Antioxidant activity, phenol and flavonoid contents of plant and callus cultures of *Plectranthus barbatus andrews*

Mona M. Ibrahim, Nermeen M. Arafa, Usama I. Aly

Department of Plant Biotechnology, Genetic Engineering and Biotechnology Division, National Research Center, Dokki, Giza, Egypt

Correspondence to Mona M. Ibrahim, PhD, Department of Plant Biotechnology, Genetic Engineering and Biotechnology Division, National Research Center, Dokki 12311, Giza, Egypt
e-mail: usamajp@yahoo.com

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Background and objective
Plectranthus barbatus is cultivated in many parts of the world for healing and food tradition. This study describes a protocol for the establishment of callus cultures of *P. barbatus* and examines their content of active compounds as well as their effects as antioxidants compared with *in-vitro* plants.
Materials and methods
For obtaining callus cultures, three different explants were tested on MS medium with different growth regulators. Growth index was calculated for the best explant which gave the highest percentage of callus induction. Two different solvents were used for extraction. 2,2′-Diphenyl-1-picrylhydrazyl-scavenging activity, total phenolic and flavonoid contents were determined. Gas chromatography–mass spectroscopy analysis was performed to detect the different components.
Results and conclusion
Maximum callus induction (100%), fresh weight (3.5 g), and growth index (16.5) were obtained from cotyledon explants cultured on MS medium supplemented with 2.0 mg/l naphthalene acetic acid+2.5 mg/l benzyl adenine. Aqueous methanol extracts exhibited higher 2,2′-diphenyl-1-picrylhydrazyl radical scavenging activity than hexane extracts at all tested concentrations. Likewise, methanolic extract of *in-vitro* plant and callus cultures gave the highest values of total phenolic (1.39 and 1.19 mg/g dry weight, respectively) and total flavonoid contents (4.87 and 1.14 mg/g dry weight, respectively). Thirty-one bioactive ingredients have been identified in the hexane extract of *in-vitro* plant and callus cultures of *P. barbatus* by gas chromatography–mass spectroscopy analysis.

Keywords:
2,2′-diphenyl-1-picrylhydrazyl, gas chromatography–mass spectroscopy, *in-vitro* culture, *Plectranthus barbatus*

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Introduction

Plectranthus barbatus, also known as *Coleus barbatus* (*Andr.*), is a member of *Lamiaceae* family [1]. *P. barbatus* is a tropical perennial plant used medicinally in Africa, Arabia, and India and grows spontaneously throughout many countries around the world. It has a wide range of therapeutic applications and used for body weight control, heart failure, hypertension, eczema, colic, respiratory disorders, sore urination, insomnia, and convulsions [2]. Moreover, medical studies also indicated that it may have a therapeutic benefit in asthma, angina, and psoriasis [3]. The leaves of *C. barbatus* are used medicinally in Egypt and Africa as an expectorant, emmenagogue, and diuretic [4]. *P. barbatus* has a significant economic impact worldwide due to its nutritive and therapeutic values [5].

Scientists have become persuaded that the compounds of plant origin play an important role for healing as well as for curing of human diseases [6]. *P. barbatus* has been found to be a rich source of bioactive metabolites such as phenols, alkaloids, terpenoids, flavonoides, and antioxidants [7–11]. Nowadays it has been studied extensively for novel biologically active constituents.

For the production of bioactive plant ingredients, biotechnological approaches, mainly plant tissue culture tools, seem to be an important stride to illuminate the suitable morphogenetic structure for that purpose. In plant tissue culture, biosynthesis of bioactive ingredients is occasionally differentiation dependent [12] and thus linked with the types and concentrations of growth regulators added to the culture medium [13–15]. In view of that, fitting of the culture medium and growth circumstances is the key for the biosynthesis of plant metabolites [16,17]. In the present research, the effect of different plant growth regulators on callus induction has been clarified.

The ability of scavenging 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radicals, total phenolic and flavonoid contents were also examined. Finally, the chemical composition of the hexane extract of *in-vitro* plant and callus cultures of *P. barbatus* was analyzed using gas chromatography–mass spectroscopy (GC-MS).

