Elicitation of Secondary Metabolites Production from *Thevetia Neriifolia Juss* in *Vitro* Cultures Using Chemical Elicitors

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Abstract: **The current study aimed to promote the production of active secondary metabolites from *Thevetia neriifolia* callus using cholesterol and salicylic acid individually. The callus of this plant was stimulated from internodes on basal Murashige and Skoog (MS) medium with a combination of 1.0 mg.l^{-1} 2,4-Dichlorophenoxy acetic acid(2,4-D) + 0.3 mg.l^{-1} Kinetin (Kin). A weight of 350 mg of induced callus from previous experiment was grown on MS medium with the same combination of growth regulators and supplemented with 50,100, 150 or 200 mg.l^{-1} from cholesterol or salicylic acid (SA) individually. After three weeks of culture, the fresh and dry weights of callus, relative growth rate(RGR) and callus diameter were calculated. The callus samples from all elicitors treatments were harvested, dried, crushed and extracted with hexane. Then, the bioactive compounds in these samples were detected using Gas Chromatography-Mass Spectrometry(GC-MS) analysis. The results showed that the concentrations of cholesterol caused a significant decrease in the means of fresh weight and callus diameter from control treatment, while, the dry weight and RGR did not differ from those of control. Results also showed there was no significant difference in fresh and dry weight means among SA concentrations, whereas, RGR of callus decreased significantly in the treatment 150 mg.l^{-1}, as well as for callus diameter in the treatment of 200 mg.l^{-1}. The analysis using GC-MS showed a clear difference in the quality and ratios of active secondary compounds in the callus samples treated with various concentrations of chemical elicitors. Results showed the dominance of certain compounds such as vitamin E, beta-Amyrin, alpha-Amyrin, beta-Sitosterol, Nonacosan, Eicosan, Ergost-en-3-ol,(3.beta.) and 12-Oleanen-3-yl acetate (3.alpha.).**

Keywords: **Callus, Chemical elicitors, Secondary metabolites, *Thevetia neriifolia*.**

I. Introduction

The plant *Thevetia neriifolia* Juss. is called yellow oleander, due to the yellow color of its flowers is belonging to the Apocynaceae family, which **has** high importance for its medicinal and ornamental value, and distributed worldwide in tropical and subtropical regions [1], [2].

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Thevetia was named to commemorate the explorer Andre Thevet (1502-1590), who discovered it as genuine plant during his trip to Brazil and transported it to Europe [3].
Most parts of plant are used in folk treatments, because they contain natural products that facilitate their pharmaceutical uses in the treatment of heart failure, microbial inflammatory, anti-cancer and rheumatism, etc.[4], [5]. This plant has been introduced into Iraq in the last 15 years as ornamental plant in nurseries and gardens without knowledge of its medicinal importance [6].

The production of secondary metabolites can be enhanced by the exogenous supplying of elicitors or precursors that may improve the accumulation of desired compounds from plant tissue cultures. Elicitors are compounds triggering the biosynthesis of secondary metabolites, and these elicitors can be abiotic or biotic [7]−[9]. These elicitors can be apply alone or in combinations to enhance the formation of bioactive compounds from different plant tissue cultures [10]. Cholesterol is one of the compounds that can be introduced into the medium to stimulate the explants, especially, the callus and cell suspension on the production of effective secondary metabolites. Soladonine compound content has increased in cell suspensions of *Solanum xanthocarpum* and *S. aviculare* , when adding cholesterol to the culture medium of these suspensions [7], also the addition of cholesterol to cell cultures of *Withania somnifera* increased the content of Withaferin A compound as compared with untreated control cultures [11]. On the other hand, most of studies supplied culture media with salicylic acid to enhance the *in vitro* accumulation of secondary metabolites in many plant species; such as hyoscyamine and scopolamine compounds in *Datura metel* root cultures [12]; trigonelline compound in *Trigonella foenum-graecum* cell suspension [13], and also enhanced the synthesis of alkanes and fatty acids in cell cultures of *Jatropha curcas*[14]. There are no studies or reports available about the production of secondary metabolites from callus cultures of *Thevetia neriifolia* . Consequently, the current study aimed to investigate the ability of *Thevetia neriifolia* Juss. callus to grow and accumulate of effective secondary metabolites when culturing on different concentrations of cholesterol and salicylic acid and the evaluation of these metabolites using gas chromatography mass spectrometry (GC-MS) technique.

II. Material And Methods

A. Selection of explants and callus induction medium

This research was achieved in the laboratory of plant tissue culture in Al-Musaib Technical College. For the gaining of enough amounts of callus, fresh branches were

taken from ten years old trees of *Thevetia neriifolia* and in the laboratory they were cut into segments of 2cm long(were used as explants), then washed with liquid soap under running tap water, then explants were transferred into laminar air flow cabinet where they immersed for 5 minutes in solution of $HgCl_2$ (0.1% w/v) for sterilization. Finally, they were rinsed three times with sterile distilled water (3 minutes for each one).The explants were trimmed into pieces of 1.5 cm long and cultured on MS medium [15] fortifying with 30 $g.l^{-1}$ sucrose, 7 $g.l^{-1}$ agar , 2,4-Dichlorophenoxy acetic acid(2,4-D) at 1.0 $mg.l^{-1}$ and Kinetin (Kin) at 0.3$mg.l^{-1}$ (as an optimum for callus induction) . The pH of medium was adjusted to 5.7 ± 0.1 with 0.1N of NaOH or HCl before autoclaving at 121ºC and 1.04 $kg.cm^{-1}$ pressure for 20 minutes. The cultures were incubated in growth room under 16hrs. photoperiod (1000 lux) and 25 ± 2ºC.

