *U. gambir* extract exhibited concentration dependent scavenging activity against hydroxyl radical generated in a Fenton reaction system. The IC_{50} value was found to be 14.83 µg/ml while IC_{50} value for ascorbic acid was 1.27 µg/ml.

Nitric oxide scavenging activity assay

The percentages of inhibitions were increased with increasing concentration of the extracts. The IC_{50} value for scavenging of nitric oxide for *U. gambir* extract was 34.20 µg/ml, while the IC_{50} value for ascorbic acid was 3.90 µg/ml [Figure 4].

Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical

Figure 5 shows the DPPH radical scavenging activity of the

Table 2: Total phenolic and flavonoid content of *Uncaria gambir* extract

Total phenolic content (mg GAE/g dry weight)	Mean±SEM	Total flavonoid content (mg RE/g dry weight)	Mean±SEM
18.6		5.70	
17.8		6.26	
18.6		5.41	
	18.37±2.79		5.82±2.23

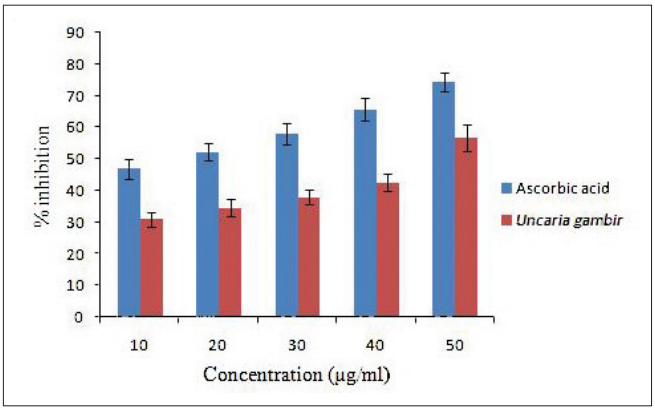

Figure 1: Reducing power assay

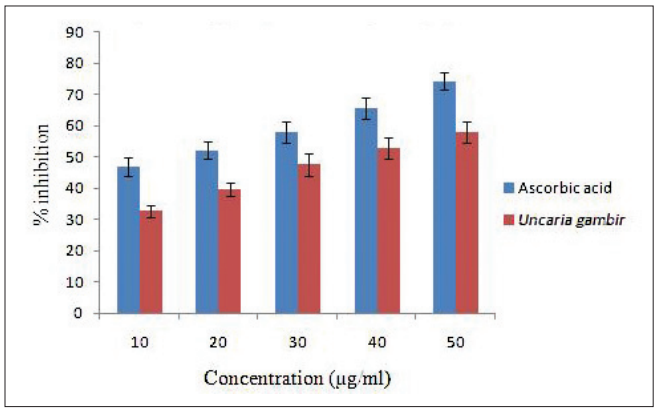

Figure 2: Superoxide anion scavenging activity assay

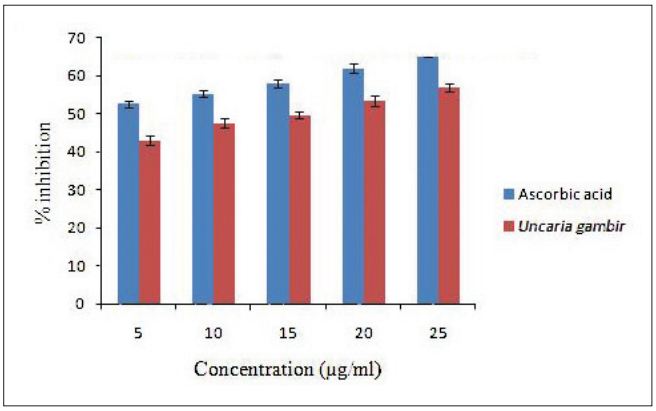

Figure 3: Hydroxyl radical scavenging activity assay

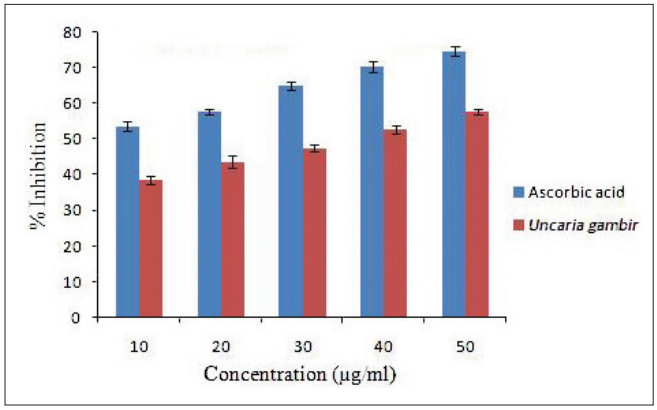

Figure 4: Nitric oxide scavenging activity assay

U. gambir extract, compared with ascorbic acid, as standard. The IC_{50} values of extract and standard were 18.27 µg/ml and 7.79 µg/ml, respectively.