Materials and methods

Plant material

Seeds of *P. barbatus* were supplied from SEKEM Company, Cairo, Egypt.

Sterilization and incubation conditions

Seeds of *P. barbatus* were washed in current tap water, then surface sterilized in 70% (v/v) ethanol for 30 s, and immersed in 50% Clorox solution of household bleach (5.25% sodium hypochlorite) with a drop of Tween-20 for 15 min. After thorough washing four times in sterile water, the seeds were cultured on basal MS medium [18] supplemented with 0.7% (w/v) agar and 3% (w/v) sucrose. The cultures were incubated under controlled light regime (16/8 h photoperiod and 2000 lux) at 25±1°C.

In-vitro plant formation

Shoot tip explants were excised from growing seedlings and cultured on a solidified MS basal nutrient medium supplemented with 0.5 mg/l kinetin, after one month, the formed shoots were subcultured on the same medium for multiple plant formation.

Callus induction and growth dynamics

Cotyledon, leaf, and root segments were excised from the *in-vitro* growing seedlings and cultured on solidified MS basal nutrient medium supplemented with 2.0 mg/l naphthalene acetic acid (NAA) +2.5 mg/l benzyl adenine (BA) and 2.0 mg/l dichlorophenoxy acetic acid (2,4-D)+2.5 mg/l BA. Cultures were kept under a controlled temperature of 26±1°C and light conditions of 16/8 h photoperiod. Data were recorded after 4 weeks of culture period; callus induction percentage and growth index [19] were calculated based on the following equations:

$$\text{Callus induction}(\%) = \frac{\text{Total number of explants produced callus}}{\text{Total number of cultured explants}} \times 100.$$

$$\text{Growth index} = \frac{\text{Final callus fresh weight} - \text{Initial callus fresh weight}}{\text{Initial callus fresh weight}}.$$

Preparation of extracts

Dried powdered samples of the *in-vitro* plants and callus culture of *P. barbatus* were extracted using methanol (85%) and hexane for 24 h at room temperature. The extracts were collected, filtered, and evaporated to dryness. Each residue was dissolved in the same extract solvent and stored at 4°C until further use.

Extraction yield (%) of the extract was calculated using the formula:

$$\text{Extraction yield } (\%) = \frac{\text{Weight of the extract}}{\text{Weight of sample}} \times 100.$$

2,2′-Diphenyl-1-picrylhydrazyl radical scavenging capacity

Radical scavenging capacity of the extracts against stable DPPH was determined by a slightly modified method [20]. Different concentrations of each extract (2.0, 4.0, 6.0, 8.0, and 10 mg/ml) were used to evaluate the antioxidant capacity. 500 µl of each extract were added at 2.5 ml of methanolic solution of DPPH (0.3 mM). After 30 min at room temperature, the absorbance values were measured at 517 nm on the spectrophotometer. Radical scavenging capacity (%) was calculated by the following formula:

$$\text{RSC } (\%) = [(A_{\text{DPPH}} A_s / A_{\text{DPPH}}) \times 100].$$

where A_s is the absorbance of solution with extract and A_{DPPH} is the absorbance of DPPH solution.

Total phenolic and total flavonoid contents

The concentration of phenolic compounds was determined using Folin–Ciocalteu reagent according to Singleton *et al.* [21]. A calibration curve of gallic acid (20, 40, 60, 80, and 100 µg/ml) was prepared. The absorbance of the samples and standard solutions were determined against a reagent blank at 550 nm with an ultraviolet/visible spectrophotometer. Total phenolic content was expressed as milligram of gallic acid equivalent per gram of dry weight (DW).

Total flavonoid content was measured using a modified colorimetric method according to Vabkova and Neugebauerova [22]. The standard curve was prepared using different concentrations of quercetin. The flavonoid content was expressed as milligram quercetin equivalents per gram of DW.

