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Original Article

A Comparative Study of Phytochemical Constituents of *Benkara malabarica* (Lam.) Leaf and Leaf Callus Extracts

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ABSTRACT

Callus induction was initiated in *Benkara malabarica* leaf cultured on Murasique and Skoog medium with growth regulators. The highest frequencies of callus induction (91%) was achieved by using MS medium augumented with BAP (8.88 µm) + TDZ (0.91 µm) + NAA (2.68 µm). Comparative study of FTIR and GCMS analysis was conducted in leaf and leaf callus ethanol extracts. FTIR spectrum of leaf extract showed 6 peaks and callus extract showed 2 peaks. GCMS profile of ethanol extract of leaf and callus resulted 75 and 136 compounds with 10 bioactive compounds each. Among these 8 bioactive principles are present in both the extracts.

Keywords: *Benkara malabarica,* FTIR, callus, GCMS, phytochemical, *in vitro.*

1. INTRODUCTION

Many secondary metabolites have great importance for humans. They are widely used as active drug ingredients in medicine [1,2], as herbicides or phytotoxins in agriculture[3], as food additives[4], fragrances, and even as precursors for the synthesis of plastics[5]. The rapid development of genomics in the last years has helped to reveal that many organisms encode the potential to produce many more secondary metabolites than was originally expected. Most of these new secondary metabolites are only predicted by bioinformatics analysis of putative secondary metabolite gene clusters in

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sequenced genomes, but are not produced naturally under laboratory conditions or are present at levels that are too low to be detected by standard methods. In some cases, the production of such cryptic or sleeping secondary metabolites has been successfully induced by genetic manipulations[6,7]. The emerging methods of Synthetic Biology have recently resulted in renewed interest in the discovery of novel bioactive secondary metabolites from a wide variety of sources[8,9,10].

Rubiaceae of various flowering plants called the madder family, bedstraw family or coffee family. It is an essentially tropical woody family. It comes among the six largest angiosperm families having 637 genera and 10700 species[11]. Many Rubiaceae family plants exhibited antimalarial, antimicrobial, antihypertension, antidiabetic, antioxidant, anti-inflammatory, antitumor, larvicidal, gastrointestinal, anti-ulcer, and hepato protective activities[12].About 3000 plants of this family has anticancer properties are subsequently used as potent anticancer drugs[13].

Benkara is a genus of flowering plants in the Rubiaceae family. It is found in tropical and subtropical Asia from India east to China and the Ryukyu Islands, south to Java and the Philippines[14].*Benkara malabarica* parts are used by folklore of Jharkhand, India for wide variety of illnesses, such as an emetic, as an astringent, as sedative and as nervine tonic[15]. Tribes of Mesra, Jharkhand, India are using its root as an anticonvulsant for many years. In spite of these, the plant species is relatively unexplored with only few reports like used in arthritis[16], antimicrobial[17], and anticonvulsant[18], and this species were reported to contain scopoletin[19]. Scopoletin was reported to have anticonvulsant property[20]. So, in this study, callus induction and FTIR, GCMS analysisof leaf and callus was studied and compared.

2. MATERIAL AND METHODS

Plant collection and authentication

The plant species of *Benkara malabarica* (Lam.) Tirv. were collected from the Madukkari region of Western Guards, Coimbatore, Tamil Nadu. The collected plant material of leaves were dried well under shade and powdered through mortar and pestle and it stored for further uses.

Explants selection and mode of sterilization

The leaf explants were harvested from *in vivo* plant was treated with ten percent (w/v) of (Bavistin)-methyl-3-benzimidizole carbonate solution and washed thoroughly in running tap water with teepol. The explants were subsequently surface sterilized with 0.12% (w/v) mercuric chloride solution for 4-8 minutes and washed 3-4 times in sterile distilled water. The surface sterilized explant was trimmed gently with the help of sterile surgical blade and aseptically inoculated.

