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CHEMICAL COMPOSITION OF ESSENTIAL OIL FROM *IN VITRO* GROWN *PEPEROMIA OBTUSIFOLIA* THROUGH GC-MS

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Abstract

Apical meristems and nodal plant parts were used for mass propagation of *Peperomia obtusifolia*. Different concentrations of BAP (6-benzylaminopurine), TDZ (Thidiazuron) and KIN (Kinetin) were used in MS medium. The highest shoot proliferation and multiplication formation occurred in the MS basal medium containing 1.0 mg/mL BAP. Maximum number of shoots and shoot lengths were 9.80 ±0.50 and 35.40 ± 2.92 cm respectively. Rooting response was the best in MS basal medium fortified with1.0 mg/mL BAP plus 0.5 mg/mL NAA (α-naphthalene acetic acid). For acclimatization, the rooted plantlets were transferred to the greenhouse. The volatile oil of this *In vitro* grown *P. obtusifolia* was extracted by hydro-distillation and investigated by gas chromatography/mass spectrometry (GC–MS). The chromatographic analysis of oil showed 35 constituents of which, 16 volatile compounds contributing 65.0% of the total oil constituents could be identified. The major components identified in this oil were sesquiterpenes such as caryophyllene (17.17%), apiol (16.65%), α-cardinol (2.12%) and α-caryophyllene (1.90%). The monoterpenes such as R-α-Pinene (1.00%), camphene (0.84%), borneal (0.32%) and limonene (0.25%) were also identified. The tau.-muurolol (0.68%), τ-Elemene (0.63%), copaene (0.43%) and tau.-cadinol (0.27%) were present comparatively in minor percentages. The caryophyllene oxide (2.95%) was oxygenated sesquiterpene, 3,5-Dimethoxy-4-hydroxycinnamic acid (0.53%) and asarone (0.41%) were phenyl-propanoids.

Introduction

The *Peperomia* genus belongs to *Piperaceae* family that comprises some 600 species (Mabberley, 1993) widely distributed in tropical regions. The chemical studies carried out on *Piperaceae* species have revealed the occurrence of a variety of compounds including essential oils, pyrones, lignoids, polyphenols, unsaturated amides and alkaloids (Parmar *et al*., 1997; Moreira *et al*., 1998a, 1998b; Baldoqui *et al*., 1999). These species have been extensively investigated as a source of new natural products with potential antimicrobial, anti-tumor and insecticidal activities (Cost- antin *et al*., 2001; Min *et al*., 2004; Konishi *et al*., 2005; Sacchetti *et al*., 2005). Some biological activities were found in compounds isolated from the *Peperomia* genus (Mahiou *et al*., 1995, 1996) as well as analgesic activity in extracts of aerial parts from *P. pellucida* (Aziba *et al*., 2001). Many species of *Piperaceae* family have medicinal importance and have been used for the treatment of many diseases like vaginitis, psychotropic, antioxidant, gynaecological maladies, intestinal disorders and cytotoxic effects (Morandim *et al*., 2010).

P. obtusifolia or baby rubber plant is a popular houseplant that can grow up to a height of 1.0 foot. The plant is characterized by oblong, glossy green foliage and has short, somewhat brittle stems. Quickly growing into spreading clumps, it is ideal for tropical ground cover use, as well as container culture or raised planters. It also makes a durable houseplant and will cascade over the side of a hanging basket. It also prefers bright, but indirect sunlight, and a moderate level of humidity. The ideal temperature for growing this plant is 60 to 85^{o}F. Misting can be done occasionally, especially during the summer, to maintain the appropriate level of humidity. *P. obtusifolia* acts as ornamental plant due to both its beautiful succulent leaves which vary in shape and variegation present in many of its diverse varieties (Knauss, 1972).

Essential oils are actually volatile oils that have specific odour, flavour or scent. These secondary metabolites (by-products) are produced in glandular hairs or secretory cavities of plant cell wall as well as are found as droplets of fluid in the leaves, stems, bark, flowers, roots or fruits in different plants. Essential oils are usually extracted through hydro-distillation of aromatic plants (Kour *et al*., 2008). Essential oils and extracts from aromatic plants have long been used for a wide variety of medicinal and domestic purposes (Brown, 1995).

The objective of this paper is to optimize the protocol for plant regeneration through tissue culture techniques and to study the chemical composition of the essential oil extracted from leaves of tissue cultured *P. obtusifolia*.

