

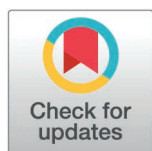
RESEARCH ARTICLE

Phytochemicals and antioxidant activity of leaf extract and callus cultures of *Cinnamomum camphora* L

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Abstract

Cinnamomum camphora L. is highly significant landscape tree known for its medicinal values and presence of secondary metabolites that have antioxidant, antimicrobial, anticancer, anti-inflammatory effects and widely utilized in pharmaceutical and cosmetic industry. Callus cultures of *C. camphora* have better antioxidant activity than extracts of naturally grown tree leaves. While there is significant lack of research about the potential of its *in vitro* callus cultures as a controlled and sustainable biotechnological alternative for mass production of bioactive compounds. The present work was aimed at comparative analysis of phytochemicals (phenolic and flavonoid) and antioxidant activities of calli and leaf extract of field grown camphor plant. To get *in vitro* germplasm, callus formation and direct shoot initiation was carried out and it was observed that MS medium supplemented with 0.1 mg L⁻¹ thidiazuron (TDZ) + 0.5 mg L⁻¹ 2, 4-dichlorophenoxyacetic acid (2, 4-D) proved best for shoot initiation from nodal explant. MS medium fortified with various plant growth regulators was used for callus formation and best callus induction response (100%) from nodal and leaf explants was observed on 0.5 mg L⁻¹ 2, 4-D + 2.0 mg L⁻¹ 6-Benzylaminopurine (BAP). Callus was successfully sub-cultured and this *in vitro* proliferated calli and fresh leaf extract of field grown plant were used for comparative study of phytochemicals. Results revealed that callus culture exhibited highest antioxidant enzyme activities compared to leaf extract and hence there was statistically significant ($P \leq 0.05$) difference. Similarly, peroxidase (31.12 U mL⁻¹ of enzyme), superoxide dismutase (35.24 U mg⁻¹ of protein), and catalase (58.6 U mL⁻¹ of enzyme) also showing highest values. In contrast, glutathione peroxidase activity (0.552 U mg⁻¹) was comparatively higher in leaf extract. Additionally, callus cultures accumulated higher phenolic contents (1.106 mg GAE g⁻¹ of FW) while flavonoid contents (7.87 mg CatE g⁻¹ of FW) were higher in leaf extract. This investigation showed that *in vitro* conditions and the use of plant growth regulators in various combinations might be work as elicitors to

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enhance the phytochemicals and antioxidant enzymes in callus culture as compared to the leaf extract.

Introduction

Phytochemicals are the most significant group of secondary metabolites that are produced by plants for their reproduction, symbiotic relationships, growth and development [1]. Although they are already present but stressful growth environments or modifications to the growth medium may further trigger their synthesis like plant grown under *in vitro* conditions [2,3]. The majority of these chemicals are produced constitutively but their synthesis can be increased under stress in a way that it depends on the growth circumstances and the stressor [4,5]. Phenolic content and antioxidant activity in plant products have been shown to be positively correlated with each other [6]. One of the major subgroups of phytochemicals with potential antioxidant benefits and positive impacts on human health is the polyphenol [7] that have significant role in determining the flavour, texture, color, and sensory perception of food [8,9]. Phenolics are the most promising phytochemical for further studies that have vital role in the detoxification of H_2O_2 in plants, as well as in UV protection and enzyme modulation [10,11]. Some researches claim that the anticancer properties of phenolic compounds are associated to apoptosis [12] and the identification with standardization of phytochemicals play crucial role for its effective treatment [13].

Similarly, flavonoids are actively present polyphenolic phytochemicals secreted by plants [14] that have been utilized in a variety of herbal remedies since ancient times as they have antiviral, anti-bacterial, anti-inflammatory, anticarcinogenic and antimutagenic properties [15]. Phenylpropanoid pathway synthesizes flavonoid [16], while they are synthesized in response of any microbial infection or disease and mainly accumulated in the edible sections of the plants [17]. There are many studies in literature, conducted on various medicinal plants to explore the phytochemicals and their pharmaceutical effects on diseases. Traditional medicines made from a variety of medicinal plants are widely used in countries; Pakistan, China, India, Bangladesh, Korea, Taiwan, Japan, and Sri Lanka. Among many of medicinal plants *C. camphora* bearing with high significance because of its herbal and ritual use especially in China and Sub-continent.

Cinnamomum camphora L. Presl is a perennial tree that belongs to the family *Lauraceae*. It is naturally cultivated as a landscaping tree, and is used as an important herbal medicine in southern China [18,19]. It is also known as Camphor tree, Camphor, Camphorwood or Camphor laurel, and found in temperate to subtropical regions of East Asian countries, i.e., China, Japan, Korea, Vietnam, especially along the coast from Cochin China (Vietnam) and also belongs to the estuary of the Yangtze River (river), adjoining islands; Hainan and Taiwan and the naturally grown range bounds to just about 10–36°N and 105–130°E [20]. Leaf essential oil (CEO) has potent antioxidant, antimicrobial, anticancer, strong insecticidal [21] and anti-inflammatory effects [22]. The CEOs are being widely utilized in pharmaceutical, food

and cosmetics industries as significant raw resources [23]. Camphor is helpful in evaluating more possible pharmaceuticals and creating new anti-inflammatory medications [24]. In Chinese tradition, camphor leaves may treat gastrointestinal problems like diarrhea as well as mental problems like hysteria, anxiety, and neuralgia [25]. Further, various species of the genus *Cinnamomum* are also utilized as significant condiments [26]. According to some studies, ethanolic extract from leaves of camphor plant is significantly effective in treating atopic dermatitis [27]. It has been showed that leaves of *C. camphora* have remarkable anti-inflammatory capability in adult human [28]. Extracted essential oils from various parts of *C. camphora* such as twigs, leaves, and seeds, has notable insecticidal potential, leaves essential oil is additionally used as fruits and vegetables preservative and is predicted to have a lot of other applications. In future, the researcher hopes to develop bio-based products by using *C. camphora* leaves in medicine industry [29].

Besides this huge pharmacological, industrial and environmental significance, *C. camphora* is not propagated at large scale in Pakistan due to several issues regarding its plantation. *C. camphora* is an exotic species in the area of sub-continent and there's a problem with the traditional methods of propagation for instance from seeds propagation. Seeds are produced in huge amount within fruits (approximately up to 100,000 seeds per adult tree) [30] but in Pakistan production of flowers are rare and non-viable therefore seeds are not produced. Cuttings and layering are also methods that are frequently used in propagation but the limitations of resources and poor response prevent these techniques from being widely utilized at commercial level. Consequently, the conventional methods of breeding which are described above are no longer be able to satisfy the rising demand of *C. camphora* seedlings in therapeutical and pharmaceutical industries. While on the other hand, because of its versatile therapeutic properties, the annual demand of camphor is rising day by day. The global camphor market predicts that between 2020 and 2030 its demand will expand at compound annual growth rate of 11–13% (<https://www.lucintel.com/camphor-market.aspx>).

