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STUDY OF ANTIOXIDANT ACTIVITY AND ESSENTIAL OILS COMPOSITION FROM THE FLOWERS AND FRUITS OF CRYPTOCARYA STOCKSII MEISN

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Abstract:

The aim of this study was to investigate the flowers and fruits essential oil composition and antioxidant activity of Cryptocarya stocksii, an endemic and vulnerable tree grows in the Western Ghats of India. The essential oils were obtained by hydrodistillation using a Clevenger apparatus and analyzed by GC/MS. GC-MS analysis of essential oil of flower and fruit revealed the presence of individual 24 different compounds corresponding to 99.87% and 99.99% of the total oil respectively. The flower and fruit oil were dominated by α-Pinene (39.42% and 8.84%), β-Pinene (17.96% and 4.87%), 3-Carene (29.52% and 1.92%) and Longifolene (6.53% and 19.12%) respectively. Antioxidant activities were determined by four different test systems, namely, DPPH, FRAP, H_2O_2, and anti-lipid peroxidation assay and result found to be dose dependent manner. This work represents the first study of the chemical composition and anti oxidant of essential oils from flowers and fruits of C. stocksii.

Keywords: *Cryptocarya stocksii, Lauraceae, essential oils, antioxidant activity, GC-MS.*

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INTRODUCTION:

Essential oils are complex mixtures of volatile organic compounds produced in the form of secondary metabolites in plants. They may consist of monoterpenes, sesquiterpenes, and phenylpropanes, which may contain different functional groups (alkanes, alcohols, aldehydes, ketones, esters and acids) [1, 2]. Essentials oils are accumulated in cells, secretory cavities or glandular hairs of plants. They are globules with impermeable cells (stomata) whose interior have essentials oils [3]. In nature, Essential oils plays an important role in the protection of plants such as antifungal, antiviral, antibacterial, insecticide and also against herbivores, reducing their appetite for these plants. They are also responsible for the characteristic smell of plants, which can attract some insects to favor the dispersal of pollen and seeds, or repel other undesirable ones [4]. Around 3000 of the essential oils are known and 10% of them having commercial significance in the cosmetic, food and pharmaceutical industries, and in agriculture [4, 5]. Therefore, they are generally recognized as safe by the FDA (Food and Drug Administration). Its composition can differ considerably between species of aromatic plants and varieties and within the same variety of different geographical areas [1].

Essential oils possess antifungal, antibacterial, antiseptic, antiviral, antioxidant, anti-parasitic and insecticidal activities properties and have been screened on a global scale as potential sources of novel antimicrobial compounds, agents promoting food preservation, and alternatives to treat infectious diseases [6, 7, 8, 9, 10]. Various studies have demonstrated the antioxidant properties of essential oils. The antioxidant capability of an essential oil relies upon its composition. It is well established that phenolic and secondary metabolites with conjugated double bonds generally show generous ant oxidative properties [11]. The greater parts of the essential oils are ruled by oxygenated monoterpenes [12]. Volatile oils with scavenging ability of free radicals may play important role in some disease prevention, such as heart disease, brain dysfunction, cancer, and immune system decline. In fact, these diseases may result from cellular damage brought about by free radicals [13, 14].

Lauraceae are one of the major families of angiosperms, with around 2500–3500 species in around 50 genera, predominantly distributed in tropical and subtropical regions [15]. Lauraceae family members are characterized by a woody habit and are of extraordinary monetary significance around the world, as they give profitable timber, fragrant oils and significant substances that are broadly utilized in the pharmaceutical and food industries [16, 17]. The genus *Cryptocarya* comprises about 350 species, most of which are confined to tropical Asia [18]. In the Indian subcontinent, the genus is represented by 15 species, of which 6 are known to be distributed in the Western Ghats of southern India [19].

Cryptocarya stocksii Meisn. (Family: Lauraceae) is medium sized tree occur mainly in the Western Ghats region of India. It has been included in the world list of threatened trees under vulnerable category by International Union for Conservation of Nature and Natural Resources [20]. *C. stocksii* has been also considered as one of the rare and endemic threatened tree of south Western Ghats of India [21].

