Phytochemical Analyses and Evaluation of Antioxidant Efficacy of *in vitro* Callus Extract of East Indian Sandalwood Tree (*Santalum album* L.)

Biswapriya B. Misra¹*, Satyahari Dey²

1. Post-Doctoral Fellow, Center for Chemical Biology, Universiti Sains Malaysia [CCB@USM], 1st Floor Block B, No 10, Persiaran Bukit Jambul, 11900 Bayan Lepas, Penang, Pulau Pinang, Malaysia. [E-mail: bbmisraccb@gmail.com; Tel: +6-0103700201]
2. Professor, Plant Biotechnology Laboratory, Department of Biotechnology, Indian Institute of Technology Kharagpur, Kharagpur-721302, West Bengal, India.

The phytochemical constitution and antioxidant activity of *in vitro* grown callus cultures of East Indian Sandalwood tree (*Santalum album* L.) were investigated. The extractive yield for a dichloromethane-methanol (1:1) solvent mixture was 4.3 %. The phytochemical screening revealed the extract’s richness in phenolics (18.2 µg), terpenoids (16.4 µg), saponins (9.4 µg) and flavan-3-ols (7.4 µg) per milligram of extract, as major constituents. This extract showed antioxidant activity in ferric reducing assay power (FRAP), total antioxidant capacity (TAC), metal ion chelation, inhibition of lipid peroxidation and in scavenging of hydroxyl radical (OH.), 2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS.), di (phenyl) (2, 4, 6-trinitrophenyl) imaginazium (DPPH.) and nitric oxide (NO.) free radical scavenging and reducing power assays that was comparable to sandalwood oil and reference antioxidant such as quercetin, gallic acid and α-tocopherol. We conclude that *in vitro* propagated callus shows immense potential as a renewable resource of antioxidant constituents.

**Keyword:** Antioxidant, Callus, *In vitro*, Phenolics, Sandalwood, Terpenoid

1. Introduction

*Santalum album* L., the East Indian Sandalwood tree is an important medicinal tree. This woody and tropical member of Santalaceae is the major source of sandalwood essential oil, a mixture of sesquiterpenoid alcohols, i.e., 90% santalols. Deposited in the core of heartwood of the tree, a 50 year old matured tree may yield 2.5-6% of essential oil, and is influenced by several factors. The oil finds use in traditional medicine system Ayurveda as an antiseptic, antipyretic, antiscabetic, diuretic, expectorant, stimulant and for the treatment of bronchitis, dysuria, urinary infection and gonorrhea as it seems to contain antibacterial and antifungal properties [1]. The hydrolyzed exhausted sandalwood powder demonstrates anti-remorogic, anti-inflammatory, anti-mitotic, anti-cancer, anti-hypertensive, anti-pyretic and sedative properties [2]. The oil also possess antiviral activity against herpes simplex virus [3] and anti-*Helicobacter pylori* properties [4], the causative organism for gastric cancer and peptic ulcer.
Epidemic phytoplasmal ‘spike’ disease leading to severe destruction of natural population, illegal poaching and over exploitation owing to increased global demand are the reasons of the tree being inducted into IUCN, Red List of Threatened Species [5] as vulnerable. Unsurprisingly, the first in vitro micropropagation study on any woody forest tree was reported in sandalwood (callusing from embryos) followed by further advances in biotechnological routes of micropropagation i.e., somatic embryogenesis, regeneration, suspension cultures, somatic embryo production and maturation in air lift bioreactors [6]. Furthermore, in vitro callus is known to yield sandalwood oil constituents [7].

However, till date there are no reports available that investigated the antioxidant potential of sandalwood oil or the in vitro callus of sandalwood. This comparative study was undertaken to probe the antioxidant properties of a dichloromethane: methanol extract from in vitro callus, with sandalwood oil and reference antioxidants. Moreover, it is important to establish appropriate means to evaluate and quantify effective antioxidant principles of economically viable resources for plant-based therapeutics. To our knowledge, this is the first time effort towards evaluation of biological activities of any in vitro material from sandalwood tree.