Culture media and culture conditions

MS[21] basal medium (MS) with macro elements, micro elements and 3% w /v sucrose (Hi Media, India) and solidified with agar 0.8 % (Hi Media, India) was used as a basal medium along with plant growth regulators. The pH of the medium was adjusted to 5.8 with 1N NaOH or 1N HCl. The media were steam sterilized in an autoclave under 15 psi and 121ºC for 20 min. All the cultureswere incubated under 50µ mol m-2 s-1 light provided by coolwhite fluorescent lamps for 16 h photo period at temperature 24 ± 2ºC.

Callus induction

MS medium enriched with BAP (2.22 – 8.88 µg/l) with NAA (2.68 µg/l) or TDZ (0.91 µg/l) or kinetin (1.16 µg/l) in the respective concentration were tested for callus induction. Percentages of callus induction of six week old cultures were calculated. Calli were sub cultured regularly at an interval of three weeks. The percentage of callusing was recorded at the end of fifth week. Frequency of callus induction was calculated and was represented as percentage.

Extract preparation

The powdered leaf material (100 g in 1000 ml solvent) and callus (20 g in 500 ml) was extracted with ethanol using Soxhlet extractor. The solvents were partly removed in vacuo at room temperature and freeze-dried to give extracts.

Preliminary phytochemical screening

Phytochemical examinations were carried out for ethanol extract as per the standard methods. The extract was subjected to preliminary phytochemical tests to determine the group of secondary metabolites present in the plant material. Condensed extracts were used for preliminary screening of phytochemicals such as alkaloids[22], flavonoids[23], tannins[22],steroids[22], triterpenoids[24], saponins[25], glycosides[26].

FTIR

FTIR analysis of the ethanol extract of leaf and leaf callus were carried out through the potassium bromide (KBr) pellet (FTIR grade) method in 1:100 ratio and spectrum was recorded using Jasco FT/IR-6300 Fourier transform infrared spectrometer equipped with JASCO IRT-7000 Intron Infrared Microscope using transmittance mode operating at a resolution of 4 cm−1 (JASCO, Tokyo, Japan).

GC-MS analysis

The Clarus 680 GC was used in the analysis employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run. The 1µL of extract sample injected into the instrument the oven temperature was as follows: 60 °C (2 min); followed by 300 °C at the rate of 10 °C min−1; and 300 °C, where it was held for 6 min. The mass detector conditions were: transfer line temperature 240 °C; ion source temperature 240 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 600 Da. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

3. RESULTS

Callus culture

The effect of various concentration and combinations of cytokinins and auxin, employing leaf explant is summarized in table 1. On MS medium supplemented with BAP (2.22 to 8.88 µm) with TDZ (0.91 µm) or KIN (1.16 µm) or NAA (2.68 µm) combinations, callus was initiated to begin with at the cut ends which gradually extended to the entire leaf surface within a week of culture. In all the combinations the callus was compact, friable and green or friable and dark green depends upon the growth hormones (Fig 1). Among the different combination tried the medium supplemented with BAP (8.88 µm) together with TDZ (0.91µm) and NAA (2.68 µm) had the ability to produce the excellent callus with 91.0±0.18response percentage. (Fig 1)

While the explant cultured on medium with BAP (4.44 µm) TDZ (0.91 µm) and NAA (2.68 µm) also produced excellent callus with 89.1±0.12 percentage of callus induction. In all the cases the callus is green and friable except in BAP (8.88 µm) with TDZ (0.91 µm) and BAP (6.66 µm) with NAA (2.68 µm) and KIN (1.16 µm) combination, here the callus is dark green and friable in nature.

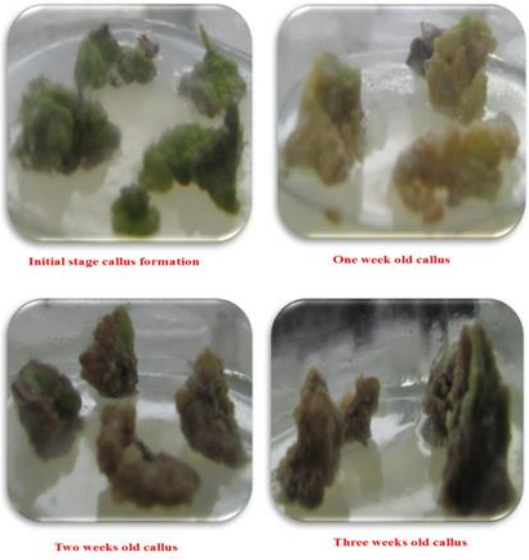

Fig 1: Different stages of callus formation of *Benkara malabarica*

Table 1: Effect of MS medium and different concentration and combination of BAP, TDZ, KIN, NAA on callus induction in leaf explant of *Benkara malabarica*

