ORIGINAL ARTICLE

Influence of altitudinal variation on the anti-oxidant capacity of essential oil of *Syzygium densiflorum* from Southern Western Ghats, India

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Background: *Syzygium densiflorum* is a vulnerable tree species belonging to the Myrtaceae family. **Objective:** To investigate the influence of altitudinal variation on the anti-oxidant potential of leaf essential oil of *Syzygium densiflorum*. **Materials and Methods:** The leaf essential oil has been isolated using hydrodistillation process and their scavenging ability was determined using five *in vitro* assays such as 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing anti-oxidant power (FRAP), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), hydrogen peroxide (H_2O_2) and hydroxyl radical scavenging assays. **Statistical analysis:** The results were analysed statistically using one-way analysis of variance (ANOVA) following Duncan's multiple range test. **Results:** Leaves from lower altitude showed higher activity against hydrogen peroxide, hydroxyl radical and ferric ion and moderate activity against DPPH and ABTS free radicals. Leaves from higher altitude essential oil exhibits potent activity against hydrogen peroxide and hydroxyl radical compared with the standard. **Conclusions:** Comparatively lower altitude leaf essential oil showed potent anti-oxidant activity confirming the fact that altitudinal variations have profound effect on the anti-oxidant potential of *Syzygium densiflorum*.

Key words: Altitudinal variations, anti-oxidant capacity, essential oil, hydrodistillation, myrtaceae, *Syzygium densiflorum*, vulnerable tree

INTRODUCTION

Syzygium densiflorum is one of the tree species belonging to the family Myrtaceae that has been categorised as vulnerable under the IUCN[1] red list of threatened species and endemic to India. *Syzygium densiflorum* is a large canopy tree growing in high elevation evergreen forests between the altitudes 1500-2300 m.[2] *Syzygium* species are reported to exhibit anti-diabetic,[3] anti-fungal,[4,5] anti-inflammatory,[6] anti-bacterial,[5,7,8] anti-oxidant,[9] anti-hyperlipidemic[10] and growth inhibitory activity.[11]

In recent years, research on natural anti-oxidants from plants is increased owing to their free radical scavenging potential. A number of plants have been screened for investigating their anti-oxidant and radical scavenging activities.[12] Reports on distributions of phenolics and flavonoids in nature as anti-oxidants were wide-reaching.[13,14] Anti-oxidant properties of plants also depends on environmental factors such as mean temperature, sunlight hours and altitude.[15] Altitudinal variations have profound effects on the polyphenol content and anti-oxidant activity.[16] Interestingly, *Syzygium densiflorum* tree grows at different altitudes and also exhibit morphological variations. Leaves of the tree growing in higher altitudes are small, while leaves from lower altitude are broader. Hence, we attempted to analyse the anti-oxidant capacity of leaf essential oil of *Syzygium densiflorum* tree growing at 1500 and 1800 m altitudes above mean sea level.

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MATERIALS AND METHODS

Matured broad sized fresh leaves of *Syzygium densiflorum* from an 80-year-old tree growing at 1500 m altitude and a 75-year-old tree with narrow small leaves at 1800 m altitude were collected from evergreen forest at Chembra, Wayanad, Kerala. Higher and lower altitude trees were identified as *Syzygium densiflorum* and the voucher specimens deposited at Kerala Forest Research Institute (KFRI) herbarium (KFRI No. 23312 (1500 m ASL) and KFRI No. 23367 (1800 m ASL)). Five hundred grams of fresh leaves from each tree were coarsely chopped and subjected to hydrodistillation for

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4 h using a Clevenger apparatus.[17] The crude essential oil from broad and narrow small leaf extracted using hexane was stored in glass screw amber cap bottles and refrigerated at 4°C until use.