2. Materials and Methods
2.1 Reagents
The chemical reagents were obtained as follows, i.e., 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), linalool and butylated hydroxy toluene (BHT) were obtained from Fluka, Switzerland; 2-deoxy-2-ribose, trichloroacetic acid (TCA), sodium nitroprusside (SNP), sulfanilamide, naphthylethylenediamine dihydrochloride (NED), rutin, catechin, gallic acid, quercetin, ferrozine, Bradford reagent and Phytagel were obtained from Sigma, St. Louis; Folín-Ciocalteu reagent, dimethylamino cinnamaldehyde (DMACA), aluminum chloride (AlCl3), ferric ammonium sulfate [NH4Fe(SO4)2·12H2O], sodium carbonate [Na2CO3·10H2O], ammonium molybdate [(NH4)6Mo7O24·4H2O], sodium nitrite [NaNO2], ferric chloride [FeCl3], ferrous chloride [FeCl2], dichloromethane (HPLC grade), methanol (HPLC grade) and n-butanol (Spectroscopy grade) were procured from Merck, India; sapogenin, bovine serum albumin (BSA), polyvinylpolypyrrolidone (PVPP), thiobarbituric acid (TBA), di (phenyl) - (2, 4, 6-trinitrophenyl) imaginazium (DPH), casein, vanillin, potassium persulfate, potassium ferricyanide [K3Fe (CN)6], ethylene diamine tetraacetic acid (EDTA), ascorbic acid, Woody Plant Medium (WPM) and 2,4-dichlorophenoxyacetic acid [2, 4-D], 2, 4, 6-tripyridyl-s-triazine (TPTZ), were purchased from HiMedia, India while authentic sandalwood oil samples were procured from Cauvery™, Bangalore, India.

2.2 Plant Materials
In vitro callus was from a highly proliferating cell line (IITKGP/ 91), grown aseptically on solid media i.e. Woody Plant Medium [8] supplemented with 2, 4-D (1 mg/ L), 3 % sucrose, and 0.35 % Phytagel, pH 5.8±0.5 in culture tubes, in dark 25±2 °C and were maintained by sub culturing at intervals of 3 weeks, in the laboratory in the plant tissue culture facility of Department of Biotechnology.

2.3 Preparation of Extracts
In vitro grown callus (100 g) was collected, washed in ddH2O, freeze dried and pulverized into fine powder using a mortar and pestle, and extracted for 18 h in dichloromethane: methanol (1:1, v/ v) [9] at 40 °C in a ratio of 1: 200 (w/ v) of plant material and solvent. Post- extraction, solid materials were excluded by filtration using a Whatman No. 1 filter paper and were centrifuged at 5, 000 g for 10 min. The supernatant was concentrated using an Eyela, N- N series, rotary evaporator connected to an Eyela aspirator, Model: A 3S (Rikakikai Inc., Tokyo, Japan) at 40 °C under reduced pressure. The extract obtained was stored at -20 °C until further use.
Table 1. Summary of methods followed for phytochemical characterization of sandalwood callus extract.

