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Chemical profiling of bioactive compounds through gas chromatography-mass spectrometry in the methanolic extract of wild leaf and callus of *Vitex negundo*

Garg G *et al*. GC-MS analysis wild leaf and callus of *Vitex negundo*

Abstract

BACKGROUND

The investigation of plant based therapeutic agents among medicinal plants reflected their presence in the extracts and provide the vision to formulate novel techniques for drug therapy. A perennial herb belongs to family *Varbanaceae* is *Vitex negundo (L.),* employed extensively in conventional medication.

AIM

To correlate the existence of components with the presence of callus extracts *via* a gas chromatography-mass spectrometry (GC-MS) investigation of leaf extracts from wild plants.

METHODS

In this study, we conduct the GC-MS study of wild plant leaf extracts and correlate the presence of the constituents with the presence of callus extracts. Different growth regulators such as 6-benzylaminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthylacetic acid (NAA), diphenylurea (DPU) were added to plant leaves with *in-vitro* callus and grown on MS medium.

RESULTS

The result clearly indicates that addition to BAP (2.0 mg/L) along with 2,4-D (0.2 mg/mL) and DPU (2.0 mg/L) containing 2,4-D (0.2 mg/mL) in MS medium results in rapid callus development. GC-MS analysis was performed on the plant profile of *Vitex* callus extract; here 24; 10; 14 bioactive constituents were detected in the methanol extract of leaf, green callus and methanolic extract of white loose callus, respectively.

CONCLUSION

The outcome of methanol leaf extract showed that octadecadienoic acid, hexadecanoic acid and methyl ester were the major constituents in the leaf and callus methanolic

extract. Octadecadienoic acid is the most commonly present in all the samples. The maximum concentration of octadecadienoic acid in leaves, green callus and white loose callus was recorded as 21.93%, 47.79% and 40.38%, respectively. The outcome of the present study endorses that the concentration of octadecadienoic acid doubles *in-vitro* compared to *in-vivo*. In addition to octadecadienoic acid; butyric acid, benzene, 1-methoxy-4-(1-propenyl), dospan, tridecanedialdehyde, methylcyclohexenylbutanol, chlorpyrifos, n-secondary terpene diester, anflunine and other important active compounds were also detected. All these components were only available in callus formed *in-vitro*. The outcome of this study showed that the callus contained additional botanical characteristics in comparison to wild plants. Due to the presence of numerous bioactive compounds, the medical use of *Vitex* for various diseases has been accepted and the plant is considered an important source of therapeutics for research and development.

Key Words: Leaf extracts; Callus extracts; Methanolic extract; Octadecadienoic acid; Hexadecanoic acid; Methyl ester and gas chromatography-mass spectrometry analysis

Garg G, Bharadwaj A, Chaudhary S, Gupta V. Chemical profiling of bioactive compounds through gas chromatography-mass spectrometry in the methanolic extract of wild leaf and callus of *Vitex negundo*. *World J Exp Med* 2024; In press

INTRODUCTION

India has always been considered a treasure trove rich in medicinal plants and the application of medicinal plants in various forms has been considered a way of life[1]. 80% of the world's population relies on herbal medicine as primary healthcare. This medicinal plant is an important source of secondary metabolites (SMs). These SMs are small organic molecules that are important for the longevity of plants, but they do not play a role in the growth and development of plants. On the basis of their structures and biosynthesis, these SMs are divided into three categories like: (1) Phenolic

compounds; (2) Terpenoids; and (3) Alkaloids[2]. These are beneficial to human health and effective in preventing diseases. The use of *in-vitro* propagation techniques facilitates the rapid propagation of rare and commercially important plants. The massive expansion of medicinal plants through the use of plant biotechnology has the potential to meet the pharmaceutical industry's need for raw materials for herbal preparations. Numerous medicinal plants have been extracted from the natural flora for commercial drug production[3]. Among plant tissues, the collection of phytochemicals has been studied for over 30 years, and the information obtained contributes to the use of cell cultures to produce the desired phytochemicals. In the last few years, the development of plant tissue culture has been noted, and the corporate utility of this technique as a measure of producing precious phytochemicals has received feedback from the scientific community. Therefore, cell suspension culture is found to be the best method for the study of biosynthesis, and callus tissue is the most abundant cell mass during culture production. Cell cultures are useful tools for studying and producing important SM[4]. Chemical evaluation of plant SM includes qualitative, quantitative, and biochemical tests. Qualitative and quantitative drug testing can be used to identify the important bioactive components in the plant samples.