In spite of the relatively large number of *Cryptocarya* species known, little work on the essential oils of the genus has been reported. Phytochemical investigations of the essential oils of *C. mandioccana*, leaf oil yielded 64 compounds with predominance of δ -cadinene, β-caryophyllene, spathulenol, benzaldehyde caryophyllene oxide, β germacrene-D, and bicyclogermacrene [22]. Analysis of the essential oil from the *Cryptocarya* species, endemic to the Brazilian Atlantic rainforests, were examined by Marcelo *et al*. [23] shows monoterpene oil with linalool, α-terpinene and γ-terpinene as major compounds in *C. moschata*. Leaf oil of *C. botethensis* gave trans-verbenol α-pinene and β-pinene and were the major, components, while in *C. saligna* spathulenol, germacrene- D and bicyclogermacrene predominated.

Analysis of the leaf oil from *C. cunninghamii* showed the presence of bicyclogermacrene, benzyl benzoate (80.2%), β -phellandrene (11.8%), viridiflorene (9.1%), phenylethyl benzoate (1%) and methyl benzoate (trace). There were lesser amounts of the two furanoid linalool oxides (4.1 and 3.6%, respectively) and linalool (4.5%). Lesser amounts of caryophyllene oxide and spathulenol (1.3 and 1.2%, respectively) were also present [24]. Naves *et al.* investigated the leaf oil of *C. moschata* and *C. aschersoniana*, *C. aschersoniana* has been investigated and found to oil contained myrcene, 1,8-cineole, (+)-linalool and the two stereoisomeric linalool oxides, while *C. moschatum* contained (S)-linalool as its major constituent. [25].

The component that is most commonly associated with *Cryptocarya* is that derived from *C. massoia* bark, viz. massoia lactone, which has a pleasant peach smell. This compound (combined with its 6-heptyl- homologue) has previously has been obtained

from the bark and heartwood of this species [26-28]. *C. cocosoides* produced oil in which bicyclogermacrene (3-26%), spathulenol (16-47%), massoia lactone (11-15%), and benzyl benzoate (0.2-5%) were the principal components. *C. bellendenkerana* gave leaf oil in which the major components were the terpenes viridiflorene (9.1%) limonene (8.3%) and β-phellandrene (11.8%). The principal components of the leaf oil of *C. lividula* were spathulenol (21.1%), bicyclogermacrene (26.1%), and β-eudesmol (6.1%). acetophenone and Benzaldehyde and were both present in amounts of less than 0.7%.[29].In recent study Seventy one components were identified in the essential oil from leaves of *C. alba* composed mainly of (E)-βbergamotene , viridiflorol and germacrene-D, sabinene , camphene , β-eudesmol , eucalyptol , terpin-4-ol, p-cymol, cineol, α-pinene, β-pinene and borneol-terpineol [30].

Further, phytochemical investigation of *C. stocksii* bark revealed the presence of significant levels of alkaloids, flavonoids and moderate amounts of steroids, and phenols. It shows moderate to strong antioxidant activity in terms of FRAP, DPPH and analgesic activity [31, 32].Considering the interest in species of the family Lauraceae, the objective of this study was to describe, for the first time, the chemical composition and their antioxidant activities of the flower and fruit essential oil *C. stocksii* developed in the South India.

MATERIALS AND METHODS:

Isolation of essential oil using Clevenger's apparatus:

The flower and fruit *of C. stocksii* was collected from Western Ghats region of Karnataka, India. The botanical identifications were carried out at Bioscience Department in P.G.Centre, Hemagangothri, Hassan and the voucher specimen number PS175/30.2014 were deposited in the herbarium of the institute. The fresh flower and fruit (200 g) was cut into small pieces were subjected to hydrodistillation for 4 h using a Clevenger type apparatus [33].The oils were dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4 °C prior to analysis and antioxidant tests. The yields (w/w) were calculated according to the weight of fresh plant material.