B. Effect of cholesterol and salicylic acid (SA) on callus biomass and accumulation of secondary metabolites in vitro

In order to enhance the growth and development of callus biomass and to increase the accumulation of metabolites in it, about 350 mg of induced callus was taken and sub-cultured on MS medium containing the same combination of growth regulators (1.0 $mg.l^{-1}$ 2,4-D + 0.3 $mg.l^{-1}$ Kin) and supplemented with various concentrations of cholesterol or SA (0.0, 50, 100, 150, or 200 $mg.l^{-1}$) individually. Each treatment including 10 replicates. Cultures were incubated in growth room at the same conditions that mentioned above. After 3 weeks of culture, the fresh weight(FW), dry weights(DW)and diameter of callus were determined. The relative growth rate (RGR) of callus was determined according to the method of [16].

C. Extraction of secondary metabolites from callus

The calli from all treatments of cholesterol and salicylic acid were collected and dried in an oven at 60ºC for 48 h. and then, they were crushed into a powder. Five milliliters of hexane was added to 5 mg of dried powder for 6hrs. and centrifuged at 4000 rpm for 10 minutes. The extracts were collected in glass vials and stored at 4ºC for further analysis.

D. Evaluation of secondary metabolites in callus using GC-MS technique

The samples were analyzed using GC-MS(Agilent 19091S-33UI) apparatus equipped with National Institute of Standard and Technology (NITS)Library; column HP-5MS capillary column (cross bond 5% diphenyl-95% dimethylpolysiloxane); 30m(L)× 250µm(i. d.) with a 0.25 µm film thickness; injection temperature, 290ºC; column temperature, 40ºC held to 2 min., rising 4ºC/min, then rising to 290ºC 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, 40-600m/z. The active compounds of all extracts were identified by comparing their retention indices with NIST library.

E. Statistical analysis

A completely randomized design was used and data were subjected to analysis of variance (ANOVA). Significant differences were assessed using least significant difference (LSD) test at $P \leq 0.05$ [17].

III. Results And Discussion

A. Effect of cholesterol and salicylic acid on callus growth parameters

The MS medium with 1.0 $mg.l^{-1}$ 2,4-D+ 0.3 $mg.l^{-1}$ Kin was optimum for increasing of callus biomass of *T. neriifolia* . This combination was used in all treatments that containing different concentrations of cholesterol and salicylic acid to investigate the effect of these elicitors on callus growth and accumulation of metabolites. An inoculum callus about 350mg was used for this experiment. At the beginning of culture, callus growth was slowly, but from the 7th day, there was a rapid growth of callus biomass up to 3 weeks (Fig. 1). After that , there was a decreasing in callus biomass, so the best period for callus biomass production was 3 weeks.

Results in Table I. revealed the effect of cholesterol during callus growth showed varied responses in terms of fresh and dry weights, RGR and diameter of callus. Results showed there was a sharp and significant decline in fresh weight with the increasing of cholesterol concentrations as compared with control that gave the highest value (695 mg), whereas there were no significant differences in callus dry weight and RGR among cholesterol concentrations. On the other hand, there was a significant increasing in callus diameter to reach 9.93 mm as compared with other concentrations of cholesterol, in which the lowest diameter (5.52 mm) was recorded at concentration 200 $mg.l^{-1}$. These results were in agreement with [11] who observed slowly growth of callus on different concentrations of cholesterol, this may be suggested that cholesterol may be stressful for *in vitro* growing of cultures. Furthermore, in previous study of [18], they found that the exogenously applied of cholesterol to culture medium of cell cultures of rice, maize and soybean helped in regulation of rate of cell division that inhibited by growth retardant tetcyclacis. Incorporation of different concentrations of salicylic acid (SA) on callus features after 3 weeks of culture(Table I). The maximum increasing in fresh and dry weights of callus was observed in control treatment reached to 695 and 49 mg respectively, but these values were not significant as compared with the other salicylic acid treatments. Concerning the relative growth rate (RGR) of callus , the lowest rate was 2.43 $mg.day^{-1}$ was recorded in the concentration 150 $mg.l^{-1}$ SA which is significantly decreasing from control (7.56 $mg.day^{-1}$). Moreover, the highest value of

callus diameter was obtained in control treatment (9.93 mm) which is not significantly different from other SA concentrations, except from the concentration 200 mg/l that gave 7.49 mm. These results were in agreement with [19], who referred that in the absence of SA, typical growth could be noted with control cultures of callus and cell suspension. Previous studies proved that SA in moderate concentrations can influence a wide range of metabolic and physiological processes in plants affecting their growth and development [20]−[22], whereas higher concentrations of SA proved to be highly toxic and affected the growth parameters of callus. This means that higher concentrations of SA induced accumulation of ethylene, adversely affecting the *in vitro* growth of cultures [23]. Also, in the study of [24] on *Ziziphus spina-christa* tissue culture, has recorded that lower concentrations of SA improved culture proliferation, while its higher concentrations adversely affected it.

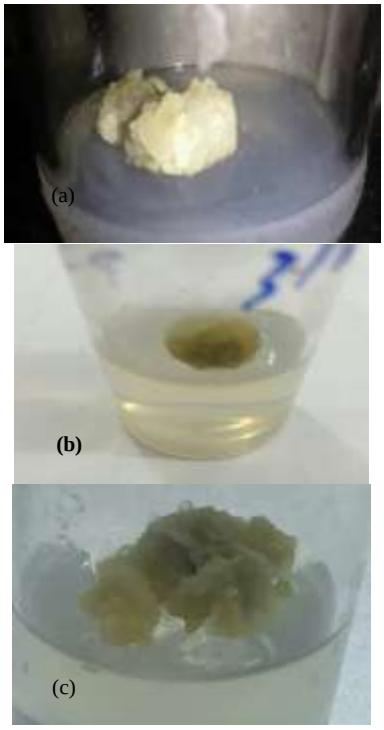

Fig. 1. Callus growth after three weeks of subculture on: (a)Control , (b)Cholesterol and (c)Salicylic acid.