Materials and Methods

Plant material: *P. obtusifolia* were collected from the garden of Bagh-e-Jinnah PHA Lahore. The voucher specimens were deposited in the Herbarium of Botany Department in Lahore College For Women University, Lahore, Pakistan in 2012.

Surface sterilization of explants: About three hundred explants (apical meristems and nodal parts) were washed with running tap water and dipped in detergent for five min. Then thoroughly rinsed with tap water and again immersed in 30% sodium hypochlorite for twenty minutes. These explants were washed twice or thrice with autoclaved distilled water.

Micropropagation and acclimatization of microplants: After surface sterilization, the explants (both) were

inoculated on MS (Murashige & Skoog, 1962) basal medium supplemented with various concentrations (0.1, 0.5, 1.0, 1.5, 2.0 2.5mg/mL) of cytokinins (TDZ (Thidiazuron), BAP (6-Benzylaminopurine) and KIN (Kinetin)) and auxins (NAA) alone and in combinations (Table 1 & Fig. 2) for initiation and production of multiple shoots (Fig. 1a-c). MS basal mineral formulation containing standard salts, vitamins, 30g/L sucrose and 1.5g/L phytagel. Before adding phytagel, the pH of each medium was adjusted to 5.5±0.1 and each medium was autoclaved at 121°C for 20 min at 15 lb/inch^2. The cultures were grown at 20 ± 2°C and 2000-3000 lux light intensity with a photoperiod of eight hours dark and sixteen hours light.

In the present study, microplants of *P. obtusifolia* were then shifted to green house for their acclimatization (Fig. 1d-f). At early stages, plantlets were potted in autoclaved sand for at least 30 days. After that *In vitro* grown plants were transferred to soil containing 50% leaf mould. Survival percentage of these plants was 95%. These hardened plants were then transferred to trial field for further growth.

Essential oil analysis

Extraction of oil by hydro-distillation: Weighed amount (500g) of *In vitro* grown plant leaves were used in reaction vessel and vessel is attached to steam generator. A water cool condenser was also attached with reaction vessel. Steam generator produced the steam which passed through the sample condensed and collected with essential oils. The oil was dried over anhydrous sodium sulphate and stored in a sealed vial at 4°C till GC-MS analysis was carried out. The yield of the oil was calculated on the basis of fresh weight of sample.

Gas chromatography-mass spectrometry: GC-MS of Varian, Saturn model 2000, equipped with ion trap detector (ITD) was used for the identification of different components of the essential oil of *P. obtusifolia*. Two samples (2µL) were injected on a DB-5MS (30mx 0.25mm i.d., 0.25µm film thickness) column. Helium was used as a carrier gas with a flow rate of 7.0-9.5 psi and split ratio 1:5. The column temperature was maintained at 75°C for five minutes with a 2.5°C rise per minute to 250°C.

Various components were identified by their retention time and peak enhancement with standard samples in gas chromatographic mode and MS library search from the derived mass fragmentation pattern of various components of the essential oil.

Results and Discussion

For micropropagation initially both explants apical meristems and nodal segments were inoculated in all possible combinations of hormones with MS basal media. Nodal segments showed 80.0% while apical meristems showed 30.0% proliferation rate. The multiplication efficiency of nodal segments from plants was significant as compared to apical meristems, when estimated four to five weeks. So, the nodal segments were chosen for further studies. Different concentrations of growth regulators showed significantly different responses in terms of average shoots length per explants (cm), average number of shoots per explants (Table 1), average root lengths per explants (cm) and average number of roots per explants (Fig. 2).

Table 1. Effect of various concentrations of TDZ, BAP and KIN on shoot multiplication and elongation of *P. obtusifolia*.