Plant tissue culture has been widely used for the multiplication of this important tree species because this technique has very high regenerative efficiency. Moreover, in micro-propagation, there are no limitations of climatic conditions, seasonal affect and regional restrictions, although application of this technique is very limited with regard to *C. camphora* tree. A desirable method for plant regeneration and for conservation of genetic diversity as well as use of genetic resources like bioactive substances is possible by tissue culture technique [31]. For this purpose, the growth of plant is regulated by utilizing PGRs (plant growth regulators) in various concentrations and combinations while using various explants (nodal, leaves, internodal). Externally applied PGRs have potential to alter the physiology as well as internal polarity of explant. *C. camphora* can be regenerated by two pathways; somatic embryogenesis or organogenesis under controlled conditions. Based on the mentioned methods and strategies, there is a dire need to consider *in vitro* propagation strategies for this economically significant medicinal plant. Environmental conditions also effects on the phytochemical compositions of plants as well as the antioxidant capacity [32]. However, the composition of phytochemicals may vary with plant species based on the specific effect and type of plant growth regulators [33].

Because of its high antioxidant content and diverse phytochemical profile, *C. camphora* has substantial therapeutic value and is a prime target for biotechnological research. Although the bioactivity of its natural leaf extracts has been proven, but the potential of *in vitro* callus cultures as a sustainable and regulated method for increased metabolite production is not explored as much. A significant research gap exists in the comparative assessment of phytochemical and antioxidant enzyme activities between field-grown plants and callus cultures of *C. camphora*. To address this, we hypothesized that callus cultures induced under optimized *in vitro* conditions would exhibit a significantly higher phytochemical yield and enhanced antioxidant enzyme activity as compared to extracts from field-grown leaves. Therefore, the main objective of this research was to establish callus cultures via a novel protocol and conduct a comparative analysis of their phytochemical and antioxidant profiles against fresh leaf extracts. Our findings support the hypothesis that controlled *in vitro* conditions can significantly enhance the accumulation of bio-active compounds, offering a viable biotechnological platform for the mass production of valuable antioxidants from *C. camphora*.

Materials and methods

Plant material and culture conditions

Disease-free young nodes and fresh juvenile leaves of *Cinnamomum camphora* L. were used as an explant during this research work. These explants were procured from a healthy 20-year-old single tree of *C. camphora* grown at Quaid-e-Azam Campus, University of Punjab, Lahore, Pakistan (31.50° N, 74.30° E), in November and December 2023. Murashige and Skoog [34] (MS) medium and Woody Plant Medium (WPM) [35] with 30 gL⁻¹ sucrose, 8.0 gL⁻¹ agar were used with different concentrations of 2, 4-D (2, 4-dichlorophenoxyacetic acid), BAP (6-Benzylaminopurine), NAA (1-Naphthaleneacetic acid), KIN (Kinetin), IBA (Indole butyric acid), TDZ (Thidiazuron), IAA (Indole- acetic-acid), and Zeatin for this experiment on the basis of our preliminary experiments. Explants were cleaned with running tap water 4–5 times to remove all the particles of dust. They were then dipped in a solution of household detergent for 15 minutes while being continuously stirred. After rinsing with distilled water these segments were treated with 15% (v/v) bleach for 15 minutes with gentle agitation and washed with autoclave distilled water in laminar air flow chamber. Explants were also treated with 0.1% solution of mercuric chloride (HgCl₂), depending on maturity of explant, with gentle agitation and washed with autoclave distilled water in laminar air flow chamber then immersed in 70% ethanol for 1 minutes and then blot-dried using sterilized blotting paper after rinsing again with autoclave distilled water. These explants were then inoculated into the test tube containing medium while keeping them near spirit lamp to avoid any contamination. These cultures were then kept under 16-hours photoperiod (40 μmol m⁻² s⁻¹) and 8 hours dark period provided by white fluorescent light (36 Watt) at 25 ± 2°C temperature in the culture room.

Data collection for morphological attributes

Data were recorded in terms of callus texture, days and frequency of calli formation, growth index and color of calli and also for the number of shoots, percentage response and number of leaves for all the tested media.

Shoot induction and root formation in MS and WPM medium

Nodal segments were used as an explant in MS and WPM supplemented with various combinations and concentrations of plant growth regulators for shoot induction and root formation. The detail of various combinations used were as follows: M0 (MS medium without PGRs); M1 (2, 4-D 0.5 mg L⁻¹ + BAP 2.0 mg L⁻¹); M2 (2,4-D 0.9 mg L⁻¹ + KIN 1.0 mg L⁻¹); M3 (BAP 1.0 mg L⁻¹ + KIN 1.0 mg L⁻¹); M4 (TDZ 0.1 mg L⁻¹ + 2,4-D 0.5 mg L⁻¹); M5 (Zeatin 1.0 mg L⁻¹ + KIN 1.0 mg L⁻¹); M6 (IBA 1.0 mg L⁻¹ + AC 2.0 g); M7 (IAA 0.4 mg L⁻¹ + BAP 2.0 mg L⁻¹) and same concentrations and combinations of PGRs were used for WPM. The media combinations were selected on the basis of preliminary experiment (data not shown here). After inoculation, the data were recorded for % response (Eq. 1), number of shoots and leaves, days for shoot initiation and leaves formation.

$$\% \text{ Response} = \frac{\text{no. of grown explant}}{\text{total no. of cultured explants}} \times 100 \quad (\text{Eq. 1})$$

Callus initiation and its proliferation

Leaves and nodal segments were used as explants for initiation of callus in MS media supplemented with various concentrations of plant growth regulators, C0 (MS medium without PGRs); C1 (2,4-D 0.5 mg L⁻¹ + BAP 2.0 mg L⁻¹); C2 (Zeatin 1.0 mg L⁻¹ + KIN 1.0 mg L⁻¹); C3 (BAP 2.9 mg L⁻¹ + KIN 1.0 mg L⁻¹); C4 (TDZ 0.1 mg L⁻¹ + BAP 2.0 mg L⁻¹); C5 (IBA 0.5 mg L⁻¹ + NAA 0.5 mg L⁻¹) used for callus induction from leaf while M0 (MS medium without PGRs); M1 (2, 4-D 0.5 mg L⁻¹ + BAP 2.0 mg L⁻¹); M2 (2,4-D 0.9 mg L⁻¹ + KIN 1.0 mg L⁻¹); M3 (BAP 1.01.0 mg L⁻¹ + KIN 1.0 mg L⁻¹); M4 (Zeatin 1.0 mg L⁻¹ + KIN

1.0 mg L⁻¹); M5 (IBA 0.5 mg L⁻¹ + BAP 1.0 mg L⁻¹). After 30 days of inoculation the data were recorded for % frequency of callus formation (Eq 2), Growth index (Eq. 3), days for callus initiation, callus texture and color.

$$\text{Frequency of Callus formation (FCF)\%} = \frac{\text{no. of Callus initiation}}{\text{total no. of cultured explants}} \times 100 \quad (\text{Eq. 2})$$

The fresh weight of callus formation/growth index was measured using following formula:

$$\text{Growth Index (GI)} = \frac{\text{Final weight (Wf)} - \text{Initial Weight(Wi)}}{\text{Initial Weight(Wf)}} \quad (\text{Eq. 3})$$

Wi = initial weight.