TABLE I: EFFECT OF DIFFERENT CONCENTRATIONS OF CHOLESTEROL AND SALICYLIC ACID ON CALLUS GROWTH PARAMETERS OF *THEVETIA NERIIFOLIA*AFTER 3 WEEKS OF CULTURE(INITIAL INOCULUM WEIGHT 350 mg)

Type of Elicitor ($mg.l^{-1}$)	Callus FW (mg)	Callus DW (mg)	Callus RGR ($mg.day^{-1}$)	Callus Diameter (mm)
Cholesterol				
0.0	695	49	7.56	9.93
50	453	38	6.63	6.00
100	462	40	8.10	6.20
150	401	33	6.36	5.96
200	402	32	6.03	5.52
LSD at $P \leq 0.05$	189.8	*NS**	*NS**	1.99
Salicylic acid(SA)				
0.0	695	49	7.56	9.93
50	523	40	4.70	7.95
100	558	38	3.20	8.28
150	556	41	2.43	8.09
200	602	36	3.23	7.49
LSD at $P \leq 0.05$	*NS**	*NS**	5.02	2.16

*NS**= Not statistically different at $P \leq 0.05$.

B. Effect of cholesterol and salicylic acid on production of secondary metabolites detected with GC-MS analysis

The hexane extracts of cholesterol and salicylic acid treated calli were analyzed using GC-MS, which showed that the control contained 35 compounds(Fig. 2), and the major of these compounds are: Olean-12-ene-3-methoxy-(3.beta.) (4.767%),12-oleanen-3-yl acetate(.3beta.) (7.546%),Urse-12-ene-3-ol,acetate (.3beta.) (18.641%),.alpha.-Amyrin (15.6385), Benzo(b)naphtha(1,2-d)furan (10.715), Trans-Geranylgeraniol(1.046%), Eicosane(3.676%), Vitamin E (1.831%), (+)-trans-3,4-Dmethyl-2-phenyl tetrahydro-1,4-thiazine(4.019%), .beta.-Sitosterol(2.091%), Pyrrolo [2,3-b],indole,1,2,3,3a,8,8a-hexahydro-5-methoxy-3a,8-dimethyl-(7.291%), Taraxasterol(2.544%), .beta.-Amyrin(1.852%) and other different compounds. While the different concentrations of cholesterol gave different numbers and ratios of active secondary metabolites and some of them appeared for the first time in concentrations of cholesterol(Table II. and Fig. 3), in

which the concentration 50mg.l^{-1} gave 30 compounds,100mg.l^{-1} gave 34 compounds,150mg.l^{-1} gave 31 compounds and 200mg.l^{-1} gave 37 compounds.

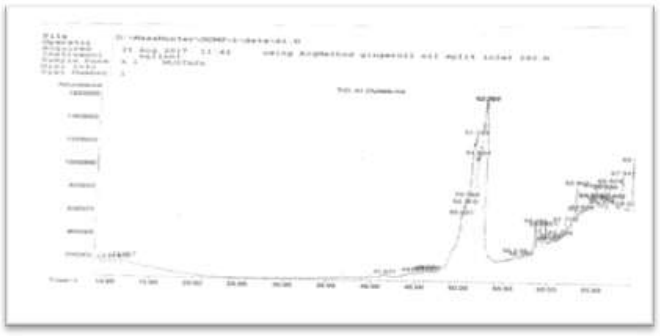

Fig. 2. The GC-MS chromatogram of *in vitro* control callus extracted with hexane.

Results in Table II. showed that the compound 12-Oleanen-3-yl acetate,(3.alpha.) reached the highest ratio 10.344%in the concentration 200 mg.l^{-1} , while it is absent in 150 mg.l^{-1}, whereas the compound Urs-12-en-24-oic acid, 3-oxo-,methyl ester appeared in concentrations cholesterol to reach 10.412% at 50 mg.l^{-1}, while it absent from the concentration 150 mg.l^{-1} and control. The compound .alpha.-Amyrin was abundance in all treatments with ratios of 15.638, 6.014, 11.446 and 9.358 % at control, 50, 100 and 200 mg.l^{-1} respectively, while it is not found in concentration150 mg.l^{-1} .Vitamin E was appeared in all treatments with ratios 1.831, 3.268, 1.433, 3.430 and 3.682% at control, 50, 100, 150 and 200 mg.l^{-1} respectively. Furthermore, Ergost-5-en-3-ol,(3.beta.) was not found in control and first appeared at all cholesterol concentrations to reach highest ratio (7.400%) at 150 mg.l^{-1}. In addition, the compound .beta.-Sitosterol was appeared in control (2.091%), but the addition of cholesterol to the medium enhanced the synthesis of this compound to reach 3.012, 6.168, 10.499 and 7.795% at 50, 100, 150 and 200 mg.l^{-1} respectively. Also, .beta.-Amyrin was found in all treatments with ratios 1.852, 7.815, 15.152, 22.021 and 5.258% at control, 50, 100, 150 and 200 mg.l^{-1} respectively. From these results, we can see an important effect of cholesterol concentrations in stimulating the production of secondary compounds in *Thevetia neriifolia* callus such as alkaloids, terpenes, phenols, fatty acids and lipids, as the cholesterol can enter as a precursor for biosynthesis of some phytosteroids [25], [26]. These bioactive compounds play important roles as anti-inflammatory, anti-microbes and anticancer [27].