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Assay</th>
<th>Extract amounts</th>
<th>Reagents</th>
<th>Conditions</th>
<th>Monitoring system</th>
<th>Standard curve</th>
<th>Calculation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total terpenoid content</td>
<td>100 µg in methanol</td>
<td>500 µl of 2 % vanillin-H2SO4 in cold.</td>
<td>60 °C for 20 min, cooled at 25 °C for 5 min and within 20 minutes, absorbance measured</td>
<td>Blue-green color, absorbance at 608 nm</td>
<td>Linalool (20 – 100 mg/l)</td>
<td>as µg/ mg extract</td>
<td>[11]</td>
</tr>
<tr>
<td>2</td>
<td>Total anthocyanins content</td>
<td>100 µg in methanol</td>
<td>a. 500 µl extract + 6 ml of n-butanol: HCl (95:5, v/v) b. 0.2 ml of 2 % (v/ v) solution of NH4Fe(SO4)3, 12H2O in 2 M HCl</td>
<td>Heated tubes at 92-95 °C for 40 min</td>
<td>Deep green/ brown solution, absorbance at 550 nm</td>
<td>Multiplied OD650 by 33.3 (ε= cyanidin chloride of 30,000)</td>
<td>as mg of cyanidin chloride/ 100 g extract</td>
<td>[12]</td>
</tr>
<tr>
<td>3</td>
<td>Total oligomeric proanthocyanidins content</td>
<td>1 mg/ ml in methanol</td>
<td>a. 5 ml of 0.5% vanillin reagent. b. 5 ml volume of 4% methanolic HCl</td>
<td>Incubation for 20 min at room temperature</td>
<td>Dark greenish-blue color, absorbance at 500 nm</td>
<td>Catechin (100-500 µg)</td>
<td>as mg or g catechin equivalents per 100 g extract</td>
<td>[13]</td>
</tr>
<tr>
<td>4</td>
<td>Total phenolics content</td>
<td>1 mg/ ml in 30 % methanol</td>
<td>a. 100 µl of extract + 50 µl of Folin Ciocalteau reagent (FCR), 10 min wait and up to 1 ml with Na2CO3,10H2O and well shaken.</td>
<td>Incubation for 2 hours in dark at room temperature</td>
<td>Blue solution, absorbance at 720 nm</td>
<td>Gallic acid (10-50 µg)</td>
<td>as mg gallic acid/g extract</td>
<td>[14]</td>
</tr>
<tr>
<td>5</td>
<td>Total polyphenols and Casein/ BSA/ PVPP-Bound Tannins</td>
<td>1 mg/ ml in 30 % methanol (total polyphenols)</td>
<td>a. 200 µl of extract + 800 µl of water. b. 100 mg of BSA/ casein or PVPP added.</td>
<td>a. Shaken for 1 h at room temperature and then 1-2 h incubation at 4°C b. Filtered or centrifuged at 4000 rpm/ 15 min. Filterate (= Unbound polyphenols</td>
<td>Blue solution, absorbance at 720 nm</td>
<td>Catechin (100-500 µg)</td>
<td>Bound tannins= Total polyphenols- Unbound polyphenols; as mg catechin/g extract</td>
<td>[14]</td>
</tr>
<tr>
<td>6</td>
<td>Total flavonoid content</td>
<td>100 µg/ ml in methanol</td>
<td>1 ml of 2% methanolic AlCl3, 6 H2O + 1 ml extract</td>
<td>Incubation at 10 min.</td>
<td>Yellow color, absorbance at 430 nm</td>
<td>Rutin (10-50 µg)</td>
<td>as mg rutin/g extract</td>
<td>[15]</td>
</tr>
<tr>
<td>7</td>
<td>Total flavan-3-ols content</td>
<td>1 mg/ ml in methanol</td>
<td>200 µl sample + 1 ml of p-dimethylamino cinnamaldehyde (DMACA) (0.1% in 1 N HCl in methanol)</td>
<td>Sample vortexed, 10 min at room temperature.</td>
<td>Blue color monitored at 640 nm</td>
<td>Catechin (25-150 µg)</td>
<td>as mg catechin/g extract</td>
<td>[16]</td>
</tr>
<tr>
<td>8</td>
<td>Total saponin content</td>
<td>1 mg / ml in methanol</td>
<td>10 µl extract in methanol+ 50 µl 8% vanillin in ethanol + 500 µl 72 % H2SO4</td>
<td>Heated to 60 °C for 20 min, followed by 4 °C for 5 min.</td>
<td>Yellow/ green color, absorbance at 544 nm</td>
<td>Sapogenin (10-100 µg)</td>
<td>as mg saponin/g extract</td>
<td>[17]</td>
</tr>
</tbody>
</table>