Free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH).[18] The stock solution was prepared by dissolving 24 mg DPPH with 100 ml methanol and then stored at −20°C until use. The working solution was obtained by mixing 10 ml stock solution with 45 ml methanol. An aliquot (150 µl) of each sample (with different concentrations) was added to 2850 µl of working DPPH solution and allowed to stand for 1 h in dark condition. Absorbance was read at 517 nm.[19]

$$\text{DPPH Scavenging ability (\%)} = [A_{control} - A_{sample}/A_{control}] \times 100$$

The ferric reducing anti-oxidant power (FRAP) assay was done according to Benzie and Strain[20] with some modifications. The stock solutions included 300 mM acetate buffer with pH 3.6, 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. Fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3.6H_2O$ solution and then warmed at 37°C before use. The oil sample (150 µl) was allowed to react with 2850 µl of the FRAP solution for 30 min in dark condition. Readings of the coloured product [ferrous tripyridyltriazine complex] were then measured at 593 nm.

$$\text{Reducing anti-oxidant power (\%)} = [A_{control} - A_{sample}/A_{control}] \times 100$$

The 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation de-colourisation method is based on the reduction of ABTS•+ radicals by anti-oxidants of the essential oil tested. The stock solution was prepared by adding 7.4 mM ABTS solution to 2.6 mM potassium persulphate solution. The working solution was obtained by mixing the two stock solutions in equal measure and allowing them to stand for 12 h in dark at room temperature. The solution was then diluted by mixing 60 ml methanol to 1 ml ABTS solution. An aliquot of 150 µl at different concentrations was added to 1425 µl of the ABTS solution and allowed to incubate for 2 h in dark. Absorbance was read at 734 nm using a spectrophotometer with methanol as blank.[21]

$$\text{ABTS scavenging activity (\%)} = [A_{control} - A_{sample}/A_{control}] \times 100$$

The ability of the oil to scavenge hydrogen peroxide was determined according to the method described by Ruch *et al*.[22] A solution of 2 mM H_2O_2 was mixed in phosphate buffered saline (PBS) (pH 7.4). The essential oil at different concentrations was added to 600 µl of hydrogen peroxide and allowed to stand for 10 min. Absorbance was then measured at 230 nm.

$$\text{Hydrogen peroxide activity (\%)} = [A_{control} - A_{sample}/A_{control}] \times 100$$

The scavenging activity of oil on hydroxyl radical was determined according to the prescribed method by Halliwell *et al*.[23] The reaction mixture contained 1750 µl PBS (pH 7.4), 50 µl of each of 2-deoxy-2-ribose (80 mM), EDTA (4 mM), $FeCl_3$ (4 mM), H_2O_2 (20 mM), ascorbic acid (4 mM) and 200 µl of various concentration vortexed and allowed to incubate for one hour at 37°C. One millilitre of 2% trichloroacetic acid (TCA) and 1 ml of 1% thiobarbituric acid (TBA) were added and kept in a boiling bath for 15 min. After cooling, the pink chromogen revealing the formation of thiobarbituric reactive substances (TBARS) was read at 532 nm using UV-spectrophotometer.

$$\text{Hydroxyl scavenging activity (\%)} = [A_{control} - A_{sample}/A_{control}] \times 100$$

Results were calculated as mean ± standard deviation ($n = 3$) and focussed to one-way analysis of variance (ANOVA). The significance of the difference between means was determined by Duncan's multiple range test ($P < 0.05$) using SPSS 17.0 statistical software (SPSS South-Asia Pvt Ltd., Bangalore).

RESULTS AND DISCUSSION

The anti-oxidant activity of *Syzygium densiflorum* collected at higher and lower altitudes are presented in Tables 1 and 2. Anti-oxidant capacities of essential oils from aromatic plants are mainly due to the active compounds present in them. From the earlier published study, it is clear that *Syzygium*

Table 1: Anti-oxidant activities of leaf essential oil of *Syzygium densiflorum* from lower altitude

Concentration (µg/ml)	Percentage activity*				
	DPPH	FRAP	H_2O_2	Hydroxyl scavenging	ABTS
1000	11.73±0.39	31.07±0.43	44.01±0.41	29.18±0.30	7.53±0.22
2000	29.71±0.62	37.10±0.81	80.39±0.78	56.17±0.34	29.3±0.28
3000	34.19±0.85	78.55±1.25	88.08±0.38	93.18±0.36	30.96±0.88
4000	49.74±0.51	81.23±0.77	93.15±0.87	96.00±0.99	37.39±0.89