S. No	BAP µm	TDZ µm	KIN µm	NAA µm	Days taken for Initiation	% Explant Callus	Callus amount	Nature of the Callus
1	2.22	-	-	-	20	22.1±0.17	+	YF
2	4.44	-	-	-	20	21.3±0.18	+	YF
3	6.66	-	-	-	16	29.4±0.13	+	YF
4	8.88	-	-	-	15	35.0±0.16	+	YF
5	2.22	0.91	-	-	19	59.0±0.20	++	WF
6	3.33	0.91	-	-	17	59.7±0.15	++	WF
7	4.44	0.91	-	-	18	63.3±0.11	++	WF
8	8.88	0.91	-	-	16	68.3±0.24	++	WF
9	2.22	-	1.16	-	20	54.7±0.18	++	GF
10	3.33	-	1.16	-	21	55.3±0.23	++	GF
11	4.44	-	1.16	-	16	59.0±0.17	++	GF
12	8.88	-	1.16	-	18	60.3±0.01	++	GF
13	2.22	-	-	2.68	17	72.1±0.19	++	GF
14	3.33	-	-	2.68	18	75.0±0.15	++	GF
15	4.44	-	-	2.68	15	76.6±0.16	++	GF
16	8.88	-	-	2.68	14	80.5±0.18	+++	**GF**
17	2.22	0.91	-	2.68	17	78.0±0.25	+++	GF
18	3.33	0.91	-	2.68	16	88.2±0.17	+++	GF
19	**4.44**	**0.91**	-	2.68	**15**	**89.1±0.12**	**+++**	**GF**
20	**8.88**	**0.91**	-	2.68	**14**	**91.0±0.18**	**+++**	**GF**
Basal medium	-	-	-	-	-	-	-	-

GF= Green Friable, WF= White Friable, YF= Yellow Friable

Extract yield

The powdered *B. malabarica* leaf (100 gm) and leaf callus (20 gm) was extracted using 1000 ml and 500 ml ethanol by Soxhlet apparatus for 18 h and 12 hours respectively. After extraction the leaf sample 9.71 gm and callus sample 1.37 gm was stored at 4^0C until for further experiment.

Phytochemical Study

Qualitative phytochemical study

The leaf and leaf callus extracts were subjected to chemical test as per the standard methods for identification of the different constituents. Qualitative phytochemical analysis of various extracts of *Benkara malabarica* showed the presence of the compounds, including alkaloids, flavonoids,tannins, steroids and triterpenoids. Some of the compounds saponins, glycosides, gum and mucilages and anthraquinones were absent (Table 2).

Table 2: Preliminary phytochemical analysis of ethanol extracts of leaf and leaf callus of *Benkara malabarica*

S. No	Compounds	Tests	Leaf	Leaf callus
1	Alkaloids	Dragendroff's test	+	+
		Mayer's test	_	_
		Wagner's test	+	+
		Hager's test	+	+
2	Flavonoids	10% HCl &5% NaOH test	+	+
		Alkaline test	+	+
3	Tannins	5% $FeCl_3$ test	+	+
4	Steroids	Libermann - Burchard's test	+	+
5	Triterpenoids	Libermann - Burchard's test	+	+
		Salkowski's test	+	+
6	Saponins	Foam test	-	-
7	Glycosides	Whistler &BeMiller test	_	_
8	Gum &Mucilages	Spot test	_	_

9	Fixed oils	NH_4OH test	_	_
10	Anthraquinones	Dragendroff's test	_	_

+ Denotes Present - Denotes Absent

Fourier-Transform Infrared Spectroscopy Analysis (FTIR)