Gas chromatography–mass spectrometric analysis

The hexane extract of plants and callus cultures of *P. barbatus* were analyzed in National Research Center, using Gas Chromatography–Mass Spectrometry with

the following specifications; a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ_Single Quadrupole Mass Spectrometer). TG-5MS-fused silica capillary column (30 m, 0.251 mm, 0.1 mm film thickness). For GC-MS detection, an electron ionization system with an ionization energy of 70 eV was used. Helium gas was used as the carrier gas at a constant flow rate of 1 ml/min. The injector and MS transfer line temperature was set at 280°C. The quantification of all the identified components was investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC-MS system.

Results and discussion

Seeds germination of *Plectranthus barbatus*

The sterilized seeds of *P. barbatus* were grown on basal MS medium. Seedlings were fully germinated in a range of 3–4 weeks (Fig. 1).

In-vitro plant formation

Shoot tip explants cultured on MS medium containing 0.5 mg/l kinetin succeeded in shoot and root formation after 1 month. Formed shoots were subcultured on the same medium, after 3 months the formation of new shoots with rooting was observed (in-vitro plants, Fig. 2).

Callus induction and growth dynamics

Callus cultures were initiated form cotyledon, leaf, and root explants. Data presented in Table 1 observed that callus have been formed from all tested explants (cotyledon, leaf and root). Maximum callus induction percentage observed with cotyledon explants in the media containing NAA+BA and 2,4-D+BA (100 and 80%, respectively) was notably higher than that of the root explants (50 and 40%, respectively), whereas the leaf explants were shown to have the least response in the two used media (30 and 20%, respectively).

The copious callus induction was obtained with the MS medium containing 2.0 mg/l NAA+2.5 mg/l BA followed by the MS medium containing 2.0 mg/l 2,4-D+2.5 mg/l BA. The callus nature was compacted and yellow to green in color (Fig. 3).

Callus induction percentages in *P. barbatus* were 100, 50, and 30% with cotyledon, root, and leaf explants, respectively, in an MS medium containing 2.0 mg/l NAA+2.5 mg/l BA, whereas the MS medium containing 2.0 mg/l 2,4-D+2.5 mg/l BA was shown the lower response with cotyledon, root, and leaf explants (80, 40, and 20%, respectively; Table 1). Initiated calli derived from different explants (cotyledon, leaf, and root) were subcultured on the best combination medium which contains 2.0 mg/l NAA+2.5 mg/l BA for callus fresh weights and

Figure 1

Germination of *Plectranthus barbatus* seedlings on basal MS medium after four weeks of cultivation.

Figure 2

Multiple plants formation of *Plectranthus barbatus* from shoot tip explant after sub-cultured on MS-medium containing 0.5 mg/l kinetin.

Table 1 Effect of different growth regulators and explant types on callus induction percentage of *Plectranthus barbatus*

Growth regulators (mg/l)			Explants	Callus induction (%)
NAA	BA	2,4-D		
2.0	2.5	0.0	Cotyledon	100
			Leaf	30
			Root	50
0.0	2.5	2.0	Cotyledon	80
			Leaf	20
			Root	40

2,4-D, dichlorophenoxy acetic acid; BA, benzyl adenine; NAA, naphthalene acetic acid.

growth index evaluation. Browning of initiated callus was detected with leaf and root calli during the second subculture. Therefore, the callus derived from the cotyledon explant was relied on in the rest of the trials.