Scavenging of hydrogen peroxide

As shown in Figure 6, *U. gambir* extract also demonstrated H_2O_2 decomposition activity in a concentration dependent manner with an IC_{50} of 11.75µg/ml, while IC_{50} value for ascorbic acid was 2.60µg/ml.

DISCUSSION

Phenolic compounds are known to be powerful chain breaking antioxidants and are important constituents of plants. Phenolic compounds may contribute directly to antioxidative action. It is suggested that phenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1.0 g daily from a diet rich in fruits and vegetables.[28] It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process.[29,30] In reducing power assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, by measuring the formation of Perl's Prussian blue at 700 nm, we can monitor the Fe^{2+} concentration. The reducing properties are generally associated with the presence of reductants[31] which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[32] It is well known that superoxide anions damage biomolecules directly or indirectly by forming H_2O_2, OH, peroxy nitrite or singlet oxygen during aging and pathological events such as ischemic reperfusion injury. Superoxide has also been observed to directly initiate lipid peroxidation.[33] Hydroxyl radical is highly reactive oxygen centred radical formed from the reaction of various hydroperoxides with transition metal ions. It attacks proteins, DNA, polyunsaturated fatty acid in membranes and most biological molecule. In addition to ROS, nitric oxide is also implicated in inflammation, cancer and other pathological condition. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability.[34] Although the DPPH radical-scavenging abilities of the extracts were less than those of ascorbic acid, the study showed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. H_2O_2 is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. H_2O_2 can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form

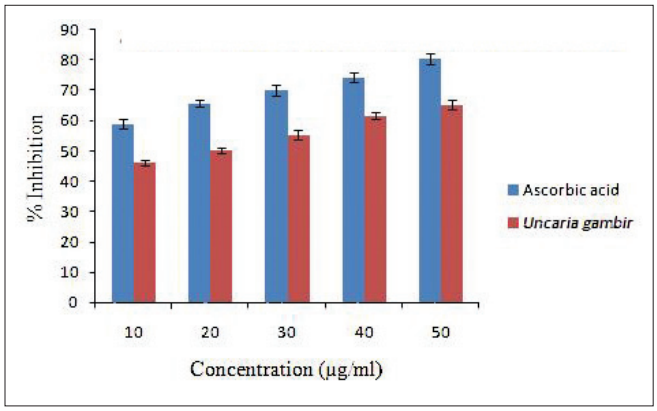

Figure 5: Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical

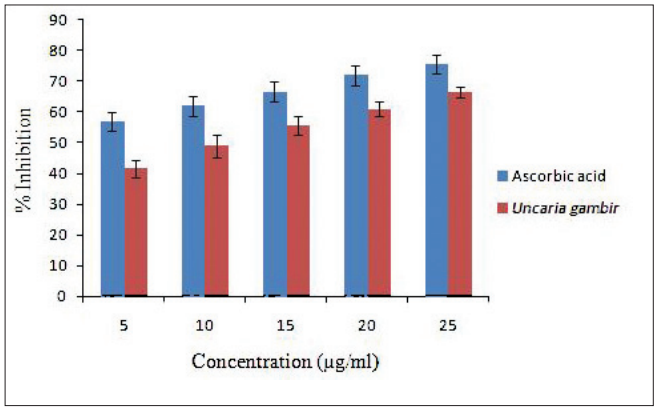

Figure 6: Scavenging of hydrogen peroxide

hydroxyl radical and this may be the origin of many of its toxic effects.[35] Phytochemical screening of the *U. gambir* revealed the presence of phenolic and flavonoid glycosides. Flavonoids have been shown to have antioxidant, antibacterial anti-inflammatory, antiallergic activity, etc.[36] The presence of phenolic and flavonoids in the drug extract is likely to be responsible for the antioxidant activity. These compounds are reported to be antioxidant or free radical scavengers.[37]

CONCLUSION

The results obtained in the present study indicate that *U. gambir* extract exhibits free radical scavenging, reducing power. The overall antioxidant activity of *U. gambir* extract might be due to its flavonoid, polyphenolic and other phytochemicals constituents. The findings of the present study suggested that *U. gambir* could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of aging and age associated oxidative stress related degenerative diseases. Hence, it is worthwhile to isolate and elucidate the bioactive principle that one responsible for antioxidant activity that is under process in our laboratory.

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