FTIR spectroscopy analysis carried out to identify functional groups present in the leaf and leaf callus extracts of *B. malabarica.* Figure 2 and 3 refers FTIR spectrum of the leaf and leaf callus ethanol extracts of *B. malabarica*. In leaf extract spectrum prominent bands of absorbance were observed at around 447.89, 483.73, 1032.33, 1642.33, 2933.93 and 3284.15 in the region of 500 to 3500 cm^{-1}were as the callus extract the intense peaks are 442.93 and 482.37 observed between 500 to 3500 cm^{-1}. The observed peaks denote -C-OC-, ether linkages, -C-O-, germinal methyls, -C=C- groups or from aromatic rings and alkyne bonds, respectively.

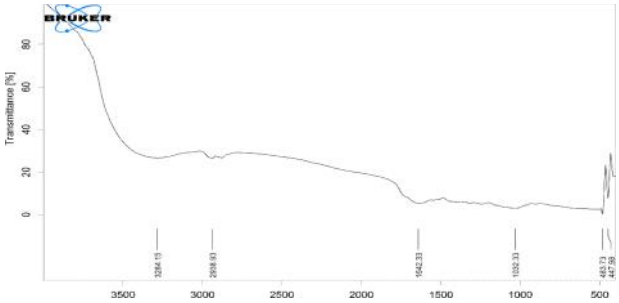

Fig 2: FTIR analysis of leaf ethanol extract of *Benkara malabarica*

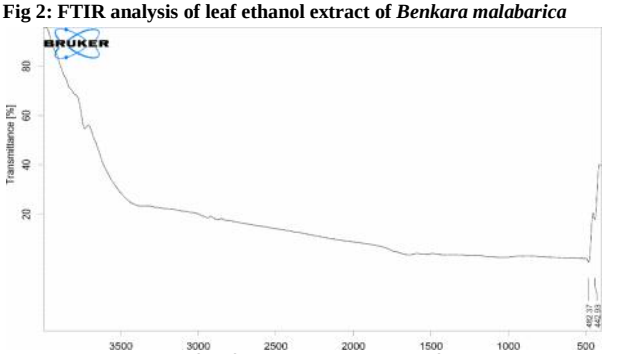

Fig 3: FTIR analysis of leafcallus ethanol extract of *Benkara malabarica*

Gas Chromatography Mass Spectrometer (GCMS)

The GCMS analysis of *B. malabarica* leaf ethanol extract exhibited the presence of 75 compounds with 10 known bioactive principles (Table 3, Fig 4). Whereas the leaf callus ethanol extract showed the presence of 136 compounds with 10 reported biologically active compounds (Table 3, Fig 5). Among the 10 bioactive compounds eight compounds namely N-Hexadecanoic acid, Eicosanoic acid, Octadecanoic acid, Tridecanoic acid, L-(+)-Ascorbic acid 2,6-dihexadecanoate, Tetradecanoic acid, Pentadecanoic acid and Dodecanoic acid were present in both leaf and callus ethanol extracts. The identification of the phytochemical compounds was comfirmed based on the peak area, retention time and molecular formula.

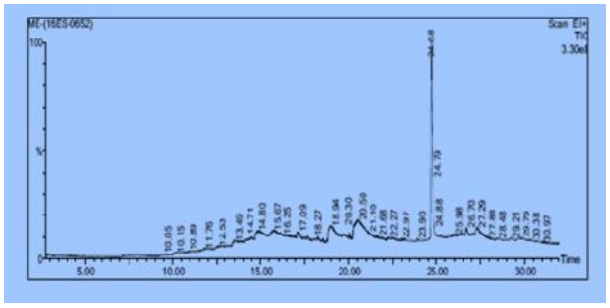

Fig 4: GCMS analysis of leaf ethanol extract of *Benkara malabarica*

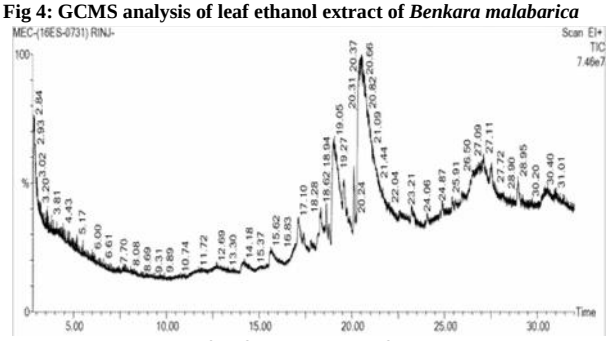

Fig 5: GCMS analysis of leaf callus extract of *Benkara malabarica*

Table 2: List of bioactive compounds in GCMS analysis of leaf ethanol extract of *Benkara malabarica*

