

Full Length Research Paper

Effect of antioxidant and anti-aggregating properties of micro-propagated plantlets of *Ruta graveolens*

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In the present study, we developed a protocol for *in vitro* propagation and evaluated antioxidant and anti aggregating properties of micro propagated leaf and callus extract of *Ruta graveolens*. The maximum number of callus induction was achieved from leaf explants on Murashige and Skoog (MS) medium enriched with 0.5 mg/L IAA and 1.0 mg/L of 2,4-D which yielded morphogenic compact yellowish green calli formed after 4 weeks interval. The highest shoot multiplication (82 ± 0.31) was observed on MS medium with 0.5 mg/ L 6-BA. The regenerated shoots were rooted *in vitro* on rooting medium containing 6-benzyl adenine and Indole-3- acetic acid (6-BA, 1.5 mg/l and 0.5 IAA mg/l showed better results of 9.08 ± 0.12 number of roots per shoots grew normally. The rooted plants were hardened in polythene bags containing sterile soil and vermiculite were successfully acclimated (at a frequency of 80%) and finally well established in the field. The enzymatic antioxidants viz: catalase, super oxide dismutase, glutathione reductase, glutathione peroxidase and glutathione-S-transferase; and non enzymic antioxidants, ascorbic acid, α - tocopherol, reduced glutathione, total carotenoids and flavonoids were found to be present. The free radicals scavenging (DPPH, hydroxyl radical and nitric oxide) activity were also observed. These extracts were evaluated for hemolytic activity in human blood, 500 μ g/ml concentration showed moderate hemolytic action. Further studies isolation and clinical trials will proved *R. graveolens* as a potent source for antioxidant and antiplatelet aggregating agent.

Key words: *Ruta graveolens*, aromatic, antioxidant, antiplatelet aggregation, compact.

INTRODUCTION

Ruta graveolens L. (Rue) is a medicinal and aromatic plant, mostly grown in the Mediterranean region as an ornamental plant because of its yellow-cupped beautiful flowers. The herb contains many secondary metabolites such as furocoumarins, furoquinolines and acridone alkaloids are used as many therapeutic applications including anthelmintic, antispasmodic, antiepileptic; rube-facient also used for preparation of synthetic perfumes (Anonymous, 2003). The essential oil and some of its

constituents were tested for their allelopathic activity *in vitro* on radish germination and radicle growth in light and darkness (Vincenzo et al., 2002). Direct shoot bud induction in nodal segments of *R. graveolens* through auxiliary shoot multiplication had been reported earlier (Faisal and Anis, 2005). *R. graveolens* has several pharmacological effects which include antimicrobial, antispasmodic, photosensitizing and an abortifacient. The alkaloid (commercial product name Pilocarpine) of this plant has been successfully used in treating glaucoma and atropine poisoning (Thoma, 2000). Furanocoumarins was synthesized from *R. graveolens* which are used in dermatology (Poutaraud et al., 2000). *Rue* has emmenagogue, ecbolic, anthelmintic and antispasmodic properties. Infusions and decoctions of aerial parts of *Rue* were used as anti-inflammatory and anti-rheumatic medicine and for the treatment of hypertension, skin illness and rhinitis (Parajapati and Kumar, 2003). The

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Abbreviations: IAA, Indole-3-acetic acid; 2,4-D, dichlorophenoxyacetic acid; BA, benzyl adenine; DPPH, diphenyl-1-picrylhydrazyl.

Table 1. Effect of different concentration of IAA and 2,4-D for callus induction from *Ruta graveolens* leaf explants.

Growth hormones concentration (mg/L) (IAA+2,4-D)	Degree of callus formation
0.5 + 0.25	+
0.5 + 0.5	++
0.5 + 1.0	+++
0.5 + 1.5	++

There were ten explants for each treatment and data were taken after three weeks of culture.

+++ indicates excellent degree of callus, ++ indicates good degree of callus and + indicates poor degree of callus.

aerial parts and roots of this plant reported potential anticonceptive activity in rats (Gandhi et al., 1991). The antitumor activity of this plant has been scientifically proved on Dalton's lymphoma ascites (DLA), Ehrlich ascites carcinoma (EAC) and L929 cells (Preethi et al., 2006). Also *R. graveolens* was reported to have the phytochemicals which includes flavonoids rutin, alkaloids quinolone, furoquinolone, acridone, pyranocoumarin and essential oils (Sinshemko et al., 2000).