2.4 Physiochemical Characterization
Dry weight, moisture and ash content of callus were determined according to the AOAC methods [10]. The pH of the obtained extract was measured using a pH meter (model: 720 A, Orion) as well as pH paper strips (1-14, HiMedia, India). The yield of extract was calculated based on the following equation shown below: Yield (%) = (W1 x 100)/ W2, where W1 was the weight of extract after evaporation of solvents (mg) and W2 was the dry weight of the fresh callus (mg).
2.5 Phytochemical Analyses
The standard phytochemical quantification procedures followed are mentioned in Table 1.

2.6 Screening of Antioxidant Efficacy
The antioxidant efficacy of the callus extracts were compared against sandalwood oil by the following nine methods, essentially following the exact procedures as mentioned by the authors. Positive controls (reference antioxidants) used across all the assays were gallic acid, \(\alpha\)-tocopherol, quercetin, squalene and EDTA (for metal ion chelating assay), while only the best results were reported.

2.7 Reducing power determination
Reducing power of the callus extract and sandalwood oil were tested as described \[18\].
Hydroxyl radical scavenging activity (deoxyribose method). Hydroxyl radical scavenging activity of callus extract and sandalwood oil were performed as described \[19\].
Metal (ferrous) ion chelating activity Metal ion chelating activity was screened for the callus extract and sandalwood oil as described \[20\].
Ferric reducing antioxidant power (FRAP) assay Ferric reducing antioxidant power (FRAP) was measured for the callus extract and sandalwood oil as described \[21\].

Total antioxidant capacity (TAC)
Total antioxidant capacity was tested by the phosphomolybdenum method for the callus extract and sandalwood oil as described \[22\].

Nitric oxide free radical scavenging activity
Nitric oxide free radical scavenging activities were performed for the callus extract and sandalwood oil as described \[23\].

ABTS free radical scavenging activity
ABTS free radical scavenging activity was tested for the callus extract and sandalwood oil by the ABTS- potassium persulfate method\[24\].

DPPH free radical scavenging activity
The DPPH free radical scavenging activity was performed for the callus extract and sandalwood oil as described \[25\].
Lipid peroxidation inhibition in mice liver microsomes (TBA method).
Inhibition of lipid peroxidation in isolated mice liver microsomes were tested for the callus extract and sandalwood oil as described \[26\].
Protein concentrations were measured by Bradford method, using BSA as a standard \[27\].

2.8 Statistical Analysis
Six independent assays were performed and the results were expressed as mean±S.D. Data analysis was performed using Microsoft Excel (Microsoft Corp., Redmond, WA). Student's t-tests were used for determining the levels of significance between the control and the test values.

3. Results and Discussion
3.1 Physico-Chemical Characteristics
In vitro grown callus cultures provided a renewable resource of biomass which is easily culturable and the conditions are very much standardized under laboratory conditions. The moisture content of the callus is found to be 93.7±8.7%, with 4.1% ash content. The dichloromethane: methanol (1:1) extracts from callus was found to be a standardized solvent for optimal extraction and hence, a process for enrichment of both polar and non-polar constituents from sandalwood. The extract yield was 4.3%, and the pH of the extract was slightly acidic at 6.6 as shown in Table 2.

Identification of new effective antioxidants has recreated interest in plant extracts and secondary metabolites, as they protect against oxidant-induced damage. Owing to differences between the tests systems investigated, it has been recommended that at least two methods be used to evaluate antioxidant efficacy. Thus the antioxidant activity of callus extract was tested by nine different methods.
Table 2: Physico-chemical and phytochemical characterization of sandalwood callus extract.

