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Identification of Active Pharmaceutical Ingredients in *Thevetia neriifolia* Juss Leaf Callus using Analysis of GC-MS

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ABSTRACT

Plants of *Thevetia neriifolia* are recorded from the most important medicinal plants that used in folklore medicine in different countries. Most of its parts were used to treat different diseases and having antioxidant, antimicrobial and anti-inflammations activities. Callus induction was occurred by culturing of leaf explants that were taken from eight years old trees on Murashige and Skoog(MS) medium supplemented with the auxin 2,4-D at 3.0 mg. l^{-1}. After the gaining of suitable amount of callus and extracted with hexane, the screening of active phytochemica1s was achieved by using the analysis of GC-MS. The results showed the presence of forty different compounds that diagnosed in the callus extract with different retention times and peak areas, the main compounds that having various pharmaceutical features including: Hexatriacontane (23.095%), Nonacosane(8.849%), .beta-Amyrin(8.060%), 2-Methylhexacosane(6.870%), .alpha.-Amyrin(4.446%), Octacosane (4.309%), Phenol,2,2'-methylenebis-(1,1-dimethylethyl)-4-methyl- (4.030%), Vitamin E (3.134%), .beta.-Sitosterol(3.254%), Isopropyl myristate(2.777%), 1,2-Benzisothiazol-3-amine tbdms (2.886%), Pentatriacontane(1.716%) Squalene(1.170%).

Keywords: *Phytochemicals, medicinal plants, GC-MS technique, Thevetia neriifolia, callus.*

INTRODUCTION

Plant tissue culture is an important and potent source for production of effective secondary metabolites (or phytochemicals). This technique carries many important benefits in the production and extraction of active compounds from small explants in small areas with short periods of time over the year without resorting to the significant depletion of large-scale cultivated plants that caused by traditional methods [1,2]. The medicinal plants play an important vital-role in everyday human life as they are natural factories for the production of many effective compounds that contribute to the treatment of various infections and diseases. Several studies have been carried out on most medicinal plants for the purpose of increasing the production of active phytochemicals (like phenolic compounds, alkaloids, terpenoids, tannins, steroids, flavonoids and glycosides) on the commercial and pharmaceutical scale. These studies included: the selection of plants with high productivity for effective compounds and manipulation of the conditions of culture or using of precursors, biotic and abiotic elicitors, metabolic engineering and culturing of plant tissue and organs through the use of different techniques of plant tissue culture [3,4]. *Thevetia neriifolia* Juss (belongs to the family Apocynaceae) is one of the medicinal plants, and most of its parts were used in folk medicine as anti-septic, disinfectant, anti-bacterial and anti-fungal infections. It is ; therefore, used in the treatment of acute heart disorders, arthritis, gout, ulcers, tooth-ache, acne, nausea, abdominal-pain, diarrhea, dysrhythmias and as strong anti-oxidants. The medicinal importance of this plant is due to its containment of medically effective compounds, such as alkaloids, phenols, glycosides and terpens [5,6]. Several organic solvents have been used to extract the active secondary metabolites from medicinal plants, including chloroform, methanol, ethanol, hexane and petroleum ether. These active compounds have been quantified using various chromatographic techniques based on standard compounds of the active compounds [7]. Gas chromatography-mass spectrometry (GC-MS) technique is one of the most advanced and effective techniques for screening and identifying the active secondary metabolites that exist in any plant sample without resorting to the use of standard compounds. It provides a library that contain a wide range of mass-

spectrometers for compounds that can be compared with crude materials separated from plant samples [8,9]. The main objective of this study is focused on the extraction and investigate the active secondary metabilites (phytochemicals) in the cal lus induced from leaves of *Thevetia neriifolia* plant using GC-MS technique.

MATERIALS AND METHOD

In vitro-induction of cal lus from leaf explants

This research was carried out in the plant tissue culture laboratory of Department of Plant Production Techniques at Al Musaib Technical Col lege. Intact and healthy leaves were taken from the *T. neriifolia* plant (eight-years old) and washed wel l with running-water and liquid soap to remove dirt from them. Then, they were immersed in a 2% solution of sodium- hypochlorites in the laminar-air-flow cabinet for 15 min., after that, they were rinsed thoroughly with sterile distil led water twice for 2 min. each. The steri1e leaves were cut into pieces of 5-l0 mm long used as exp lants and cultured in glass-jars (3*15 cm) containing 20 ml of full-strength MS medium [10] supplemented with 30 g. l^{-1} sucrose, 3.0 mg. l^{-1} 2,4-Dichlorophenoxy acetic acid (2,4-D) and 7.0 g. l^{-1} agar for solidifying before autoclaving at 121 ºC and 1.04 Kg. cm^{-1} for 20 min. Cultures were incubated in the growth-room at 25 ± 2 ºC and light- intensity of 1000 lux for photo-period of 14 h. After five weeks, the cal lus that formed on the explants was sub-cultured on the same formula of medium to increase the biomass of cal lus for drying and extraction.

