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Research article

IN VITRO ANTIOXIDANT ACTIVITY OF METHANOL EXTRACT OF THE INNER BARK OF *GUETTARDA SPECIOSA* L. (RUBIACEAE)

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ABSTRACT

The aim of present study was to estimate the Antioxidant activity of methanol extract of the inner bark of *Guettarda speciosa L.* (Rubiaceae) was assessed by using 2,2- diphenyl-1-picryl-hydrazyl (DPPH•) assay and reducing power activity. Here, ascorbic acid was used as standard antioxidant. The results of the study indicate that the methanolic extracts of the inner bark of *Guettarda speciosa L.* (Rubiaceae) possess significant scavenging activity against DPPH• and reducing power activity. The free radical scavenging and antioxidant activities may be attributed to the presence of adequate flavonoid compounds in methanol extract of the inner bark of *Guettarda speciosa L.* (Rubiaceae). This study revealed that the methanolic extract of methanol extract of the inner bark of *Guettarda speciosa L.* (Rubiaceae) has demonstrated significant antioxidant activity.

Key Words:- DPPH assay, Reducing power activity, *Guettarda speciosa L.*

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INTRODUCTION

Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (Sies, 1997). Plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells.

Oxidative stress might be an important part of many human diseases; the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke, dementia and neurodegenerative diseases. Antioxidants are widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease. In addition antioxidants are added to a variety of foods to prevent the lipid peroxidation. These compounds have many industrial uses, such as preservatives in food and cosmetics and preventing the degradation of rubber and gasoline. Antioxidant compounds are obtained from natural foods and β-carotene, vitamin-C, vitamin-E and tocopherol.

MATERIALS AND METHODS

Materials Used

- Powdered plant material.
- Soxhlet apparatus.
- **Solvents:** Methanol
- **Reagents:** Mayer's reagent, Dragendroff's reagent, Hager's reagent, Wagner's reagent and Fehling's A & B.
- **Chemicals:** HCl, H_2SO_4, Tannic acid, α-Napthol, Acetic anhydride, Glacial acetic acid, NaOH, Pyridine,

Sodium nitroprusside, Dil. Ammonia, Dil. Ferric chloride, 10% NaCl, 10% Lead acetate solution and Zinc dust.

Methodology

Extraction of Plant Material

The inner bark of *Guettarda speciosa L.* (Rubiaceae) were collected, cleaned, dried in shade and pulverized in a grinder-mixer to obtain a coarse powder and then passed through a 40-mesh sieve. The air-dried powdered plant material (200gm) was extracted with methanol by using Soxhlet apparatus for 48hrs. The solvent is removed from extracts by distillation under reduced pressure. The concentrated extract was kept in a dessicator and was used for further experiment. The extract was weighed and its percentage in terms of air-dried weight of plant material was calculated and also the consistency of the extracts was noted.

Phytochemical Tests

The methanol extracts of *Guettarda speciosa L.* (Rubiaceae) obtained were subjected to preliminary phytochemical screening for the detection of various phytochemical constituents such as alkaloids, carbohydrates, steroids, triterpenoids, flavonoids, glycosides, saponins, tannins, fixed oils, gums and mucilages. The preliminary phytochemical screening was performed according to standard procedures (Harborne, 1998; Khandelwal, 2003; Kokate *et al*., 2004).

1. Test for Alkaloids

A small portion of extracts were stirred separately with few drops of dilute hydrochloric acid and filtered. The filtrate was tested with various reagents for the presence of alkaloids.

- **Mayer's reagent** (Potassium mercuric iodide)**:** Formation of Cream colored precipitate
- **Dragendroff's reagent** (Potassium bismuth iodide)**:** Formation of Orange red colored precipitate
- **Hager's reagent** (Saturated solution of picric acid)**:** Formation of Yellow colored precipitate
- **Wagner's reagent** (Iodine in potassium iodide)**:** Formation of Reddish colored brown precipitate
- **Tannic acid solution:** Formation of Buff colored precipitate.

