International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
www.florajournal.com
IJHM 2020; 8(4): 49-54
Received: 28-05-2020
Accepted: 30-06-2020

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Standardization of ginger callus culture and its comparison with ginger rhizome and callus treated with extrinsic molecule on their antioxidant activity

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Abstract
Zingiber officinale as a medicinal plant exhibits good antioxidant properties. In this study, the ginger leaves and ginger rhizome were selected as explants for the callus induction and plantlet production and were cultured on MS medium supplemented with various growth regulators. In addition to the induced callus, elicitors such as Salicylic acid, Yeast extract and Glycine were used as extrinsic molecules to check for the enhancement of antioxidants and phytochemicals compared to the callus and rhizome produced cultures without elicitors The highest significant amount of antioxidant was (45% to 50%) recorded by DPPH assay. This proved the effect of the elicitation of ginger cultured tissues in the antioxidant study that would be of immense importance for pharmacological, cosmetic and agronomic industries in the future as induction of callus reduces time.

Keywords: Tissue culture, callus, elicitors, rhizome, antioxidant, phytochemicals

1. Introduction
The plant *Zingiber officinale* which is commonly known as Ginger belongs to the family *Zingiberaceae*, has its origin in South East Asia. *Zingiber officinale* is a medicinal herb that has been broadly used in Chinese, Ayurvedic and Tibb-Unani herbal medicines. The rhizome of this plant has been used as a medicine in Indian, Asian and Arabic herbal traditions since ancient times [1]. It is widely used in the treatment of many unrelated ailments like arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever and infectious diseases. The plant has its own specific pharmacological importance. It has immuno-modulatory, anti-tumorigenic, anti-inflammatory, anti-apoptotic, anti-hyperglycemic and anti-lipidemic properties [2].
The leaves are linear and the flowers are yellowish green, oblong and ensheathed in a few scarious bracts. It is one of the most widely used spice all over the world. Leaves and rhizomes of ginger have been used as condiment for various foods and beverages [3]. Ginger finds its applications in food, and pharmaceutical industries. Studies revealed that the plant is a rich source of alkaloids, saponins, tannins, flavonoids, terpenoid and phlobotannins. Rhizomes of ginger act as a strong anti-oxidant thus resulting in the prevention or mitigate the generation of free radicals in the body. Antioxidants are compounds capable of inhibiting the oxidation processes and protecting your cells against free radicals. They have industrial applications for the stabilization of polymeric products of petrochemicals, foodstuffs, cosmetics, and pharmaceuticals [4]. Antioxidants are involved in the defense mechanism of the organism against the pathogenesis associated with the attack of free radicals. Plant tissue culture technology has been successfully used for germplasm conservation of rare and endangered species and the commercial production of pathogen-free plants. *In vitro* plant culture techniques provide an alternative means of plant propagation and a tool for crop improvement.
Plant tissue culture techniques also help in the production of the improved disease-free cultivar with a rapid multiplication rate. The propagation method is a best method that increases large scale production of planting material in a short period of time with high phytochemical property. Therefore, it produces large amounts of disease-free clones. *In vitro* culture technique of axillary and adventious shoot system was attempted by callus culture. The *In vitro* culture of callus tissues from ginger with the supplement of extrinsic molecules increases antioxidant property when it is compared with antioxidant property of the ginger plant.

2. Scientific classification

Kingdom	:	Plantae
Clade	:	Tracheophytes
Clade	:	Angiosperms
Clade	:	Monocots
Clade	:	Commelinids
Order	:	Zingiberales
Family	:	Zingiberaceae
Genus	:	*Zingiber*
Species	:	*Z. officinale*

3. Materials and Methods

3.1 Explant Collection

Young disease free rhizomal explants (rhizomal buds of 2.5 - 3 cm) were collected along with the leaves as explants. Leaves were chosen for callus formation.

3.2 Surface Sterilization

The leaves and the rhizome were then subjected to surface sterilization.

Mortality rate was calculated for the explants by using the formula,

% Mortality = Total number of explant dead/ Total number of explants x 100

3.3 Initiation

Surface sterilized rhizomal segments (1.2 - 1.5 cm) and leaves were cultured on MS basal medium containing 3% (w/v) sucrose for culture initiation and served as explant sources for subsequent experiments. The pH of the medium was adjusted to 5.8 before gelling with 0.8% (w/v) agar (Himedia, Mumbai, India). The explants initially were implanted vertically on the culture medium in bottles and capped tightly.

