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

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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-remorogenic, 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; Folin-Ciocalteu reagent, dimethylamino cinnamaldehyde (DMACA), aluminum chloride (AlCl3), ferric ammonium sulfate [$NH_4Fe(SO_4)_2.12H_2O$], sodium carbonate [$Na_2CO_3.10H_2O$], ammonium molybdate [$(NH_4)_6Mo_7O_{24}.4H_2O$], sodium nitrite [$NaNO_2$], ferric chloride [$FeCl_3$], ferrous chloride [$FeCl_2$], 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 (DPPH), casein, vanillin, potassium persulfate, potassium ferricyanide [$K_3Fe\ (CN)_6$], 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 ddH_2O, 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 ºC 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 ºC under reduced pressure. The extract obtained was stored at -20 ºC until further use.

Table 1. Summary of methods followed for phytochemical characterization of sandalwood callus extract.

Serial No.	Assay	Extract amounts	Reagents	Conditions	Monitoring system	Standard curve	Calculation	Reference
1	Total terpenoid content	100 µg in methanol	500 µl of 2 % vanilin-H_2SO_4 in cold.	60 °C for 20 min, cooled at 25 °C for 5 min and within 20 minutes, absorbance measured	Blue-green color, absorbance at 608 nm	Linalool (20 – 100 mg/ l)	as µg/ mg extract	[11]
2	Total anthocyanins content	100 µg in methanol	a. 500 µl extract + 6 ml of n-butanol: HCl (95:5,v/ v) b. 0.2 ml of 2 % (w/ v) solution of $NH_4Fe(SO_4)_2$, $12H_2O$ in 2 M HCl	Heated tubes at 92-95 °C for 40 min	Deep green/ brown solution, absorbance at 550 nm	Multiplied OD_{530} by 33.3 (ε= cyanidin chloride of 30,000)	as mg of cyanidin chloride/ 100 g extract	[12]
3	Total oligomeric proanthocyanidins content	1 mg/ ml in methanol	a. 5 ml of 0.5% vanillin reagent. b. 5 ml volume of 4% methanolic HCl.	Incubation for 20 min at room temperature	Dark greenish-blue color, absorbance at 500 nm	Catechin (100-500 µg)	as mg or g catechin equivalents per 100 g extract	[13]
4	Total phenolics content	1 mg/ ml in 30 % methanol	a. 100 µl of extract + 50 µl of Folin Ciocalteau reagent (FCR), 10 min wait and up to 1 ml with $Na_2CO_3.10H_2O$ and well shaken.	Incubation for 2 hours in dark at room temperature.	Blue solution, absorbance at 720 nm	Gallic acid (10-50 µg)	as mg gallic acid/g extract	[14]
5	Total polyphenols and Casein/ BSA/ PVPP-Bound Tannins	1 mg/ ml in 30% methanol (total polyphenols)	a. 200 µl of extract + 800 µl of water. b. 100 mg of BSA/ casein or PVPP added.	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. Filtrate (= Unbound polyphenols	Blue solution, absorbance at 720 nm	Catechin (100-500 µg)	Bound tannins= Total polyphenols- Unbound polyphenols; as mg catechin/g extract	[14]
6	Total flavonoid content	100 µg/ ml in methanol	1 ml of 2% methanolic $AlCl_3$, 6 H_2O + 1 ml extract	Incubation at 10 min.	Yellow color, absorbance at 430 nm	Rutin (10-50 µg)	as mg rutin/ g extract	[15]
7	Total flavan-3-ols content	1 mg/ ml in methanol	200 µl sample + 1 ml of p-dimethylamino cinnamaldehyde (DMACA) (0.1% in 1 N HCl in methanol)	Sample vortexed, 10 min at room temperature.	Blue color monitored at 640 nm	Catechin (25-150 µg)	as mg catechin/ g extract	[16]
8	Total saponin content	1 mg / ml in methanol	10 µl extract in methanol+ 50 µl 8% vanillin in ethanol + 500 µl 72 % H_2SO_4	Heated to 60 °C for 20 min, followed by 4 °C for 5 min.	Yellow/ green color, absorbance at 544 nm	Sapogenin (10-100 µg)	as mg saponin/ g extract	[17]

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 (%) = (W_1 x 100)/ W_2 , where W_1 was the weight of extract after evaporation of solvents (mg) and W_2 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, α-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.