Plant growth hormones mg/mL			% Response	Mean no. of shoots per explants	Mean shoot length (cm)
TDZ	BAP	KIN			
0.1	-	-	44.3	$2.20^{j} \pm 0.17$	$22.00^{j} \pm 0.63$
0.5	-	-	35.0	$3.31^{ij} \pm 0.12$	$28.30^{defghi} \pm 1.45$
1.0	-	-	38.8	$4.10^{hi} \pm 0.33$	$34.70^{ab} \pm 0.79$
1.5	-	-	56.0	$7.40^{bcd} \pm 0.31$	$26.10^{ghij} \pm 0.80$
2.0	-	-	49.1	$6.20^{def} \pm 0.30$	$31.90^{abcd} \pm 1.43$
2.5	-	-	52.8	$6.90^{cdef} \pm 0.29$	$24.50^{ij} \pm 1.48$
-	0.1	-	51.1	$7.01^{cde} \pm 0.63$	$31.70^{abcde} \pm 0.38$
-	0.5	-	66.7	$8.00^{bc} \pm 0.40$	$30.70^{bcdefg} \pm 0.38$
-	1.0	-	86.0	$9.80^{a} \pm 0.50$	$35.40^{a} \pm 2.92$
-	1.5	-	56.3	$8.50^{b} \pm 0.44$	$33.10^{abc} \pm 1.18$
-	2.0	-	50.4	$7.80^{bc} \pm 0.33$	$30.00^{cdefgh} \pm 0.56$
-	2.5	-	47.5	$6.10^{def} \pm 0.29$	$29.20^{cdefghh} \pm 0.76$
-	-	0.1	57.7	$4.70^{gh} \pm 0.26$	$25.50^{hij} \pm 0.71$
-	-	0.5	64.3	$5.60^{fg} \pm 0.35$	$31.40^{abcdef} \pm 1.33$
-	-	1.0	59.0	$4.40^{ghi} \pm 0.32$	$27.10^{efghi} \pm 1.17$
-	-	1.5	43.0	$3.40^{ij} \pm 0.21$	$26.90^{fghi} \pm 0.57$
-	-	2.0	31.3	$2.80^{j} \pm 0.33$	$30.30^{bcdefg} \pm 2.17$
-	-	2.5	53.1	$6.02^{ef} \pm 0.63$	$33.20^{abc} \pm 1.14$
LSD				**1.17 4.003**	

Each value is mean of three replicates with standard error (Mean ± S.E), Mean with same superscript are not significantly different by Duncan test ($p<0.05$)