2. Test for Carbohydrates

- **Molisch's test:** To the test substance few drops of α-Napthol solution in alcohol was added, shaken well and concentrated H_2SO_4 was added from sides of the test tube. Violet colour ring formed at the junction of two liquids indicates the presence of carbohydrates.
- **Fehling's test:** To the substance Fehling's A& B solutions were added and boiled for 5-10 minutes in a water bath. Brick red precipitate indicates the presence of reducing sugars.

3. Test for Steroids and Triterpenoids

- **Libermann Burchard test:** The substance was dissolved in few drops of chloroform, 3ml of acetic anhydride, 3ml of glacial acetic acid were added warmed and cooled under the tap. Then few drops of concentrated H_2SO_4 were added along the sides of test tube. Appearance of bluish green colour indicates the presence of Steroids and red colour indicates presence of terpenoids.
- **Salkowski test:** The extract was treated with few drops of concentrated H_2SO_4. Appearance of red colour at lower layer indicates presence of steroids and formation of yellow coloured lower layer indicates presence of triterpenoids.

4. Test for Flavonoids

- **Shinoda's test:** To the substance in alcohol, a few magnesium turnings and few drops of concentrated hydrochloric acid were added and boiled for five minutes. Red or pink coloration shows the presence of flavonoids.
- **Alkaline reagent test:** To the test solution few drops of sodium hydroxide solution was added, intense yellow colour is formed which turns to colourless on addition of few drops of dilute acid indicates presence of flavonoids.
- **Zinc hydrochloride test:** To the test solution a mixture of zinc dust and concentrated hydrochloric acid were added. It gives red colour after few minutes, indicates presence of flavonoids.

5. Tests for Glycosides

A portion of the extract was hydrolyzed with hydrochloric acid for few hours on a water bath and hydrolysate was subjected to legals test and born-tragers test.

- **Legals test:** To the hydrolysate, 1ml of pyridine and few drops of Sodium nitroprusside solutions were added and it was made alkaline with Sodium hydroxide. Appearance of pink to red colour, indicates the presence of glycosides.
- **Borntragers test:** Hydrolysate was treated with chloroform and the chloroform layer was separated. To this, dilute ammonia solution was added. Pink color in the ammonia solution indicates the presence of glycosides.

6. Test for Saponins

- **Froth formation test:** The extract was diluted with distilled water and it was agitated in a graduated cylinder for 15 minutes. The formation of 1cm layer of stable froth (foam) indicates the presence of saponins.

7. Test for Tannins

A small quantity of extract was taken separately in water and tested for the presence of tannins, with the following reagents.

- **Dilute ferric chloride solution:** Appearance of Violet colour indicates the presence of tannins.
- **1 % solution of gelatin containing 10% sodium chloride:** Formation of White precipitate indicates the presence of tannins.

➢ **10% lead acetate solution:** Formation of White precipitate indicates the presence of tannins.

8. Test for Fixed Oils

A small quantity of various extracts was separately pressed between two filter papers. Appearance of stain on the paper indicates the presence of fixed oil.

9. Test for Gums and Mucilage

A small quantity of extract was slowly added into a test tube containing alcohol with constant stirring. Formation of precipitate indicates the presence of gums and mucilages.

EVALUATION OF ANTIOXIDANT ACTIVITY

DPPH Radical scavenging test

The free radical scavenging activity of the methanol extract of inner bark of *Guettarda speciosa L.* (MEGS) was determined by using 2, 2 Diphenyl-1-picryl hydrazyl radical (DPPH) using UV-Spectrometry (Mathiesen *et al*., 1995) at 517nm. The DPPH solution was prepared in 95% methanol. The MEGA was mixed with 95% methanol to prepare the stock solution (10mg/100ml or 100µg/ml). From the stock solution 2ml, 4ml, 6ml, 8ml and 10ml of this solution were taken in five test tubes and by serial dilution with same solvent were made the final volume of each test tube up to 10ml whose concentration was then 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml and 100µg/ml respectively. Freshly prepared DPPH solution (0.004% w/v) was added in each of these test tubes. Containing MEGA (20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml and 100µg/ml) and after 10 min, the absorbance was taken at 517nm, using a spectrophotometer (SHIMADZU UV-1700, UV-visible spectrophotometer). Ascorbic acid was used as a reference standard. It is dissolved in distilled water to make stock solution with the same concentration of MEGA control sample was prepared without extract and reference ascorbic acid. 95% methanol was used as blank % scavenging of the DPPH free radical was measured using following equation.