Vitex negundo (L), is a small perennial, woody, aromatic and flowering-ornamental medicinal herb, commonly known as chaste tree, belonging to the Verbenaceae family. This plant is deciduous in nature and has a medium height (4-5 m). It has 3 or 5 palmate-sized flowers, with deep-violet purple aromatic flowers and rounded black-ripe fruit. Plants of *Vitex* species have different ethno-botanical and pharmacological applications. *Vitex negundo* contains many bioactive phytochemicals such as glycosides, phenolic compounds, flavonoids, terpenes and phytosteroids. Terpenoids are thought to be one of the most abundant metabolites in the *Vitex* plant. Chemically, they contain five carbon isoprene units, forming monoterpenes (five carbon atoms), hemiterpenes (C5), sesquiterpenes (fifteen carbon atoms), diterpenes (twenty carbon atoms), and triterpenes (thirty carbon atoms). The leaves of *Vitex* are used to reduce the tenderness of breasts, regulate hormones related to fertility, menstrual cycle, menstrualpain and

amenorrhea symptoms. Pharmacologically, this species showed anti-oxidative, antimicrobial, anti-shows inflammatory and anti-tumor properties. It reduces the level of serum-prolactin hyper-prolactinemia and mastodyn. Leaves of *Vitex negundo* are aromatic, bitter, pungent, having astringent, analgesic, anti-inflammatory, antipyretic, anthelmintic, and anthelmintic properties. Literature revealed that leaves and flowers of *Vitex* are the main sources of SMs. Leaves are the good source of alkaloids, vitamin C, carotene, glycol-nonanol, benzoic acid, β-sitosterol, flavonoids (such as flavonoids, luteolin-7-glycosides, ricin, iridoid glycosides, C-glycosides), terpenes oil (such as caryophyllene epoxide, δ-guaiene, and ethyl-hexadecenoate), while flowers contain oil like α-selinene, (E)-nerolidol, carryophyllene epoxide, and germacren-4-ol. Till date, there is no comprehensive record available on the phyto-chemical study on wild leaf and callus culture (*in-vitro*) samples obtained from this plant. Thus, seeking the therapeutic importance of *Vitex negundo*, detailed comparative study of phyto-chemical constituents is very essential. Hence, the aim of this study was to analyze and identify the phyto-constituents of wild leaves and *in-vitro* cultured leaf calluses and to spectroscopically identify bioactive compounds in crude extracts prepared in methanol by gas chromatography-mass spectrometry (GC-MS) analysis.

MATERIALS AND METHODS

Collection of plant sample

The fresh young leaves of *Vitex negundo* (*L.*) were collected from National Bureau of Plant Genetic Resources (NBPGR), New Delhi. The leaves are carefully rinsed with distilled water to eliminate dust particles and dried at room temperature for 15 d until the dry weight stabilises. Put the dried herb in liquid nitrogen, grind it into a fine powder, and store it in an airtight container until later.