Antioxidant and anti-aggregating properties

The imbalanced dietary and body free radicals react with biomolecules, causing cellular injury and death which lead to the development of chronic diseases such as cancers and those that involve the cardio- and cerebrovascular systems. Numerous scientific studies had been evaluated as an antioxidant property of coastal medicinal plants (Bandaranayaka, 2002; Ramanathan, 2010). Also hemolytic activities are flattering a modern area of research in drug lead discovery. The natural anti-aggregant agents are investigating ethno botanically important coastal plants in our laboratory. These studies are essential because some patients had become resistant to the already exiting drug aspirin (Undas et al., 2007) and conventional medication in association with medicinal plant formulation. According to the literature survey, *R. graveolens* is a potential medicinal plant and being endangered in southeast coast of India; hence the present study regenerated *R. graveolens* for conservation also lack scientific validation on antioxidants and antiaggregant effect of this plant, we evaluated it for pharmaceutical applications.

MATERIALS AND METHODS

Source of explants

R. graveolens were freshly collected from the Southeast coast of India and kept under shade net (50%) house environment. The specimen was certified by the Herbaria of C.A.S. in Marine Biology

(AUCASMB69), Annamalai University and Botanical Survey of India (BSI) Coimbatore, Tamil Nadu.

Surface sterilization

The leaf was used as explants material for initiating the organogenic calli. The explants were surface sterilized with 70% (v/v) ethyl alcohol for 1 to 5 min followed by 0.1% HgCl₂ for 3 min. The explants were then washed 4 times with sterile distilled water to remove traces of HgCl₂. The experimental chemicals were purchased from Hi-Media, Mumbai.

Plantlet regeneration

The leaf explants (one leaf divided into two segments) were cultured for callus induction in 250 ml conical flask and 25 ml Petri plates containing Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 30 g l⁻¹ sucrose, 8 g l⁻¹ and various concentration of auxin and cytokinin such as 6-BA and auxin such as IAA and 2,4-D (Table 1). There were ten explants for each treatment and data were taken after three weeks of culture. Control cultures were initiated on MS medium with growth regulators. The pH was adjusted to 5.8 prior to the addition of agar. Media were autoclaved at 15 lbs for 15 min. Culture was incubated at 25 ± 2°C and relative humidity of 60 ± 10%. An 8 h photoperiod (16 h dark) with light intensity of 20 to 30 μmol/m²s⁻¹ was provided by cool day light fluorescent tubes; 40 W cool-white fluorescent tubes (Phillips Electronics India Ltd.). Multiple shoots obtained were transferred to elongation medium supplemented with different growth regulators (such as 6-BA, Kn and BAP). The cultures were regularly subcultured on fresh medium at four weeks intervals and observation was recorded. Elongated and healthy plantlets were transferred to rooting medium containing different combinations of 6-BA and IAA. Regeneration plantlets with well developed shoot and root systems were removed from the culture, washed carefully with tap water and transferred to pots containing sterilized soil and vermiculite (3:1). The plantlets after 10 to 15 days in green house; they were then placed in the normal environment for 1 h and assessed for signs of wilting. The exposure was increased daily until the plants were established fully under normal environmental conditions; they were then transferred into the field for normal growth. Experiments were set-up in completely randomized design. Each treatment had 10 replicates.

Significance of the treatment effects was determined using analysis of variance (ANOVA, p≤0.05) and comparison between mean values of treatments were made by Tukey's test. All statistical analysis were performed using the software SPSS (version 14, USA).

Evaluation of antioxidant and ANTI aggregating properties

Preparation of extract

Micro-propagated leaves (50 g) of *R. graveolens* were extracted with methanol using a Soxhlet assembly for 48 h, filtered and last traces of the solvent were evaporated under reduced pressure in a rotary evaporator. Also, approximately 20 g of callus was grinded in 100 ml of sterile distilled water in mortar and pestle. The resulting extract was filtered through filter paper (What man No.1). The experimental chemicals were purchased from Sigma Chemicals, Mumbai.

Assessment of the activities of enzymic and non-enzymic antioxidants

The leaf and callus extracts of *R. graveolens* was analyzed for the enzymic and non-enzymic antioxidants such as catalase (Luck, 1974), superoxide dismutase (Mirsa and Fridovic, 1972), glutathione reductase (David and Richard, 1983), glutathione peroxidase (Rotruck et al., 1973), glutathione-s-transferase (Habig et al., 1974), ascorbic acid (Roe and Kuether, 1953), α -tocopherol (Rosenburg, 1992), reduced glutathione (Moron et al., 1979), total carotenoids (Zakaria et al., 1979) and flavonoids (Cameron, 1943).