S. No	Compound Name	Molecular Mass	Molecular Weight	Bioactive Uses
1	N-HEXADECANOIC ACID	$C_{16}H_{32}O_2$	256	Antioxidant, hypocholesterolemic nematicide, pesticide, lubricant, antiandrogenic, flavor, hemolytic 5-alpha reductase inhibitor, Anti-inflammatory, potent mosquito larvicide[27,28,29,30].
2	EICOSANOIC ACID	$C_{20}H_{40}O_2$	312	No activity reported[31].
3	OCTADECANOIC ACID	$C_{18}H_{36}O_2$	284	Antibacterial, Antifungal, 5-α reductase inhibitor, hypo cholesterolemic, suppository, cosmetic, lubricant, surfactant & softening agent, perfumery, propecic, flavour[32,33].
4	TRIDECANOIC ACID	$C_{13}H_{26}O_2$	214	Anthelminthic, Anti-inflammatory and Antimicrobial activities and anti-cancerous activity[34].
5	L-(+)-ASCORBIC ACID 2,6-DIHEXADECANOATE	$C_{38}H_{68}O_8$	652	anti-scorbutic activity, anti-stress and protects against colds, chills and dumps, hemorrhages, bleeding gums, fragile bones, anemia and pains in the joints and defects in skeletal calcification, wound healing[35].

6	TETRADECANOIC ACID	$C_{14}H_{28}O_2$	228	Antioxidant Cancer preventive Cosmetic Hypercholesterolemic Nematicide, Lubricant, Larvicidal and repellent activity[36,37].
7	PENTADECANOIC ACID	$C_{15}H_{30}O_2$	242	Antioxidant[38].
8	DODECANOIC ACID	$C_{12}H_{24}O_2$	200	Flavour, Antimicrobial, anti-inflammatory[38,39].
9	SQUALENE	$C_{30}H_{50}$	410	anticancer and antioxidant activity, good marker for postprandial lipoproteinemia, cosmetic[40].
10	VITAMIN E	$C_{29}H_{50}O_2$	430	Antiageing, Analgesic, Antidiabatic, Antiinflammatory, Antioxidant, Antidermatitic, Antileukemic, Antitumor, Anticancer, Hepatoprotective, Hypocholesterolemic, Antiulcerogenic, Vasodilator, Antispasmodic, Antibronchitic, Anticoronary[41].

Table 3: List of bioactive compounds in GCMS analysis of leaf callus of *Benkara malabarica*