Wf = final weight.

Sub-culturing of Calli for their Proliferation on MS medium supplemented with various combinations of Plant Growth Regulators

In vitro grown calli were divided into small segments by excising the calli established on basal medium (MS medium) supplemented with different growth regulators. These calli were shifted on fresh MS medium containing various growth regulators, designated as S1 (0.5 mg L⁻¹ 2,4-D + 2 mg L⁻¹ BAP), S2 (0.5 mg L⁻¹ 2,4-D + 0.1 mg L⁻¹ TDZ) and S3 (1.7 mg L⁻¹ IAA + 2 mg L⁻¹ BAP) with the interval of 20 days to maintain healthy calli. After shifted calli on media, they were placed under white fluorescent light (36 Watt) at 25 ± 2°C temperature. Before exposing to light [(14-hour light) (36 Watt)], they were maintained in the dark for five days at 25 ± 1°C temperature in the culture room. After one month, calli were well-established and they were further used for various biochemical analysis.

Comparative Analysis of antioxidant enzyme activities and phytochemicals of leaf extract and callus culture of *C. camphora* L

Extraction and estimation of antioxidant enzymes. Healthy and fresh leaves of *C. camphora* were collected and packed in polythene bags and brought to Plant Developmental and Regenerative Biology Laboratory, Institute of Botany, University of the Punjab, Lahore. This plant material was rinsed under running tap water thoroughly and dried with the help of tissue paper by gentle tapping. Afterwards, leaves (4g) were placed in -80°C for 24 hrs. Similarly, 4g calli were used for the extraction of enzymes. Plant material and calli were crushed with the help of pestle and mortar separately. Phosphate buffer (7.2 pH) was used with 1:2 of crude extract and 0.1g PVP (*Polyvinyl polypyrrolidone*; Sigma Aldrich) was added to make homogenous slurry. Slurry was centrifuged at 4°C and 14000 rpm for 15 minutes. After centrifugation, supernatant was used for estimation of antioxidant enzyme activities and phytochemical analysis. Extraction percentage was calculated by using following formula (Eq. 4):

$$\text{Extraction percentage(\%)} = \frac{W1}{W2} \times 100 \quad (\text{Eq. 4})$$

W1 = weight of crude extract

W2 = weight of sample

Estimation of peroxidase (E.C 1.11.1.7) activity (POD). The 'Guaiacol-H₂O₂' method described by Luck [36] was modified for quantitative estimation of peroxidases. Two test tubes were used, one with a reaction mixture; 3.0 mL of phosphate buffer (0.1M; pH 7.2) with 0.05 mL of guaiacol (20 mM; 2-methoxyphenol) and 0.1 mL of crude enzyme extract. Instead of crude enzyme extract, 0.1 mL of distilled water was used in the second test tube. In both test tubes, 0.03 mL

H₂O₂ (12.3mM) was added for the reaction. Peroxidase activity was estimated as the time required for increase in absorbance of 0.1 (0.4–0.5) at 240 nm and represented as U mL⁻¹ of enzyme (Eq. 5).

$$\text{Enzyme activity} = \left(\frac{500}{\Delta}t\right) \times \left(\frac{1}{1000}\right) \times \left(\frac{TV}{UV}\right) \times \left(\frac{1}{fwt}\right) \quad (\text{Eq. 5})$$

Δt=change in time; TV=total prepared volume (mL); VU=volume of the sample used (mL); fwt=fresh weight (g).

Estimation of catalase (E.C 1.11.1.6) activity. The activity of catalase was measured using the method described by Beers and Sizer [37], with some modifications. The reaction was conducted with two buffer solutions (A and B). Reagent A contained 50 mM potassium phosphate at pH 7.0 while B was 0.036% H₂O₂ solution with 50mM potassium phosphate buffer. The reaction mixture of catalase was composed of 0.1 mL of enzyme extract and 2.9 mL of Reagent B, while the blank contained only 3.0 mL of Reagent A. The enzyme activity was determined by measuring the required time for decrease in absorbance from 0.45 to 0.40 (at 240 nm) and was expressed as U mL⁻¹ of enzyme (Eq. 6).

$$\text{Catalase activity} \left(\frac{\text{units}}{\text{ml}}\text{enzyme}\right) = \frac{3.45 \times df}{\text{Min} \times 0.1} \quad (\text{Eq. 6})$$

3.45=Decomposition of 3.45 micromoles of hydrogen peroxide in a 3.0 ml. of reaction mixture that produce decrease in the A_{240nm} from 0.45 to 0.40 units.

df=dilution factor (mL); Min=Time in minutes required for the A_{240nm} to decrease from 0.45 to 0.40 absorbance units; 0.1=Volume of enzyme used (mL).

Estimation of superoxide dismutase (E.C 1.15.1.1) Activity (SOD). The activity of superoxide dismutase was measured by using a modified method developed by Maral and co-researchers [38]. Reaction mixture was prepared by adding 1 mL of sodium cyanide (NaCN), 10 ml of Methionine, 10 mL of EDTA, 1mL of NBT (Nitroblue tetrazolium), and 1 mL of Riboflavin as a substrate. The volume of the mixture was made up to 100 mL by adding buffer solution. Two test tubes (15×150 mm) were used for the assay; one contained 5 μL of extract and reaction mixture, while the other tube served as a blank, contained 2 mL of reaction mixture only. Both the tubes were placed under fluorescent bulbs (30 Watt) for 15 minutes. By using spectrophotometer (UV- 4000S), the absorbance of both test tubes was measured at 560 nm. SOD activity was further calculated on the basis of this fact that 50% inhibition is caused by one unit of SOD. SOD activity was expressed as units mg⁻¹ of protein. The % inhibition of NBT was calculated to determine SOD as follows (Eq. 7):

$$\% \text{ inhibition} = \frac{\text{Absorbance of control sample} - \text{Absorbance of experimental sample}}{\text{Absorbance of experimental}} \times 100 \quad (\text{Eq. 7})$$