Leaves

Medium 1(M_1) = MS + 3%Sucrose + 6BAP – 3 mg/l

Medium 2(M_2) = MS+3% Sucrose + 6BAP – 3 mg/l + NAA – 1 mg/l

Observations were done for the callus formation daily

Rhizomes

Medium 3(M_3) = MS+3% Sucrose + Kinetin – 3 mg/l

Medium 4(M_4) = MS+3% Sucrose + Kinetin – 3 mg/l + NAA – 1 mg/l

3.4 Culture Conditions

All the cultures were subjected to light intensity in the growth room for 10-12 hours photoperiod provided by cool white fluorescent lamps of 1500-3000 lx, temperature of about 25 ± 2 °C and humidity of 35- 40%. The observation of the shoot induction was recorded after 4-5 weeks.

After the observation of callus from leaves and the response using the rhizome explant, elicitors were used in contact with the callus formed to see the response of the antioxidant activity. The formed callus and the plant from the rhizome explants were kept ready for the analysis of comparison of the antioxidant activity after the treatment with the elicitors for the callus.

3.5 Callus with extrinsic molecules

Freshly proliferated weighed callus of about 500 mg were transferred to MS medium supplemented with different concentration of elicitors namely yeast extract (500 mg/L), glycine (500 mg/L) and salicylic acid (100 mg/L) separately. All cultures of the elicitor treatment were maintained at 25 ± 2 °C, photoperiod of 16/8h light/dark for three weeks. Calli were freeze dried and powdered.

3.6 Preparation of Leaf extract

The leaves collected from the rhizome propagated ginger plant extract were prepared using Soxhlet apparatus using the solvents Water, Acetone.

Similarly, after the callus formation, the callus was collected and extract was prepared using the water and acetone as solvents and same procedure was followed for the callus induced with the extrinsic molecules [3].

3.7 Qualitative analysis – phytochemicals study

Different phytochemical tests were performed to estimate the presence of antioxidant in the explant.

3.7.1 Test for alkaloids

Wagner's and test for saponins were carried out to check the presence of alkaloids.

3.7.2 Test for carbohydrates

Molisch's test, Fehling's test and Benedict's test were carried out to reveal the presence of sugars [5].

3.7.3 Test for tannins

The test for tannins was carried out by following the procedure of (Fear *et al.*, 1929) [6].

3.7.4 Test for flavonoids

Presence of flavones was confirmed by shinoda's test [7].

3.7.5 Test for fats and oils

A little amount of drug sample was placed on the filter paper and stand for 15 minutes. A greasy spot was observed due to presence of fats. Thus, the presence of fats in the drug sample was tested

3.8 Quantitative analysis - antioxidant study

3.8.1 Estimation of total flavonoids content

In order to compare the total flavonoid contents between leaf-derived calli and *in vivo* leaves of *zingiber officinale*, aluminum (III) chloride colorimetric assay was carried out. The total flavonoids content of the samples were then estimated from the standard curve and further expressed in milligram of catechin equivalent per gram of sample fresh mass (mg/g) [8].

3.8.2 Estimation of total phenols

Total phenol was estimated by the method of [9]. The total phenol was determined using a standard curve prepared with different concentration of gallic acid. The results were expressed in milligrams per gram fresh weight [10].

3.8.3 Estimation of carbohydrates

The estimation of carbohydrates including glucose, fructose and sucrose were carried out using anthrone as a reagent.

3.8.4 Estimation of protein

The dried and powdered samples were extracted by stirring with 50 ml of 50% methanol (1:5 w/v) at 25 °C for 24 h and centrifuged at 7,000 rpm for 10 min. 0.2 ml of extract was pipette out and the volume was made to 1 ml with distilled water. 5 ml of alkaline copper reagent was added to all the tubes and allowed it to stand for 10 min followed by the addition of 0.5 ml of Folin'sCiocalteau reagent followed by

the incubation at dark for 30 min. The intensity of the colour developed was read at 660 nm [11].