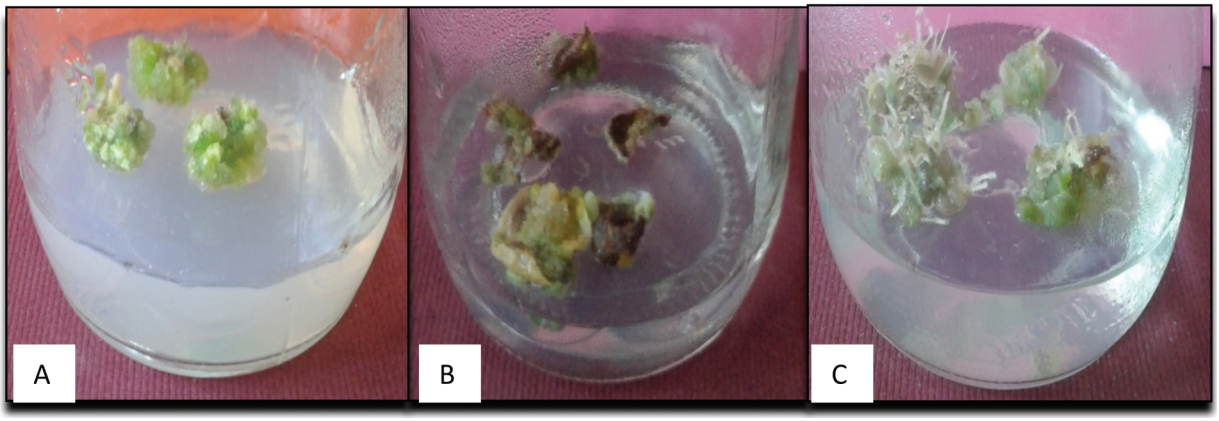
Callus fresh weight as well as callus growth index increased gradually until the maximum values of 3.5 g and 16.5, respectively, were recorded at the fifth week and then declined at sixth week of cultivation (Fig. 4). So it needs to be subcultured every 5-week intervals. After three subcultures (with 5-week intervals), the calli resulted from the cotyledon explant were proliferated and enlarged (Fig. 5) and were used in chemical composition evaluation compared with the in-vitro plants.

The change in cell metabolism from a stationary state to one of active cell division is required for callus induction, which often means the reverse of cell differentiation and specialization [23]. To induce callus from explants owing to its effect on cell growth, auxin is usually required to achieve this, whereas cytokinins stimulate cell division [24]. The capacity for callus induction seems to be highly dependent on the explant nature and the type of growth regulators. A seedling was found to be the optimal source for plant segments used in callus induction [25]. The use of 2,4-D has been notarized in various *Plectranthus* species, and was used in *P. barbatus* by the studies of Tripathi *et al.* [26].

2,2′-Diphenyl-1-picrylhydrazyl radical scavenging capacity and extraction yield

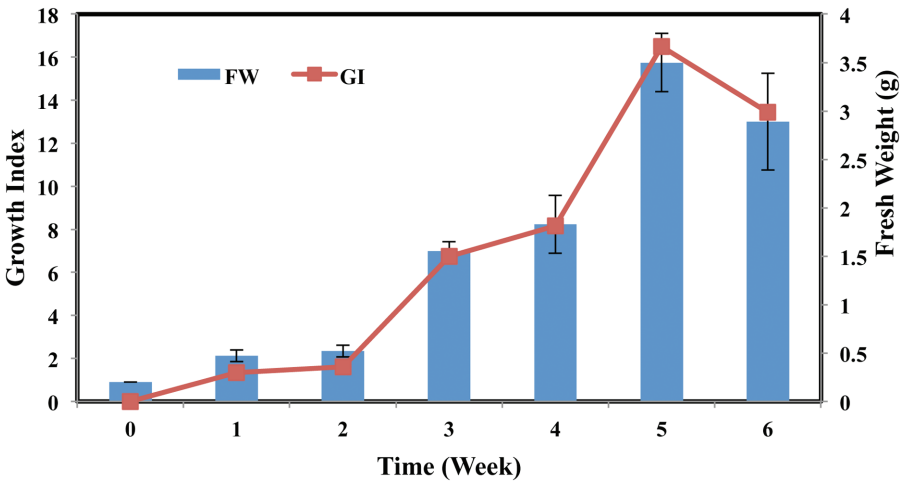
The accumulation of active ingredients in cell cultures at a higher level than those in native plants has been observed in *Panax ginseng* through optimization of cultural conditions [27], rosmarinic acid in *Coleus blumei* [28], shikonin in *Lithospermum erythrorhizon*

Figure 3



Callus induction of *Plectranthus barbatus* on MS-medium containing 2.0 mg/l NAA+2.5 mg/l BA from cotyledon (A), leaf (B) and root (C) explants.

Figure 4



Growth dynamics of *Plectranthus barbatus* callus obtained from cotyledon explants for six weeks.