GC-MS analysis:

The essential oils of *C. stocksii* were analyzed on an Aligent gas chromatograph model 7890A, coupled to Agilent MS model 5975C MSD with data analysis and AMDIS software along with Quadrupole mass analyzer and electron impact ionization . GC equipped with a DB5 MS Column (30mL X 0.25mm ID X 0.25 µl thickness), programming from 50^0C (1min) to 300^0C at 10^0C/min, 5 min hold time. Helium as carrier gas at a flow rate 1.0 ml/min. Samples (1 µl) were injected manually in the split mode (10:1).Mass spectra data were acquired in the scan mode in m/z range 30-600. The components were identified based on their retention time, abundance and fragmentation patterns by reference to the NIST 2011 library. The percentages of each component are reported as raw percentages based on total ion current without standardization.

Antioxidant activity of the essential oil isolated from *C. stocksii*:

Anti-lipid peroxidation activity:

Anti-lipid peroxidation activities of essential oil were estimated by TBARS method [34]. 0.5 ml of egg homogenate and essential oil with different concentrations (1-50µl) were made up to 1 ml by adding distilled water. 100µl of 0.07M $FeSO_4$ was added to the above mixture and incubated for 30 min at room temperature, to induce lipid peroxidation. Thereafter, to all test tubes 1.5 ml of 20% acetic acid, 1.5 ml of 0.8% TBA (w/v) and 50µl of TCA were added, Vortexed and then heated in a boiling water bath for 60 min. After cooling, 5.0 ml of butanol was added to each tube and centrifuged at 3000 rpm for 10 min. Absorbance of supernatant was measured at 530 nm. α-lipoic acid standard was used for comparison. The percent inhibition activity was calculated using the formula:

% Anti lipid peroxidation= (1−Extract absorbance)/ (Control absorbance) ×100

DPPH scavenging activity:

Antioxidant activity by 2, 2-diphenyl-1-picrylhydrazyl radical method:

Antioxidant activity of essential oil was determined using DPPH according to Giresha *et al.* [35]. The essential oil were taken at different concentration (1-50µl) and mixed with 5 ml of 0.1 mM methanolic solution of DPPH˙ and incubated at 20°C for 20 min in darkness. The control was prepared as above without any oil and methanol was used for the baseline correction. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as percentage activity using the following formula. Ascorbic acid was used as standard was used for comparison. IC_{50} values denote the concentration of the sample, which is required to scavenge 50% of DPPH free radicals.

Percentage radical scavenging activity = ([control absorbance − sample absorbance] /

[Control absorbance]) × 100.

Hydrogen peroxide scavenging activity:

Hydroxyl radical-scavenging by phenanthroline - Fe (II) oxidation assay

The Fenton reaction is a key reaction in organisms which produces hydroxyl radicals. Hydroxyl radicals can attack aromatic compound hydroxylation to form hydroxylation products, which can be detected by colorimetric methods. The adding of antioxidants can reduce hydroxyl radicals and hydroxylation so that the efficiency of scavenging hydroxyl radical can be determined. The ability of the hydroxyl radical-scavenging was carried out as described previously [36]. Briefly, 600 µl of (5 mM) phenanthroline, 600 µl (5 mM) $FeSo_4$, 600 µl of EDTA (15 mM), 400 µl phosphate buffer (0.2 M, pH =7.4) and 800 µl (0.01%) H_2O_2 were added into 10-100 µl various concentration of the oil. After 1h of incubation at 37°C, the absorbance at 536 nm was recorded. Quercetin was used as standard.

The percentage hydrogen peroxide scavenging activity of samples was calculated as follows: Scavenged % Hydrogen peroxide = $(A_0 - A_1 / A_0) \times 100$

Here, A_0 was the absorbance of the control and A_1 was the absorbance of the samples.

Reducing power assay

Ferric reducing antioxidant power assay

The reducing power of essential oil was determined according to the method of Giresha *et al*., [35]. The essential oil were taken at different concentration (1-50µl) and mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated at 50°C for 20 min. The reaction was terminated by adding 2.5 ml of 10% TCA and the mixture was centrifuged at 3500 rpm for 10 min. An aliquot of supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride to form a colored complex which is measured at 700 nm against blank. Absorbance increased by the reaction mixture indicated increased reducing power. Butylated hydroxyanisole was used as a positive control.

STATISTICAL ANALYSIS:

The experimental results were performed in triplicate. The results were analyzed by the Student t-test ($P < 0.05$) and experimental results were expressed as Mean ± Standard error (SE) for each experiments. The regression curve analysis was used to calculate IC_{50} using Graph Pad Prism version 5.02 statistical software. IC_{50} value is a concentration of essential oil required to scavenge 50% free radical and is inversely proportional to the activity of the essential oil.