3.9 Determination of total reducing power

The reducing power of the sample was determined according to the method of Jayanthi [12]. 1ml of the extract was mixed with 1ml of 200mM of sodium phosphate buffer (pH- 6.6) and1ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min and 1ml of 10% trichloroacetic acid (w/v) was added. The mixture was centrifuged at 2000rpm for 10min. The upper layer solution (2.5ml) was mixed with 2.5ml of double deionised water and 1ml of fresh ferric chloride solution (0.1%). The absorbance was measured at 700nm. The higher absorbance indicates the higher reducing power.

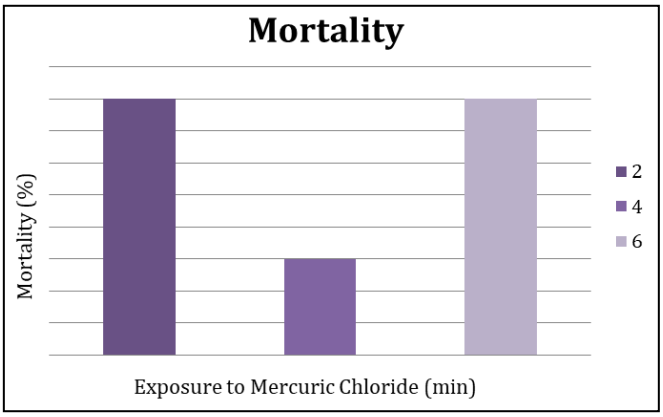
3.10 DPPH free radical scavenging assay

This method was used to determine the antioxidant property in the callus of the Ginger explant. 1 g of sample was taken and extracted with 3 different solvents such as water, acetone, ethanol and 1 ml was filtered for the test which was then mixed with 3ml of DPPH solution in separate tube. The tube was incubated in dark at room temperature for 30 minutes. The optical density was measured at 517 nm using spectrophotometer. Ascorbic acid was used as reference standard [13].

4. Results and Discussions

4.1 Effect of Surface Sterilization

The surface sterilization done for the Rhizome explants with different concentrations of mercuric chloride and mortality rate was calculated.



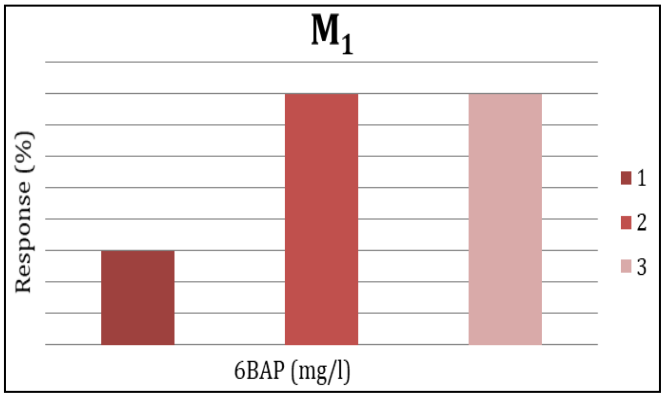
Graph 1: Graph showing the mortality of ginger rhizomes

To overcome contamination problem, surface sterilization of ginger explants was done with 0.1% concentration of Mercuric chloride ($HgCl_2$) for 2, 4, 6 minutes. Mercuric chloride ($HgCl_2$) is a very strong sterilant. When the explants sterilization was done with 0.1% concentration of $HgCl_2$ for 2, 4 min, 80 *et al*. % of the explants got contaminated. Exposure of 0.1% concentration of $HgCl_2$ above 6 minutes, prove to allow death or contamination of explant respectively. 6 minutes exposure was found to be apt for sterilization.

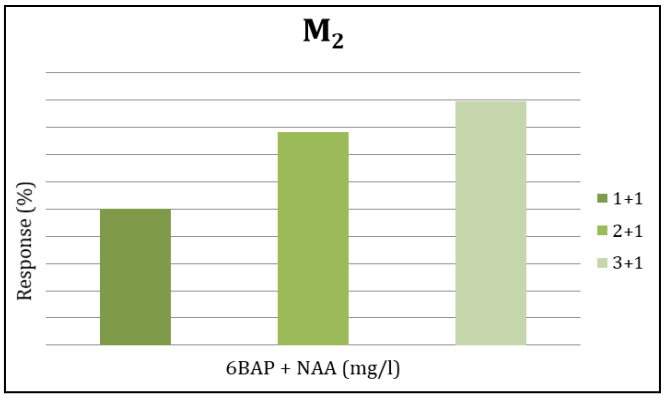
4.2 Initiation

MS medium supplemented with different concentrations and combinations of Cytokinins and auxins showed variation in the regeneration percentage.