% DPPH radical-scavenging=

$$\frac{(Absorbance\ \ of\ \ control\ -\ Absorbance\ \ of\ \ test\ \ sample)}{(Absorbance\ \ of\ \ control)} \times 100$$

Reducing Power Method

The assay of reducing power method (Koleva *et al*., 2002, Makari *et al*., 2008) is one to determine the antioxidant activity. In this 1 ml of plant extract of MEGS solution mixed with 2.5 ml phosphate buffer (0.2M, pH 6.6) and 2.5 ml Potassium Ferricyanide [K_3Fe (CN6)] (10g/l), the mixture was incubated at 50°C for 20 minutes. 2.5 ml of Tri chloroacetic acid (100g/l) was added to mixture. This was centrifuged at 3000 rpm for 10 min. Finally 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (lg/L) and absorbance measured at 700nm in UV-visible spectrophotometer (SHIMADZU UV-1700, UV-visible spectrophotometer). Ascorbic acid was used as standard and phosphate buffer used as blank.

RESULTS

The percentage yield and consistency of various extracts of inner bark of *Guettarda speciosa L.* (Rubiaceae) were presented in the table 1. The methanol extract gives the high percentage of yield and it was found to be 12.95%w/w.

The results of phytochemical studies of various extracts of inner bark of *Guettarda speciosa L.* were presented in the table 2. The various extracts revealed the presence of phytoconstituents such as alkaloids, carbohydrates, steroids, triterpenoids, flavonoids glycosides, saponins, tannins, fixed oils, gums and mucilages.

From the results of the phytochemical screening of extracts of *Guettarda speciosa L.* (Rubiaceae), it is concluded that the medicinal value of this plant may be attributed due to the presence of various phytoconstituents viz., alkaloids, carbohydrates, triterpenoids, flavonoids, tannins, gums and mucilages. The methanol extract gives high percentage yield. Hence, we have evaluated the methanol extract of the inner bark of *Guettarda speciosa L.* (Rubiaceae) for anti-oxidant potential.

Antioxidant activity of MEGS by DPPH method

As shown in table 3, methanol extract of inner bark of *Guettarda speciosa L.* (MEGS) exhibited the highest activity of more than 90.28%. The DPPH activity of standard ascorbic acid showed higher degree of free radical-scavenging activity (94.28%) than MEGS at different concentrations.

Antioxidant activity of MEGS by reducing power method

As seen in table 4, reducing power of the methanol extract of inner bark of *Guettarda speciosa L.* (MEGS) increased with increasing concentration from 20 to 100µg. These results clearly indicated that MEGS possesses antioxidant activity in dose dependently. The antioxidant activity of ascorbic acid was significantly higher than MEGS in reducing power method.

DPPH radical scavenging activity of methanol extract of inner bark of *Guettarda speciosa L.* (MEGS) added to methanol solution of DPPH and radical scavenging activity was measured at 517 nm as compared to standard ascorbic acid. Values are the average of triplicate experiments.

Reducing power of methanol extract of inner bark of *Guettarda speciosa L.* (MEGS) of was comparable with that of Ascorbic acid at 700nm. Values are the average of triplicate experiments.