RESULTS AND DISCUSSION:

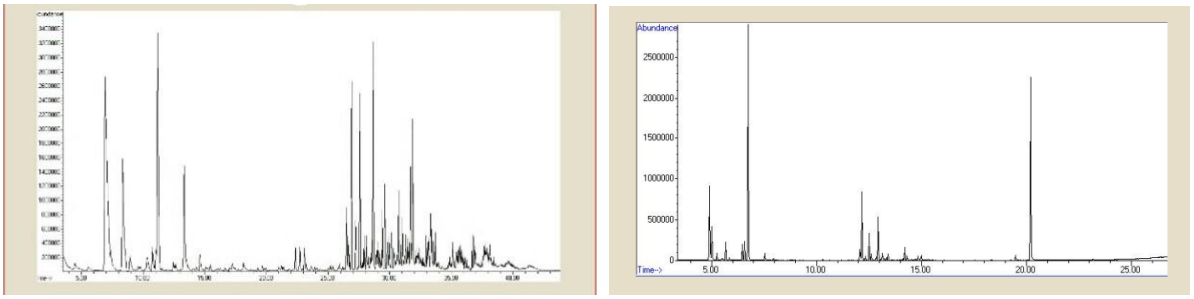
Chemical constituents of the essential oil

This study aimed, for the first time, to characterize the chemical composition of the essential oil isolated from *C. stocksii* flower and fruit. Two essential oils were obtained from fresh *C.stocksii* flower and fruit by hydro-distillation using Clevenger's apparatus yielded 0.05 % and 0.1% (w/w) of colorless oil respectively. Fruit essential oil had the highest yield of 0.1% while flower had lowest yield 0.05 %. Each of the oils had characteristic aroma and the chromatograms of these essential oils are shown in Figures 1. Comparative data on the different essential oil constituents in the flower and fruit of *C.stocksii* is presented in Table 1.

Flower essential oil contained 24 compounds, accounting for 99.78% of the total oil. α-Pinene (39.42%), and β-Pinene (17.96%), is a major constituent of flower oil which is a bicyclic monoterpene. It acts as a natural insecticide [37]. Another interesting compound in flower oil is 3-Carene (29.52%) which is, 4, 7, 7-Trimethyl-3-norcarene. 3-carene, is a bicyclic monoterpene which occurs naturally as a constituent of turpentine and has a sweet and pungent odor. It is used as a raw material in perfumes, cosmetics, flavors and terpene resins [38].

Fruit oil had 24 major compounds accounting for 99.99% of the total oil were characterized. The Fruit oil is dominated by Tetracosane (55.21%), Longifolene (19.12%) and α-Pinene, (8.84%). β-Pinene (4.87%) is also in appreciable amount. Nerolidol (3.57%), a notable compound also known as peruviol, is a naturally occurring sesquiterpene alcohol present in the EO of various plants with a floral odour [39,40] .Nerolidol was found to exist as one of the bioactive compounds responsible for the biological activities demonstrated by the EOs plants. For instance, nerolidol is frequently incorporated in cosmetics (e.g., shampoos and perfumes) and non-cosmetic products (e.g., detergents and cleansers) [41]. The other compounds identified in the Fruit oil are presented in Table 3. The chemical composition observed in the present study was almost similar to the chemical composition already described in the literature for other species belonging to the same genus [22]. Although some changes in the essential oil compositions might arise from several factors

such as geological, geographical, seasonal and climatic[42].