S. No	Compound name	Molecular weight	Molecular mass	Bioactive uses
1	N-HEXADECANOIC ACID	$C_{16}H_{32}O_2$	256	Anti-inflammatory, Antioxidant, hypocholesterolemic nematicide, pesticide, anti androgenic flavor, hemolytic, 5-Alpha reductase inhibitor, potent mosquito larvicide, lubricant[28,29,30,27].
2	L-(+)-ASCORBIC ACID 2,6-DIHEXADECANOATE	$C_{38}H_{68}O_8$	652	anti-scorbutic activity, anti-stress and protects against colds, chills and dumps, hemorrhages, bleeding gums, fragile bones, anemia and pains in the joints and defects in skeletal calcification, wound healing[35].
3	OCTADECANOIC ACID	$C_{18}H_{36}O_2$	284	5-α reductase inhibitor, hypo cholesterolemic, suppository, cosmetic, lubricant, surfactant & softening agent, perfumery, propecic, flavour, Antibacterial, Antifungal[33,32].
4	TETRADECANOIC ACID	$C_{14}H_{28}O_2$	228	Larvicidal and repellent activity, Antioxidant Cancer preventive Cosmetic Hypercholesterolemic Nematicide, Lubricant[37,36].
5	TRIDECANOIC ACID	$C_{13}H_{26}O_2$	214	Anthelminthic, Anti-inflammatory and Antimicrobial activities and anti- cancerous activity[34].
6	PENTADECANOIC ACID	$C_{15}H_{30}O_2$	242	Antioxidant[38].
7	DODECANOIC ACID	$C_{12}H_{24}O_2$	200	Antimicrobial, anti-inflammatory, Flavour[39,38].
8	EICOSANOIC ACID	$C_{20}H_{40}O_2$	312	No activity reported[31].
9	OLEIC ACID	$C_{18}H_{34}O_2$	282	5-α reductase inhibitor, allergenic, α- reductase inhibitor, anti-inflammatory, anti-androgenic, cancer preventive, anemiagenic, anti-alopecic, antileukotriene-D4, choleretic, dermatitigenic, hypocholestrolemic, insectifuge, perfumery, propecic, flavour[33].
10	PENTADECANAL	$C_{15}H_{30}O$	226	Nutrient, Stabilizers, Surfactants and Emulsifier[42].

4. DISCUSSION

In vitro techniques have been found to be effective in overcomming crossability barriers encountered in traditional methods. A wide range of plants have now been successfully propagated using *in vitro* techniques. This technique facilitates the distribution of material of these species to other institutions around the world, because the cultures do not require quarantine due to their sterile nature. In some cases *in vitro* propagation has also allowed material to be stored in *in vitro* gene banks and this will increase the further developments in cryopreservation technology[43,44,45,46,44,47] have already succeeded in the *in vitro* propagation methods for various species.

The combination of BAP, KIN and NAA induced greater amount of callus from the leaf explant of *B. malabarica* and the morphology of the callus was green and friable and nodular in nature. There was a wide range of variation on percentage of callus induction according to the concentration of hormones. The caulogenic effect of BAP along with NAA observed in the present study is in consonance with other reports[48,49,50],. Generally cytokinin stimulate plant cell division, control the cell cycle also engage in the DNA synthesis. This might be the reason for initiation of callus and adventitious bud[51]. In the present study BAP is found effective along with TDZ and NAA. Similar results have also been reported in *Capparis decidua*[52] but in *Cadaba fruticosa* the multiple shoot induction was observed in BAP alone[53].Best growth of callus however occurred on MS + BAP (8.88µM) + NAA (2. 68 µM) + TDZ(0.91 µM). Similar observation was reported in *Tylophora indica*[49] and *Ceropegia pusilla* from the cell layer explants[48] which indicate that BAP+NAA are basically involved in the development of callus. Callusing started at the cut ends or along the entire surface after 8 days of culture and after 18 to 21 days the entire segment turned with a mass of green soft and friable callus. The leaf derived callus is highly viable, whereas the callus derived from the leaf bits was soft and could not maintain beyond the second or third subcultures. Similar observations were also made in *Tylophora indica*[54], *Ceropegia jainii*, and *C. bulbosa* var. *bulbosa*[55]. The combination of BAP with NAA and TDZ had the organogenic ability for certain extent. The leaf explants are

cultured on to the medium supplemented with BAP, NAA and TDZ produced higher amount of callus and few shoots; the callus is very competent and friable in nature.

The medicinal and pharmacological actions of medicinal herbs are often depended on the presence of bioactive compounds, the secondary metabolites[56]. The use and search for, drugs and dietary supplements derived from plants have accelerated in recent years. Pharmacologists, microbiologists, biochemists, botanists and natural-products chemists all over the world are currently investigating medicinal herbs for phytochemicals and lead compounds that could be developed for treatment of various diseases[57]. Phytochemical constituents are responsible for medicinal activity of plant species. In the present study, preliminary phytochemical screening of *B. malabarica* for leaf and leaf callus was carried out. Qualitative phytochemical analysis of this plant confirm the presence of various secondary metabolites like alkaloids, glycosides, tannins, saponins, flavonoids, steroids, triterpenes and phenols. The results suggest that the phytochemical properties for curing various ailments and possess potential anti-inflammatory, antimicrobial and antioxidant and leads to the isolation of new and novel compounds.