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Physico-chemical parameters</th>
<th>Quantity in Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Moisture Content (%)</td>
<td>93.7±8.7</td>
</tr>
<tr>
<td>2</td>
<td>Ash Content (%)</td>
<td>4.1±0.2</td>
</tr>
<tr>
<td>3</td>
<td>Yield of extract (%)</td>
<td>4.3±1.3</td>
</tr>
<tr>
<td>4</td>
<td>pH of extract</td>
<td>6.6±0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phytochemical parameters</th>
<th>Quantity in Extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Terpenoid content (µg linalool equivalence / mg)</td>
<td>16.4±2.7</td>
</tr>
<tr>
<td>6 Total soluble proanthocyanidin (flavan-3-ols) content (µg catechin equivalence / mg)</td>
<td>7.4±1</td>
</tr>
<tr>
<td>7 Total insoluble proanthocyanidin content (µg catechin equivalence / mg)</td>
<td>0.6±0.08</td>
</tr>
<tr>
<td>8 Total anthocyanin content (µg cyanidin chloride equivalence / mg)</td>
<td>0.4±0.09</td>
</tr>
<tr>
<td>9 Total flavonoid content (µg rutin equivalence / mg)</td>
<td>0.07±0.002</td>
</tr>
<tr>
<td>10 Total condensed tannin content (µg catechin equivalence / mg)</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>- BSA bound condensed tannin (µg catechin equivalence / mg)</td>
<td>-</td>
</tr>
<tr>
<td>- PVPP bound condensed tannin (µg catechin equivalence / mg)</td>
<td>-</td>
</tr>
<tr>
<td>- Casein bound condensed tannin (µg catechin equivalence / mg)</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>11 Total phenolic content (µg gallic acid equivalence / mg)</td>
<td>18.2±1.8</td>
</tr>
<tr>
<td>12 Total saponin content (µg saponin equivalence / mg)</td>
<td>9.4±0.5</td>
</tr>
</tbody>
</table>

3.2 Phytochemical Composition Analyses
Phytochemical evaluations done by spectrophotometric methods. Standard curves for linalool \[y = 0.002x, R^2 = 0.988\], sapogenin \[y=0.017x- 0.197, R^2= 0.988\], gallic acid \[y = 0.871x, R^2 = 0.968\], rutin \[y=0.0448x, R^2= 0.968\] and catechin \[y=0.0006x, R^2= 0.944\] were used to quantify the amounts of terpenoids, saponins, phenolics, flavonoids and soluble proanthocyanidins, flavan-3-ols and polyphenols, respectively. The BSA standard curve \[y = 0.609x, R^2 = 0.971\] served to quantify the proteins in mice liver microsomal fractions. Results suggested higher yields of phenolics (18.2 µg), followed by terpenoids (16.4 µg), saponins (9.4 µg) and soluble proanthocyanidins (7.4 µg) per mg of total extract. Nevertheless, condensed tannins, anthocyanins and flavonoids were also quantified in the extract (Table 2).

Phenolics are the most plentiful classes of constituents in the plant kingdom. In sandalwood callus extract, 18.2 mg gallic acid equivalents (GAE)/ g of total phenolics was quantified. Tannins are widely distributed among the angiosperms. In fact, presence of tannins such as gallic acid, catechins and tannin- glycosides in callus cultures of the woody species, *Quercus acutissima* is well known[28]. The fact, that saponins are well-represented in sandalwood callus extract, is further corroborated by the fact that the plant order Santalales are known to contain oleane- type triterpene saponins. Moreover, the callus of common hawthorn, *Crataegus monogyna* accumulates proanthocyanidins[29]. Recently, flavonoids were reported from *Santalum insulare* leaves i.e., chlorogenic acid, luteolin, apigenin and apigenin- and luteolin- glucopyranosides[30].