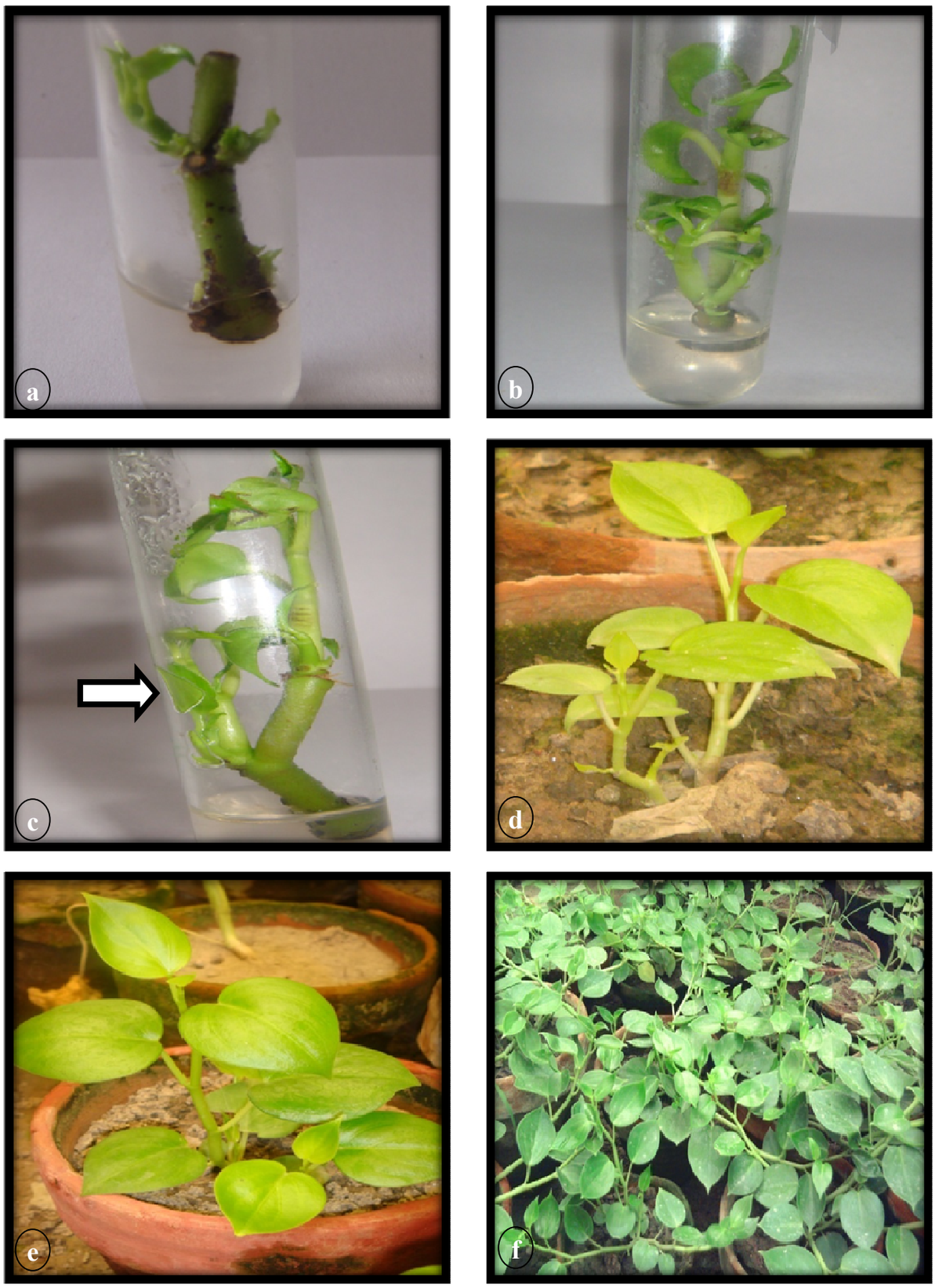

Fig. 1(a-f): Various stages of *In vitro* propagation: (a). Initiation from surface sterilized nodal explants. (b). Development of shoot proliferation. (c). Shoot multiplication in MS medium supplemented with BAP 1.0 mg/ml. (d, e & f). Various stages of acclimatization of plantlets in green house.

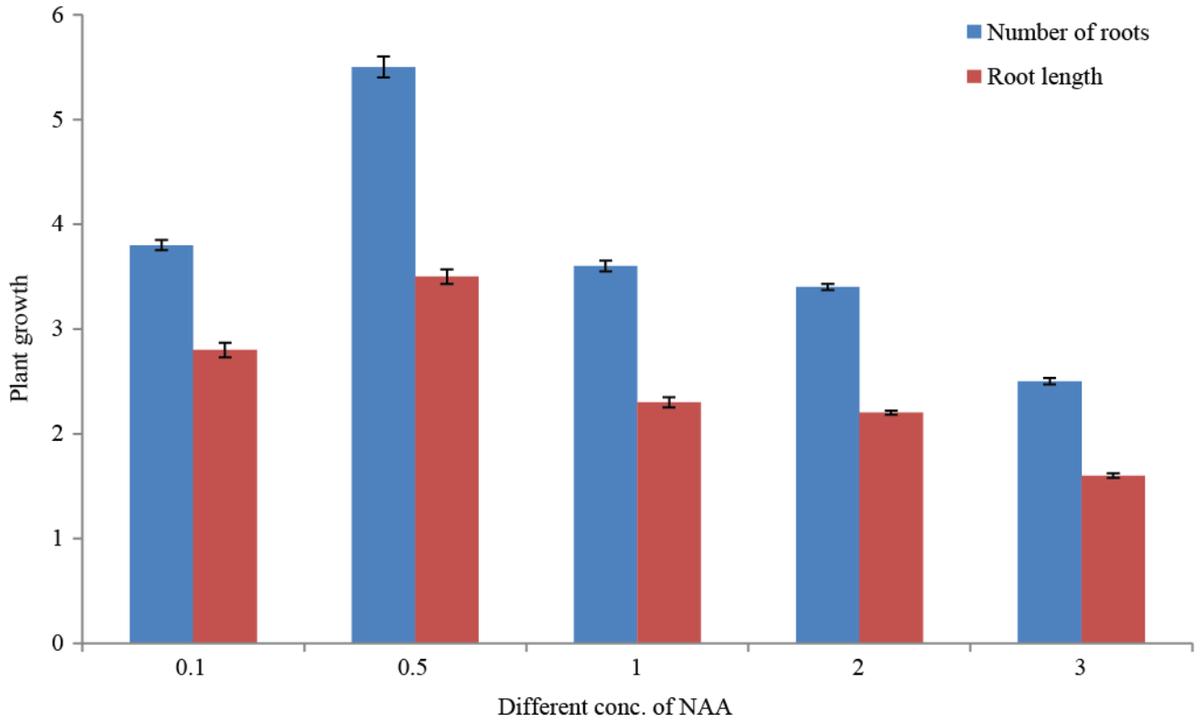

Fig. 2. Effect of different concentrations of NAA on root formation of *P. obtusifolia* growing on MS medium fortified with BAP (1.0 mg/mL).

