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Research Article

IN VITRO ANTIOXIDANT AND *IN VITRO* ANTI INFLAMMATORY ACTIVITY OF *RUTA GRAVEOLENS* METHANOL EXTRACT

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ABSTRACT

Ruta graveolens commonly called as Ruta is one of the oldest and widely used herbs in traditional medicine. The powdered extract of *Ruta graveolens* was prepared using methanol and its antioxidant and membrane stabilizing properties were evaluated employing various established in vitro systems,DPPH,superoxide radical scavenging, hydroxyl radical scavenging ,nitric oxide radical scavenging,lipid peroxidation and reducing power along with polyphenols and flavonoids. Membrane stabilizing assay was also carried out. The study revealed a notable inhibitory activity of methanol extract.

Keywords:*Ruta graveolens,* free radicals,superoxide,hydroxyl,nitric oxide radical,lipid peroxidation,reducing power,membrane stabilizing.

INTRODUCTION

Majority of the oxidative diseases are mainly linked to oxidative stress due to free radicals(Gutteridgde,1995).In treatment of these diseases antioxidant therapy has gained an immense importance. Antioxidants have been reported to prevent the occurrence of disease, cancer and aging. Reactive oxygen species (ROS), which consist of free radicals such as superoxide anion,hydroxyl radicals and non radical species such as hydrogen peroxide,singlet oxygen are different forms of activated oxygen(Halliwell and Gutteridge 1999,Yildirim et al 2000,Gulcin et al 2002b).ROS are produced by all aerobic organisms and can easily react with most biological molecules including proteins,lipids lipoproteins and DNA. Thus ample generation of ROS proceed to a variety of pathophysiological disorders such as arthritis ,diabetes ,inflammation ,cancer and genotoxicity (Kourounakis et al 1999,Gulcin et al 2002 a).

The mechanism of inflammation injury is attributed in part to release of ROS from activated neutrophil and macrophages. This overproduction leads to tissue injury by damaging the macromolecule and lipid peroxidation of membranes.ROS propagate inflammation by stimulating the release of the cytokines such as tumor necrosis factor α,interleukine 1,which stimulate recruitment of additional neutrophil and macrophages. Thus free radicals are important mediators that provoke or sustain inflammatory processes and concequently,their neutralization by antioxidants and radical scavengers can attenuate inflammation(Lavanya et al 2010).Inflammation is a complex process is associated with pain and involves occurrences such as, the increase of vascular permeability,increase of protein denaturation and membrane alteration(Umapathy et al 2010).

Ruta graveolens is a hard, evergreen shrub of up to one meter tall with a characteristic grayish color and a sharp unpleasant odor. The leaves are small, oblong, deeply divided; pinnate, glandular dotted. The stems are much ramified. The flowers are small (13 mm), yellow and in clusters during spring and summer. They have four petals, except for the central flower, which has five petals. The fruits are round, small and four- or five-lobulated. The taste is slightly stinging but is masked by the strong bitter odor (Font-Quer, 1979). Ruta is an ornamental, aromatic, culinary and medicinal plant, cultivated in gardens. It prefers rocky, well-drained soils and it resists dry weather. It is native to Europe, specially the Mediterranean region, but widely distributed into all the temperate and tropical regions. It is a very popular and attractive garden shrub in South America, where it is grown not only for ornamental and medicinal reasons but also because of the belief that it provides protection against evil.

MATERIALS AND METHODS

Plants

Ruta graaveolens plants were collected from SDM Ayurveda College, Hassan and Curzon Park Mysore.

Chemicals

DPPH, Folin ciocalteu reagent, ferrous sulphate,ascorbic acid,gallic acid, ferric chloride, trichloroaceticacid, sodium carbonate, sodium nitroprusside, NED, sulphonillic acid, quercitin, dextrose, sodium citrate, citric acid, sodium chloride, thiobarbutaric acid, potassium ferricyanide, ammonium molybdate, sulphuric acid, potassium acetate, TBA, deoxy ribose, EDTA, PMS, NBT, NADH were of analytical grade and are obtained from SRL, Rankem and Merck.

Preparation of the extract

Fresh leaves were collected, washed, shade dried and powdered mechanically.The air dried and powdered plant material (150 gm) was defatted using 500 ml of petroleum ether in Soxhlet apparatus. Then the plant material was extracted with 500 ml methanol for 8 hours. The residue was dried over night. The methanolic extracts were filtered and evaporated. The dried extracts were stored.