Callus extraction and GC-MS analysis conditions

The callus from previous experiment was dried at a temperature of 70 ºC in the oven and grinded to fine powder. Extraction process was performed by adding 5 ml of n-hexane to 5 mg of callus powder and left for 6 hrs, the mixture was then centrifuged at 4000 rpm for 10 min. The phytochemicals were identified in the crude-extract of callus using GC-MS(Agilent 1909lS-33UI) apparatus equipped with Nationa1Institute of Standard and Technology (NITS)Library; column HP-5MS capillary column (cross-bond 5% dipheny1-95% di-methylpolysiloxane): 30m(L)× 250µm(i. d.) with a 0.25 µm film thickness; injection temperature: 290 ºC; column temperature: 40 ºC held to 2 min., rising 4 ºC. min^{-1}, then rising to 290 ºC and held for 5 min.; injection mode: split: split at ratio 1 : 20; injected volume: 5 µl. The carrier-gas was Helium (99.99 %); acquisition mass range: 40-600 m. z^{-1}. The compounds of the extract were identified by comparing their retention indices with NIST library.

RESULTS AND DISCUSSION

The results indicated the efficacy of the auxin 2,4-D in the induction of the cal lus from leaves of *T. neriifolia* and increase its biomass, and this is in agreement with many previous studies in the induction of callus using 2,4-D alone and from different exp lants and different plant species they studied, such as the induction of cal lus from leaves and stems of *Gymnema sylvestre* [11]; seeds of *Boerhaavia paniculata* (Souza *et al.*, 2014) and leaves, petioles and internodes of *Terminalia arjuna*. Results presented in Table –1 and Figure – 2, revealed the presence of 40 compounds of active phytochemicals that found in the hexane-fraction of the leaf callus of *T. neriifolia* and detected with GC-MS, from which the major dominant compound was Hexatriacontane(23.095 %), followed by the compounds: Table 1 Other compounds that were appeared in less than one-percentage were: Henteriacontane (0.377 %); 1-Decanol, 2-hexyl- (0.657 %); Indazol-4-one,3,6,6-trimethyl-1-phthalazin-1-yl-1,5,6,7-tetrahydro- (0.262 %); 2-Dodecen-l-y1(-)succinic anhydride(0.274 %); Nonadecy1 trif1uoroacetate (0.224 %); lH-Indene,5-butyl-6-hexyloctahydro- (0.173 %); l-Nonadecene (0.327 %); Oleic acid (0.202 %); Ethyl iso-allocholate (0.130 %); Benzene,2-f1uoro-1-methyl-4-nitro (0.578 %); 1,2-Cyclohexanedicarboxylic acid, hexyl isohexyl ester (0.801 %); 1,2-Cyclohexanedicarboxylic acid, hexy1 nony1 ester (0.924 %); N-(3-Chlorophenyl)-bis(2,2,3,3-pentafluoropropane)amide (0.385 %); Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15 - hexadecamethy1- (0.236 %); lH-Indole-2-carboxylic acid,6-(4-ethnoxyphenyl)-3- methyl-4-oxo-4,5,6,7-tetrahydro-, isopropyl ester (0.105 %); Batilol (0.447 %); Cyclobarbital (0.319 %) and Propiophenone, 2'-(trimethylsiloxy)-(0.257 %). Chandar and Ramasamy (2016) [12] referred in their study that the compound Indazol-4-one,3,6,6-trimethyl-1-phthalazin-1-yl-1,5,6,7- tetrahydro-, was isolated for the first time from the ethanolic-extract of *Combretum albidum* leaves. In the current study, this compound was isolated from the hexane extract of *Thevetia neriifolia* leaf callus, this displays the efficacy of this method for extraction of different active phytochemicals from various plant parts and callus. It was known that the plants belonging to the family Apocynaceae, including the genus *Thevetia*