Callus preparation and culture

To prepare callus, collect fresh leaves from plants and wash thoroughly with water to remove dust, then disinfect the surface with 0.1% $HgCl_2$. Leaf explants were excised

aseptically and cultured on MS medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP), and di-phenyl urea (DPU). The highest and fastest response for callogenesis with a green and friable callus was observed in the MS medium with the addition of BAP (2.0 mg/L) with 2,4-D (0.2 mg/mL) (*i.e.,* M2 media); and DPU (2.0 mg/L) with 2,4-D (0.2 mg/L) (*i.e.,* M4 media). The peculiarity of the combination is the difference between the formation of green brittle and white loose calluses, respectively (Table 1). The callus of *Vitex negundo* (*L.*) was produced from the primary callus of leaf explants through sub-culturing on MS media (M2 and M4) enriched with standard growth regulators, *i.e.,* M2-media: BAP (2.0 mg/L) with 2,4-D (0.2 mg/mL); and M4-media: DPU (2.0 mg/L) with 2,4-D (0.2 mg/L).

Preparation of plant extract and GC-MS analysis of bioactive compounds present in the sample

The preparation of plant extract was done by following the modified method of Anwar *et al*[5]. The fresh young leaves of *Vitex negundo* (*L.*) were collected from the herbal garden, NBPGR, New Delhi, and callus were produced by subculture from leaf explants of *Vitex negundo* in M2 and M4 medium, were selected for further quantitative analysis by GC-MS. For leaf sample preparation (LS), the leaves were carefully rinsed in tap water to eliminate dust particles, then rinsed with sterilized distilled water, dried at room temperature for 24 h, and ground into a fine powder in a mechanical grinder. For the callus sample preparation, we select 6-8-wk-old green friable callus (CS1) and white loose callus (CS2) from the M2 and M4 medium, respectively. Remove the medium particles properly through washing with double distilled water and then drying in an oven at 62 ± 5 °C for 28 h. Dried samples were grinded to make a fine powder and stored in sterilised, airtight polythene bags until use. For the methanolic extraction, 2 g of the powder of the plant samples (LS, CS1, and CS2) were weighed and soaked in 10 mL of GC-MS grade methanol for 14 h (or incubated overnight) in the flask. The solution was mixed properly with the shaker. After mixing, filter the solution through a

Whatman No. 41 filter paper and collect the filtrate. This solution was further used for GC-MS analysis.

GC analysis was performed using an Agilent gas chromatograph equipped with a split/split less injector (230 °C) and mass spectrometer detection (230 °C). The gas used was helium (1 mL/min), and HP-5MS 5% Phenyl Methyl Silox (325 °C: 30 m × 250 µm × 0.25 µm) was employed as the capillary column. Inject 2 µL of sample through a split-less system with the following program: 170 °C for 1 min, 250 °C for 2 min at 8 °C/min, and finally 3 °C/min at 310 °C for 2 min. The MSD was operating at 70 eV in the 70-600 amu range. The final running time was 39 min, and the data was analyzed using total ion count for identification and quantification of compounds. Each extract spectrum reflects the retention time in the column, and the peaks detected correspond to the relative abundance (%) of bioactive compounds found in specific regions. Through comparison between the mass spectra of the detected components and the mass spectra of known components available in the National Institute of Standards and Technology library, the bioactive phytochemical compounds of *Vitex negundo* were identified. Compound concentrations were determined from the GC peak area of the total ion current.

RESULTS

The GC-MS chromatogram spectra were achieved in all three extracts of *Vitex negundo i.e.,* wild *in-vivo* leaves, green-compact and white-loose *in-vitro* callus. Results showed that bioactive compounds were present in both *in-vivo* and *in-vitro* sample extracts (Figure 1A-C). Through GC-MS analysis we detected twenty-four, ten, and fourteen bioactive compounds in the methanolic extract of wild-leaves, green, and white loose callus, respectively (Tables 2-4). The results showed that octadecadienoic acid, hexadecanoic acid and methyl esters were the main components in the methanol extract of leaves and callus. Among these, octadecadienoic acid is the most common in all samples[7,8]. The maximum concentration of octadecadienoic acid in leaves, green callus and white loose callus is 21.93%, 47.79% and 40.38%, respectively. Our results confirm

that the concentration of octadecadienoic acid doubles *in-vitro* compared to *in-vivo*. In the earlier research, the results of GC-MS analysis reflected the similar compounds as obtained in the present study[9].