Among the different combinations of Cytokinin and Auxin tested, the best response was obtained in the presence of 6BAP 4 mg/l after 22 days of incubation. The response was about 94%. The other concentrations showed response of about 70-80%. The combinations of 6BAP and NAA showed about 50-70% whereas Kinetin concentrations showed response of about 60-70%. The combinations of Kinetin and NAA showed response of 30-50%



Graph 2: Graph showing the efficiency of shoots in M_1 media



Graph 3: Graph showing the efficiency of shoots in M_2 media

(a) (b)

Fig 1: (a) Callus formation from leaves (b) Plantlet formation from rhizome

The developed callus was then extracted separately and in addition to elicitors for the detection of secondary metabolites.

4.3 Test for phytochemicals

Phytochemical analysis was carried out using the selected samples such as leaves from the rhizome propagated plant, callus and callus induced with elicitor molecules using water and acetone as solvents and the results were tabulated as below

Table 1: Phytochemical analysis

Test	L-w	L-a	C-w	C-a	Ce-w	Ce-a
Saponins	+	+	+	+	+	+
Fats and oils	+	-	+	-	+	+
Alkaloids Wagner's test	+	+	+	+	+	+
Carbohydrates						
Fehling's Test	-	-	-	+	-	-
Benedict's Test	+	+	+	+	-	-
Molisch's Test	-	+	+	+	+	+
Tannins						
Lead acetate	+	+	+	+	+	+
Ferric chloride	+	+	+	+	+	+
Flavonoids Shinoda's Test						
NaOH	+	+	+	+	-	-
H_2SO_4	+	+	+	+	-	+

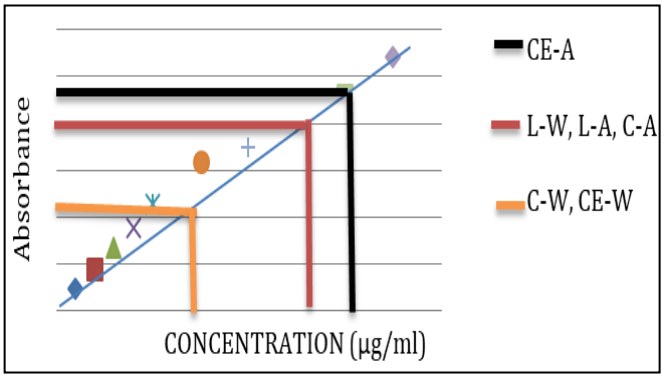
L-W - Leaf extract treated with water, **L-A** - Leaf extract treated with acetone, **C-W** - Callus treated with water, **C-A** - Callus treated with acetone, **CE-W**- Callus exposed to extrinsic molecule and treated with water **CE-A** - Callus exposed to extrinsic molecule and treated with acetone

At the end of phytochemical analysis, it was inferred that the Saponins were present in all sample extracts. The phytochemicals were detected in almost both the solvents. Thus, it is inferred that the phytochemicals could be detected in callus stage itself in the Ginger plant as the leaves part.

4.4 Antioxidant analysis

4.4.1 Determination of total flavonoid content

The Flavonoid content was analyzed using Quercetin as standard at 510 nm and the concentration of flavonoids present in the samples were tabulated.

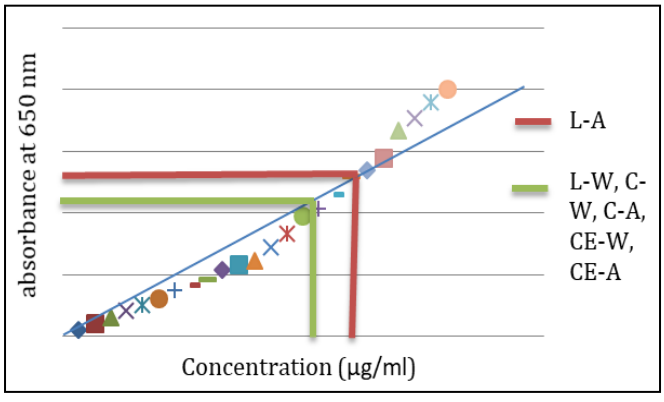


Graph 4: Graph showing the total Flavonoids content of extracts

From the standard graph, the total flavonoids content was determined as 30.5 µg/ml in the Acetone extracts of callus induced with elicitors followed by 27 µg/ml in water extract of leaves and acetone extracts of leaf, and callus and 14 µg/ml in the water extracts of callus and callus induced elicitors.