possess characteristics and recipes make them from the important medicinal plants in treating diseases or as antimicrobial for many pathogenic bacteria such as: *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhi*, *Klebsiella phenonemia* and some fungal strains such as: *Aspergillus niger*, *Candida albicans*, *Fusarium oxysporum* and *Penicillium spp.*(**13**). This may be due to the presence of various active phytochemicals in such plants with medicinal and pharmaceutical uses. The current study showed that there are various medicinal and pharmaceutical ly active compounds that have been diagnosed in the crude extract of leaf cal lus of *T. neriifolia* plant using GC-MS analysis. Sixteen different compounds of the identified phytochemicals that have been reported to possess different pharmaceutical and therapeutic activities such as anti-inflammatory, anti-oxidant, anti-microbial and anti-cancer activities, making them of special pharmaceutical importance compared to synthetic drugs and anti-oxidants [13-16]. Also, the compound 2-Methylhexacosane has been reported as a reducer of blood-cholesterol and as an anti-microbial [17]. In addition, Isopropyl myristate (is an ester of isopropyl-alcohol with myristic acid; a common-fatty acid) which enters into different pharmaceutical industries as a thickening-agent, emollient and skin-enhancer or as anti-oxidant and anti-microbial [17]. On the other hand, the compound 2-Dodecen-1-yl(-) succinic anhydride has a structure similar to phenytoin that used as anticonvulsant-agent, thus making it as a natural medicine for treating convulsion, as well as for its activity as anti-oxidant and anti-microbial [18]. Oleic acid is a mono-unsaturated fatty acid which has many important therapeutic benefits including anti-cancer, anti-androgenic, anti-oxidant, anti-fungal, anti-bacterial, anemia-genic, anti-inflammtory, inhibition of 5-.alpha.-reductase and dermatili-genic [19]. Moreover, Nikalje *et al.*(2017) revealed that the oleic acid can delay or hinder the progression of the disease adreno-leuko dystrophy(ALD), the deadly disease that causes damages to the brain and the adrenal glands. Additionally, oleic acid has capability of the regulation of many functions in human body such as blood-clotting, blood-pressure and immune response to the infections resulting from wound-injuries, also, it is possibly responsible for the increasing of high-density lipoprotein-cholesterol (HDL) and decreasing low-density lipoprotein-cholesterol (LDL) [20]. In the study of Ju *et al.*(2014), they revealed to the isolation of the Batilol compound for the first time from *Styela clava* plant. In the current study , this compound was also diagnosed in the leaf cal lus extract of *T. neriifolia*. Batilol has a medical importance by giving it to prevent radiation disease with X-ray and radio-therapy, as well as anti-oxidant activity. Results also showed the presence of the Cyclobarbital, which is used medical ly in the treatment of insomnia and as anesthetic , anti-convulsant, neuro-transmitter antagonist, skeletal-muscle relaxant [21]. Another important compound is Nonacosane, which has multiple activities including anti-hypertensive activity, also shows angiotensin, vasodilator, AT2-receptor antagonist, Sal-uretic effects, anti-oxidant and anti-bacterial. Vitamin E is one of the most effective compounds that has been diagnosed in the current study and is necessary for a large number of important activities. It reduces the risk of Alzheimer's and Parkinson's disease symptoms and as anti-tumor agent. Also, it has anti-inflammatory and analgesic activity, protects cell-membranes from damages and preventing the formation of plaque in the arteries by preventing of low-density lipoprotein-cholesterol oxidation, thus leads to the lowering of cardio-vascular disease risk. Additionally, its effectiveness as anti-oxidant is represented by delaying or preventing the agerelated growth of cataracts of eye. Moreover, it is commonly used in skin-care creams and lotions.

Table 1. The phytochemicals of *Thevetia neriifolia* leaf cal lus analyzed with GC-MS

R.T.*	Compounds	Molecular Formula	MW*	**Area**	**%of Total**
34.239	Henteriacontane	$C_{31}H_{64}$	436	9 144 983	0.377
36.833	l-Decano1, 2-hexy1-	$C_{16}H_{34}O$	242	15 933 717	0.657
37.596	Isopropyl myristate	$C_{17}H_{34}O_2$	270	67 356 788	2.777
41.770	Tetrapentacontane, l,54-dibromo-	$C_{54}H_{108}Br_2$	917	9 743 469	0.402
41.944	Indazo1-4-one,3,6,6-trimethy1-l-phthalazin	$C_{18}H_{18}N_4O$	306	6 359 926	0.262
46.168	2-Dodecen-l-y1(-) succinic anhydride	$C_{16}H_{26}O_3$	266	6 650 811	0.274
46.552	Nonadecy1 trif1uoroacetate	$C_{21}H_{39}F_3O_2$	380	5 421 072	0.224

Cont... **Table 1. The phytochemicals of *Thevetia neriifolia* leaf cal lus analyzed with GC-MS**