After four weeks of culture, MS basal medium fortified with 1.0 mg/mL BAP gave maximum proliferation rate (86%) with cultured nodal segments as compared to apical meristems (Table 1). This optimized medium produced maximum no. of shoots ($9.80^{a} \pm 0.50$) and maximum shoot length ($35.40^{a} \pm 2.92$). BAP was proven the best among the other cytokinins for multiple shoot induction from shoot tips of *Scrophularia takesimensis* reported by Sivanesan *et al.*, (2012). Aslam *et al*., (2013) gave similar results on micropropagation of *Lilium longiflorum and L. orientalis* as well and maximum frequency of shoot formation was reported with MS basal medium supplemented with 0.1 mg/L BAP and 0.1 mg/L NAA. According to the Memon *et al*., (2013) MS medium fortified with BAP 4.0 mg/L was chosen the best for shoot regeneration (98.33%) of Gladiolus from explants cormel sprouts (twelve days old). Media containing TDZ and Kin had the least effectiveness in inducing regeneration. TDZ with 1.5 mg/ml and Kin with 2.5 mg/ml produced $7.4^{bcd} \pm 0.31$ no. of shoots, $26.10^{ghij} \pm 0.80$cm shoot length and $6.02^{ef} \pm 0.63$ no. of shoots and $33.20^{abc} \pm 1.14$cm shoot length respectively. After shoot multiplication, various concentrations of NAA (0.1, 0.5, 1.0, 2.0 and 3.0 mg/ml) were tested for maximum production of roots (Fig. 2). The maximum number of roots per explant ($5.5^{a} \pm 0.12$) was obtained in MS basal medium combined with BAP (1.0 mg/mL) plus 0.5 mg/mL NAA. Highest root length per explants was $3.5^{a} \pm 0.43$ on optimized rooting medium. The success of any process of plant cloning can be evaluated by the number of regenerated plants that can survive in field conditions and hardening (Naz *et al*., 2009).

Fresh leaves of *In vitro* grown plants of *P. obtusifolia* were submitted to hydro-distillation for extraction of the essential oil and the percentage yield of oil was 0.731%. The composition of the essential oil from leaves of *P. obtusifolia* is summarized in Table 2. Analysis of oil revealed the presence of 35 chemical constituents, out of which 16 have been identified on the basis of their fragmentation pattern by mass spectroscopy.

The major constituents of oil were sesquiterpenes which contain caryophyllene (17.17%), apiol (16.65%), α-cardinol (2.12%) and α-caryophyllene (1.90%). The other sesquiterpenes were tau.-Muurolol (0.68%), τ-Elemene (0.63%), copaene (0.43%) and tau.-cadinol (0.27%). The oxygenated sesquiterpene present in this oil was caryophyllene oxide (2.95%). The monoterpenes such as R-α-Pinene (1.00%), camphene (0.84%), borneal (0.32%) and limonene (0.25%) were also identified. Moreover, 3,5-dimethoxy-4-hydroxycinnamic acid (0.53%) and asarone (0.41%) were the members of phenyl-propanoids. Another identified compound was 1-Octen-3-ol (0.07%), a secondary alcohol derived from 1-octene which inhibits spore germination (Chitarra *et al*., 2004).

The chemical composition of oil from several other *Peperomia* species from South America have been previously reported by many authors (Díaz *et al*., 1988; Bessiere *et al*., 1994; Silva *et al*., 1999; Moreira *et al*., 1999; Santos *et al*., 2001). Morandim *et al*., (2010) reported the essential oil composition of *P. obtusifolia* (*In vivo*) from Brazil and analyzed constituents that were valerianol (29.40%), guaiol (8.65%), bulnesol (3.86%), δ-cadinene (3.33%), β-farnesene (2.42%), β-caryophyllene (2.13%), germacrene (1.35%), E-nerolidol (0.94%), γ-cadinene (0.75%), α-cadinene (0.72%) and γ-muurolene (0.54%).