TABLE II: SECONDARY METABOLITES OF *THEVETIA NERIIFOLIA* CALLUS ELICITATED WITH CHOLESTEROL ANALYZED WITH GC-MS.

S.	R.	Compounds	Cont (%	50mg (%	100m (%	150m (%	200m (%
	10.	1-pentene,2-methyl-	0.09	-	-	-	-
	11.	Cyanic acid,2-methylpropyl ester	-	-	-	0.11	-
	11.	Cyclotetrasiloxane,octamethyl-	-	-	-	-	0.04
	11.	Aziridine,2-methyl-	0.22	-	-	-	-
	12.	2-Heptenal, (E)-	-	-	-	0.20	-
	12.	1-Hexene,3,4-dimethyl-	0.10	-	-	-	-
	25.	Melamine, tris(trimethylsilyl)derivative	-	-	-	0.20	-
	34.	.beta.-Phenyl butyrate	-	0.05	-	-	-
	36.	Cyclobutane, 1,2-diphenyl-	-	0.33	-	-	-
	39.	1-Octadecanesulphonyl chloride	-	-	-	-	0.17
	40.	Hexadecanoic acid, methyl ester	-	-	-	-	0.20
	41.	1-(4-Methoxy-phenyl)-5,5-dioxo-hexahydro-5.lambda (6)-thieno[3,4-b]pyrrol-2-one	-	-	0.07		
	41.	Phthalic acid, di(8-chlorooctyl) ester	0.19	-	-	-	-
	44.	17-Pentatriacontene	-	-	-	0.08	-
	44.	9-Octadecenoic acid, methyl ester, (E)-	-	-	-	-	0.92
	44.	d-Norpregnane (5.alpha.,14.alpha.)	-	-	-	-	0.12
	44.	6-Octadecenoic acid, methyl ester	-	-	-	-	0.14
	44.	Octasiloxane,1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	-	-	0.22	-	-
	44.	Oleic acid	0.31	-	-	-	-
	45.	Heptadecafluorononanoic acid, tridecyl ester	0.18	-	-	-	-
	46.	9-Undecen-2-one,6,10-dimethyl-	0.15	-	-	-	-
	46.	5-(1H)-Azulenone,2,4,6,7,8,8a-hexahydro-3,8-dimethy -4-(1-methylethylidene)-,(8S-cis)-	-	-	-	-	1.79
	47.	.alpha.-Amyrin,trimethylsilyl ether	0.15	3.38	7.50	-	3.99
	47.	Olean-12-ene,3-methoxy-,(3.beta.)-	4.76	-	2.77	-	3.72
	47.	8-Amino-2.6-dimethoxylepidine	-	-	1.18	-	-
	48.	12-Oleanen-3-yl acetate,(3.alpha.)-	7.54	0.34	1.78	-	10.3
	49.	Urs-12-ene-3-ol,acetate,(3.beta.)	18.6	-	9.20	-	3.73
	49.	Urs-12-en-24-oic acid,3-oxo-,methyl ester	-	10.4	1.82	-	2.52
	50.	.alpha.-Amyrin	15.6	6.01	11.4	-	9.35
	51.	Phenol,2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl	-	-	0.42	0.90	-
	51.	3-Phenylthiane,S-oxide	-	9.22	-	3.50	2.17