For the experimental use the methanolic extract was dissolved in methanol and stored.

Estimation of total polyphenolic content (Singleton, et al., 1965)

The total polyphenolic content was determined colorimetrically using folin ciocalteau's method for extracts. Aliquotes (0-50µl) of Gallic acid was taken in test tubes. Volume of all the tubes was made up to 1ml with distilled water. Extract was also diluted accordingly; 1 ml of 1:1 FC reagent and 2ml of 10% sodium carbonate was added to each of the tubes. After 30 mins absorbance was read at 760 nm against a blank. Concentration of polyphenol in extract was calculated using standard curve and expressed as %concentration.

Estimation of flavanoids (Zhishen , et al., 1999)

The aluminum chloride colorimetric method was modified from the procedure reported by Woisky and Salatino Quercetin was used to make the calibration curve. Ten milligrams of Quercetin was dissolved in 80% ethanol and the working solution contained 100µg/ml. 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water was added. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Plant extract reacted with aluminum chloride for determination of flavonoid content as described above.

Antioxidant assay

Measurement of antioxidant activity by reducing power assay (Jayaprakash, et al., 2001)

The reducing power ability was determined by the method of Yen and Chen. The extract (50-250µg/ml) was mixed with equal volume of 0.2M phosphate buffer pH 6.6 and potassium ferricyanide. The

mixture was incubated at 50^0 C for 20 min., an equal volume of 10%TCA was added to the mixture, centrifuged at 3000g for 10min. Upper layer of the solution was mixed with distilled water and 0.1% ferric chloride in a ratio of 1:1:2(v/v/v) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture compared to blank indicated increased reducing power.

Free radical scavenging activity (Bondet, et al., 1997)

Herb extracts were mixed with DPPH (0.1mM) in ethanol solution. After 20 min incubation at room temperature absorbance was read at 517 nm. The inhibitory percentage of DPPH was calculated according to the following equation:i

$$\% = [(A_o - A_1) / A_o] 100$$

Where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the samples of methanol extract.

Reduction of phosphomolybdate by antioxidants (Kanner et al.,1994)

Standard working solution of ascorbic acid is taken in different aliquots (0-1ml, 0-10µg).and the volume is made up to 1 ml using water.1 ml of coloring reagent is added to all tubes including unknown. Different aliquots of extracts are taken as the unknown .The tubes are shaken vigorously for 15 min and then kept on boiling water bath for 90mins .The tubes were cooled and the absorption was measured at 695nm.

Hydroxyl radical scavenging (Elizabeth and Rao, 1990)

The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (the Fenton reaction). The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM); KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 µM); EDTA (100 µM); H_2O_2 (1.0 mM); ascorbic acid (100 µM) and concentrations (500 µg/ml) of the test sample or reference compound. After incubation for 1 h at 37°C, 0.5 ml of the reaction mixture was added to 1 ml 2.8% TCA, then 1 ml 1% aqueous TBA was added and the mixture was incubated at 90°C for 15 min to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed six times. Quercetin was used as a positive control. Percentage inhibition was evaluated by comparing the test and blank solutions.

Superoxide radical scavenging (Dasgupta & De, 2004)

This activity was measured by the reduction of NBT according to a previously reported method the non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan. The 1 ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM) and concentrations (500µg/ml) of sample solution. After incubation for 5 min at ambient temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated. All tests were performed six times. Quercetin was used as positive control.

Nitric oxide radical scavenging (Garratt , 1964)

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions, which may be quantified by the Griess Illosvoy reaction .The reaction mixture contained 10 mM SNP, phosphate buffered saline (pH 7.4) and concentration (500µg/ml) of the test solution in a final volume of 3 ml. After incubation for 150 min at 25°C, 1 ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Then 1 ml of napthylethylenediamine dihydrochloride (NED) (0.1% w/v) was added and the mixture was incubated for 30 min at 25°C. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against a blank sample. All tests were performed six times. Quercetin was used as positive control.