Estimation of glutathione peroxidase (E.C 1.11.1.9) activity (GPX). To measure the activity of GSH-Px, the method of Flohé and Günzler [39], was slightly modified by using H₂O₂ (Duksan reagent) as a substrate. To conduct the enzyme reaction, 200 μL of the supernatant was mixed with 400 μL of 0.1mM GSH (reduced glutathione; Sigma) and 200 μL of Na₂HPO₄ (0.067 M) in a test tube. For the non-enzyme reaction, the same reagents were used without the supernatant. The mixture was pre-heated on a water bath at 25°C temperature for 5 minutes, followed by the addition of 200 μL of 1.3mM H₂O₂ to initiate the reaction. This reaction was lasted for 10 minutes and then stopped by adding 1 mL of 1% trichloric acetic acid (TCA; Merck) and by placing the mixture into an ice bath for 30 minutes. The supernatant was collected by centrifuging the mixture for 10 minutes at 3000 rpm, 480 μL of the supernatant was added into a test tube with 2.2 mL of Na₂HPO₄ (0.32 M) and 0.32 mL of 1.0mM 5,5-dithio-bis (2-nitrobenzoic acid; DTNB) was added for development of color. The absorbance was measured at 412 nm with a spectrophotometer within 5 minutes. GPX activity is expressed as U mg⁻¹. Residual glutathione concentration was calculated by using standard curve of the glutathione. GPX activity is equals to the no. of μmol consumed glutathione (Eq. 8).

$$\text{Glutathione peroxidase activity} \left(\frac{\text{units}}{\text{mg}} \right) = \frac{\text{Conc. of GSH in STD} - \text{Conc. of residual}}{\text{time (10 minutes)}} \times \frac{\text{Total volume (ml)}}{\text{volume of sample (ml)}} \times \text{D.f} \quad (\text{Eq. 8})$$

One unit of GPX is defined as the amount of enzyme that has capability to oxidize 1.0 μM GSH to GSSG per minute at temperature 25°C.

Estimation of total phenolic contents. Chun and Kim [40] method was used to determine the total phenolic content involved mixing of 0.2 mL of the extract with 2.6 mL of distilled water and adding the 0.2 mL of Folin-Ciocalteu reagent after 5 minutes, then 2 mL of 7% Na_2CO_3 was added and stirring for 30 seconds. This solution left in the dark for almost 90 minutes and then absorbance was measured at 750 nm against blank (without enzyme extract). The amount of total phenolic contents was measured in mg GAE g^{-1} . This measurement was obtained by using a standard curve of Gallic acid calibration at 750 nm (Eq. 9).

$$\text{TPC} = c \frac{V}{m} \quad (\text{Eq. 9})$$

c = concentration of gallic acid obtained from calibration curve in mg/mL

v = volume of extract in mL; m = mass of extract in gram

Determination of total flavonoid contents

To measure the amount of flavonoids, Ivanova [41] method was employed that involved using the AlCl_3 - NaNO_2 -NaOH complex. The test tube contained 0.2 mL of the extract with 3.5 mL of distilled water, 0.15 mL of 5% NaNO_2 , 0.15 mL of 10% AlCl_3 , and 1 mL of 1 M NaOH, with the interval of 5 minutes. The reaction mixture was left to react for 15 minutes, and the absorbance was measured at 510 nm. Flavonoid content was reported in mg CatE g^{-1} , which was calculated from the standard curve of catechin calibration at 510 nm (Eq. 10).

$$\text{TFC} = c \frac{V}{m} \quad (\text{Eq. 10})$$

c = concentration of catechin obtained from calibration curve in mg/mL

v = volume of extract in mL; m = weight of plant extract in g

Experimental layout and statistical analysis

All experiment was performed with 10 replicates for each treatment of calli induction and shoot formation. For the analysis of phytochemical contents and antioxidant activity 3 readings were recorded for each sample of all treatments. By using statistical program SPSS (Version 25.0.0), all the recorded data were analyzed to find out means and standard errors conducting ANOVA. LSD and Duncan's Multiple Range test was also used to find statistically significant differences among the means of various treatments. Differences were considered as significant at $P \leq 0.05$.

Results

Standardization of the medium for shoot initiation of *C. camphora*

Young juvenile explant of *C. camphora* was collected during the months of November and December 2023. Nodal sections were inoculated on MS media containing various combinations of growth regulators. *In vitro* plants were raised to get healthy germplasm under the controlled environmental conditions. Explant treated with 10% bleach gave best surface sterilization of explant as compared to other sterilant used during this investigation and only 20% contamination

was observed. For *in vitro* establishment of plant only nodal sections were used as explant during this investigation. After reviewing literature, two types of media, MS and WPM with various growth regulators were tried alone and in combination with PGRs however, only MS media fortified with different concentrations of PGPRs were proved effective. Shoot formation and callus induction were not observed on WPM media. So, here we presented the results regarding to MS media only.

Shoot initiation of *C. camphora* in MS medium supplemented with different plant growth regulators

For shoot initiation of *C. camphora*, MS media were used with various combinations of PGRs and activated charcoal (2.0 gL⁻¹) was also used for reducing hyperhydricity (vitrification). Eight media were used for shoot initiation from nodal segment. Best results were observed on M1 medium with 100% response (Fig 1C), although it took more time (24.67 days) to initiate shooting after inoculation but it produced more number of leaves (11.00) and shoots (1.33) as compared to other tested media. Similarly, M4 medium also showed 100% response within 18.00 days but number of leaves (6.33) and number of shoots (1) were less (Fig 1D) as compared to M1. Shoot initiation response was also 100% on M6 media within 19 days of inoculation. Here, the number of leaves (5.66) and number of shoots (1) were also less as compared to M1 medium (Fig 1B). M7 showed 60% response after 14.33 days of inoculation and produced 4.33 leaves and 0.67 shoots (Fig 1A). During this investigation, M0, M2, M3, and M5 media were not responded regarding shoot formation (Table 1).

Standardization of Medium for Callus Induction and Maintenance of *C. camphora*

MS media in combination with various growth regulators were tried for callus induction and proliferation of *C. camphora*. Seven media were used for callus induction from leaf explants as mentioned in Table 2. From these media best responded medium was used for further proliferation of callus.