Serial No.	Physico-chemical parameters	Quantity in Cell
1	Moisture Content (%)	93.7±8.7
2	Ash Content (%)	4.1±0.2
3	Yield of extract (%)	4.3±1.3
4	pH of extract	6.6±0.3
	Phytochemical parameters	**Quantity in Extracts**
5	Terpenoid content (µg linalool equivalence / mg)	16.4±2.7
6	Total soluble proanthocyanidin (flavan-3-ols) content (µg catechin equivalence / mg)	7.4±1
7	Total insoluble proanthocyanidin content (µg catechin equivalence/ mg)	0.6±0.08
8	Total anthocyanin content (µg cyanidin chloride equivalence/ mg)	0.4±0.09
9	Total flavonoid content (µg rutin equivalence/ mg)	0.07±0.002
10	Total condensed tannin content (µg catechin equivalence / mg)	2.4 ±0.2
	- BSA bound condensed tannin (µg catechin equivalence / mg)	-
	- PVPP bound condensed tannin (µg catechin equivalence / mg)	-
	- Casein bound condensed tannin (µg catechin equivalence / mg)	2.4 ± 0.2
11	Total phenolic content (µg gallic acid equivalence/ mg)	18.2±1.8
12	Total saponin content (µg saponin equivalence/ mg)	9.4±0.5

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].

Serial No.	Antioxidant Activity	Callus Extract	Sandalwood Oil	Reference compounds
1	Reducing Assay Power (100 µg /ml, OD-700 nm)	0.13±0.01**	0.14±0.01**	0.53±0.05 (Quercetin)
2	Hydroxyl Radical Scavenging Activity (% inhibition at 100 µg /ml)	94.5***	98.3***	78.5 (Gallic Acid)
3	Metal Ion Chelating Activity (IC_{50} in µg/ ml)	1025.5±43.5	23.5±2.8 **	0.61±0.1 (EDTA) 37±4.5 (Gallic acid)
4	FRAP (µM Fe (II)/ g extract)	55±5 **	1056±106.9***	29.7±7.7 (α-tocopherol)
5	Total Antioxidant Capacity Assay (µmol equivalents of ascorbic acid/ g extract)	61.2±5**	22.4±1.8	37.3±5.1 (Gallic Acid)
6	NO Free Radical Scavenging Assay (% inhibition at 100 µg/ml)	0.5±0.02	4.2±0.5	67.9±9.3 (Gallic Acid)
7	ABTS Free Radical Scavenging Activity (mM L-ascorbic acid equivalent/ 100 mg extract)	175±19***	4.5±0.8	11.66±0.9 (L-ascorbic acid)
8	DPPH Radical-Scavenging Capacity (IC_{50} in µg/ ml)	1.17±0.2**	0.87±0.07**	4.05±0.7 (Quercetin)
9	Lipid peroxidation inhibition (TBRS) (IC_{50} in µg/ ml)	4.8±0.71**	12.4±1.4	0.84±0.09 (α-tocopherol)

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 Fe^{2+}-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 Fe^{2+} 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 Fe^{2+}-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 IC_{50} 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 $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ 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$^{\bullet}$ radicals. The results are expressed as IC_{50}values. The lower the IC_{50} 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 IC_{50} 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 Fe^{2+}-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 Fe^{2+}- polyphenol or Fe^{2+}- 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 IC_{50} values for inhibition of metal ion chelation by the extract. In contrast, IC_{50} 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 IC_{50} 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 peroxyl 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_{50} values of 13 µg/ ml were obtained for *Emblica officinalis*[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 [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, *in vitro*. The findings from this study are summarized in Figure 1.

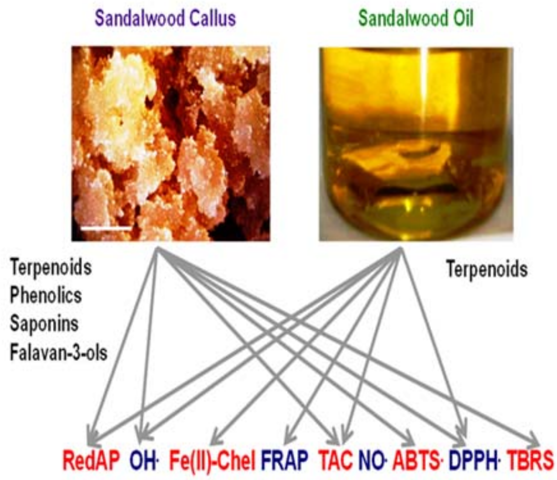

Figure 1. Summary of comparative antioxidant efficacy of sandalwood callus and oil. The illustration provides over view of the phytochemical constituents quantified in the callus and sandalwood oil, and their bioactive potential across nine antioxidant screening assays. Scale bar for callus (on left)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

