Hindawi Publishing Corporation
Journal of Botany
Volume 2012, Article ID 685427, 6 pages
doi:10.1155/2012/685427

Research Article

Phytochemical Composition and Antioxidant Potential of *Ruta graveolens* L. *In Vitro* Culture Lines

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Received 20 July 2011; Accepted 14 January 2012

Academic Editor: Gaoming Jiang

Ruta graveolens L. is a medicinal plant used in traditional systems of medicine for treatment of psoriasis, vitiligo, leucoderma, and lymphomas with well-known anti-inflammatory and anticancer properties. Therefore antioxidant potential of *R. graveolens* (*in planta* and *in vitro*) was investigated. As antioxidants present in plant extracts are multifunctional, their activity and mechanism depends on the composition and conditions of the test system. Therefore, the total antioxidant capacity was evaluated using assays that detect different antioxidants: free radical scavenging (DPPH and ABTS), transition metal ion reduction (phosphomolybdenum assay), reducing power, and nitric oxide reduction. Content of furanocoumarin-bergapten in the extracts showed good corelation with free radical scavenging, transition metal reduction and reducing power, while total phenolic content showed good corelation with nitric oxide reduction potential. Antioxidant activity of *in vitro* cultures was significantly higher compared to *in vivo* plant material. The present study is the first report on comprehensive study of antioxidant activity of *R. graveolens* and its *in vitro* cultures.

1. Introduction

Free radicals, together with secondarily formed radicals, are known to play an important role in the pathogenesis of many chronic conditions like atherosclerosis, arthritis, diabetes, ischemia, reperfusion injuries, central nervous system injury, and cancer [1, 2]. Hence, the study of antioxidant status during a free radical challenge can be used as an index of protection against the development of these degenerative processes in experimental condition for therapeutic measures.

Ruta graveolens is used in homeopathic, ayurvedic, and unani preparations [3] because this herb is so efficacious in various diseases (*Ruta* derived from Greek "reuo" means to set free). It has been extensively used in treatment of leucoderma, vitiligo, psoriasis, multiple sclerosis, cutaneous lymphomas, rheumatic arthritis and recently reported to possess anti-inflammatory and anticancer activity [4, 5]. Antioxidants in plants are affected by area, climatic conditions, and pest attack [6, 7]; therefore *in vitro* cultures are being investigated as alternate source of natural antioxidants [8]. For estimation of total antioxidant potential many authors have stressed the need to perform more than one type of antioxidant activity measurement to take into account the various mechanisms of antioxidant action [9]. With this perspective the present study investigates the total antioxidant activity evaluated using DPPH, ABTS, phosphomolybdenum complex, reducing power, and Nitric oxide reduction assay of six selected culture lines of *R. graveolens*. Correlation between activity and phytochemical composition of extract (for total phenolics, flavonoids, flavonols, and furanocoumarin) was determined. To our knowledge, the present study is the first report on comprehensive study of antioxidant activity of *R. graveolens* and its *in vitro* cultures.

2. Methodology

2.1. Plant Material. Fruits, shoots, and roots were obtained from *Ruta graveolens* L. (Rutaceae) plants grown in Botanic garden, Department of Botany, University of Pune.

2.2. In Vitro Cultures. Three cell culture lines with varying degree of differentiation (dispersed cell line RC1,

aggregated cell line RC3 and differentiated cell line RC6) [10] were selected for evaluating antioxidant potential. Shoot line RS2 [11] and genetically transformed clone Ia3 (Manuscript under revision by Acta Physiologiae Plantarum) were selected due to their lower doubling time and high furanocoumarin productivity.

2.3. Preparation of Extracts. *In vivo* fruits, shoots, and roots were used as reference. Finely pulverized plant material (100 mg) was cold-extracted in ultrapure methanol overnight and centrifuged at 10,000 rpm for 20 min and supernatant was filtered. Supernatant was evaporated to dryness at room temperature and dissolved in methanol (ultrapure) to achieve final concentration of 1 mg/mL.