[29], diosgenin in *Dioscorea* spp. [30], and ubiquinone-10 in *Nicotiana tabacum* [31], whereas plant cell cultures sometime produce lower quantities of secondary metabolites with different profiles when compared with the intact plant [32].

Bioactive compounds extracted using two different solvents are methanol (85%) and hexane. Table 2 shows the highest extraction yield with in-vitro grown plants (38.5%) followed by callus cultures (13.2%) with methanolic extract. Likewise, the same trend was observed with hexane extraction but with a lower extraction outcome with both plant and callus (8.1 and 4.8%, respectively). The difference in extraction yield may be attributed either to the solvent used for extraction and/or to the source of the plant part. To develop the production of plant metabolites, a lot of organic compounds were included to the culture medium [33]. The concept is that an intermediate compound of a metabolic route is expected to raise the yield of final products [34].

Figure 5

Callus culture of *Plectranthus barbatus* from cotyledon explant after three sub-cultures.

The free radical scavenging method explains and evaluates the antioxidant potential of a compound, an extract, or other biological sources. Table 3 shows the activity of free radical scavenging of different concentrations of *P. barbatus* extracts. Except with hexane extract from callus cultures, radical scavenging activity increases with increasing the concentration of the extract. It is important to mention that methanol extracts exhibited higher activity than hexane at all tested concentrations.

Methanol extract of the callus showed the highest DPPH radical scavenging activity (94.5%) among all tested extracts, followed by methanol extract of the in-vitro plant (92%) at the maximum used concentration of the extract (10 mg/ml). While the highest DPPH radical scavenging activity with hexane extracts was recorded with the in-vitro plant (64%) at 10 mg/ml of the extract concentration, the lowest value was observed with callus culture extracts (44.4%) (Table 3).

The model of scavenging the stable DPPH radical is a method used extensively to evaluate the antioxidant activity in a comparatively short time [35].Antioxidant activity from different parts of *C. forskohlii* has been studied [36]. Ethanolic extract of *P. barbatus* is being widely used in African countries as a herbal treatment to reduce oxidative stress [37]. The extract represented significant free radical scavenging activity [38]. A comparative study has been made between the callus extract and leaf extract of C. *forskohlii* and

Table 2 Extraction yield of plants and callus cultures of *Plectranthus barbatus* extracted with methanol (85%) and hexane solvents

Source	Extraction yield (%)	
	Methanol (85%)	Hexane
Plant	38.5	8.1
Callus	13.2	4.8

Table 3 2,2′-Diphenyl-1-picrylhydrazyl antioxidant capacity (%) *in vitro* plant and callus extracts of *Plectranthus barbatus* using methanol (85%) and hexane solvents

Concentration of extract (mg/ml)	DPPH antioxidant capacity (%)				
	Plant methanolic extract	Plant hexane extract	Callus methanolic extract	Callus hexane extract	l-Ascorbic acid
2	85.5±0.17	31.3±0.60	82.1±0.77	44.6±1.10	93.6±0.03
4	91.0±0.33	40.0±3.00	88.6±2.00	44.1±1.40	93.9±0.07
6	91.2±0.04	43.3±1.00	93.8±0.04	45.1±1.10	94.5±0.09
8	91.4±0.07	43.3±1.00	94.1±0.57	43.6±0.92	94.2±0.02
10	92.0±0.50	64±0.26	94.5±0.26	44.4±0.51	93.9±1.00

A well-known radical scavenger l-ascorbic acid was used as standard; Values are represented as mean±SE; DPPH, 2,2′-Diphenyl-1-picrylhydrazyl.

found that the antioxidant activity of the callus extract was more compared with the leaf extract, they showed that this result may be due to more accumulation of active phenolic compounds in the callus [39].

Table 4 Total phenolic and total flavonoid contents of aqueous methanol and hexane extracts of *Plectranthus barbatus*

Extracts	Total phenolic (mg/g DW)	Total flavonoid (mg/g DW)
Plant methanolic extract	1.39±0.050	4.87±0.010
Plant hexane extract	0.25±0.009	0.31±0.005
Callus methanolic extract	1.19±0.050	1.14±0.020
Callus hexane extract	0.09±0.005	0.06±0.001

Values are represented as mean±SD; DW, dry weight.