A **B**

Fig.1-GC-MS chromatogram of (A) flower and (B) fruit oil of *Cryptocarya stocksii*

Table.1- Percentage composition of the essential oils isolated from *C.stocksii*

			Composition (%)	
Sl.No	**Retention Time(Min)**	**Compound name**	**Flower**	**Fruit**
1	5.0108	α-Pinene	39.42	8.84
2	5.2435	Camphene	3.37	1.78
3	5.4409	Benzaldehyde		0.7
4	5.6721	β-Pinene	17.96	4.87
5	5.9449	1,3,5-trimethylbenzene	0.03	
6	6.1352	4-Methyldecane		0.01
7	6.4643	Limonene	0.39	0.57
8	6.4672	Terpinolene	0.16	
9	6.5231	Eucalyptol	0.21	
10	7.2226	Undecanol	0.02	
11	7.401	α-Terpinene	0.01	0.16
12	7.4888	3-tert-Butylphenol		0.01
13	7.4966	Promecarb		0.01
14	7.9939	α-Terpinolene		0.05
15	8.8723	Carvacrol		1.07
16	8.9502	α-Terpineol	0.53	0.05
17	9.3756	Methyl Alcohol		0.01
18	9.4819	3-Methyl-2-cyclopenten-1-one		0.02
19	10.2977	Borneol	0.03	0.2
20	11.1312	3-Carene	29.52	1.92
21	12.0161	p-Xylene		0.02
22	12.1627	Longifolene	6.53	19.12
24	12.7908	Benzeneacetic acid	0.02	
25	13.2035	2,4-Di-tert-butylphenol		0.02
26	13.8372	Nerolidol	1.32	3.57
27	14.4201	N,N-Diethyl-p-phenylenediamine	0.01	
28	14.5126	Pyrethrin I	0.03	
29	17.2671	1-Hexadecanol	0.04	
30	19.4758	γ-Decalactone	0.06	1.55
31	20.2006	Tetracosane		55.21
32	21.1167	Tridecane		0.02
33	23.1806	Bis(2-ethylhexyl) phthalate		0.21
34	25.4033	2,5-Methylvinylpyridine	0.06	
35	25.4139	tert-Butylbenzene	0.02	
36	26.6857	Pentamethylbenzene	0.02	
37	31.0701	2-Methylbenzothiazole	0.02	
38	37.1791	Ethylene glycol n-hexyl ether	0.03	
		Total oil (%)	**99.81**	**99.99**

Anti-lipid peroxidation activity:

Lipid peroxidation is broadly recognized as primary toxicological event, which caused by the generation of free radicals from a variety of sources including organic hydro peroxides, redox cycling compounds and iron-containing compounds. In present work the TBARS assay was utilized to measure the degree of lipid peroxidation. TBA reacts specifically with malondialdehyde (MDA), a byproduct of lipid peroxidation to give a red chromogen, which can be read by spectrophotometrically [43]. According to the obtained results, *C.stocksii* essential oil and its component significantly inhibited the formation of MDA at dose dependent manner (Fig.2) and suppressive power on the lipid peroxidation of flower and fruit essential oil were found to be the most potent (IC_{50} value 71.14±1.85µl/ml and 39.72±1.59µl/ml). α-lipoic acid showed significant suppressive power on lipid peroxidation with IC_{50} value of 3.29±291µg/ml (Table 2). These result recommend that, the oil might prevent reactive radical species from damaging biomolecules such as DNA, proteins, amino acids, sugar, lipoprotein and PUFA in biological and food systems.

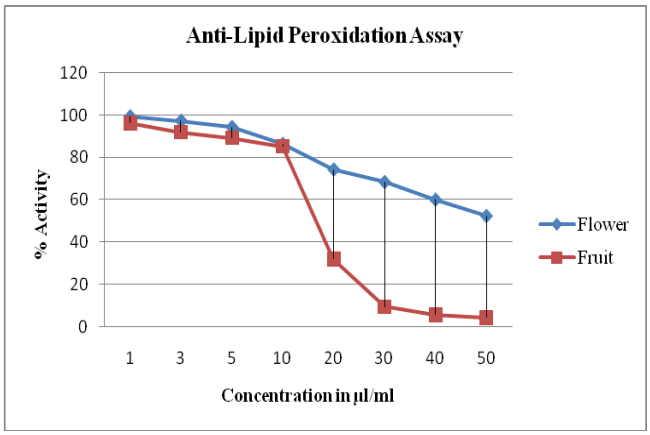


Fig-2. Anti-lipid peroxidation activity of flower and fruit essential oil (µl/ml), isolated from *C.stocksii.*

DPPH free radical scavenging activity:

DPPH assay has been broadly used for screening plant extracts because many samples can be accommodated in short period and are sensitive enough to identify active ingredients at low concentrations [44]. The antioxidant activity of *C.stocksii* flower and fruit essential oil result showed a dose-dependent inhibition of DPPH radical and is summarized in (Fig.3). DPPH free radical is stable nitrogen centered free radical commonly used for testing radical scavenging activity of the plant extracts. The violet colour of the DPPH radical reduced to yellow colored diphenylpicrylhydrazine radical after accepting an electron from the antioxidant compound, which is measured spectrometrically. Substances which are able to perform this reaction will be considered as antioxidants and therefore radical scavengers [45]. It was found that the radical-scavenging activity of the flower and fruit essential oil (1-50 µl/mL) from *C.stocksii* was showed increased with increasing concentration and the IC_{50} value was found to be 41.09±1.61µl/ml and 32.95±1.51µl/ml flower and fruit respectively Correspondingly, IC_{50} value for ascorbic acid, used as standard, was 19.59±3.23 µg/ml (Table.2). The high terpenes contents of this plant may lead to its good DPPH-scavenging activity.

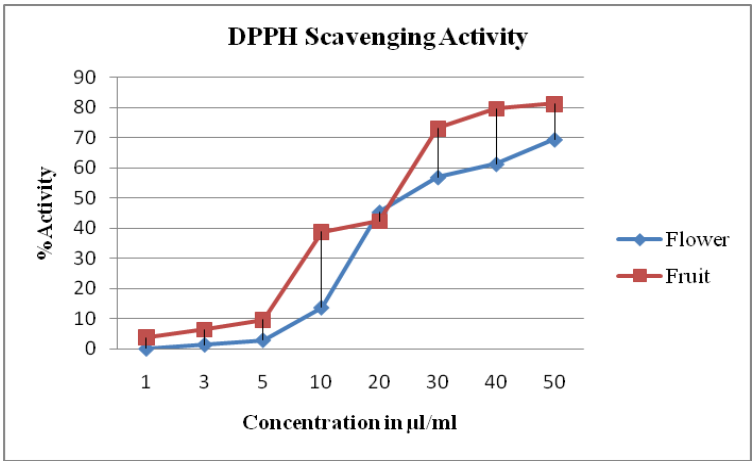


Fig-3.DPPH radical scavenging activity of flower and fruit essential oil (µl/ml), isolated from *C.stocksii.*

Hydrogen Peroxide assay:

Several investigations reveals that superoxide anions breakdown the biomolecules directly or indirectly by forming H2O2, ¯OH, singlet oxygen during aging and pathological events such as ischemic reperfusion injury. Superoxide has also been observed to directly initiate lipid peroxidation [46, 47]. H_2O_2 is a non-radical reactive oxygen species and the most stable intermediate in the four-electron reduction of O_2 to water [48]. Since H_2O_2 is uncharged, it easily passes through cell membranes by diffusion, and when inside the cells it can react with transition metals generating hydroxyl radicals. At high concentrations, these radicals induce peroxidation of lipids and proteins, affecting cell integrity [49]. Thus, exploring essential oil behavior in the presence of H_2O_2 remains of high interest. The hydrogen peroxide scavenging ability of *C.stocksii* flower and fruit essential oil is shown in Figure 3. However, the required concentrations (Fig.4) for the inactivation of 50% of the H_2O_2 are: flower 95.1±1.97 µl/ml and fruit 77.46±1.88 µl/ml for flower and fruit essential oil respectively. Whereas, IC_{50} value for quercetin, used as standard, was 12.76±1.45 µg/ml (Table.2).