2.4. Phytochemical Composition. Estimation of total phenolic, flavonoids, flavanols, and furanocoumarins. Total phenols were estimated as gallic acid equivalents (GAEs), expressed as mg gallic acid/g extract by Singleton et al. [12]. The content of flavonoids was determined by method described by Miliauskas et al. [6] using rutin as a reference compounds. The amount of flavonoids in plant extracts was expressed in rutin equivalent (RE). The flavonols content was determined by Miliauskas et al. [6] method. The content of flavanols was expressed in rutin equivalents (REs) as described above. Furanocoumarins psoralen, bergapten, and xanthotoxin were extracted and estimated according to method described elsewhere [11].

2.5. Antioxidant Activity

(1) DPPH. Free radical scavenging estimated according to Szabo et al. [13].

(2) ABTS. The free radical scavenging was measured by method of Teow et al. [14].

(3) Phosphomolybdenum Reduction. The total antioxidant capacity of extracts was evaluated by method of Prieto et al. [15] and expressed as equivalents of ascorbic acid (μmol/g of extract).

(4) Reducing Power. It was determined using method described by Oyaizu [16].

(5) Nitric Oxide Scavenging Activity. The nitric oxide scavenging activity was measured according to the method described by Marcocci et al. [17].

2.6. Statistical Analysis. The influence of various treatments on growth and phytochemical content was analyzed by one-way analysis of variance (ANOVA). Free radical scavenging activity (DPPH and ABTS), reduction of transition metal ions by phosphomolybdenum complex, reducing power, nitric oxide reduction assay, and total phenolics, flavonoids and flavonols were determined in triplicates.

3. Results and Discussions

3.1. Phytochemical Composition of In Vitro Lines. Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical scavengers. Flavonoids are one of the most diverse and widespread group of natural compounds and are one of the most important natural phenolics. These compounds possess a broad spectrum of activities including radical scavenging properties [7]. Therefore it was necessary to determine total amounts of phenolics, flavonoids, and flavanols in the selected extracts.

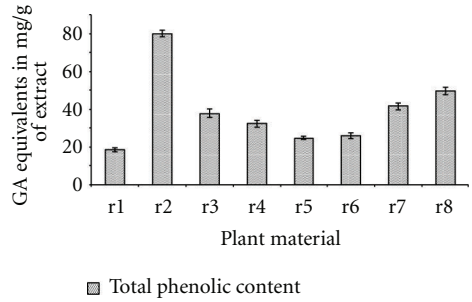
In vivo root extracts has the highest phenolic content (80 mg GAE/g dry wt). *In vivo* shoot extracts showed lower phenolic content (37 mg GAE/g dry wt) as compared to *in vitro* shoot line RS2 (41 mg GAE/g dry wt) and transformed shoot clone Ia3 (50 mg GAE/g dry wt) (Figure 1(a)). The total flavanoid content in the *in vivo* plant extract ranged from 0.4 to 0.8 mg/g plant extract in RE, whereas total flavanoid content in the *in vitro* cultures ranged from 1 to 17.7 mg/g plant extract (Figure 1(b)). *In vitro* shoot cultures showed 2.18-fold increase in flavanoid content as compared to *in vivo* shoots. The total flavanol content in the *in vivo* plant extract ranged from 0.36 to 0.68 mg/g plant extract in RE, whereas total flavanol content in the *in vitro* cultures ranged from 0.5 to 2.4 mg/g plant extract (Figure 1(b)).

Furanocoumarins are one of the main active constituents of *Ruta graveolens* and are also reported to be potent antioxidants [18, 19]. Potent antioxidant activity (DPPH, APPH) of *Angelica dahuricae* extracts was attributed to the furanocoumarins [20]. Therefore amount of psoralen, bergapten, and xanthotoxin in the extracts were determined (Figure 1(c)). Fruits showed highest furanocoumarins content *in planta*. Amongst *in vitro* cultures differentiation dependent accumulation of bergapten and xanthotoxin was seen [11, 12] with high bergapten and xanthotoxin accumulation in culture lines RC5, RS2, and Ia3, and high psoralen accumulation in RC1 and RC3 (Figure 1(c)).