	52.	2-methylhexacosane	-	5.01	0.34	0.80	-
	52.	Benzo(b)naphtha(1,2-d)furan	10.7	-	-	-	-
	53.	Bis(2-methylhexy)phthalate	-	-	0.35	-	5.05
	53.	Lanosta-8,24-dien-3-ol	-	-	-	-	8.59
	53.	Methadone N-oxide	-	7.42	-	2.95	1.99
	53.	1H-Indole,5-methyl-2-phyneyl-	-	12.5	1.09	0.48	-
	56.	Bendazol	-	5.23	-	-	-
	56.	Tetracosane	-	0.86	0.43	0.21	-
	56.	Triacontane	0.29	-	-	-	1.71
	56.	(4-Oxo-4H-quinazolin-3-yl)-acetic acid, methyl aster	0.07	-	-	-	-
	57.	7-Methyl-z-tetradecene-1-ol acetate	-	0.53	0.14	0.44	0.26
	58.	Squalene	-	-	-	-	0.16
	58.	Supraene	-	0.54	0.80	0.49	0.15
	58.	Trans-Geranylgeraniol	1.04	-	-	-	-
	58.	1-Pyrrolidinebutanoicacid,2-[(1,1-dimethylethoxy)-carbonyl]-.alpha.-nitro-,2,6-bis(1,1-dimethylethyl)-4-methox ester,[S-(R*,R*)]-	-	0.50	0.70	0.02	-
	58.	Lup-20(29)-en-3-ol,acetate,(3.beta.)-	1.80	-	-	-	6.91
	59.	Silane,dimethylhexadecyloxy(2-phenylethoxy)-	-	-	0.70	0.47	-
	59.	Nonacosane	0.96	1.35	2.60	2.72	3.30
	60.	Cyclobarbital	-	0.18	-	-	-
	60.	3-N-Nitroso-solanocapsine	0.32	-	-	-	-
	61.	1.2.4-Benzenetricarboxylicacid,4-dodecyldimethyleste	-	-	0.12	0.18	-
	61.	2,2-Dimethyl propionic acid	0.25	-	-	0.44	-
	61.	1,2-Benzisothiazol-3-amine tbdns	-	-		-	-
	61.	Pentacyclo[19.3.1.1(3,7).1(9,13).1(15,19)]octacosa-1(25),3,5,7(28),9,11,13(17),15,17,19(26),21,23-dodec aene-25,25,27,28-tetrol,5,11.17,23-tetrakis	0.97	-	1.16	0.73	1.70
	62.	(E)-2-bromobutyloxychalcone	-	-	0.41	0.18	0.31
	62.	Hentriacontane	-	-	7.81	-	-
	62.	Eicosane	3.67	-	-	-	-
	62.	Hexatriacontane	-	0.10	-	9.62	0.07
	63.	Vitamin E	1.83	3.26	1.43	3.43	3.68
	63.	2-Ethylacridine	0.99	-	-	-	0.05
	64.	Benzo[h]quinoline,2,4-dimethyl-	-	1.70	1.13	1.50	0.25
	64.	(+)-trans-3,4-Dimethyl-2-phenyltetrahydro-1,4-thiazin	4.01	-	-	-	-
	64.	Ergost-5-en-3-ol, (3.beta.)	-	1.04	4.04	7.40	4.11
	64.	Unknown	5.20	-	-	-	-
	65.	Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silan	-	3.57	1.77	2.68	-
	65.	Prepiophenone,2'-(trimethylsiloxy)-	-	-	0.19	0.31	0.55
	65.	Unknown	1.96	-	-	-	-
	66.	Eicosane,2-methyl-	1.81	-	-	-	-
	66.	Pentatriacontane	-	2.09	5.78	7.87	4.66
	66.	.beta.-Sitosterol	2.09	3.01	6.16	10.4	7.79
	66.	Ethanone,2-(2-benzothiazolylthio-1-(3,5-dimethyl -pyrazolyl)-	-	4.45	-	-	-
	66.	Pregna-5,16-dien-20-one,3-hydroxy-,(3.beta.)-	-	-	-	2.05	0.49
	66.	Cholest-5-en-3-ol,24-propylidene-,(3.beta.)-	0.85	-	4.11	6.00	-
	66.	Anthracene,9-(2-propenyl)-	-	1.01	-	-	-
	66.	Pyrrolo[2,3-b]indole,1,2,3,3a,8,8a-hexahydro-5-meth -oxy-3a,8-dimethyl-	7.29	-	-	-	1.13
	67.	Unknown	1.06	-	-	-	-
	67.	[1,2,4]Triazolo[1,5a]pyrimidine-6-ino-,ethyl ester	-	0.02	0.73	-	-
	67.	Pyrene, hexadecahydro-	-	-	6.40	11.2	2.64
	67.	Taraxasterol	2.54	3.05	-		-
	68.	Bisphenol,bis(tert-butyldimethylsilyl) ether	0.35	4.94	-	-	-
	69.	.beta.-Amyrin	1.85	7.81	15.1	22.0	5.25
Total (%)			100.	100.	100.	99.9	99.7

R.T.*(Retention time)