DPPH free radical scavenging activity

The free-radical scavenging activity of *R. graveolens* leaf and callus extract was measured by the decrease in absorbance of methanolic solution of DPPH (Sreejayan and Rao, 1996). A stock solution of DPPH (33 mg l⁻¹) was prepared in methanol and 5 ml of this stock solution was added to 1 ml of the leaf and callus (0.5 ml) extract solution at concentration of 250 mg ml⁻¹. After 30 min, absorbance was measured at 517 nm. Scavenging activity was expressed as the percentage inhibition.

Hydroxyl radical scavenging activity

The leaf and callus extract at concentrations of 250 mg ml⁻¹ was placed in a test tube and evaporated to dryness. 1 ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of 0.018% EDTA, 1 ml of DMSO - 0.85%, V/V, in 0.1 mol l⁻¹ phosphate buffer, pH 7.4 and 0.5 ml of 0.22% ascorbic acid were added to each tube (Klein et al., 1981). The tubes were capped tightly and heated in a water bath at 80 to 90°C for 15 min. The reaction was terminated by adding 1 ml of ice-cold TCA (17.5% m/V). 3 ml of Nash reagent (75.0 g ammonium acetate, 3 ml glacial acetic acid and 2 ml acetyl acetone were mixed and water was added to a total volume of 1 L) was added to each tube; the tubes were left at room temperature for 15 min for colour development. The intensity of the yellow colour formed was measured at 412 nm against a blank of the reagent. Percentage inhibition was determined by comparing the results of the test and standard compounds.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was measured spectrophotometrically (Govindarajan et al., 2003). Sodium nitroprusside (5 mM l⁻¹) in phosphate buffered saline pH 7.4 was mixed with different concentrations of the extract (250 mg ml⁻¹) prepared in methanol and incubated at 25°C for 30 min. A control without the test compound, but with an equivalent amount of methanol was taken. After 30 min, 1.5 ml of the incubated solution was removed and

diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthylethylenediamine-dihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-1-naphthylethylene diamine dihydrochloride was measured at 546 nm and the percentage scavenging activity was measured with reference to the standard.

Hemolytic activity

1% red-blood cell suspension in pH 7.4 phosphate buffer was used throughout in the preparation of experimental (test) and control tubes (WHO, 1998). Extracts prepared as described earlier were initially dissolved in dimethylsulphoxide (DMSO) at known sample concentrations. These sample solutions were transferred by micropipette to test tubes containing a fixed volume of red-blood cell suspension. The extracts were screened attest concentrations of 50, 250 and 500 µg/ml. Hemolytic activity of extracts was evaluated by comparison with the effects of the commercially available positive control saponin from quillaja bark (Sigma, U.S.A.). This control provides total hemolysis of red-blood cell suspensions at concentrations of 10 to 20 µg/ml. Negative controls (blanks) contained 1% solvent in red-blood cell suspension.

Statistical analysis

Values were represented as mean \pm SD of three parallel measurements and data were analyzed using the t-test.

RESULTS AND DISCUSSION

Leaf explants of *R. graveolens* expanded approximately fourfold of their initial size after 1 week of culture. The maximum number of callus induction was achieved from leaf explants on MS medium enriched with 0.5 mg/L IAA and 1.0 mg/l 2,4-D which yielded morphogenic compact yellowish green calli and transferred to the shoot induction medium. At lower concentration of IAA and 2,4-D (0.5 and 0.25 mg/L) only indicate poor degree of calli induction. However, the best frequency of compact yellowish green callus production increased with the concentration of IAA (0.5 mg/L) with 2,4-D (1.0 mg/L) after 4 weeks interval as shown in Table 1 and Figure 1. Similar observation had been reported in *Citrullus colocynthis* (Satyavani et al., 2011). Ahmad et al. (2010) reported light yellow friable callus induction in *R. graveolens* using MS medium supplemented with 10 µM 2, 4, 5- trichlorophenoxyacetic acid (2, 4, 5-T). As shown in Table 2, combinations of cytokinins (without auxin) is capable of promoting shoot differentiation at various frequencies from morphogenic calli in shoot induction MS medium. The best results for shoot induction were obtained in the medium containing 2.5 mg/l 6-BA and 0.5 mg/l BAP. The combination of 6- BA with Kn did not show significant shoot induction compared to BAP. The effect of IAA in combination with 6-BA depended on the concentration used. The lowest concentration did not alter the response compared to the media without auxins. The highest concentration decreased both calli and shoot



Figure 1. The best frequency of compact yellowish green callus production increased with concentration of IAA (0.5 mg/l) with 2,4-D (1.0 mg/l) after 4 weeks interval.