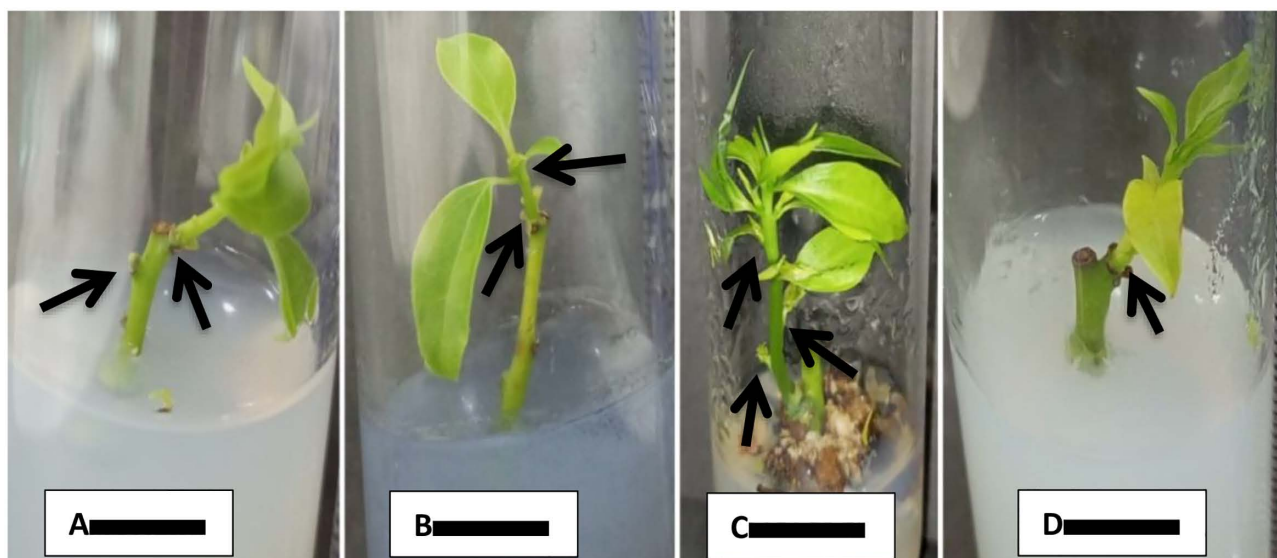


Fig 1. A: Shoot initiation from nodal segment on MS medium fortified with BAP and IAA (2.0 mg L⁻¹ + 0.4 mg L⁻¹) after 22 days. B: On MS basal media fortified with IBA (1.0 mg L⁻¹) and AC 2.0g after 20 days. C: On MS basal media fortified with BAP and 2, 4-D (2.0 mg L⁻¹ + 0.5 mg L⁻¹) after 30 days. D: On MS basal media fortified with TDZ and 2, 4-D (0.1 mg L⁻¹ + 0.5 mg L⁻¹) after 22 days. Bar = 1 cm.

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Table 1. Shoot initiation from nodal segment of *C. camphora* on MS medium supplemented with various concentrations of plant growth regulators.

Medium MS	Treatments	Days for Shoot Initiation	% Response	No. of Leaves	No. of shoots
M0	0	0	0	0	0
M1	2, 4-D (0.5 mg L ⁻¹) + BAP (2.0 mg L ⁻¹)	24.67 ± 1.76 ^a	100	11.00 ± 2.64 ^a	1.33 ± 0.33 ^a
M2	2, 4-D (0.9 mg L ⁻¹) + KIN (1.0 mg L ⁻¹)	0	0	0	0
M3	BAP (1.0 mg L ⁻¹) + KIN (1.0 mg L ⁻¹)	0	0	0	0
M4	TDZ (0.1 mg L ⁻¹) + 2, 4-D (2.26 mg L ⁻¹)	18.00 ± 1.52 ^{ab}	100	6.33 ± 1.45 ^b	1.00 ± 0.00 ^{ab}
M5	Zeatin (1.0 mg L ⁻¹) + KIN (1.0 mg L ⁻¹)	0	0	0	0
M6	IBA (1.0 mg L ⁻¹) + AC (2.0 g)	19.00 ± 0.57 ^{ab}	100	5.66 ± 0.88 ^b	1.00 ± 0.00 ^{ab}
M7	IAA (0.4 mg L ⁻¹) + BAP (2.0 mg L ⁻¹)	14.33 ± 7.17 ^b	60	4.33 ± 2.96 ^{bc}	0.67 ± 0.33 ^b

Values are the mean ± S.E of ten replicates for each treatment.

Different alphabetical letters in each column shows the significant differences at $P \leq 0.05$ according to Duncan's multiple range test.

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Table 2. Callus induction from leaf explant of *C. camphora* on MS medium fortified various combinations of plant growth regulators.

Medium	Treatments	Days to callus induction	FCF %	Morphology
C0	0	0	0	
C1	2,4-D (0.5 mg L ⁻¹) + BAP (2.0 mg L ⁻¹)	26.2 ± 1.46 ^b	100	Brownish in color with dark patches, friable and compact
C2	Zeatin (1.0 mg L ⁻¹) + KIN (1.0 mg L ⁻¹)	0	0	
C3	BAP (2.9 mg L ⁻¹) + KIN (1.0 mg L ⁻¹)	0	0	
C4	TDZ (0.1 mg L ⁻¹) + BAP (2.0 mg L ⁻¹)	57.2 ± 1.39 ^a	100	Soft callus, Whitish Yellow, friable
C5	IBA (0.5 mg L ⁻¹) + NAA (0.5 mg L ⁻¹)	0	0	
C6	IAA (1.7 mg L ⁻¹) + BAP (2.0 mg L ⁻¹)	0	0	

Values are the mean ± S.E of ten replicates for each treatment.

Different alphabetical letters in each column shows the significant differences at $P \leq 0.05$ according to Duncan's multiple range test.

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Effect of different combinations of plant growth regulators on the leaf explant for callus induction

From all the seven media only two were proved effective for callus induction with 100% response. C1 medium showed best results as compared to C4 with regards to days for callus induction. In case of C1 medium, calli were induced within 26.2 days after inoculation (Fig 2B). Morphologically, callus was brown in color with dark patches on it, and friable in texture. Callus was induced after 57.2 days on C4 culture medium (Fig 2A). Callus produced on this medium was soft, whitish yellow in appearance and friable in texture. The entire calluses were produced from the leaf discs on both C1 and C4 medium were non-embryogenic.

Effect of different combinations of plant growth regulators on nodal segment for callus induction and maintenance of *C. camphora*

Six media combinations were used for callus induction from nodal explants, calli were induced in two (M5 and M1) combinations, M1 media showed 100% callus induction after 22.70 days of inoculation. Calli derived from M1 medium were brownish yellow and leathery in texture after 22 days of inoculation, but after maintaining, it turned into dark brown and friable texture (Fig 2D). It was observed that the hard calli produced from nodal segment in the presence of 2, 4-D and BAP were non-embryogenic with no regenerative ability. Callus Initiation was supported about 60% in M5 media within 17.40 days but the rate of callus induction was slowest as compared to M1. Calli derived from M5 were also non-embryogenic.