1. Dikshit A, Hussain A. Antifungal action of some essential oils against animal pathogen. Fitoterapia 1984; 55:171-176.
2. Desai VB, Hiremath RD, Rasal VP, Gaikwad DN, Shankaranarayana KH. Pharmacological screening of HESP and sandal oils. Indian Perfumer 1991; 35:69-70.
3. Benencia F, Courreges MC. Antiviral activity of sandalwood oil against herpes simplex viruses-1 and -2. Phytomedicine 1999; 6:119-123.
4. Ochi T, Shibata H, Higuti T, Kodama KH, Kusumi T, Takaishi Y. Anti-*Helicobacter pylori* compounds from *Santalum album*. J Nat Prod 2005; 68(6):819-824. DOI: 10.1021/np040188q
5. IUCN. Asian Regional Workshop 2006. (Conservation and Sustainable Management of Trees, Vietnam) 1998, *Santalum album* L. In: IUCN Red List of Threatened Species (www.iucnredlist.org), 2006.
6. Das S, Das S, Mujib A, Pal S, Dey S. Optimisation of sucrose and dissolve oxygen level for somatic embryo production of *Santalum album* in airlift bioreactor. Prens Aromatica 1998; 14:12-13.
7. Y. Yamashita. Production of essential oils by culture of the callus of sandalwood tree, Patent No: JP09023892; 1997.
8. Lloyd G and Mc Cown B. Commercially-feasible micropropagation of Mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Int Plant Prop Soc Proc 1981; 30:421-427.
9. Balick MJ. Ethnobotanical screenings of medicinal plants most often yield higher hit rates than random screenings. Ethnobotany, drug development and biodiversity conservation exploring the linkages. Ciba Foundation Sym 1994; 185:4-18.
10. AOAC. Method 920. 153. Ash content. In Official Methods of Analysis, Ed 17th, Assoc. of Official Analytical Chemist, Gaithersburg, Maryland, 2002.
11. Doneva-Šapceska D, Dimitrovski A, Bojadžiev T, Milanov G, Vojnovski B. Free and potentially volatile monoterpenes in grape varieties from the republic of Macedonia. Maced J Chem Chem Eng 2006; 25(1):51-56.
12. Porter LJ, Hrstich LN, Chan BG. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry 1986; 25:223–230. DOI: 10.1016/S0031-9422(00)94533-3
13. Price ML, Scoyoc SV, Butler LG. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. J Agric Food Chem 1978; 26(5):1214-1218. DOI: 10.1021/jf60219a031
14. Schneider G. Zur Bestimmung der Gerbstoffe mit Casein. Arch Pharm 1976; 309:38-44.
15. Lamaison JLC, Carnet A. Teneurs en principaux flavonoids des fleurs de *Crataegeus monogyna* Jacq et de *Crataegeus laevigata* (Poiret DC) en fonction de la vegetation. Pharm Acta Helv 1990; 65:315-320.
16. Arnous A, Makris DP, Kefalas P. Effect of principal polyphenolic components in relation to antioxidant characteristics of aged red wines. J Agric Food Chem 2001; 49(12):5736–5742. DOI: 10.1021/jf010827s
17. Makkar HPS, Siddhuraju S, Becker K. Methods in Molecular Biology, Plant Secondary Metabolite. Humana Press Inc., NJ, 2007.
18. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. Japanese J Nutr 1986; 44(6):307-315.
19. Nagai T, Myoda T and Nagashima T. Antioxidative activities of water extract and ethanol extract from field horsetail (tsukushi) *Equisetum arvense* L. Food Chem 2005; 91(3):389-394. DOI: 10.1016/j.foodchem.2004.04.016
20. Decker EA and Welch B. Role of ferritin as a lipid oxidation catalyst in muscle food. J Agric Food Chem 1990; 38(3):674-677. DOI: 10.1021/jf00093a019
21. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: The FRAP assay. Anal Biochem 1996; 239(1):70-76. DOI: 10.1006/abio.1996.0292.
22. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E_1. Anal Biochem 1999; 269(2):337-341. DOI: 10.1006/abio.1999.4019
23. Geetha S, Ram MS, Singh V, Ilavazhagan G, Sawhney RC. Effect of seabuckthorn on sodium nitroprusside induced cytotoxicity in murine macrophages. Biomed Pharmacother 2002;