DISCUSSION

In *Vitex negundo* plants, octadecadienoic acid is present along with plant glycosides[10]. Plant glycosides are a diverse group of natural compounds found in various parts of plants, including leaves, stems, roots, and seeds. It consists of a sugar molecule (glycone) attached to a non-sugar, biologically active compound (aglycone or genin), which serves both medicinal and toxic properties[11]. Along with the plants' glycosides, octadecadienoic acid develops bitterness in leaves, which frightens herbivores by being toxic or unpleasant when ingested[12]. Some important glycosides, *e.g.*, anthocyanin glycosides, along with octadecadienoic acid are responsible for the pigmentation (red, blue, and purple) in leaves, which act as stressors in plant defense against temperature stress[13].

Other bio-active constituents like 1-oxo-dimethyl-methylene-hexahydrocyclopentanol pyran (7.91%), veridiflorol (6.79%), pyrrolo-carbazole (CAS) (6.79%), and dimethyl-phenyl (6.79% peak area) were descending in the methanolic leaf extract. It has been observed that some other bio-active compounds like dursban, butyric acid, benzene, 1-methoxy-4-(1-propenyl), nor-ses-terterpene-diester, tri-decanedial, chlorpyrifos, methyl-cyclohexenyl-butanol, and anhalonine were present in good amount in *in-vitro* developed callus (both green-compact and white-loose) along with the major (like: Octadecanoic acid, hexadecanoic acid, and methyl ester) compounds (Tables 3 and 4). The result obtained in the present study was analogous with the data obtained by Kaliyannagounder *et al*[9]. In medicinal plants, octadecadienoic acid is an important polyunsaturated fatty acid. It is also known as linoleic acid and plays several important roles in plant growth and development. Octadecadienoic acid triggers the production of defensive compounds in plants and plays a major role in plant defense mechanisms. It is considered as a core-structural integral element of phospholipids that make up the

lipid bi-layer of plant cell membranes. It maintains the integrity of the plant cell and controls the movement of molecules in and out of the cell along with the other fatty acids. Finally, it adjusts the fluidity and stability of membranes under different environmental stresses, such as temperature extremes, drought, and pathogen attacks. Further, it acts as a precursor for jasmonic acid, which is a signaling molecule involved in the plant's response to herbivores, pathogens, and other stressors in plant defense mechanisms. All these findings were similar with the results obtained by Ahuja *et al*[14].

Hexadecanoic acid and methyl esters have antioxidant, cholesterol-lowering, antiandrogenic, hemolytic, and alpha-reductase inhibitory properties. Hexadecanoic acid (palmitic acid) is a common saturated fatty acid, while hexadecanoic acid methyl ester (methyl palmitate) is a chemically modified derivative of palmitic acid. On the basis of data available in the literature, plant biomass having good amounts of methyl palmitate (a chemically modified derivative of hexadecanoic acid) is used in biodiesel production. Present findings reported that *in-vitro* raised callus (white-loose) is a good medium for the synthesis of methyl esters, and we can consider this approach in near future as a renewable fuel source for rewarding our future energy demand, if the research at the higher level goes in the right direction[15].

In present research, two major compounds were found *i.e.,* sesquiterpenoid compound "viridiflorol" and anhalonine (naturally occurring alkaloid) in the *in-vitro*-derived white loose callus extract of *Vitex negundo*[16,17]. Viridiflorol showed anti-inflammatory, antioxidant, and anti-mycobacterium tuberculosis activity. Anhalonine is considered an important naturally occurring alkaloid. A very small amount of this compound is obtained from *Lophophora williamsii* (a rare species of cactus). It may serve as a chemical defense mechanism in some plants, and may be toxic to herbivores and pathogens. Hence, by producing anhalonine, plants may daunt herbivores and reduce the risk of damage from grazing animals. It also seems that anhalonine showed allelopathic symptoms. Such compounds are released by the plants into the soil through their root system and inhibit the growth of nearby competing plants and provide a competitive advantage for the producing plant. It has been noticed from present work