Most alkaloids have a strong bitter taste and are very toxic, for these reasons they are used by plant to protect themselves against herbivory, and attacks by microbial pathogens and invertebrate pests. Phenolics are a class of herbal secondary metabolites that are characterized by the presence of one or more hydroxyl (-OH) groups attached to a benzene ring or to other complex aromatic ring structures[58]. Phenolic herb secondary metabolites are widely distributed in herbs and are responsible for colour development, pollination and protection against UV radiation and pathogens[56]. Flavonoids have several proven medicinal properties, such as anti-inflammatory, antioxidant, anticancer, antibacterial and antiviral properties[59,60]. Terpenoids, also known as isoprenoids constitute the largest group of herbal secondarymetabolites. Terpenoids are involved in defense, wound scaling and thermo tolerance of plants as well as in the pollination of seed crops[56] used as an antibacterial,antifungal, antimalarial, antioxidant activity[61].

In the present study leaf ethanol extract FTIR spectrum, prominent bands of absorbance were observed at around 447.89, 483.73, 1032.33, 1642.33, 2933.93 and 3284.15 were as the callus extract the intense peaks are 442.93 and 482.37 observed between 500 to 3500 cm^{-1}. The observed peaks denote -C-OC-, ether linkages, -C-O-, germinal methyls, -C=C- groups or from aromatic rings and alkyne bonds, respectively. These bands denote stretching vibrational bands responsible for compounds like flavonoids and terpenoids[62,63].

The results pertaining to GC-MS analysis led to the identification of number of compounds from the ethanolicleaf andleafcallus extracts of *B. malabarica.* The presence of 75 and 136 phytochemical compounds with 10 each bioactive compounds could contribute the medicinal quality of the plant. The bioactive compounds are N-Hexadecanoic acid, Eicosanoic acid, Octadecanoic acid, Tridecanoic acid, L-(+)-Ascorbic acid 2,6-dihexadecanoate, Tetradecanoic acid, Pentadecanoic acid, Dodecanoic acid, Squalene and Vitamine in leaf and N-Hexadecanoic acid, Eicosanoic acid, Octadecanoic acid, Tridecanoic acid, L-(+)-Ascorbic acid 2,6-dihexadecanoate, Tetradecanoic acid, Pentadecanoic acid, Dodecanoic acid, Oleic acid and Pentadecanal in leaf callus. Among these eight bioactive principles are present in both the extracts N-Hexadecanoic acid, Eicosanoic acid, Octadecanoic acid, Tridecanoic acid, L-(+)-Ascorbic acid 2,6-dihexadecanoate, Tetradecanoic acid, Pentadecanoic acid and Dodecanoic acid. This shows that the callus cells also produced the alomost similar compounds. So, this study clearly indicates that the callus might be used in the place of leaf. The pharmaceutical companies no need to depend on the wild plantsinstead they can use callus for their medicinal purpose.

Phytochemical constituents are responsible for medicinal activity of plant species. The therapeutic value of medicinal plants lies in the various chemical present in it. The bioactivity of plant extracts is attributed to phytochemical constituents. For instant plant rich in flavonoids are reported to have major group of phenolic compounds for their antiviral properties[64] antimicrobial[65] and tannins[66] which inhibit the bacterial growth by damaging the cell membrane. Naturally accuring compounds present in plants have been shown to possess antimicrobial activities and could thus serve as a source of traditional medicine since ancient times peoples have applied herbs and its derivatives as therapeutic medicines [67].

5. CONCLUSION

In conclusion, we report here an efficient callus production system for *B. malabarica* a plant of high medicinal and industrial importance. Also the comparative study of leaf and leaf callus ethanol extract FTIR and GCMS spectrum for identification of functional group and bioactive compounds showed similarity among the extracts. This study clearly indicates that the callus might be used in the place of leaf because of the presence of similar bioactive compounds. This will help in conservation of the medicinal plant *B. malabarica*.

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