3.3 Antioxidant Potential of Callus Extract
Antioxidants act by prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical-scavenging. In all the assays, gallic acid (polyphenol), α-tocopherol (soluble form of vitamin E), quercetin (flavonoid), squalene (terpenoid), BHT (synthetic fat soluble antioxidant) were used as reference compounds. However, for comparison purposes, only the results for the reference compound with highest antioxidant potentials are provided for ease in Table 3.
Table 3. Reactive oxygen species scavenging and free radical activities of sandalwood callus extract, oil and reference compounds obtained using a variety of in vitro assays. [** p < 0.01; *** p < 0.001 against reference compounds].

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Antioxidant Activity</th>
<th>Callus Extract</th>
<th>Sandalwood Oil</th>
<th>Reference compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reducing Assay Power (100 µg/ml, OD-700 nm)</td>
<td>0.13±0.01**</td>
<td>0.14±0.01**</td>
<td>0.53±0.05 (Quercetin)</td>
</tr>
<tr>
<td>2</td>
<td>Hydroxyl Radical Scavenging Activity (% inhibition at 100 µg /ml)</td>
<td>94.5***</td>
<td>98.3***</td>
<td>78.5 (Gallic Acid)</td>
</tr>
<tr>
<td>3</td>
<td>Metal Ion Chelating Activity (IC50 in µg/ml)</td>
<td>1025.5±43.5</td>
<td>23.5±2.8 **</td>
<td>0.61±0.1 (EDTA) 37±4.5 (Gallic Acid)</td>
</tr>
<tr>
<td>4</td>
<td>FRAP (µM Fe (II)/ g extract)</td>
<td>55±5 **</td>
<td>1056±106.9***</td>
<td>29.7±7.7 (α-tocopherol)</td>
</tr>
<tr>
<td>5</td>
<td>Total Antioxidant Capacity Assay (µmol equivalents of ascorbic acid/ g extract)</td>
<td>61.2±5**</td>
<td>22.4±1.8</td>
<td>37.3±5.1 (Gallic Acid)</td>
</tr>
<tr>
<td>6</td>
<td>NO Free Radical Scavenging Assay (% inhibition at 100 µg/ml)</td>
<td>0.5±0.02</td>
<td>4.2±0.5</td>
<td>67.9±9.3 (Gallic Acid)</td>
</tr>
<tr>
<td>7</td>
<td>ABTS Free Radical Scavenging Activity (mM L-ascorbic acid equivalent/ 100 mg extract)</td>
<td>175±19***</td>
<td>4.5±0.8</td>
<td>11.66±0.9 (L-ascorbic acid)</td>
</tr>
<tr>
<td>8</td>
<td>DPPH Radical-Scavenging Capacity (IC50 in µg/ ml)</td>
<td>1.17±0.2**</td>
<td>0.87±0.07**</td>
<td>4.05±0.7 (Quercetin)</td>
</tr>
<tr>
<td>9</td>
<td>Lipid peroxidation inhibition (TBRS) (IC50 in µg/ ml)</td>
<td>4.8±0.71**</td>
<td>12.4±1.4</td>
<td>0.84±0.09 (α-tocopherol)</td>
</tr>
</tbody>
</table>

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity, as they ameliorate the damaging effect of reactive oxygen species like hydroxyl radical. At, 100 µg/ml, both, callus extract and sandalwood oil showed comparable and significantly (p<0.01) higher reducing capacity as compared to quercetin, a strong flavonoid antioxidant. The hydroxyl radical scavenging activity indicated their efficacy as chelating agents, as well as their capacity to compete with deoxyribose for OH which are produced free in solution from a Fe2+-EDTA chelate. Both, callus extract and sandalwood oil are stronger hydroxyl radical scavengers when compared to gallic acid (p<0.001).

Phytoextracts are expected to chelate Fe2+ in the absence of EDTA, which indicates the chelating ability of extracts, possibly explaining the ability of the extracts to reduce iron and then form Fe2+-extract complexes that are inert. Sandalwood oil is a strong metal ion chelator (p<0.01) compared to gallic acid, a strong phenolic antioxidant. However, the IC50 value for callus extract was much higher in comparison, and hence is a weak metal ion chelator. The measure of capacity to reduce ferric ions, expressed as FRAP value, for callus extract was comparable to that of α-tocopherol, and much lower than sandalwood oil, thus indicating higher efficacy of sandalwood oil as reducing agent. Similarly, the NO free radical scavenging potentials of callus extract and sandalwood oil were lower than gallic acid.