56(9):463-467. DOI: 10.1016/S0753-3322(02)00290-1

24. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999; 26(9-10):1231-1237. DOI:10.1016/S0891-5849(98)00315-3
25. Gulluce M, Sahin F, Sokmen M, Ozer H, Daferera D, Sokmen A *et al*. Antimicrobial and antioxidant properties of the essential oils and methanol extract from *Mentha longifolia* L. ssp. *Longifolia*. Food Chem 2007; 103(4):1449-1456. DOI: 10.1016/j.foodchem.2006.10.061
26. Okhawa H, Ohishi W, Yagi K. Assay formulation lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95:351-358.
27. Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein- dye- binding. Anal Biochem 1976; 72:248-254.
28. Tanaka N, Shimomura K, Ishimaru K. Tannin production in callus cultures of *Quercus acutissima*. Phytochemistry 1995; 40(4):1151-1154. DOI: 10.1016/0031-9422(95)00378-K
29. Bahorun T, Gressier B, Trotin F, Brunet C, Dine T, Luyckx M *et al*. Oxygen species scavenging activity of phenolic extracts from hawthorn fresh plant organs and pharmaceutical preparations. Arzneimittel-Forschung 1996; 46(11):1086–1089.
30. Butaud JF, Raharivelomanana P, Bianchini JP, Faure R, Gaydou EM. Leaf C-glycosylflavones from *Santalum insulare* (Santalaceae). Biochem Syst Ecol 2006; 34:433–435. DOI: 10.1016/j.bse.2005.11.012
31. Chyau CC, Tsai SY, Ko PT, Mau JL. Antioxidant properties of solvent extracts from *Terminalia catappa* leaves. Food Chem 2002; 78(4):483-488. DOI: 10.1016/S0308-8146(02)00162-0
32. Ningappa MB, Dinesha R, Srinivas L. Antioxidant and free radical scavenging activities of polyphenol- enriched curry leaf (*Murraya koenigii* L.) extracts. Food Chem 2008; 106(2):720-728. DOI: 10.1016/j.foodchem.2007.06.057
33. Zhang H, Chen F, Wang X, Yao HY. Evaluation of antioxidant activity of parsley (*Petroselinum crispum*) essential oil and identification of its antioxidant constituents. Food Res Int 2006; 39(8):833-839. DOI: 10.1016/j.foodres.2006.03.007
34. Borneo R, Leon AE, Aguirre A, Ribotta P, Cantero JJ. Antioxidant capacity of medicinal plants from the Province of Cordoba (Argentina) and their *in vitro* testing in a model food system. Food Chem 2009; 112(3):664-670. DOI: 10.1016/j.foodchem.2008.06.027
35. Adom KK, Liu RH. Antioxidant activity of grains. J Agric Food Chem 2002; 50(21):6182-6187. DOI: 10.1021/jf0205099
36. Stoilova I, Krastanov A, Stoyanova A, Denev P. Gargova S. Antioxidant activity of a ginger extract (*Zingiber officinale*). Food Chem 2007; 102(3):764-770. DOI: 10.1016/j.foodchem.2006.06.023
37. Grzegorczyk I, Matkowski A, Wysokinska H. Antioxidant activity of extracts from *in vitro* cultures of *Salvia officinalis* L. Food Chem 2007; 104(2):536-541. DOI: 10.1016/j.foodchem.2006.12.2003
38. Kumar KCS, Muller K. Medicinal plants from Nepal, II. Evaluation as inhibitors of lipid peroxidation in biological membranes. J Ethnopharmacol 1999; 64(2):135-139. DOI: 10.1016/S0378-8741(98)00117-2
39. Silva EM, Souza JNS, Rogez H, Rees JF, Larondelle Y. Antioxidant activities and polyphenolic contents of fifteen selected plant species from the Amazonian region. Food Chem 2007; 101(3):1012-1018. DOI:10.1016/j.foodchem.2006.02.055.