49.709	lH-Indene,5-butyl-6-hexyloctahydro-	$C_{19}H_{36}$	264	4 191 648	0.173
50.351	1-Nonadecene	$C_{19}H_{38}$	266	7 928 002	0.327
51.074	Phenol, 2,2'-methylenebis-(l,l-dimethylethy1)-	$C_{23}H_{32}O_2$	340	97 744 461	4.030
53.434	Oleic acid	$C_{18}H_{34}O_2$	282	4 898 197	0.202
53.706	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	3 153 098	0.130
55.391	Benzene,2-fluoro-1-methyl-4-nitro	$C_7H_6FNO_2$	155	14 021 273	0.578
56.047	2-Methylhexacosane	$C_{27}H_{56}$	380	166 612 218	6.870
56.655	2H-pyran-2-carboxylic acid,6-butoxy-3,6-dihydro-,ethy1 ester	$C_{10}H_{14}O_4$	198	40 454 385	1.668
57.074	1,2-Cyclohexanedicarboxylic acid, hexyl isohexy1 ester	$C_{20}H_{36}O_4$	340	19 430 664	0.801
57.189	1,2-Cyclohexanedicarboxylic acid, nonyl 3-penty1 ester	$C_{22}H_{40}O_4$	368	36 450 676	1.503
57.480	1,2-Cyclohexanedicarboxylic acid, heptyl isobuty1 ester	$C_{19}H_{34}O_4$	326	35 264 075	1.454
57.918	1,2-Cyclohexanedicarboxylic acid, hexyl nony1 ester	$C_{23}H_{42}O_4$	382	23 627 355	0.924
58.380	Squalene	$C_{30}H_{50}$	410	28 385 990	1.170
58.878	N-(3-Chlorophenyl)-bis(2,2,3,3,3-pentaf1uoropropane)amide	$C_{12}H_4ClF_{10}NO_2$	419	9 338 266	0.385
59.614	Octacosane	$C_{28}H_{58}$	394	104 492 795	4.309
60.385	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	579	5 732 530	0.236
61.045	1H-Indole-2-carboxylic acid,6-(4-ethoxyphenyl)-3-methyl-4-oxo-4,5,6,7-tetrahydro-, isopropyl ester	$C_{21}H_{25}NO_4$	355	2 545 475	0.105
61.260	Batilol	$C_{21}H_{44}O_3$	344	10 847 536	0.447
61.564	Olean-12-ene	$C_{30}H_{50}$	410	90 928 952	3.749
61.968	Cyclobarbita1	$C_{12}H_{16}N_2O_3$	236	7 739 023	0.319
62.300	Propiophenone,2'-(trimethylsiloxy)-	$C_{12}H_{18}O_2Si$	222	6 237 361	0.257
62.905	Hexatriacontane	$C_{36}H_{74}$	506	560 112 255	23.095
63.549	Vitamine E	$C_{29}H_{50}O_2$	430	76 017 136	3.134
64.106	5-Methyl-2-phenylindolizine	$C_{15}H_{13}N$	207	30 925 643	1.275
64.413	Pentatriacontane	$C_{35}H_{72}$	492	41 616 033	1.716
64.978	1,2-Benzisothiazol-3-amine tbdms	$C_{13}H_{20}N_2SSi$	264	69 994 593	2.886
65.570	Benzo[h] quinoline, 2,4-dimethyl-	$C_{15}H_{13}N$	207	31 104 573	1.283
66.109	Nonacosane	$C_{29}H_{60}$	408	214 601 483	8.849
66.507	.beta.-Sitosterol	$C_{29}H_{50}O$	414	78 908 439	3.254

CONCLUSION

The results of the current study show the medical important of the *Thevetia neriifolia* plant to contain many medically and pharmaceutically active phytochemicals that are diagnosed with GC-MS technique. These compounds can be used for curative purposes such as vitamin E, oleic acid, .beta.-sitosterol, alpha and beta-amyrin, squalene, hentriacontane, hexatriacontane, octacosane, nonacosane, isopropyl myristate, 1-nonadecene, and ethyl iso-allocholate which are useful in the treatment of many bacterial, fungal and vira1 infections and many diseases. The medical and pharmaceutical effectiveness of many compounds in this plant requires in-depth studies in various fields.

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Conflict of Interest: None to declare.

Ethical Clearance: All experimental protocols were approved under the Al-Musaib Technical College, Al-Furat Al-Awsat Technical University, Iraq and all experiments were carried out in accordance with

approved guidelines.

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