The total antioxidant capacity (TAC) of sandalwood callus extract was significantly (p<0.01) higher than gallic acid, and almost 3-times higher than sandalwood oil. In this study, synthetic ABTS⁺ and DPPH radicals were used to assess the radical scavenging abilities of samples. These radicals are widely used for the determination of radical scavenging abilities of...
plants, even though biologically not relevant, they provide an indication of hydrogen/electron-donating capacity of plants and are hence a useful measure of in vitro antioxidant activity. The ABTS free radical scavenging activity of sandalwood oil is significantly higher (p<0.01) than L-ascorbic acid (vitamin C). The callus extract exerted significant capacity to scavenge the DPPH• radicals. The results are expressed as IC50 values. The lower the IC50 value, the higher the antioxidant capacity of the extract. The activity of the callus extract in the DPPH assay indicates its strong hydrogen-donating ability, that was significantly higher (p<0.01) than quercetin, and comparable to sandalwood oil. The lipid peroxidation (TBRS) inhibition potential for callus extract is significantly higher than sandalwood oil, but weaker than α-tocopherol, as observed from the lower IC50 value of the later.

The callus extract and sandalwood oil showed excellent reducing power values that are comparable to Terminalia extracts[31]. The deoxyribose assay for hydroxyl free radical scavenging assays reveal that, sandalwood oil at 100 μg/ ml brought about 98.3 % scavenging, thus bettering curry leaf, Murraya koenigii extracts, which scavenged 51- 74 % at similar concentrations[32]. The potencies of extracts indicated their efficacy as chelating agents and capacity to compete with deoxyribose for OH−, produced free in solution from a Fe2+-EDTA chelate.

Flavonoids exhibit the antioxidative, antiviral, antimicrobial and anti- platelet activities. Non-flavonoid polyphenolics (i.e., dihydroxyl configuration in gallotannins) and flavonoids reduce iron and then form Fe2+- polyphenol or Fe2+- flavonoid complexes that are inert and hence are the best know natural metal chelators. The lower flavonoid content of the callus may be attributed to the higher IC50 values for inhibition of metal ion chelation by the extract. In contrast, IC50 value for inhibition of metal ion chelation, in case of the parsley oil was reported at 5.12 mg/ml [33], in comparison to EDTA, 1.27±0.05 μg/ml, as was in case of sandalwood oil in this study.

For Ligaria cuneifolia extracts, the FRAP values of 1862 μg Fe (II)/ g extract are reported[34], whereas the values were much lower in case of sandalwood oil. However, saponins, though antioxidants, do not contribute to the FRAP values, whereas the reductones/ reductants such as flavones and flavanones do. This could explain the higher values of saponin content and still lower FRAP values, compared to sandalwood oil where saponins are seldom detected.