Table 2. GC-MS analysis of essential oil from leaves of *In vitro* grown *Peperomia obtusifolia*.

Name of compounds	R:T	m.f.	m.w.	Percentage (%)	m/e Value
R-α-Pinene	5.396	$C_{10}H_{16}$	136.23	1.000	M^+ 53(8%), 77(31%), 80(10%), 93(100%), 105(14 %), 121(15 %), 136(7 %)
Camphene	5.854	$C_{10}H_{16}$	136.23	0.840	M^+ 53(11%), 67(24%), 79(36%), 93(100%), 107 (28%), 121(72%), 136(17%)
1-Octen-3-ol	6.810	$C_8H_{16}O$	128.21	0.077	M^+ 54(4%), 57(100%), 68(5%), 72(20%), 85(11%), 99(7%)
Limonene	7.376	$C_{10}H_{16}$	136.33	0.251	M^+ 53(26%), 65(11%), 68(100%), 79(46%), 93(89%), 107(29%), 121(32%), 136(30%)
Borneol	10.409	$C_{10}H_{18}O$	154.25	0.319	M^+ 55(8%), 67(9%), 95(100%), 110(18%), 121(6%), 139(7%)
Copaene	12.652	$C_{15}H_{24}$	204.36	0.429	M^+ 55(10%), 77(18%), 91(38%), 105(78%), 119(84%), 161(100%), 204(24%)
τ-Elemene	13.029	$C_{15}H_{24}$	204.36	0.637	M^+ 53(15%), 67(25%), 79(31%), 93(77%), 107(24%), 121(100%), 136(20%)
Caryophyllene	14.031	$C_{15}H_{24}$	204.36	17.177	M^+ 55(21%), 65(14%), 69(47%), 79(61%), 93(80%), 105(57%), 120(44%), 133(100 %), 147(37%), 161(49%), 189(35%), 204(14%)
α-Caryophellene	14.740	$C_{15}H_{24}$	204.36	1.904	M^+ 53(11%), 67(15%), 80(32%), 93(100%), 107(17%), 121(34%), 147(26%)
Caryophyllene oxide	19.490	$C_{15}H_{24}O$	220.35	2.956	M^+ 55(39%), 65(17%), 69(50%), 79(100%), 83(20%), 93(77%), 105(38%), 109(49%), 121(35%), 135(19%)
Asarone	19.707	$C_{12}H_{16}O_3$	208.25	0.412	M^+ 55(16%), 79(30%), 91(35%), 105(29%), 133(27%), 193(65%), 208(100%)
tau.-Cadinol	20.416	$C_{15}H_{26}O$	222.36	0.263	M^+ 55(9%), 81(20%), 95(16%), 105(25 %), 119(17%), 134(15%), 161(100%), 204 (43%)
Tau.-Muurolol	20.531	$C_{15}H_{26}O$	222.36	0.685	M^+ 53(11%), 58(24%), 71(30%), 79(48 %), 95(100%), 109(38%), 121(83%), 161(66%), 204(67%)
α-Cardinol	20.903	$C_{15}H_{26}O$	222.36	2.121	M^+ 55(21%), 23(71%), 79(44%), 91(34%), 95(100%), 105(42%), 121(98%), 161(58%), 204(74%)
Apiol	21.635	$C_{12}H_{14}O_4$	222.23	16.656	M^+ 77(10%), 121(12 %), 149(19%), 177(35%), 207(22%), 222(100%)
3,5-Dimethoxy-4-hydroxycinnamic acid	22.133	$C_{11}H_{12}O_5$	224.21	0.534	M^+ 53(7%), 69(10%), 79(14%), 95(20%), 109(13%), 151(21%), 166(37%), 209(100 %), 224(76%)

Few studies have been reported concerning the composition of the essential oil of this plant (*In vivo*) while it is first time that chemical composition of oil from *In vitro* grown *P. obtusifolia* is analyzed. It is evident from results that various compounds are present in high percentages like amount of caryophyllene is 17.17% contributing major part of this oil while according to Morandim *et al*., (2010), the *In vivo* percentage of β-caryophyllene is 2.13%. The essential oil of tissue cultured *P. obtusifolia* also showed the presence of many new compounds. The obtained results showed that the constituents of essential oil from *In vitro* grown *P. obtusifolia* are quite different and better in their numbers and percentages.

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