Table 2. Effect of plant growth regulators on indirect shoot morphogenesis in leaf callus of *Ruta graveolens* on MS medium.

6-BA (mg/L)	Kn (mg/L)	Mean number of shoots per/unit callus
0.5	-	37.5 ± 0.226
1.0	-	68.56 ± 0.021
1.5	-	56.38 ± 0.246
2.0	-	70.9 ± 0.412
2.5	-	82±0.31
0.5	0.5	15.9 ± 0.359
1.0	1.0	32.8 ± 0.672
1.5	2.0	43.2 ± 0.180
2.0	3.0	22.6 ± 0.336

Values are mean ± SE from 5 replicates in each treatment.

frequencies when combined with 6-BA at 0.5 or 1 mg/L. About 82% of the organogenic calli developed 3 to 5 normal shoots in elongation medium (2.5 mg/l 6-BA and 0.5 mg/l BAP), and subsequent subcultures in the same medium kept for developing shoots (Table 2 and Figure 2). In contrast, the highest number of shoot buds was achieved from MS media containing 1.0 mg/l BAP+ 0.25 mg/l IAA (Bohidar et al., 2008).

In our study demonstrated without addition, auxin number of shoot buds were produced. Similar results also showed the effective role of NAA in combination with

BAP for the induction of multiple shoots in *Basilicum polystachyon* (Chakraborty and Roy, 2006), *Musa sapientum* L. (Kalimuthu et al., 2007), *Rauwolfia serpentina* (Baksha et al., 2007), *C. colocynthis* (Meena and Patni, 2007). The stimulatory effect on IBA on root formation had also been reported in *Clitoria ternatea* (Barik et al., 2007). These shoots were transferred to root induction medium with auxins for rooting. Root formation is an energy demanding process and thus, exogenous supply of carbohydrates is required. After 4 weeks, the *in vitro* shoots were excised from shoot clumps and



Figure 2. 2.82% of the organogenic calli developed 3 to 5 normal shoots in elongation medium (2.5 mg/16-BA and 0.5 mg/l BAP).

Table 3. Effect of auxin (6-BA and IAA) on root induction shoots of *Ruta graveolens* after 4 week of culture.

6-BA (mg/l)	IAA (mg/l)	Percentage of cutting of rooted (%)	Number of roots per shoots
0.5	0.5	75	7.62 ± 0.23
1.0	0.5	65	2.5 ± 0.86
1.5	0.5	80	9.08 ± 0.12
2.0	0.5	75	3.32 ± 0.15

Values are mean ± SE from 5 replicates in each treatment.

transferred to MS rooting medium supplemented with 6-BA at different levels (0.5, 1.0, 1.5 and 2.0) and IAA (only 0.5 mg/l). The maximum frequency of plantlets (80%) was achieved on MS medium containing 1.5 mg/l 6-BA and 0.5 mg/L IAA and mean number of roots per shoot was 9.08 ± 0.12 (Table 3 and Figure 3). In MS media supplemented with 1.5 mg/l 6-BA and 0.5 mg/L IAA about 9.08 ± 0.12 number of roots per shoots grew normally.

The rooted plants were hardened in polythene bags containing sterile soil and vermiculite were successfully acclimated (at a frequency of 80%) and finally well established in the field.

Enzymatic and non-enzymatic antioxidant effect of *Ruta graveolens*

The yield was 2.50 g of dry extract (leaf) and callus was

stored at room temperature until use. Activities of various enzymic antioxidants like catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase and glutathione-S-transferase, and non enzymic antioxidant ascorbic acid, α -tocopherol, reduced glutathione, total carotenoids and flavonoids in the leaves of *R. graveolens* was tested and the results obtained are shown in Table 4. From the table, it is clear that the activity of the enzymic antioxidants was found to be higher in both the leaf and stem. The leaves exhibited the highest activity of glutathione reductase (12.3 ± 2.3) and the callus exhibited the highest activity of superoxide dismutase (11.3 ± 0.3). The aforementioned table reveals that *R. graveolens* contains moderate levels of ascorbic acid, α -tocopherol and reduced glutathione. A significant difference in the levels of α -tocopherol, reduced glutathione, carotenoids and flavonoids was seen between the leaves and callus of *R. graveolens*. Since, *R.*

Table 4. Effect of enzymic and non-enzymic antioxidant in *Ruta graveolens*.