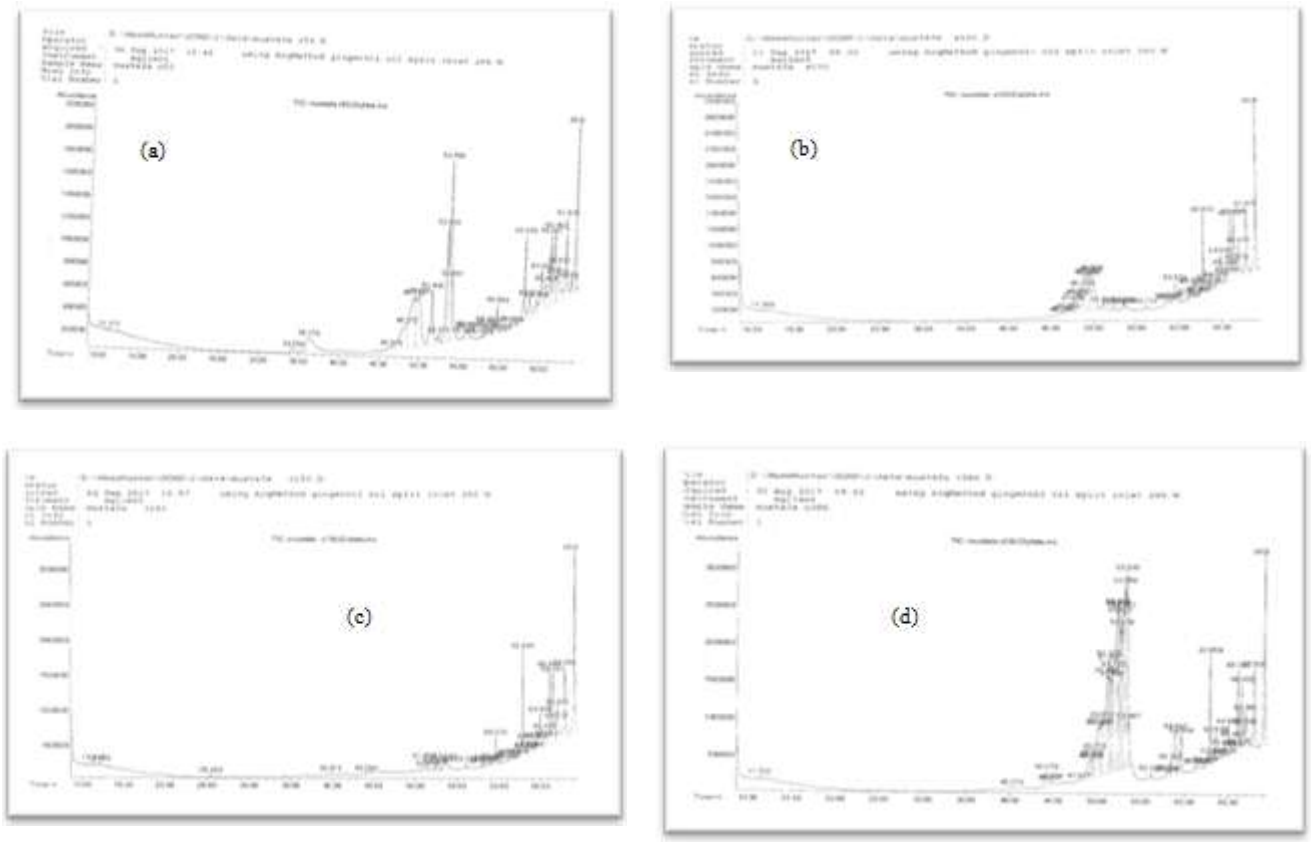

Fig. 3. The GC-MS chromatogram of hexane extracted callus samples cultured on different concentrations of cholesterol: (a) 50 mg.l^{-1}. (b) 100 mg.l^{-1} . (c) 150 mg.l^{-1} and (d) 200 mg.l^{-1}

The results in Table III. and Fig. 4. showed the influence of salicylic acid concentrations on the synthesis of secondary metabolites, in which the concentration 50 mg.l^{-1} gave 30 compounds, 100 mg.l^{-1} gave 34 compounds, 150mg.l^{-1}gave 34 compounds and 200mg.l^{-1}gave 36 compounds. The compound 12-oleanen-3-yl acetate,(3.alpha.) was found in all treatments in ratios 7.546, 10.446, 11.450, 12.582 and 5.563% at control, 50, 100, 150 and 200 mg.l^{-1} of SA, whereas, the Urs-12-ene-3-ol, acetate, (3.beta.) was absent only in concentration 150mg.l^{-1} of SA, .alpha.-Amyrin was appeared in all treatments of SA as shown in Table III. The .psi., .psi.-Carotene, 7,7',8,8',11,11',12,12',15,15'-decahydro-(1.108%) was found only in 50mg.l^{-1}of SA. Hexatriacontane was not found in control treatment, but this compound appeared with the addition of SA to the medium. On the other hand, vitamin E appeared in all treatments : control(1.831%), 50mg.l^{-1}(2.622%), 100mg.l^{-1}(0.678%), 150mg.l^{-1}(2.284%) and 200mg.l^{-1}(1.155%). In addition, .beta.-Sitosterol was appeared in all treatments except the concentration 100mg.l^{-1}of SA, whereas, .beta.-Amyrin was increased in higher ratio at SA concentrations (14.954, 13.935, 11.788 and 17.197% at 50, 100, 150 and 200 mg.l^{-1} of SA respectively) as compared with control(1.852%). From that, we can saw the importance of adding salicylic acid , which is important in the biosynthesis of many effective secondary compounds is shown by certain percentages in plant tissue cultures [13], [14].

The terpenes compounds are active secondary metabolites, the most important of which is the Amyrin compound, which is used for pharmaceuticals to treat wounds, ulcers, joint, bone and liver infections [28], [29]. Phytosterols, are the most important natural steroids of which beta-Sitosterols which are very important in the medical and cosmetic fields and added as dietary supplements to reduce the levels of high cholesterol in blood and enter as a basis in the manufacture of other secondary metabolites as well as their contribution in the transfer of acyl groups, sugar and protein [30]. Vitamin E is also an important antioxidant as it plays an important role in the treatment of tumors, Alzheimer's, infertility and Parkinson's disease symptoms [31]. On the other hand, fatty acids are very important for entry into food as antioxidants, as well as the use of pharmaceutical and entry into medical and cosmetic preparations [32].

TABLE III: SECONDARY METABOLITES OF *THEVETIA NERIIFOLIA* CALLUS ELICITATED WITH SALICYLIC ACID ANALYZED WITH GC-MS.