The TAC values of sandalwood callus extract and oil, are comparable to that of some major cereals, i.e., wheat (76.70±1.38), oat (74.67±1.49) and rice (55.77±1.62) in μM of vitamin C equivalent/ g of grain[35], thus underscoring their potential role as additives and nutritional supplements in non-cereal foods. Sandalwood oil and callus extract did not show significant NO free radical scavenging activity as compared against positive control, i.e., gallic acid. The ABTS free radical scavenging activities for many essential oil such as cinnamon, thyme, chamoline, citronella and rosemary are reported to be variable, i.e., 2120, 33.5, 0.81, 0.26 and 0.16 [Trolox eq. (mM)], respectively. Similarly, in his study we obtained a much lower ABTS free radical scavenging activity for sandalwood oil, though the callus extract was significantly (p<0.001) more active compared to α-tocopherol. Moreover, the DPPH free radical scavenging activity for callus extract and sandalwood oil were significantly (p<0.01) higher than quercetin, a strong decolorizer of DPPH•. In fact, previously terpenoid rich extracts have been reported to be strong DPPH free radical scavengers. For instance, the IC50 value for DPPH free radical scavenging activity was reported at 0.64 μg/ ml for a ginger extract rich in α-zingiberene, β- bisabolene, α- farnesene and a-curcumene[36]. Moreover, higher antioxidant activities were observed for in vitro cultures of Salvia officinalis and Rosmarinus officinalis compared to their field grown plant materials[37]. Phenolics exert their antioxidant effect via scavenging of reactive oxygen and nitrogen radicals. Additionally, phenolics inhibit the lipid peroxidation by chain termination through scavenging the peroxy
radicals and by electron donation, while terpenoids may act against lipid peroxidation owing to high lipophilicity. Using bovine brain phospholipid liposomes for inhibition of lipid peroxidation, IC\textsubscript{50} values of 13 µg/ml were obtained for \textit{Emblica officinalis}\textsuperscript{38}, a plant rich in antioxidants such as ascorbic acid, whereas in this investigation the callus culture extracts are found to be stronger antioxidant than the former.

The difference among various antioxidant activities measured by the assays for callus extract and sandalwood oil might be due to the difference in the size of radicals or in the accessibility of antioxidants to the radical center as well. Furthermore, interferences from lipid compounds such as fatty acids and waxes of plant origin might be contributory as well.

Nevertheless, positive relationships between total phenolic (polyphenol) content and antioxidant activity are reported in medicinal plants \textsuperscript{39}. In this investigation we found positive correlations between (i) flavan-3-ol content and total antioxidant capacity (TAC) \[y = 0.013x, R^2 = 0.889\], (ii) terpenoid content and total antioxidant capacity \[y = 1.297x, R^2 = 0.889\] and (iii) proanthocyanidin content and NO free radical scavenging activity \[y = 0.782x, R^2 = 0.859\] in dose-dependent manner. In addition, the levels of secondary metabolites are positively correlated in plants. In this study, positive relationships were inferred for (i) total phenolics and tannin content \[y = 2.059x, R^2 = 0.999\], (ii) total phenolics and anthocyanins \[y = 0.183x, R^2 = 0.970\] (iii) total phenolics and saponins \[y = 4.669x, R^2 = 0.834\] and (iv) terpenoid and flavan-3-ols contents \[y = 0.01x, R^2 = 1\]. These correlations indicate that in sandalwood callus, the metabolic pathways and events regulating the biosynthesis of these bioactive antioxidants are probably synchronized events in the tree life cycle, and thus presents itself towards amenable routes to biotechnological exploitation of the entire pool of antioxidant constituents in large scale, \textit{in vitro}. The findings from this study are summarized in Figure 1.

![Figure 1](image_url)

**Figure 1.** Summary of comparative antioxidant efficacy of sandalwood callus and oil. The illustration provides over view of the phytochemical constituents quantified in the callus and sandalwood oil, and their bioactive potential across nine antioxidant screening assays. Scale bar for callus (on left) measures 5 mm.

### 4. Conclusion
Results obtained in this investigation indicate that sandalwood callus extract, rich in phenolics, terpenoids and saponins exhibited comparable antioxidant activity with sandalwood oil, and better than reference antioxidant compounds in numerous instances. The findings presented also demonstrate that callus cultures producing antioxidant compounds might serve as model systems to investigate the regulation and production of these important metabolites. With an ever-expanding cosmetic industry dependent on sandalwood constituents, the \textit{in vitro} callus's phytochemical compositions and the radical scavenging activities may be utilized as antioxidants in industrial scale preparations, as an alternative or additive to sandal oil and wood components.

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### 5.1 Conflict of Interest
The authors have declared that there is no conflict of interest.

### 6. Reference
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