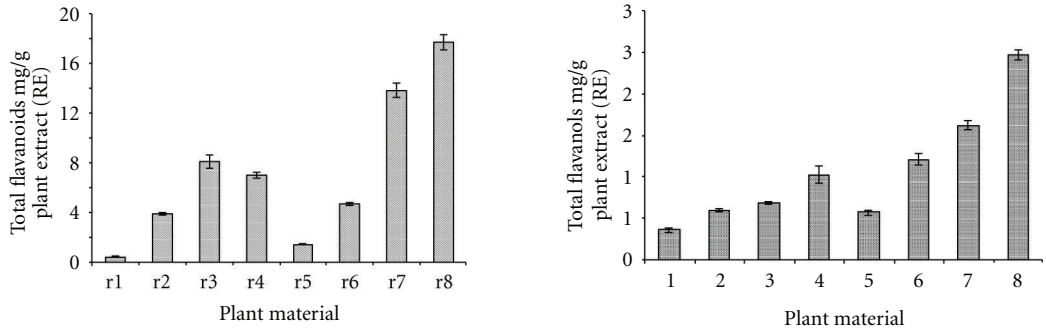
3.2. Antioxidant Activity

3.2.1. DPPH Radical Scavenging Activity. The radical scavenging activity was expressed as percentage of reduction of initial DPPH absorbance by the extracts at different concentrations (Figure 2). *In vitro* culture extracts, shoot line RS2, and transformed line Ia3 showed highest radical scavenging activity of 86.4% and 89.1% (EC_{50}: 33 and 60 μg/mL), respectively, as compared to 50% and 64% (EC_{50}: 400, 130 μg/mL) inhibition by roots and shoots. The percentages can be considered as full absorption inhibition of DPPH, because after completion of reaction the final solution always possesses some yellowish colour and therefore its absorption inhibition compared to colourless methanol solutions cannot reach 100% as shown by Miliauskas et al. [6].

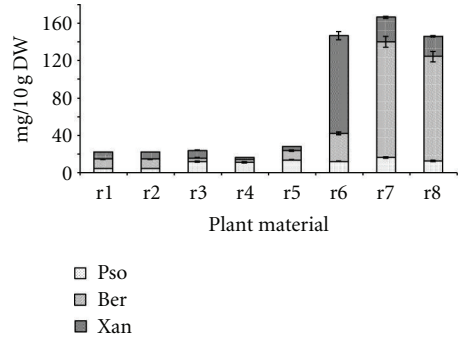
3.2.2. $ABTS^+$ Radical Scavenging Activity. Amongst all extracts tested, highest antioxidant potential was shown by



(a) Total phenolic content in *in vivo* and *in vitro* cultures of *R. graveolens*



(b) Total flavanoids and flavanols in *in vivo* and *in vitro* cultures of *R. graveolens*



(c) furanocoumarins in *in vivo* and *in vitro* cultures of *R. graveolens*

Figure 1: Values are mean of three replicates ± std. dev. values significant at $P \leq 0.095$ as calculated by two-way Anova (VassarStats), r1 = *in vivo* fruits, r2 = *in vivo* roots, r3 = *in vivo* shoots, r4 = dispersed suspension RC1, r5 = aggregated suspension RC3, r6 = differentiated suspension RC5, r7 = shoot line RS2, r8 = transformed clone Ia3.

Table 1: Correlation analysis.

Antioxidant activity	Phenolics	Flavanols	Flavanoids	Psoralen	Bergapten	Xanthotoxin
DPPH	0.433	0.089	0.124	0.407	**0.779**	0.150
ABTS	0.248	0.105	0.047	0.571	**0.865**	0.111
Phosphomolybdenum assay	0.221	0.388	0	0.221	**0.704**	0.384
Reducing power	0.163	**0.518**	0.399	0.090	**0.578**	0.151
Nitric oxide reduction	**0.83**	0.34	0.23	0.42	0.213	0.287

shoot line RS2 and transformed line Ia3 (EC50; 65 and 115 μg/mL), respectively (Figure 3).

Comparing results of the two radical scavenging tests (DPPH : ABTS) showed good correlation between their activity ($r^2 = 0.98$).