Lipid preroxidation assay (Gow-Chin Yen, et al., 1998)

For the in vitro studies, the brains of healthy animals were dissected and homogenized in 20mM, pH=7.4 Tris-HCl buffer (brain: buffer=1:9). The homogenate was centrifuged at 14000rpm for 15min. 1ml of supernatant was incubated with the test samples in the presence of 10 mM FeSO4 and 0.1mM ascorbic acid at 37^0C for 1 hour. Then 1ml TCA (trichoroacetic acid, 28%, w/v) and 1.5ml TBA (thiobarbituric acid, 1%, w/v) was added, boiled at 90^0C for 15 min and then centrifuged for 15 minutes at 4^oC. The amount of MDA produced was determined by measuring the absorbance of supernatant at 532nm.

In-vitro anti-inflammatory activity (Varadarasu et al 2007)

The human red blood cell membrane stabilization method was used for this study. The blood was collected from healthy human volunteer who was not under any NSAIDS for 2 weeks prior to the experiment and mixed with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) and centrifuged at 3,000 rpm. The packed cells were washed with isosaline and a 10% suspension was made. Various concentrations of extracts were prepared (400 and 800 µg/ml) using distilled water and to each concentration 1 ml of phosphate buffer, 2 ml hyposaline and 0.5 ml of HRBC suspension were added. Incubated at 37^0C for 30 min and centrifuged at 3,000 rpm for 20 min. The hemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. Diclofenac (100 µg/ml) was used as reference standard and a control was prepared omitting the extracts.

Indirect hemolytic assay (Bowman et al, 1957)

The substrate for indirect hemolytic activity was prepared by suspending 1ml of fresh human red blood cells and 1ml of fresh Hen's egg yolk in 8ml of phosphate buffered saline.1ml of suspension was incubated with 4-28µg of partially purified venom for 45 min at 37^0C and the reaction was stopped by the addition of 9ml of ice cold PBS. The suspension was centrifuged at 2000rpm for 20 min and then the released hemoglobin was read at 540 nm. 10µg of venom sample (*Naja naja*) was incubated with partially purified plant extract 30µg/ml and 60µg/ml for 30 min at room temperature and 1 ml of substrate was added and again incubated for 30 min at room temperature and the reaction was stopped by adding 9ml of ice cold PBS to all test tubes and centrifuged at 2000rpm for 10 min. Finally activity was measured at 540 nm.

RESULTS AND DISCUSSION

In living systems free radicals are constantly generated and they can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases and extensive lysis. Many synthetic drugs protect against oxidative damage but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicine .The antioxidant and polyphenol contents of *Ruta graveolens* have also been determined by using concentrated leaf extract.

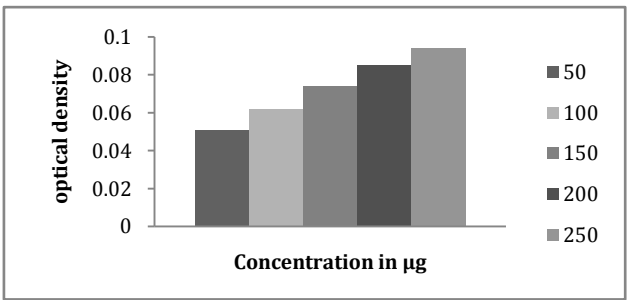

Fig 1: Measurement of antioxidant activity by reducing power assay

The methanol extract of *Ruta graveolens* was found to contain 8mg of gallic acid equivalent/ml and 1.6mg of quercitin equivalent/ml

Ruta, besides being an ornamental aromatic plant, is a medicinal plant too. The present study shows that *Ruta graveolens* has a potent antioxidant property. It has very good free radical scavenging property when compared to quercitin. Fe^{3+} was transformed to Fe^{2+} and reduction of phosphomolybdate in the presence of plant extract was observed. Increased absorbance indicated increased reducing power of the methanolic extract (Fig.1, 2).The methanol extract of *R.graveolens* showed 52% of free radical scavenging in the DPPH assay when compared to quercitin with 62% activity.

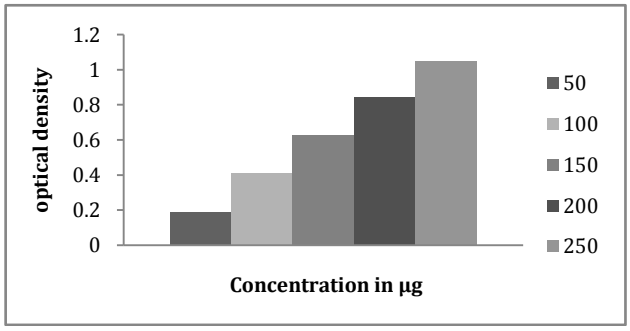

Fig2: Reduction of phosphomolybdate by antioxidants

The free radical scavenging assay shows the abilities of the extract and standard quercetin to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. At 500µg of quercitin and sample showed inhibition of 54.05% and 44.5% respectively .The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm with the plant extract and the reference compound quercitin indicates their abilities to quench superoxide radicals in the reaction mixture. At 500µg of quercitin and sample showed inhibition of 65.2% and 47.65% respectively). *R.graveolens* extract also caused a moderate inhibition of nitric oxide. 500µg of quercitin was used as reference compound. The inhibition of nitric oxide radical by plant extract was 16%, whereas that of quercitin was 22%