that plants of *Vitex* grown in the *in-vivo* field condition usually inhibit the growth of weeds in their nearby area. It may be due to the presence of anhalonine, which was not detected in the methanolic extraction of wild leaf sample. Its compound showed their presence in very good amounts (peak area 1.43%) in white loose callus. Anhalonin, used as a psychotropic drug, is used to change the function of the nervous system and results in alterations of perception, mood, cognition, and behavior in our traditional folk medicinal/herbal system. It also showed antimicrobial properties, which protect the plants from microbial infections and diseases by acting as natural antimicrobial agents[18]. The exact role of anhalonine in plants is not as well-studied. Detailed research on the functions of anhalonine in different plant species is continuing, and further studies are required to clarify its ecological and physiological significance in plant system.

Similar outcomes were attained in the previous study conducted by Lad *et al*[19]. The methanolic extract of *Vitex negundo* presents the persistence of phytol compounds, which have other biological effects such as hypocholesterolemic cancer prevention, insecticidal, hepatoprotective, 5-alpha reductase inhibitor, anti-inflammatory, anti-rash, nematocidal, antihistamine, anti-acne, and anti-inflammatory antibiotic. The same bioactive SM of phytol has been previously reported to have various medicinal properties in some aquatic plant species such as *Hydrilla verticillata*, *Gracilaria*, and *Carissa carandas*[20,21]. Octadecanoic acid is a well-known example of a saturated fatty acid and possesses antihypertensive properties along with decreasing low density lipoprotein and increasing high density lipoprotein levels of cholesterol[22].

In the present study, octadecanoic acid was present in both leaf and callus extracts of *Vitex negundo*. The results of present study were in accordance with the reports on *Cleistanthus collinus*, *Goniothalamus umbrosus*, *Kigelia pinnata*, and *Melissa officinalis* contained n-hexadecanoic and octadecadienoic acids[23,24]. The study revealed the presence of various bioactive compounds present in all the methanolic extracts of *in-vivo* and *in-vitro* plant samples[25]. However, it has become clear from the present study that the callus contained additional phytochemicals in comparison to wild types that

display significant medicinal properties of the plant. Further studies like bio-prospecting are necessary to support its biological properties and the importance of these inventive bio-molecules will be remarkable to be studied.

CONCLUSION

The screening of all the methanolic extracts of *Vitex negundo* revealed the presence of twenty-four, ten, and fourteen bioactive compounds in the wild-leaves, green, and white loose callus, respectively. The results confirmed that octadecadienoic acid, hexadecanoic acid, and methyl ester were found to be the key constituents in the methanolic extract of leaves and callus[26]. Octadecadienoic acid was the predominant potential bioactive compound identified in all samples. Results of present research confirmed that the concentration of octadecadienoic acid increases twice in *in-vitro* conditions, conditions as compared to *in-vivo* conditions. Hence, in the case of *Vitex*, we can use micro-propagated plants as a potent phyto-compounds source for commercialization without destroying the wild plant[27,28]. Recent literature reviewed showed that plant biomass having good amounts of methyl palmitate (a chemically modified derivative of hexadecanoic acid) are used in biodiesel production[29,30]. The findings of present study showed that *in-vitro* raised callus is a good medium for the synthesis of methyl esters. Hence, we can consider this approach in our near future as a renewable fuel source for rewarding our future energy demand. Some active sesquiterpenoid compound like viridiflorol and anhalonine (naturally occurring alkaloid) were found in the *in-vitro* derived white loose callus extract of *Vitex negundo*. Viridiflorol showed anti-inflammatory, antioxidant, and anti-mycobacterium tuberculosis activity, while anhalonine might be used as a psychotropic drug in animals and also responsible for showing allelopathy in plants. The outcome of this study showed that the callus contained additional botanical characteristics in comparison to wild plants. Due to the presence of numerous bioactive compounds, the medical use of *Vitex* callus for various diseases has been accepted and the plant is considered an important source of therapeutics for research and development.

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