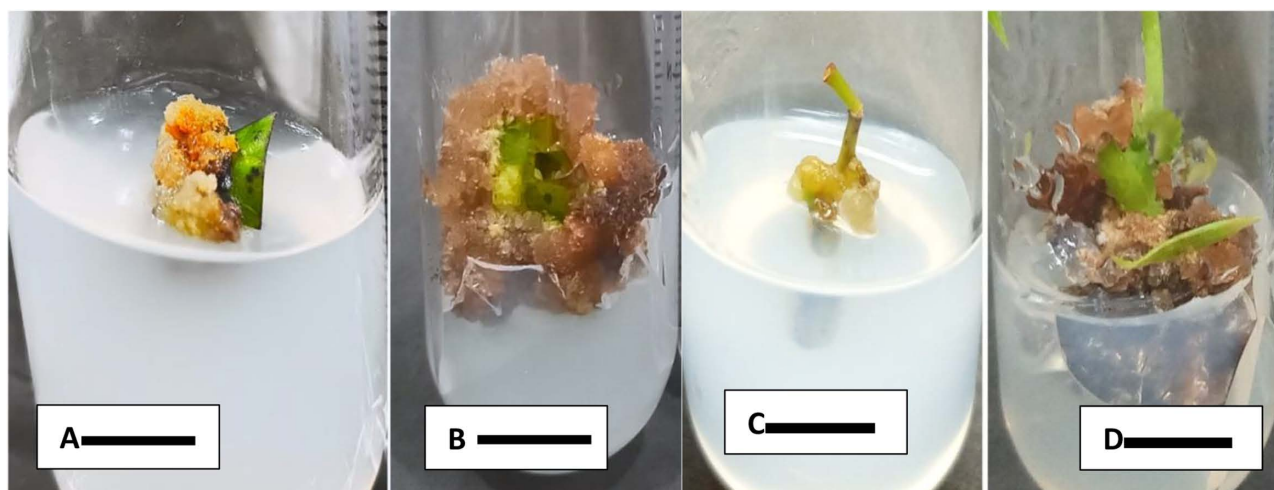


Fig 2. A: Callus induction from leaf on MS medium with TDZ and BAP ($0.1 \text{ mg L}^{-1} + 2.0 \text{ mg L}^{-1}$) after 57 days. B: On MS medium with BAP and 2, 4-D ($2.0 \text{ mg L}^{-1} + 0.5 \text{ mg L}^{-1}$) C: MS medium supplemented with BAP and IBA ($1.0 \text{ mg L}^{-1} + 0.5 \text{ mg L}^{-1}$) after 22 days. D: Callus induction in MS medium supplemented with BAP and 2, 4-D ($2.0 \text{ mg L}^{-1} + 0.5 \text{ mg L}^{-1}$) after 30 days. Bar = 1 cm.

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The deceleration in growth was observed after 50th days of inoculation. However, on M5 medium calli were creamy yellowish and leathery in texture (Table 3)

Sub-culturing of callus for maintenance and proliferation

Successful sub-culturing of callus produced from nodal segment/leaf explant was done on three media; S1, S2 and S3. Combinations and concentrations of S1 medium was same as used before for callus induction from nodal segment while two new media combination (S2 and S3) were used for further proliferation of callus cultures. S1 medium supported the production of healthy calli within 19.20 days. Morphologically, calli were soft and lush green in color, lightly friable, granular and compact in texture. Callus proliferation on S2 and S3 media took 18.40 days after inoculation (Table 4). Calli in S2 media were reddish brown in color with creamy base, lightly compact and friable in texture. On S3 media calli were whitish in color with light brown patches on it and granular in texture. The highest growth index (0.76 g) was recorded on S1 media as compared to S2 with 0.47 g and S3 with 0.66 g. These proliferated calli (0.5 g) were used for analysis of antioxidant enzyme activity and phytochemicals (Figs 3).

Table 3. Callus initiation from stem segment in MS medium supplemented with plant growth regulators.

Medium MS	Treatments	Days to callus initiation	% Response	Callus morphology
M0	0	0	0	0
M1	2, 4 D (0.5 mg L^{-1})+BAP (2.0 mg L^{-1})	22.70 ± 0.50^a	100	Hard callus, brownish yellow and compact in texture
M2	2,4-D (0.9 mg L^{-1}) +KIN (1.0 mg L^{-1})	0	0	
M3	BAP (1.0 mg L^{-1}) + KIN (1.0 mg L^{-1})	0	0	
M4	Zeatin (1.0 mg L^{-1})+ KIN (1.0 mg L^{-1})	0	0	
M5	IBA (0.5 mg L^{-1}) +BAP (1.0 mg L^{-1})	17.40 ± 7.11^b	60	Soft callus, creamy yellowish in color and leathery in texture

Values are the mean $\pm$ S.E of ten replicates for each treatment.

Different alphabetical letters in each column shows the significant differences at $P \leq 0.05$ according to Duncan's multiple range test.

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Table 4. Callus Proliferation after Sub-culturing on MS medium supplemented with various combinations of Plant Growth Regulators.

Medium	Treatments	Days to callus growth	Growth index (g)	Morphology
S1	2,4-D (0.5 mg L ⁻¹) + BAP (2.0 mg L ⁻¹)	19.20 ± 1.39 ^a	0.76 ± 0.06 ^a	Soft callus, lush green in color, lightly friable, granular and compact in texture
S2	2,4-D (0.5 mg L ⁻¹) + TDZ (0.1 mg L ⁻¹)	18.40 ± 1.63 ^a	0.47 ± 0.13 ^a	Reddish brown with creamy base, light compact and friable
S3	IAA (1.7 mg L ⁻¹) + BAP (2.0 mg L ⁻¹)	18.40 ± 0.58 ^a	0.66 ± 0.10 ^a	Whitish in color with light brown patches, granular in texture

Values are the mean ± S.E of ten replicates for each treatment.

Different alphabetical letters in each column shows the significant differences at $P \leq 0.05$ according to Duncan's multiple range test.

<https://doi.org/10.1371/journal.pone.0321155.t004>

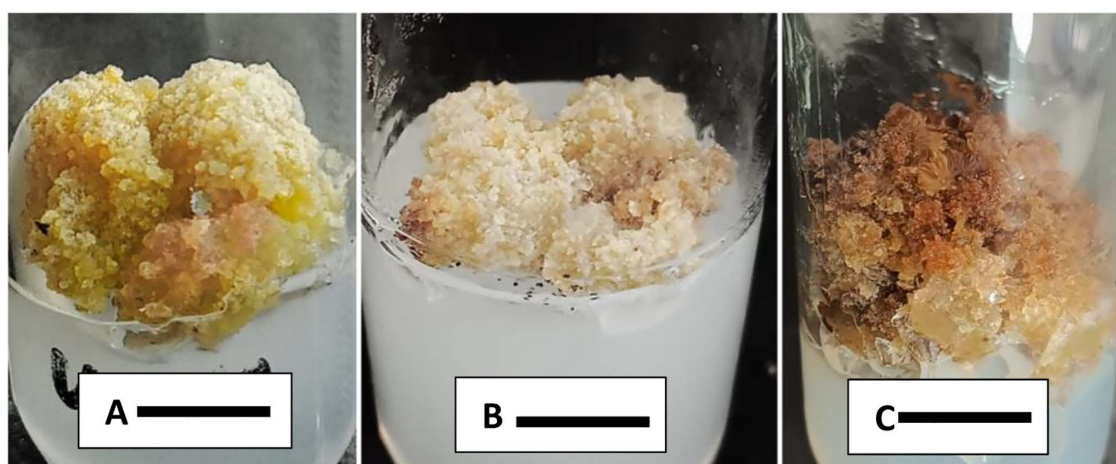


Fig 3. A: Callus proliferation, in response of sub-culturing on S1 [(MS medium with BAP + 2, 4-D) (2.0 mg L⁻¹ + 0.5 mg L⁻¹)] after 20 days. **B:** On S3 MS medium with IAA + BAP (1.7 mg L⁻¹ + 2.0 mg L⁻¹) after 20 days. **C:** On S2 MS medium with TDZ + 2,4-D (0.1 mg L⁻¹ + 0.5 mg L⁻¹) after 20 days. Bar = 1 cm.