3.2.3. Phosphomolybdenum Assay. The different extracts at a concentration of (50 μg/mL) were assayed for their antioxidant potency by the formation of green phosphomolybdenum complex (Figure 4). *In vitro* shoots line RS2 and transformed clone Ia3 extract had strongest effects on

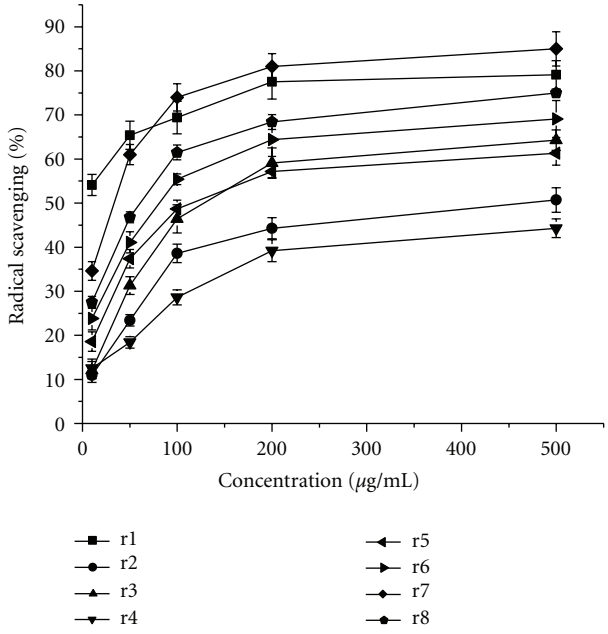


Figure 2: DPPH radical scavenging activity of *R. graveolens in vivo* and *in vitro* cultures. Values are mean of three replicates ± std. dev. values significant at $P \leq 0.095$ as calculated by two-way Anova (VassarStats), r1 = *in vivo* fruits, r2 = *in vivo* roots, r3 = *in vivo* shoots, r4 = dispersed suspension RC1, r5 = aggregated suspension RC3, r6 = differentiated suspension RC5, r7 = shoot line RS2, r8 = transformed clone Ia3.

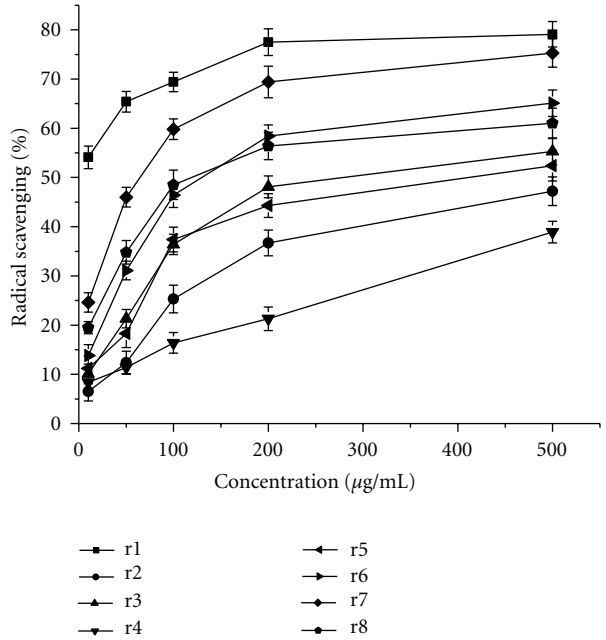


Figure 3: ABTS radical scavenging activity of *R. graveolens in vivo* and *in vitro* cultures. Values are mean of three replicates ± std. dev. values significant at $P \leq 0.095$ as calculated by two way Anova (VassarStats), r1 = *in vivo* fruits, r2 = *in vivo* roots, r3 = *in vivo* shoots, r4 = dispersed suspension RC1, r5 = aggregated suspension RC3, r6 = differentiated suspension RC5, r7 = shoot line RS2, r8 = transformed clone Ia3.