Hydroxyl radical is particularly reactive and dangerous. Hydroxyl radical damages proteins in various ways and damages membrane by initiating the oxidation of fatty acid in membrane lipids, a process termed lipid peroxidation. It also damages nucleic acid, both by causing polynucleotide strand breakage and by changing the structure of DNA bases. Ruta showed significant in vitro inhibition of lipid peroxidation in brain homogenate. Lipid peroxidation may be pro inflammatory and can damage the tissues directly. Protection against free radical lipid peroxidation by plant extract is of great significance for their traditional use against inflammatory disorders, many of which are associated with membrane damage and tissue recovery(Bonta et al 1980,Holliwell ,1990). Lipid peroxidation results in mitochondrial swellings and disintegration(Hoffsten 1962) degradation of lysosomes has been correlated with the peroxidative decomposition of lysosomal lipids(Desai 1964)The inhibition of lipid peroxidation by the plant extract was found to be 61.1% and quercitin was about 72% .

Table 1: In-vitro anti-inflammatory activity

	Conc. µg/ml	Absorbance (540nm)	% Inhibition
Control		0.29	
Methanol extract	400	0.12	59.1
	800	0.105	63
Diclofenac	100	0.83	71.7

Inflammation is a complex process and various mediators eg, prostaglandin,leucotrienes,platelet activating factor etc,have been reported to be involved in the development of inflammatory diseases. Anti-inflammatory refers to the property of a substance or treatment that reduces inflammation. Anti-inflammatory drugs make up about half of analgesics, remedying pain by reducing inflammation as opposed to opioids which affect the central nervous system. Cannabichromene, one of the many cannabinoids present in the cannabis plant, has been shown to reduce inflammation (Vani, T. et al. 1997).

In vitro anti-inflammatory studies shows that the methanolic extract exhibited membrane stabilization effect by inhibiting hypotonicity induced lysis of erythrocyte membrane. The erythrocyte membrane is analogous to the lysosomal membrane (Chou 1997) and its stabilization implies that the extract may as well stabilize lysosomal membrane. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of neutrophil such as bacterial enzymes and proteases which cause further tissue inflammation and damage, upon extra celluar release(Murugasan 1981) (Table 1). It also inhibits PLA_2 enzyme (from *Naja naja*) activity which was confirmed by direct and indirect hemolytic assays (Table 2). Methanol extract of Ruta graveolens showed significant *in vitro* anti inflammatory activity. The study also provides an evidence for the leaves Ruta in folk treatment as anti inflammatory agent.

Table 2: Direct hemolytic assay

	Conc. µg/ml	Absobance (540)	% activity	% inhibition
Control		0.649	100	
Methanol extract	30	0.268	41.29	58.71
	60	0.146	22.49	77.51

CONCLUSION

Ruta graveolens is a much known medicinal plant with enormous beneficial effects. The methanol extract of the Rue exhibits high antioxidant and free radical scavenging activities. It has a good reducing power too. The *in vitro* assays carried out indicate that this plant extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses.

Table 3: Antioxidant profile of Ruta graveolens methanol extract expressed in % scavenging.

Test	Con. µg/ml	Methanol extract	Quercitin
DPPH assay	500	52%	62%
Hydroxyl radical scavenging assay	500	44.5%	54.05%
Superoxide radical scavenging assay	500	47.65%	65.2%
Nitric oxide scavenging assay	500	16%	22%
Lipid peroxidation assay	500	61.1%	72%

The methanol extract has anti inflammatory (membrane stabilization).However, the components responsible for the antioxidative, anti inflammatory activities are currently not clear. Therefore, further investigation is needed to isolate and identify the bioactive compounds responsible for these properties. Furthermore, the *in vivo* antioxidant activity of this extract needs to be assessed prior to clinical use.

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