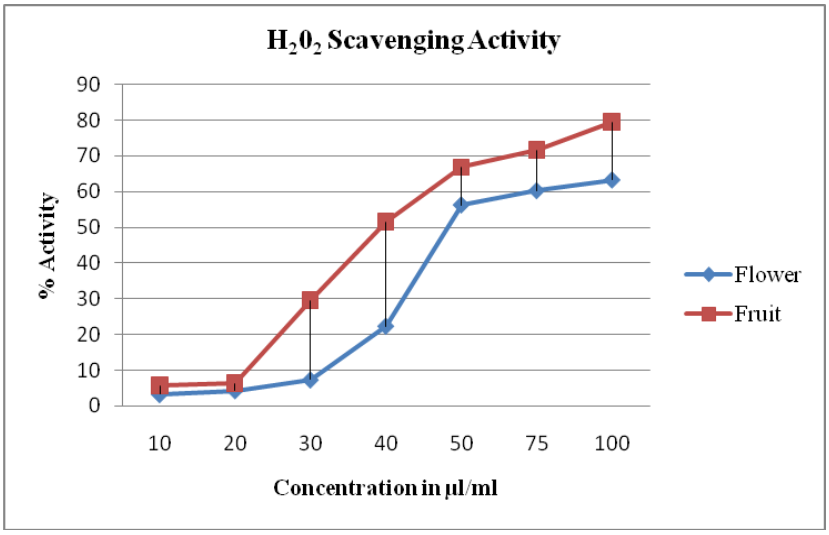


Fig-4. Hydrogen Peroxide scavenging activity of flower and fruit essential oil (µl/ml), isolated from *C.stocksii.*

Ferric reducing antioxidant power assay:

Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of antioxidant action [50]. The reducing ability of a phytochemical generally depends on the presence of reductones (antioxidants), which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom [51]. Reducing power of the essential oil increases with the increase in concentration and showed good reducing power ability in a dose dependent manner (Fig.5) with IC_{50} of flower 60.7±1.78 µl/ml and fruit 42.75±1.63 µl/ml.

IC_{50} value of Butylated hydroxyanisole which is used as standard was 9.45±2.238µg/ml (Tab.2). The antioxidant principles present in oil cause the reduction of Fe3+/ferricyanide complex to the ferrous form and thus proved essential oil isolated from *C.stocksii* has promising radical scavenging ability and potent reducing power.

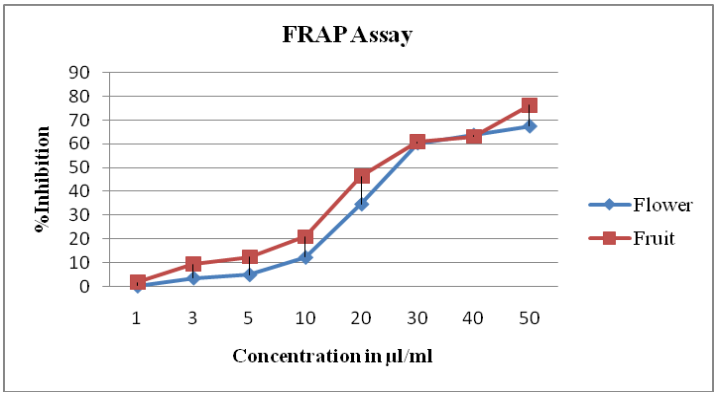


Fig-5.Reducing power activity of flower and fruit essential oil (µl/ml), isolated from *C.stocksii*.

	IC_{50} value (µl/ml)		IC_{50} value (µg/ml)
Activity	Flower	Fruit	Standard Antioxidants
FRAP Assay	60.7±1.78	42.75±1.63	9.45±2.238
ALP Assay	71.14±1.85	39.72±1.59	3.29±291
DPPH Scavenging Activity	41.09±1.61	32.95±1.51	19.59±3.23
H_20_2 Scavenging Activity	95.1±1.97	77.46±1.88	12.76±1.45

Table-2.Antioxidant capacities of essential oils of *C.stocksii* .Values are mean ± SE.

CONCLUSION:

This study has been concerned with determining the chemical composition and antioxidant activity characteristics of essential oils extracted from *C.stocksii* flower and fruit collected in the Western Ghats region of Karnataka. The presented results indicate that the *C.stocksii* essential oil could be used as potential source of natural antioxidants for the pharmaceutical and food industry. Therefore, *C.stocksii* essential oil represents the alternative to synthetic additives that exhibit toxic and carcinogenic effects. So, it is interesting to investigate its application as natural antioxidant additive in some final food and pharmaceutical products, for preservation and/or extension the shelf-life of raw and processed foods as well as pharmaceuticals. This is the first report on the antioxidant potential of the essential oils of *C.stocksii*. On the other hand, further detailed studies of essential oils of *C.stocksii* are required to determine which of their components are more responsible for its antioxidant effect and other biological properties.

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