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Comparative study of antioxidant enzymes and phytochemicals of *C. camphora* leaf extract and callus cultures

POD, CAT, SOD and GPX activity. Overall, POD activity of callus culture was found higher as compared to leaf extract of *C. camphora*. A Decreasing trend in the activity of peroxidases was recorded from 32.124 to 5.66 U mL⁻¹ of enzyme in callus as compared to leaf extract. Similar trend was also recorded in the activity of catalases, leaf extract showed 18.683 as compared to 58.6 U mL⁻¹ of enzyme activity in callus culture. SOD activity of callus cultures was 7.078 U mg⁻¹ of protein while in case of leaf extract 2.232 U mg⁻¹ of protein was recorded. Unlike the above mentioned three primary antioxidants, GPX activity of leaf extract was higher as compared to callus cultures grown under the controlled conditions. Decreasing trend in the activity of GPX was from 0.552 to 0.379 U mg⁻¹ of protein (Fig 4).

Total phenolic and flavonoid contents

The phenolic contents in callus culture were 1.106 mg GAE g⁻¹ and in leaf extract were 1.053 mg GAE g⁻¹. Overall, callus culture showed highest phenolic contents as compared to leaf extract. A decreasing trend in flavonoid contents was recorded from 7.87 to 5.189 mg CatE g⁻¹. Leaf extract of plant showed highest flavonoid contents (7.87 mg CatE g⁻¹) while in callus cultures flavonoid contents were 5.189 mg CatE g⁻¹ (Fig 5).

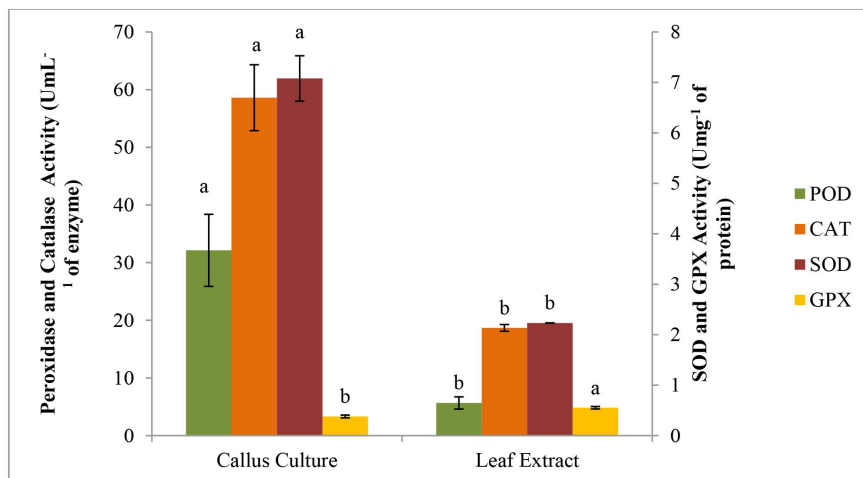


Fig 4. A comparison of antioxidant enzymes activity of callus culture and leaf extract of *C. camphora*.

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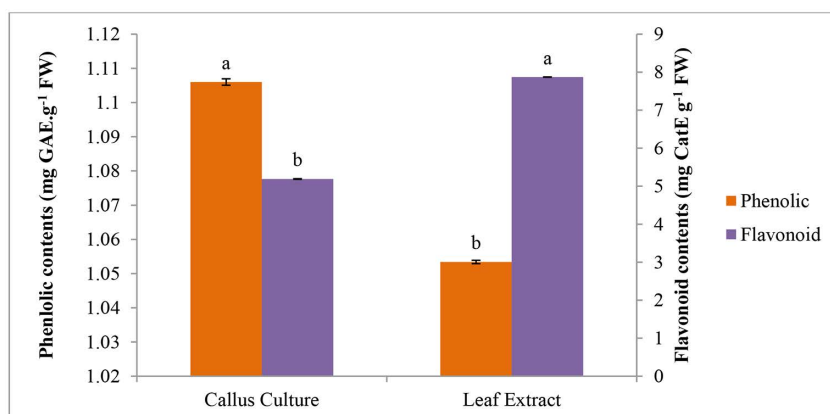


Fig 5. A comparison of phenolic and flavonoid contents of callus culture and leaf extract *C. camphora*.

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Discussion

In present study, two type of plant material (young leaves and nodal segments) were used as explant for shoot induction of *C. camphora*. Shoot initiation from nodal segments was achieved for establishment of *in vitro* plants. Different combinations of PGRs in MS media were tried for shoot induction. MS medium containing 0.5 mg L⁻¹ 2, 4-D + 2.0 mg L⁻¹ BAP provided best results with lateral shoot induction after 22 days as compared to other tested media. Similar results were recorded by Du [42] who suggested that same combination and concentration of 2, 4-D and BAP for direct organogenesis by nodal explant from seedlings of *C. Camphora*. Somatic embryogenesis in cell suspensions of *Cenchrus ciliaris* L. on the similar combination of PGRs was achieved by Goyal [43]. It is worth mentioning here that 2, 4-D and BAP were prove more effective in triggering the cell elongation as compared to other growth regulators [44,45]. When the combination of 0.1 mg L⁻¹ TDZ and 2.26 mg L⁻¹ 2, 4-D was used, shoots developed within 18 days without lateral shoot formation. Pakum [46] demonstrated similar results within 8 weeks for direct organogenesis from leaf explant of *Kalanchoe tomentosa* Baker (Panda plant) on 0.1 mg L⁻¹ TDZ and 0.5 mg L⁻¹ 2, 4-D separately while Li [47] investigated callus induction from leaf

explant of *Bupleurum chinense* on 2 mg L⁻¹ 2, 4-D and 1 mg L⁻¹ TDZ. Similarly, application of only TDZ in MS medium was also documented as effective for *C. camphora* [48]. Lower level of TDZ (cytokinin) plays an important role in shoot induction and accelerates the plant regeneration [49]. BAP in various combinations was also previously used for shoot initiation of *C. camphora* explants [50]. During this investigation, MS medium with 1.0 μM IBA + 2.0 g L⁻¹ AC (activated charcoal) was also showed 100% response of shoot initiation however, these results were contradictory to Babu [51], who reported rooting and shooting on WPM medium with similar combination of medium. The above-mentioned studies and our results confirmed that higher concentration of cytokinin's with various concentrations of auxin supports the shoot induction.