Total phenolic and total flavonoid contents

Table 4 declares that methanolic extract of *in-vitro* plant and callus cultures gave the highest values of total phenolic (1.39 and 1.19 mg/g DW, respectively) and total flavonoid contents (4.87 and 1.14 mg/g DW, respectively) compared with hexane extracts. Also, the plant extract shows higher total phenolic (0.25 mg/g DW) and total flavonoid (0.31 mg/g DW) compared with callus which recorded 0.09 and 0.06 mg/g DW, respectively, with hexane solvent.

Seeking of healthy food and dynamic medication were pressed on scientists for searching natural antioxidants from different plants. Phenolic and flavonoid compounds were known to have potential

Table 5 Gas chromatography–mass spectrometry analysis for hexane extract of *in-vitro* plant and callus cultures of *Plectranthus barbatus*

Chemical constituents	Classifications	Molecular formula	Peak area in plant extract	Peak area in callus extract
Hexadecane	Hydrocarbon	$C_{16}H_{34}$	7.41	
Tricosane	Hydrocarbon	$C_{23}H_{48}$	8.95	
2-Octadecoxyethanol	Saturated alcohol	$C_{20}H_{42}O_2$	1.11	1.90
2,6,10-Trimethyltetradecane	Hydrocarbon	$C_{17}H_{36}$	1.08	16.89
Heptadecane	Hydrocarbon	$C_{17}H_{36}$	19.65	
Myristoyl chloride	Fatty acid	$C_{14}H_{29}Cl$	1.27	1.93
Docosane	Hydrocarbon	$C_{22}H_{46}$	2.54	
1-Chloro-octadecane	Hydrocarbon	$C_{18}H_{37}Cl$	2.51	
2-Methyl-hexadecan-1-ol	Alcohol	$C_{17}H_{36}O$	1.85	3.42
11-Benzyl-heneicosane	Aromatic compound	$C_{28}H_{50}$	1.71	
Dotriacontane	Hydrocarbon	$C_{32}H_{66}$	9.40	
9,12,15-Octadecatrienoic acid (2-phenyl-1,3-dioxolan-4-yl) methyl ester	Fatty acid methyl ester	$C_{28}H_{40}O_4$	6.39	
13-Phenyl-pentacosane	Hydrocarbon	$C_{31}H_{56}$	4.51	1.72
Benzyl-olate	Fatty acid ester	$C_{25}H_{40}O_2$	2.55	
p-Toluic acid 2-ethylhexyl ester	Carboxylic acid ester	$C_{16}H_{24}O_2$	9.48	
12,15-Octadecadiynoic acid methyl ester	Fatty acid methyl ester	$C_{19}H_{30}O_2$	1.94	5.82
10,13-Octadecadiynoic acid methyl ester	Fatty acid methyl ester	$C_{19}H_{30}O_2$	2.53	
Ethyl iso-allocholate	Steroid	$C_{26}H_{44}O_5$	3.24	
18-nor-abieta-8,11,13-Triene	Diterpenoid	$C_{19}H_{28}$	4.88	
5,8,11-Heptadecatriynoic acid methyl ester	Fatty acid methyl ester	$C_{18}H_{24}O_2$	0.87	
1,1-diethyl-2,2-bis (phenyl sulfonyl) hydrazine	Inorganic compound	$C_{16}H_{20}N_2O_4S_2$	6.14	
Limonene	Cyclic terpene	$C_{10}H_{16}$		10.73
Butyl hydroxy toluene	Phenol derivative	$C_{15}H_{24}O$		4.48
Di-(2-ethylhexyl) phthalate	Organic compound	$C_{26}H_{42}O_4$		14.11
(4-Bromophenyl) bis (2,4-dibromophenyl) amine	Nitrogen compound	$C_{18}H_{10}Br_5N$		5.46
4-Pregnene-6á, 17à,21-triol 3,11,20 trione	Steroid	$C_{32}H_{58}N_2O_6Si_3$		2.28
Phorbol myristate acetate	Diester	$C_{36}H_{56}O_8$		1.39
4-Methyl 2,6-ditert-butylphenol	Phenol derivative	$C_{15}H_{24}O$		4.48
Nicotinamide adenine dinucleotide phosphate	Alkaloid	$C_{21}H_{28}N_7O_{17}P_3$		1.73
2,2′,6,6′Tetrabromo 4, 4′-tert-butylbiphenyl	Diphenol	$C_{20}H_{22}Br_4$		1.89
Nonidentified compounds				21.77