Enzymic antioxidants (U/g)	Leaf extract	Callus extract	T value
Catalase	240 ± 12.4	255 ± 25.2	1.02ns
Superoxide dismutase	4.9 ± 2.1	11.3 ± 0.3	8.60*
Glutathione reductase	12.3± 2.3	4.7± 1.03	3.35*
Glutathione peroxidase	0.49±0.03	0.5 ± 0.1	3.30*
Glutathione-s-transferase	0.5±0.1	0.2 ± 0.05	2.65*
Non-enzymic antioxidants			
Ascorbic acid	1.01±0.03	1.04 ± 0.03	1.12ns
Tocopherol	0.41±0.22	0.38 ± 0.02	6.25*
Carotenoids	2.21±0.02	1.45 ± 0.01	26.90*
Flavonoids	3.46±0.15	0.68 ± 0.23	3.46*
Reduced glutathione	0.04±0.02	0.10 ± 0.01	2.60*

Values are mean ± SD of triplicates, *Significant at 0.05 level, NS- not significant. 1) Amount of enzyme required to optical density by 0.05, 2) Amount that cause 50% reduction in the extent of NBT oxidation; 3) μ moles of CDNB conjugated/minute. 4) μ moles of NADPH utilized and 5) μ moles of GSH utilized/minute.

Table 5. Antioxidant profile of *Ruta graveolens*.

Sample	Concentration	DPPH radical scavenging (%)	Hydroxyl radical scavenging (%)	Nitrite radical scavenging (%)
Leaf extract	250	90.21 ± 2.1	61.4 ± 3.2	62.02 ± 3.5
Callus extract	250	89.13 ± 1.1	70.23 ± 2.51	86.4 ± 3.3

graveolens contains significant activities of all enzymic antioxidants analyzed; it may have the ability to reduce the risk of serious diseases caused by the reactive oxygen species like malignancy, cardiovascular and severe neural diseases. The free radical scavenging activity was evaluated by using various *in vitro* assays. DPPH radical was used as a substrate to evaluate the free radical scavenging activity of leaf and callus extract. The scavenging effect of leaf and callus extract on the DPPH radical was 90.21 ± 2.1%, 89.13 ± 1.1 ($p < 0.005$), at a concentration of 250 mg ml⁻¹ (Table 5). Hydroxyl radicals are the major active oxygen species that cause lipid oxidation and enormous biological damage (Aurang et al., 1977). The percentage of hydroxyl radical scavenging increased with the increasing concentration of leaves extract. Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, and inhibition of platelet aggregation and regulation of cell mediated toxicity (Hagerman et al., 1998). The percentage inhibition of nitric oxide generation by leaf and callus at 250 mgml⁻¹ concentration was found to be 62.02 ± 3.5%, 86.4 ± 3.3 ($p < 0.005$) respectively. At this current stage, to determine the hemolytic assay is important because whether a drug acquiring different bioactivities can be used in pharmacological applications.

The results for each test concentration of leaf and callus extract were interpreted qualitatively for *in vitro*

hemolytic action either being present or absent. Natural products had been shown to be a tremendous and consistent resource for the development of new drugs (Kalaivani et al., 2010). A large number of compounds from different sources had been found to be responsible for *in vitro* hemolysis. Among these are substances derived from plants (Gandhi and Cherian, 2000), heavy metals (Ribarov and Bemov, 1981) and pharmaceuticals (Yamamoto et al., 2001). In recent years, many plants and their active components have been isolated which are proved to have antiplatelet aggregating potency (Dong et al., 1998). Among natural compounds: quercetin, myricetin, chrysin, naringin, naringenin, hesperidin and apigenin are noted for their hemolytic activities (Hommam et al., 2000). Mono, di, tri and tetra sulphides derived from various allium plants for example *Allium cepa* and *Allium sativum* recorded with hemolytic activities (Munday et al., 2003). Reduced xenobiotic compounds such as phenols are capable of promoting hemolysis through oxidation of hemoglobin, forming methemoglobin (Bukowska and Kowalska, 2004). Bilirubin promotes the loss of lipids in the erythrocyte plasma membrane with exposure of residues of phosphatidyl serine (Brito et al., 2002). Saponins used in the present study as positive control for evaluation of hemolytic action, produce changes in the erythrocyte membrane, causing rupture and release of characteristic hemoglobin pigments.

Conclusion

In conclusion a quick, reliable and reproducible protocol for *in vitro* clonal propagation of large scale production of medicinally important plant species *R. graveolens* by indirect method without any seasonal influences and evaluation, their therapeutic potentials provide a new pathway of drug research.

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