S. No.	R.T.*	Compounds	Control (%)	50mg.l^{-1} (%)	100mg.l^{-1} (%)	150mg.l^{-1} (%)	200mg.l^{-1} (%)
1	10.313	1-pentene,2-methyl-	0.090	-	-	-	-
2	11.306	Cyanic acid,2-methylpropyl ester	-	-	0.076	-	-
3	11.316	Cyclotetrasiloxane,octamethyl-	-	-	-	0.422	-
4	11.435	Aziridine,2-methyl-	0.221	-	-	-	-
5	12.050	Silane,trichlorodocosyl-	-	-	-	0.143	-
6	12.061	1-Hexene,3,4-dimethyl-	0.101	-	-	-	-
7	25.460	Melamine, tris(trimethylsilyl)derivative	-	-	-	0.218	-
8	41.622	Phthalic acid, di(8-chlorooctyl) ester	0.190	-	-	-	-
9	43.132	Isopropyl palmitate	-	-	-	0.312	-
10	43.531	n-Hexadecanoic acid	-	-	-	0.334	-
11	43.650	Octadecanoic acid,2-hydroxy-1,3-propanediyl ester	-	-	-	0.105	-
12	44.200	Ethanone,1-(4,5-dihydro-2-thiazolyl)-	-	-	-	0.381	-
13	44.761	1-(+)-Ascorbic acid 2,6-dihexadecanoate	-	-	-	0.215	-
14	44.783	Oleic acid	0.310	-	-	-	-
15	45.765	Heptadecafluorononanoic acid, tridecyl ester	0.188	-	-	-	-
16	46.272	.alpha.-Amyrin,trimethylsilyi ether	0.159	-	2.566	-	0.506
17	46.563	9-Undecen-2-one,6,10-dimethyl-	0.150	-	0.717	-	-
18	46.574	5-(1H)-Azulenone,2,4,6,7,8,8a-hexahydro-3,8-dimethyl -4-(1-methylethylidene)-,(8S-cis)-	-	-	-	-	0.258
19	47.458	.alpha.-Amyrin,trimethylsilyl ether	-	0.699	3.202	2.657	3.099
20	47.642	Olean-12-ene,3-methoxy-,(3.beta.)-	4.767	4.708	3.562	-	2.588
21	47.954	8-Amino-2.6-dimethoxylepidine	-	-	2.994	3.966	-
22	48.278	12-Oleanen-3-yl acetate,(3.alpha.)-	7.546	10.446	11.450	12.582	5.563
23	49.163	Urs-12-ene-3-ol,acetate,(3.beta.)	18.641	1.804	4.179	-	10.187
24	49.422	Urs-12-en-24-oic acid,3-oxo-,methyl ester	-	3.532	-	-	1.928
25	50.177	.alpha.-Amyrin	15.638	13.100	8.294	14.569	6.933
26	51.094	Phenol,2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl	-	1.185	0.475	1.129	0.638
27	51.461	Azetidine,1-benzyl-3,3-dimethyl-2-phenyl-	-	-	-	1.033	-
28	52.378	2-methylhexacosane	-	0.618	0.858	0.994	0.341
29	52.389	Benzo(b)naphtha(1,2-d)furan	10.715	-	-	-	-
30	53.425	Acetamide,2-(2,4-dimethoxybenzylidenehydrazino)-N-Ethyl-2-oxo-	-	0.569	0.548	-	-
31	53.619	Methadone N-oxide	-	-	0.768	1.254	0.379
32	53.662	1H-Indole,5-methyl-2-phyneyl-	-	0.823	0.311	3.953	0.808
33	56.100	Tetracontane	-	-	-	0.273	0.606
34	56.111	Tetracosane	-	-	0.732	-	-
35	56.139	Triacontane	0.292	0.451	-	-	-
36	56.888	(4-Oxo-4H-quinazolin-3-yl)-acetic acid, methyl aster	0.070	-	-	-	-
37	57.869	Stearic acid,3-(octadecyloxy) propyl ester	-	-	-	-	0.144
38	57.880	Tetrapentacontane,1,54-dibromo-	-	0.219	-	-	-
39	58.366	.psi.,.psi.-Carotene,7,7',8,8',11,11',12,12',15,15'-decahydro-	-	1.108	-	-	-
40	58.387	Trans-Geranylgeraniol	1.046	-	-	-	-
41	58.581	A'-Neogammacer-22(29)en-3-ol,acetate,(3.beta., 21.beta.)-	-	1.003	0.478	0.419	0.842
42	58.862	1-Pyrrolidinebutanoicacid,2-[(1,1-dimethylethoxy)-carbonyl]-.alpha.-nitro-,2,6-bis(1,1-dimethylethyl)-4-methoxy ester,[S-(R*,R*)]-	-	0.640	0.501	-	0.471
43	58.959	Lup-20(29)-en-3-ol,acetate,(3.beta.)-	1.802	-	-	-	-
44	59.272	Silane,dimethylhexadecyloxy(2-phenylethoxy)-	-	0.634	1.326	-	0.774
45	59.580	Nonacosane	0.963	2.660	0.139	1.313	2.152
46	60.407	3-N-Nitroso-solanocapsine	0.324	-	-	-	-
47	61.235	2,2-Dimethyl propionic acid	0.253	0.412	-	0.397	0.222
48	61.236	1,2-Benzisothiazol-3-amine tbdns	-	-	1.523	-	-
49	61.732	Pentacyclo[19.3.1.1(3,7).1(9,13).1(15,19)]octacosa-1(25),3,5,7(28),9,11,13(17),15,17,19(26),21,23-dodecaene-25,25,27,28-tetrol,5,11.17,23-tetrakis	0.976	0.343	0.304	0.339	0.570
50	62.411	(E)-2-bromobutyloxychalcone	-	-	5.760	-	0.153
51	62.832	Hentriacontane	-	-	0.377	-	7.227
52	62.865	Eicosane	3.676	-	-	-	-
53	62.875	Hexatriacontane	-	8.253	1.231	5.470	0.198
54	63.540	Vitamin E	1.831	2.622	0.678	2.284	1.155