4.4.2 Determination of total phenolic content

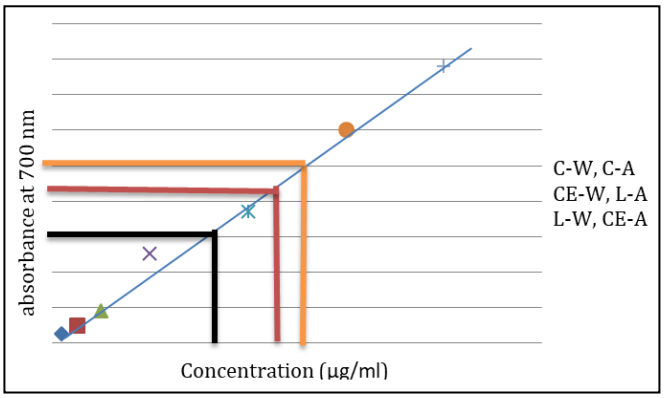
Phenolics were determined at 725 nm using Gallic acid as standard and the quantity of phenolics present in the samples were tabulated.



Graph 5: Graph showing the total phenolic content of extracts

From the standard graph, total Phenol content was found higher in acetone extract of leaves as 37 µg/ml followed by 34 µg/ml in all the other extracts. Leaves were found to possess high phenols compared to the callus.

4.4.3 Reducing Power assay



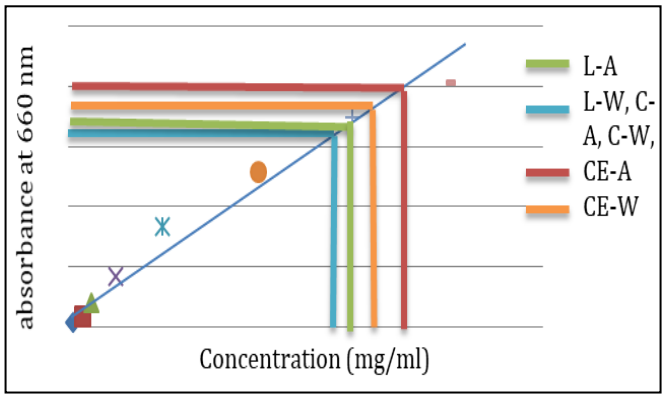
Graph 6: Graph showing the reducing power assay

From the standard graph, the reducing power assay was determined as 500 µg/ml in the acetone extract of callus induced with elicitors and water extract of leaves followed by

440 µg/ml in acetone extract of the leaves and water extract of callus induced elicitors and 370 µg/ml in water and acetone extracts of callus.

4.5 Test for proteins

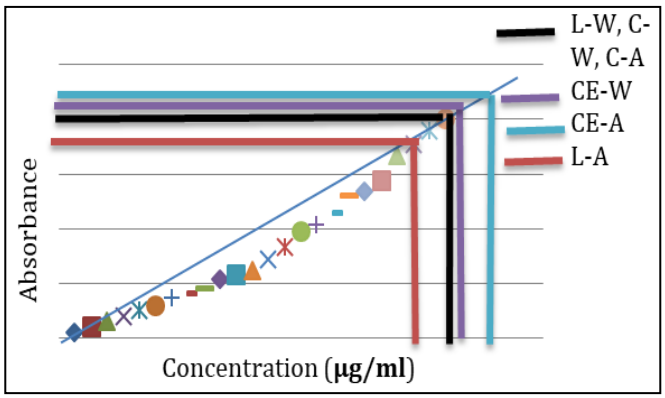
Proteins are compared in the collected sample extracts to know the amount of proteins using BSA (Bovine Serum albumin) as standard.