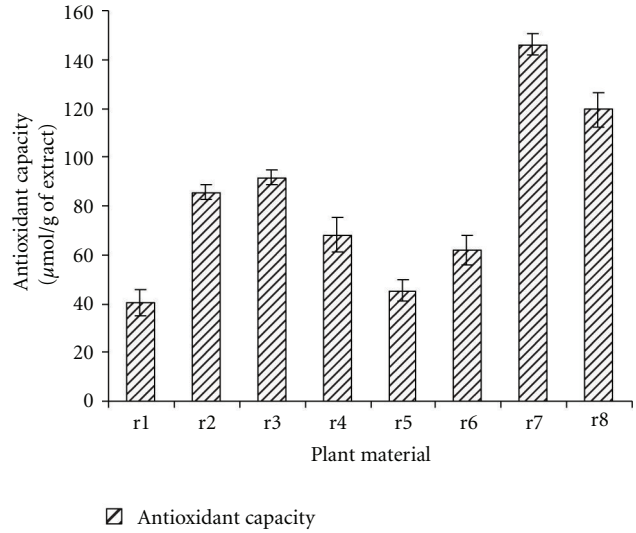


Figure 4: Phosphomolybdenum assay of *in vivo* and *in vitro* culture extracts of *R. graveolens.* Values are mean of three replicates ± std. dev. values significant at $P \leq 0.095$ as calculated by two way Anova (VassarStats), r1 = *in vivo* fruits, r2 = *in vivo* roots, r3 = *in vivo* shoots, r4 = dispersed suspension RC1, r5 = aggregated suspension RC3, r6 = differentiated suspension RC5, r7 = shoot line RS2, r8 = transformed clone Ia3.

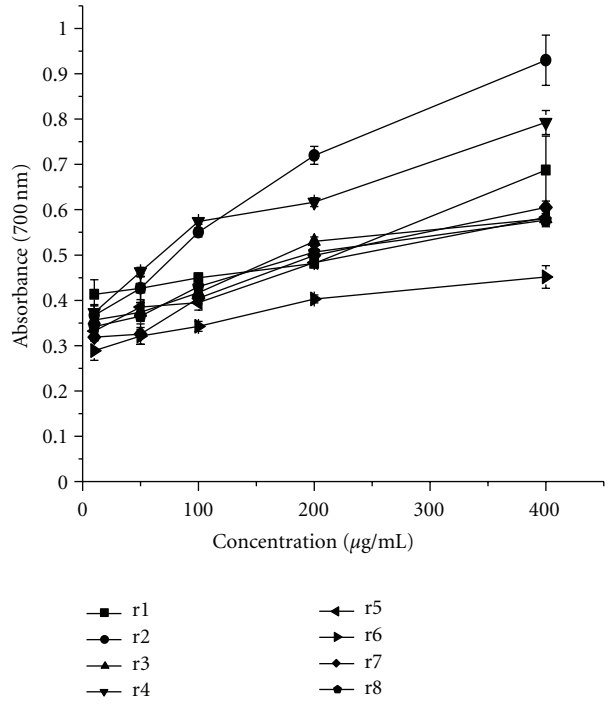


Figure 5: Reducing power *in vivo* and *in vitro* culture extracts of *R. graveolens.* Values are mean of three replicates ± std. dev. values significant at $P \leq 0.095$ as calculated by two way Anova (VassarStats), r1 = *in vivo* fruits, r2 = *in vivo* roots, r3 = *in vivo* shoots, r4 = dispersed suspension RC1, r5 = aggregated suspension RC3, r6 = differentiated suspension RC5, r7 = shoot line RS2, r8 = transformed clone Ia3.

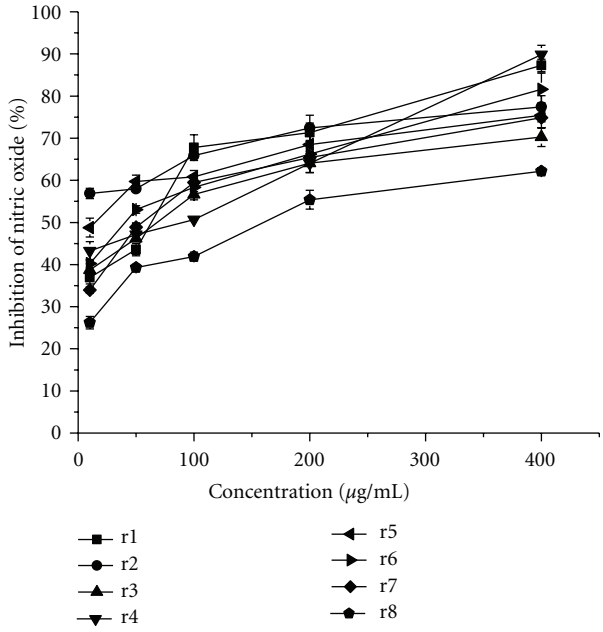


Figure 6: Percentage inhibition of nitric oxide radicals by *R. graveolens in vivo* and *in vitro* cultures extracts. Values are mean of three replicates ± std. dev. values significant at $P \leq 0.095$ as calculated by two-way Anova (VassarStats), r1 = *in vivo* fruits, r2 = *in vivo* roots, r3 = *in vivo* shoots, r4 = dispersed suspension RC1, r5 = aggregated suspension RC3, r6 = differentiated suspension RC5, r7 = shoot line RS2, r8 = transformed clone Ia3.

reducing Mo radical (146, 119 equivalents of ascorbic acid μmol/g of extract, resp.) which was 1.5-fold higher than *in vivo* shoots (91 μmol ascorbic acid/g).