55	63.814	2-Ethylacridine	0.995	-	-	-	0.082
56	64.202	Benzo[h]quinoline,2,4-dimethyl-	-	0.981	1.407	1.787	0.248
57	64.418	(+)-trans-3,4-Dimethyl-2-phenyltetrahydro-1,4-thiazine	4.019	-	4.974	-	0.989
58	64.930	1H-Indole,1-methyl-2-phenyl-	-	3.707	2.000	5.263	4.812
59	64.990	Unknown	5.206	-	-	-	-
60	65.454	Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane	-	1.286	-	2.693	2.279
61	65.648	2-[4-Cyclohexylbutanoylamino]-3-chloro-1,4-naphtho -quinone	-	-	5.225	-	-
62	65.778	Unknown	1.968	-	-	-	-
63	66.047	Eicosane,2-methyl-	1.816	-	-	-	-
64	66.187	Pentatriacontane	-	5.908	6.758	4.689	5.778
65	66.460	.beta.-Sitosterol	2.091	6.052	-	6.440	7.171
66	66.813	Ethanone,2-(2-benzothiazolylthio-1-(3,5-dimethyl -pyrazolyl)-	-	-	1.113	1.558	-
67	66.824	Pregna-5,16-dien-20-one,3-hydroxy-,(3.beta.)-	-	1.340	-	-	-
68	66.975	Cholest-5-en-3-ol,24-propylidene-,(3.beta.)-	0.851	-	6.907	-	5.381
69	66.986	Anthracene,9-(2-propenyl)-	-	-	-	3.158	-
70	66.997	Pyrrolo[2,3-b]indole,1,2,3,3a,8,8a-hexahydro-5-meth -oxy-3a,8-dimethyl-	7.291	3.189	-	-	-
71	67.018	Unknown	1.061	-	-	-	-
72	67.363	[1,2,4]Triazolo[1,5a]pyrimidine-6-ino-,ethyl ester	-	-	-	1.210	0.675
73	67.871	Pyrene, hexadecahydro-	-	-	4.051	-	7.581
74	67.900	Taraxasterol	2.544	6.754	-	6.787	-
75	68.259	5-Methyl-2-phenylindolizine	-	-	-	-	0.067
76	68.626	Bisphenol,bis(tert-butyldimethylsilyl) ether	0.358	-	-	-	-
77	69.068	.beta.-Amyrin	1.852	14.954	13.935	11.788	17.197
Total (%)			100.00	100.00	99.419	100.00	100.00

R.T.*(Retention time)

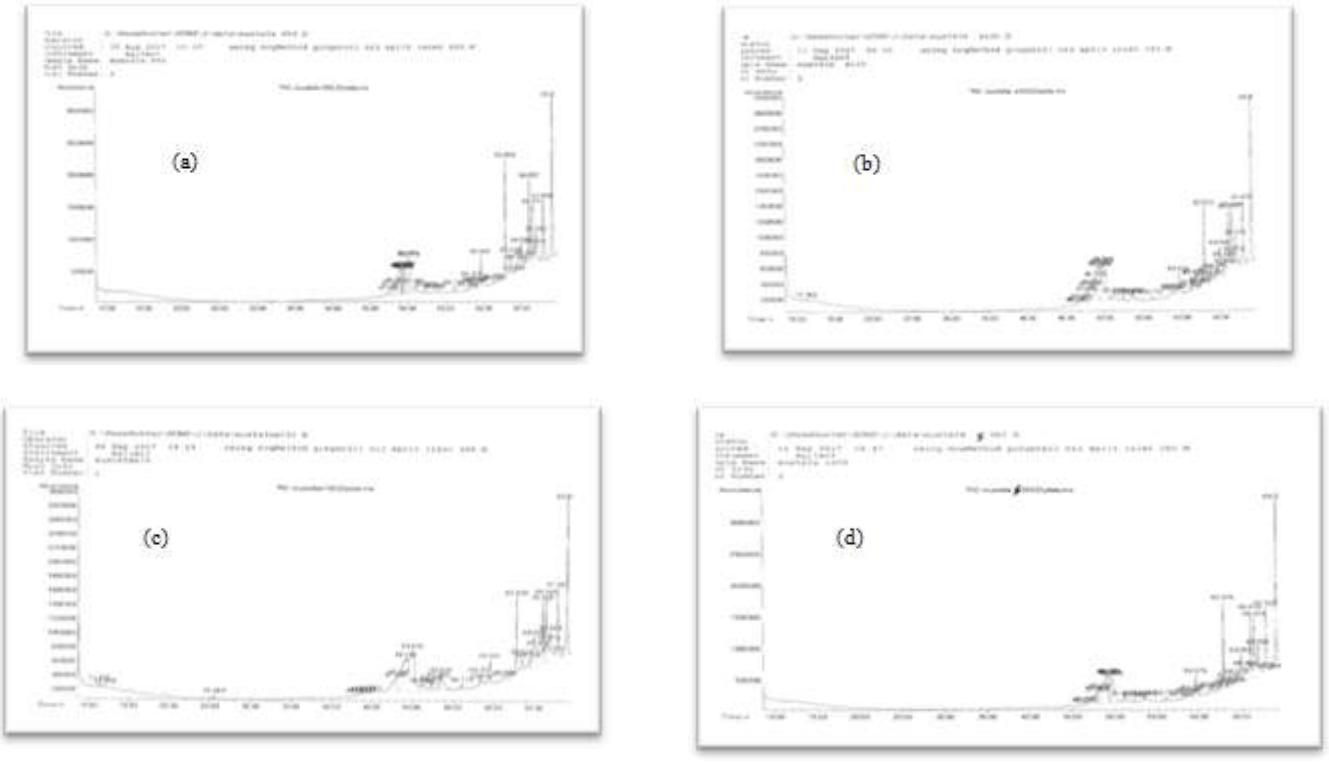

Fig. 4. The GC-MS chromatogram of hexane extracted callus samples cultured on different concentrations of salicylic acid: (a) 50 $mg.l^{-1}$. (b) 100 $mg.l^{-1}$. (c) 150 $mg.l^{-1}$ and (d) 200 $mg.l^{-1}$.

IV. . CONCLUSION

The results of the present study showed the possibility of producing callus biomass from *Thevetia neriifolia* internodes in large quantities using the combination of 1.0mg.l^{-1} 2,4-D+0.3 mg.l^{-1} Kin. Also, the addition of chemical elicitors; cholesterol and salicylic acid at different concentrations to the callus developing medium has significantly stimulated the production of bioactive secondary compound when analyzed using GC-MS. The most important of these compounds were vitamin E, alpha- and beta-Amyrin, beta-Sitosterol and Nonacosane.

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