Graph 7: Graph showing the protein content

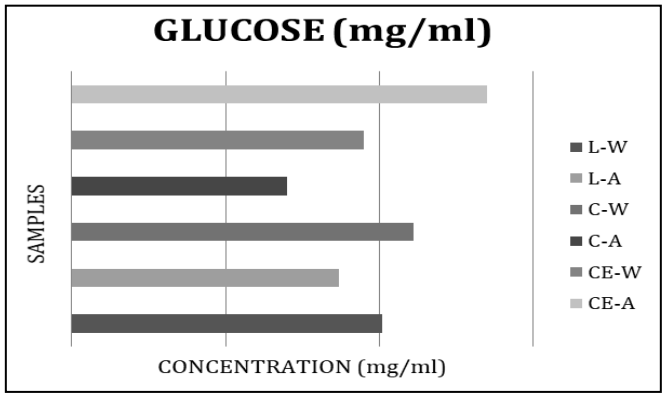
From the graph, proteins were measured higher in the acetone extract of callus induced elicitors of about 7.2 mg/ml followed by 6.2 mg/ml in the water extract of callus induced elicitors and 6 mg/ml in the acetone extract of leaves and 5.8 mg/ml in both the extracts of callus and water extract of leaves.

4.6 DPPH Radical Scavenging activity

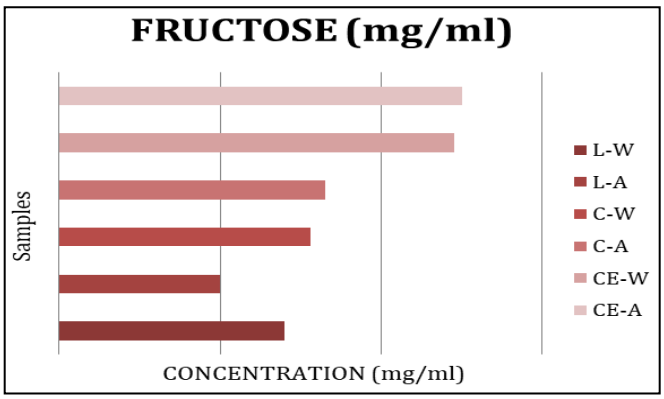


Graph 8: Graph showing the DPPH radical scavenging activity

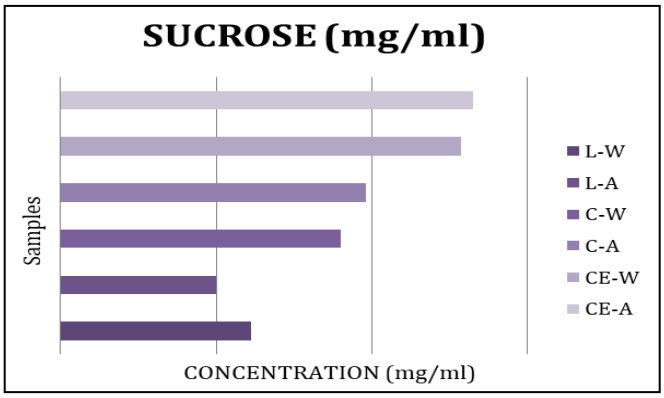
The DPPH activity was found to be highest in the callus induced elicitors acetone extract followed by callus induced elicitors water extract.



Graph 9: Graph showing the estimation of glucose



Graph 10: Graph showing the estimation of fructose



Graph 11: Graph showing the estimation of sucrose

The carbohydrates of the collected samples were tabulated showing higher amount of carbohydrates in callus and callus induced solvents compared to the leaf samples.

5. Conclusion

It is evident that the rhizome is extensively used around the world as a spice in culinary, beverages and herbal medicinal practices to treat a wide range of diseases such as rheumatic disorders, cold symptoms, fevers, gastrointestinal complications, motion sickness, bronchitis, diabetes and cancer. The demand for *Z. officinale* metabolites, mainly with higher bioactive compounds content, prompted more directed tissue culturing efforts. The present study aimed at the comparison of the rhizome propagated plants and callus produced from the leaves for the phytochemicals and antioxidant study. It was observed that the callus induced elicitors at an appropriate concentration proved antioxidant release and the phytochemicals presence as rhizome propagated culture. Thus, the elicitation of cultured tissues is necessary and could be adapted to improve the production of phytochemical compounds and to increase the antioxidant capacity of ginger callus culture in a very short time.

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