It is known that hydrogen and electron transfer from antioxidant analytes to $DPPH^-$, $ABTS^+$ and Mo(VI) complex occur in the DPPH, $ABTS^+$ and phosphomolybdenum assay methods. The transfers occur at different redox potentials in the two assays and also depend on the structure of the antioxidant. $DPPH^-$ and $ABTS^+$ scavenging assays detect antioxidants such as flavonoids and polyphenols, whereas the phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, a-tocopherol, and carotenoids [15]. Ascorbic acid, glutathione, cysteine, tocopherols, polyphenols, and aromatic amines can be detected by the two assay models [21]. Thus the antioxidant potential of the extracts differed in these two types of assays. Fruit extracts which showed highest $DPPH^-$ and $ABTS^+$ scavenging activity (EC_{50}: 9 μg/mL) showed lowest antioxidant potential in the phosphomolybdenum assay (50 equivalents of ascorbic acid μmol/g of extract). From correlation analysis it was seen that bergapten content showed good correlation with DPPH ($r^2 = 078$), ABTS ($r^2 = 0.86$) and phosphomolybdenum assay ($r^2 = 0.7$) (Table 1).

3.2.4. Reducing Power Assay. Dose-dependant increase in reducing power of Fe^{3+} to Fe^{2+} was observed in the extracts (Figure 5). Amongst all extracts tested, highest activity was observed for root extract (absorbance of 0.93 at 400 μg/mL). Dispersed suspension (RC1) extract (400 μg/mL) showed maximum absorbance (0.81) amongst all the *in vitro* cultures tested. No significant difference was observed between *in vitro* shoot cultures and *in vivo* shoots. Reducing power of BHT was observed to be 0.8 at 1 mg/mL. It was seen that reducing power showed partial correlation with total flavanol ($r^2 = 0.51$) and bergapten ($r^2 = 0.58$) content.

3.2.5. Nitric Oxide Scavenging Activity. Dose-dependant increase in nitric-oxide radical scavenging activity was observed at studied concentrations (Figure 6). Highest activity was showed by *in vivo* root extracts (EC_{50}: 10 μg/mL). Amongst *in vitro* cultures, aggregated cell suspension culture RC3 showed highest activity (EC_{50}: 13.1 μg/mL) followed by differentiated cell suspensions RC5 and dispersed cell suspensions RC1 (EC_{50}: 41.8 and 93.9 μg/mL), respectively. EC_{50} of Butylated hydroxyl toluene (BHT) was >300 μg/mL, which is considerably higher than that of *R. graveolens* extracts. Thus *in vivo* and *in vitro* extracts of *R. graveolens* are potent scavengers of NO radicals. Nitric oxide reducing ability showed good correlation with total phenolic content ($r^2 = 0.83$) of the extracts.

Results obtained here showed that extracts from *in vitro* cultures showed strong antioxidant activity (DPPH, ABTS, reducing power, phosphomolybdenum assay) as compared to *in vivo* plant material. It has been reported that dedifferentiated cultures (callus and suspension) have less antioxidant potential [22]. However, our results indicated good antioxidant potential (reducing power and nitric oxide scavenging) of *R. graveolens* cell suspension. The observed elevated activity of *in vitro* cultures can be attributed to furanocoumarins especially bergapten and phenolics (Table 1) [23].

Production of antioxidants through plant tissue cultures is an effective strategy which also offers additional advantage of optimization of production of these agents by changing culture conditions and production of active antioxidant principles throughout year [9]. Present study highlighted the use of *in vitro* cultures as a source of antioxidants. Collectively, furanocoumarin content and antioxidant activities of *R. graveolens* will help to select suitable culture type as a source of natural antioxidants and nutraceuticals to enhance health benefits.

Acknowledgment

The financial support provided by University Grants Commission (UGC), New Delhi, India is duly acknowledged. All authors contributed equally to this work.

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