antioxidant properties [40–43]. Phenolic compounds represent one of the major classes of plant-active metabolites, broadly scattered among the plant kingdom, and an essential part of the human diet. Flavonoids comprise the most studied group of plant phenolic that are effective scavengers of hydroxyl and peroxyl radicals, and of the superoxide anion [44]. Moreover, the presence of flavonoid indicates the natural occurring phenolic compound, with beneficial effects in the human diet as antioxidants and as neutralizing free radicals [45].

Correspondingly, *C. forskohlii* extracts also tested positive for phenolic compounds. The phenolic compounds are aromatic secondary metabolites that impart color, flavor, and are associated with health benefits such as reduced risk of heart and cardiovascular diseases [46,47]. Phenolic compounds account for most of the antioxidant activities in plants [48].

Gas chromatography–mass spectrometric analysis

A total of 31 bioactive ingredients have been identified in the hexane extract of in-vitro plant and callus cultures of *P. barbatus* by GC-MS analysis. Table 5 shows the constituents of the bioactive ingredients. Seven compounds representing 67.42% of the bioactive ingredients in plant cultures were identified, namely: heptadecane (19.65%), p-Toluic acid 2-ethylhexyl ester (9.48%), dotriacontane (9.40%), tricosane (8.95%), hexadecane (7.41%), 9,12,15-octadecatrienoic acid (2-phenyl-1,3-dioxolan-4-yl) methyl ester (6.39%), and 1,1-diethyl-2,2-bis(phenyl sulfonyl) hydrazine (6.14%). On the other hand, only five compounds representing 53.01% of the bioactive compounds were detected in callus culture extracts, namely 2,6, 10-trimethyltetradecane (16.89%), di-(2-ethylhexyl) phthalate (14.11%), limonene (10.73%), 12,15-octadecadiynoic acid methyl ester (5.82%), and (4-bromophenyl) bis (2,4-dibromophenyl) amine (5.46%) as shown in Table 5. Other major and minor residual compounds in both *Plectranthus barbatus* extracts ranged from 0.87 to 6.39% in plant extracts and from 1.72 to 4.48% in callus extracts. Nonidentified compounds (21.77%) were detected in GS-MS analysis of callus cultures. Eighteen essential compounds from *C. forskohlii* was detected which were hydrocarbons and oxygenated compounds in the percentage of 22 and 69%, respectively, with α-fenchyl acetate and α-pinene as the major components [49]. Four *Plectranthus* species (*P. amboinicus*, *P. neochilus*, *P. grandis*, and *P. barbatus*) were analyzed by GC/MS, they detected 14 compounds, the most common compound was sesquiterpenes, also transcaryophyllene was found in high concentrations in the extract of four species; some compounds were distinctive for each species and the others were common in the four species [50]. Also, six major components were identified in the root hexane extract of *C. forskohlii* (α-cedrene, β-cadinene, citronellal, two labdane derivatives, and β-citronellol) [51]. In this area as well, the aerial parts of six *Plectranthus* species were analyzed by GC/MS and showed that the essential oil consists mostly of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes [52].

Conclusion

P. barbatus is a prosperous plant with bioactive metabolites. True to its folk nutritive and therapeutic values, the current research has shown that the solvent extraction of the *in-vitro* plant and callus cultures of *P. barbatus* has lots of bioactive ingredients. More experimentation should be done for isolation and characterization of new antioxidant compounds.

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Conflicts of interest

There are no conflicts of interest.

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