Table 1. Percentage yield and Consistency of *Guettarda speciosa L.* (Rubiaceae) extracts

Parameter	Extracts
	Methanol
Percentage of yield (w/w)	12.95
Consistency	Sticky

Table 2. Phytochemical studies of *Guettarda speciosa L.* (Rubiaceae) extract

Tests	Methanol
Alkaloids	+
Carbohydrates	+
Steroids	-
Triterpenoids	+
Flavonoids	+
Glycosides	-
Saponins	-
Tanins	+
Fixed Oils	–
Gums & mucilage	+

– / + = Absence / Presence

Table 3. Antioxidant activity of MEGS by DPPH method

S. No	Conc. (µg/ml)	Absorbance of Ascorbic acid (Ref)	% scavenging DPPH of Ascorbic acid (Ref)	Absorbance of MEGS	% scavenging DPPH of MEGS
1	20µg/ml	0.1524±0.02	35.22±0.02	0.1685±0.01	29.64±0.02
2	40 µg/ml	0.1039±0.01	52.39±0.01	0.1094±0.02	48.22±0.02
3	60 µg/ml	0.0745±0.01	67.42±0.02	0.0835±0.01	60.18±0.02
4	80 µg/ml	0.0495±0.02	78.54±0.05	0.0522±0.02	77.22±0.03
5	100 µg/ml	0.0124±0.02	94.28±0.04	0.0246±0.01	90.28±0.02

Table 4. Antioxidant activity of MEGS by reducing power method

S. No	Concentration (µg/ml)	Absorbance of Ascorbic acid	Absorbance of MEGS
1	20µg/ml	0.41±0.01	0.33±0.02
2	40 µg/ml	0.59±0.02	0.54±0.01
3	60 µg/ml	0.74±0.02	0.69±0.01
4	80 µg/ml	1.02±0.01	0.94±0.02
5	100 µg/ml	1.19±0.02	1.13±0.01

DISCUSSION AND CONCLUSION

Two different bioassays were described, namely; scavenging of the diphenyl picrylhydrazyl (DPPH) radical method and the other reducing power of Fe^{3+} method. Methanol extract of inner bark of *Guettarda speciosa L.* (MEGS) has potent natural antioxidant and thus inhibit unwanted oxidation process.

In this method, DPPH is usually used as a substrate to evaluate anti-oxidative activity of antioxidants. It is based on the reduction of methanolic $DPPH^{-}$solution in the presence of a hydrogen donating antioxidant, due to the formation of DPPH-H (non-radical form) by the reaction. The test extract was able to reduce the stable radical $DPPH^{-}$ to the yellow coloured diphenyl picrylhydrazine. It has been reported with cysteine, ascorbic acid, tocopherol, hydroquinone, pyrogallol and gallic acid reduce and decolorize 1, 1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability (Blois, 1958).

The content of flavonoids and phenolic compound in the extracts might be their high antioxidant properties. Methanol extract of inner bark of *Guettarda speciosa L.* (MEGS) showed a significant antioxidant activity, one of the possible mechanisms is flavonoids and phenolic compound due to their redox properties, which play a key role in scavenging and neutralizing free radicals, quenching singlet and triple oxygen or decomposing peroxide (Wangsteen *et al*., 2004). The MEGS exhibited a moderate reducing power activity due to MEGS can react with free radicals, donate the electrons and convert them to stable thus, terminating the free radical chain reactions. Previous studies have reported that the reducing power of tannins

from medicinal plants prevents liver damage by inhibiting formation of lipid peroxides (Tanaka *et al*., 1988; Okuda *et al*., 1983; Frindivich, 1978).

The result of phytochemical screening of the methanol extract of inner bark of *Guettarda speciosa L.* (MEGS) revealed that presence of flavonoids and polyphenols. The presence of flavonoids has been reported to protect lipids, blood and body fluids against the attack of reactive oxygen species like superoxide, peroxide and hydroxyl radicals (So et al., 1996). Polyphenols also one of the very important plant constituents because of their free radical scavenging activity due to their hydroxyl groups (Kinsella et al., 1993; Tsao R, Akhtar MH, 2005). Despite its widespread use in traditional medicine for the treatment of liver disorders, there is scientific evidence regarding its antioxidant activity.

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