In this investigation, the response of callus induction from young juvenile leaf and the nodal segments was also evaluated and found that significant and more positive response was observed on MS medium with combination of 2.0 mg L⁻¹ BAP and 0.5 mg L⁻¹ 2,4-D as compared to other tried media. Similar results for callus initiation and proliferation from leaf disc of *C. camphora* were described by Chen [52] but in their case calli were induced on nodal explants instead of leaf segments. However, in another study, various combinations of auxins and cytokinin's were describes for callus initiation from leaf explants of *C. camphora* [50,53]. Similarly, the combination of 2,4-D and BAP was also reported as good for callus induction and proliferation in many other plant species [54–56]. Since, combination of 2,4-D and BAP synergistically more effective in promoting callus development by triggering the cell elongation [57–59]. 2, 4-D alone was also suggested as the best auxin for callus induction and proliferation in many previous studies [60–62]. In present study, the morphology of developed callus was lush green while compact in texture. Muthi'ah [55] observed similar morphology of callus on this combination of growth regulators in his study on *Calotropis gigantea*. Aref and Salem [53] found different morphology of calli of *C. camphora* on different combinations of PGRs by using different explants. According to Abu-Romman [63] and Yaroshko [64], even within the same genus, the appearance of callus depends upon the concentration and type of plant growth regulators. In present investigation, maximum proliferation response, growth index of callus (0.76g within 19 days) after callus sub-culturing was recorded at 2.0 mg L⁻¹ BAP + 0.5 mg L⁻¹ 2, 4-D. Morphologically, callus was lush green, granular, lightly friable and compact in nature. El-Kader [65] reported similar results by sub-culturing of *C. camphora* leaf callus on MS medium fortified with 2.0 mg L⁻¹ NAA and 1.0 mg L⁻¹ BAP. However, contrary to our results, best yield of 11 g was obtained after 45 days of sub-culturing in their study. For high yield of callus various combinations of auxins and cytokinin's were used in literature [66,67]. Time interval of callus sub-culture varies among various species in literature also [68].

In current study, a comparative analysis of phytochemicals and antioxidant activities was assayed in callus vs. leaf extract of *C. camphora*. The proliferated callus cultures were used for comparative analysis of phytochemical and antioxidant activity. Junairiah [69] and Astuti [70] reported that compact calli were far better regarding to production of secondary metabolites as compared friable ones. Callus culture showed great potential to produce secondary metabolites, i.e., phytochemicals and antioxidants those were also found in whole plants. Various investigations and approaches have been done for the production and to overcome the limitations of these vital secondary metabolites. For different applications of biotechnological fields these *in vitro* extract of callus cultures were utilized successfully [71,72]. In our findings, an increasing trend in the activities of peroxidase, catalase, and superoxide dismutase was observed in callus culture as compared to leaf extract. These results are in line with the previous study of Ali [73] who demonstrated that the application of GA₃ (PGR) on *sorghum* seedlings resulted in increasing SOD and POD activity. POD, SOD and catalase were considered as important enzymes which activate the defense mechanisms of plant by regulating metabolic processes [74,75]. Reason behind this increasing trend might be the inhibition of reactive oxygen species (ROS) by a variety of phytochemicals and antioxidant enzymes [76], which are essential for protecting the calli cells from various stress factors. In present study, a decreasing trend was found in Glutathione peroxidase activity. In plant, GPXs may play a variety of functions in stress tolerance and growth [77]. It was reported that GPX activity results in accumulation of high rate of ROS in zygotic or embryonic nuclei [78] and thought to have signaling factors during abiotic stress [79]. This study was in line with Passaia [80] and Madhu [81] who described that GPXs control the plant growth and development in both favorable and unfavorable environments. SOD and GPx have the ability to directly balance the oxidative stress and

provide protection to plant cells from DNA damage [82]. SOD initially, catalases the dismutation of molecular oxygen (O_2) and released hydrogen peroxide (H_2O_2) while the primary enzyme that breaks down this hydrogen peroxide into water in cells is glutathione peroxidase [83].

In present work, statistically significant increase in phenolic contents and decrease in flavonoid contents was recorded in callus cultures as compared to leaf extract grown in natural conditions. Medicinal plants with high concentrations of phenols and flavonoids have strong antioxidant effects. Our findings indicated that the phenylpropanoid pathway is activated by PGRs-containing media because this pathway causes an increase in synthesis of phenolic contents [84]. It is reported that Phenylalanine ammonia-lyase (PAL) enzyme activity increased by application of plant growth regulators exogenously and it causes the accumulation of phenols and other secondary metabolites [85,86]. As Rybin [56] has been reported the combination of 2,4-D and BAP for active production of phenolics and callus cultures of *Vaccinium corymbosum* L. Therefore, present study revealed that phenylpropanoid molecule production was positively impacted by various concentrations of PGRs and phenolic contents were produced in response of defense mechanism. Correlation between total phenolic content and antioxidants in other plants has been recorded [87]. According to Hatami [88], plant metabolism is quite complicated and depends on variety of variables, including the types and concentrations of growth hormones in the culture media, age, and type of cells or tissues. Overall results demonstrate that an increase in antioxidant activity and phenolic contents was due to various factors that might be the oxidative stress on *in vitro* callus cultures or different combinations of PGRs.

Conclusion

In conclusion, it was observed during this investigation that callus cultures extract contains higher phytochemicals and antioxidant enzymes as compared to leaf extract of *C. camphora* and this enhanced activity of enzymatic antioxidants and phenolic contents might be correlated with *in vitro* conditions and PGRs used during callus induction. The results hint the potential use of callus cultures of *C. camphora* for the production of phytochemicals and antioxidants enzymes at commercial scale. These plant-based antioxidants and phytochemicals can be used in pharmaceutical, cosmetics and food industries to meet the country demand. However, this study requires more investigations to assess the efficiency of various combinations of plant growth regulators or some other elicitors to enhance these phytochemicals and enzymes under *in vitro* conditions.

Supporting information

S1 File. Data sheet.
(XLSX)

Author contributions

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Formal analysis: Kainat Rasool.

Methodology: Sajal Rasool.

Software: Sajal Rasool, Kainat Rasool, Sheza Ayaz Khilji.

Supervision: Zahoor Ahmad Sajid.

Visualization: Sheza Ayaz Khilji.

Writing – original draft: Sajal Rasool, Sheza Ayaz Khilji.

Writing – review & editing: Zahoor Ahmad Sajid.

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