*Values calculated from three replicate (n=3) data were expressed as mean±standard deviation. All values are significant (P<0.05). DPPH – 2-diphenyl-1-picrylhydrazyl; FRAP – Ferric reducing anti-oxidant power

Table 2: Anti-oxidant activities of leaf essential oil of *Syzygium densiflorum* from higher altitude

Concentration (µg/ml)	Percentage activity*				
	DPPH	FRAP	H_2O_2	Hydroxyl scavenging	ABTS
1000	8.76±0.34	7.93±0.28	31.26±0.22	55.85±0.28	17.26±0.26
2000	27.78±0.24	9.17±0.27	66.52±0.32	66.37±0.19	26.89±0.25
3000	29.34±0.27	15.12±0.31	64.53±0.42	72.92±0.25	27.78±0.29
4000	32.09±0.22	22.01±0.29	83.02±0.29	78.02±0.15	32.14±0.21

*Values calculated from three replicate (n=3) data were expressed as mean±standard deviation. All values are significant (P<0.05). DPPH – 2-diphenyl-1-picrylhydrazyl; FRAP – Ferric reducing anti-oxidant power

densiflorum is a rich source of sesquiterpenoid compounds,[24] which in turn have efficient anti-oxidant properties.[25]

The leaf oil was evaluated for their ability to quench synthetic free radicals. The scavenging activity of leaf oil was shown to have moderate anti-oxidant activity against DPPH radical. The leaf of lower altitude tree scavenges 49.74% of the DPPH free radical and leaf of higher altitude tree scavenges 32.09% at higher concentration. The anti-oxidant capacity of both the oils increased with increasing concentration. The DPPH scavenging ability of essential oil studied was lower compared with *Syzygium benthamianum*,[26] *Syzygium caryophyllatum*[27] and *Syzygium aromaticum*.[28]

ABTS assay utilises the single electron transfer mechanism similar to DPPH radical. The leaf oil quenches only moderate amounts of ABTS free radical. Leaves from lower altitude quenches 37.39%, whereas leaves from higher altitude quenches 32.14% of ABTS radical. The leaf oils of both altitudes showed concentration-dependant scavenging activity. Both essential oils has analogous activity against ABTS but exhibits reduced activity compared with *Syzygium aqueum*[29] and comparable activity with *Syzygium cumini*.[30]

The reducing power of the leaf oil may serve as an indicator of its potential anti-oxidant capacity.[31] The oils showed concentration dependent reducing power, as the concentration increases reducing ability of essential oil also increases. The leaf oil from lower altitude showed higher reducing power with 81.23% at higher concentration, whereas the one from higher altitude is extremely lower with 22.01%. *Syzygium densiflorum* grown at lower altitude shows higher reducing ability compared with the economically important *Syzygium cumini*.[30]

Hydrogen peroxide is a weak oxidising agent and can inactivate a few enzymes and hence perturb the cell function causing toxic effects.[32] The scavenging ability of the leaf oils for hydrogen peroxide is presented in Tables 1 and 2. The leaves of both higher and lower altitude trees showed a highly potent activity against H_2O_2 with 83.02% and 93.15%, respectively, comparable with that of the standard.

The anti-oxidant activity of leaf oils against hydroxyl radical is proportional to the concentration of the oil. The leaf oil from lower altitude scavenges 96% and from higher altitude scavenges 78.02% of the hydroxyl radical at higher concentration. *Syzygium aromaticum* is one of the economically important species that scavenges 65% of the hydroxyl radical,[28] which is comparatively lower to the ability of the studied *Syzygium densiflorum*.

From the present study, it is found that the environmental factors like altitudinal variations also have its profound effects on the anti-oxidant capacity of plants. In all *in vitro* assays carried out in this study, the tree from lower altitude shows a potential anti-oxidant capacity compared with the tree from higher altitude. The higher anti-oxidant capacity of *Syzygium densiflorum* resembles the higher proportion of phenolic compounds, which in turn prone for its medicinal properties and it is clear that environmental factors highly influence accumulation of secondary metabolites in plants.

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