Eco. Env. & Cons. 25 (3) : 2019; pp. (1466-1472)
Copyright@ EM International
ISSN 0971–765X

Evaluation of antioxidant activity and phytochemicals profile in yellow oleander *in vitro* and *in* vivo cultures

Siham A. Salim*

Al-Mussaib Technical College, Al-Furat Al-Awsat Technical University, Iraq

(Received 10 March, 2019; accepted 15 May, 2019)

ABSTRACT

The current study is to compare between the callus extract and the natural plant leaves extract of the yellow oleander (*Thevetia neriifolia* Juss.) in the antioxidant activity and their contents of the phytochemicals by GC/MS analysis. Results showed that MS medium equipped with 4.52µM 2,4-D + 1.33µM BA was the best in the percentage of callogenesis (100%), fresh weight (964mg) and dry weight (98 mg) of the induced callus from internodes. The methanolic extract of callus had a significant free radicals scavenging activity through the method of DPPH free radical compared to leaves extract. IC_{50} for callus extract was 68µg.mL^{-1}, it was better to IC_{50} leaves extract (= 97 µg.mL^{-1}). Furthermore, GC/MS analysis revealed that there were 28 phytochemicals in the callus extract more than in the leaves extract (15). The most important compounds that have been diagnosed are vitamin E , Oleic acid, beta-Sitosterol alpha and beta-Amyrins, which possess antioxidant properties. It can be concluded from this study the importance of callus in the production of various bioactive phytochemicals compared to natural plants and also to keep the plant environment from damage to get these phytochemicals.

Key words : *Callus culture, Antioxidant activity, Yellow oleander, Phytochemical profile.*

Introduction

Medicinal plants have long been regarded as an essential source of human health since the passing ages because they contain many useful compounds that reflect the therapeutic importance of these plants. Field of the study of medicinal plants has increased in biomedical research due to their therapeutic properties, low cost, accessibility, heritage and a widespread belief that plant medicines are safer and courageous than synthetic drugs.

The yellow oleander (*Thevetia neriifolia* Juss) plant belongs to the Apocynaceae family, is one of the medicinal and ornamental plants. Its cultivation is spread in tropical and subtropical areas around the world (Joshi, 2000). The medical importance of this plant is that most of its parts used in the treatment of many diseases in folk medicine such as tooth ache, abdominal pain, ulcers, acne, anti-bacterial, anti-inflammatory, rheumatism, treatment of heart failure, anti-cancer and antioxidant as a result of containing of many effective compounds that act as medicinal drugs known as secondary metabolites or phytochemicals (Save *et al.*, 2015). Most of these phytochemicals produced by plants act as antioxidants to avoid damage caused by exposure to environmental stresses causing an increase in levels of free radicals, which can cause injury and damage to living cellular contents (such as proteins, nucleic acids, and lipids) (Visentin *et al.*, 2003). Antioxidants affect by inhibiting the enzymes involved in the oxidation and / or removal of free radicals forms, chelating minerals and stimulating antioxidant enzyme systems, thereby reducing the damage. These

***Corresponding author's email :** dr.sihamabdalrazzaq@yahoo.com; com.shm@atu.edu.iq

compounds including phenols of various types, tannins, terpenes, alkaloids, essential oils and glycosides.

To obtain these effective phytochemicals, large areas of cultivated plants are depleted leading to the extinction of these plants which affects the ecological balance that leads to desertification and/or limited production of these compounds is linked to the growing season of plants (Eba, 2005; Tiwari *et al.*, 2011 and Shanmuga *et al.*, 2015). Therefore, studies in recent years have resorted to the technique of plant tissue cultures being the alternative, strong and faster solution to obtain these compounds within limited areas in short periods of time and the pure production of these compounds from small explants throughout the year without adherence to the plant growing season. Beyond this, the callus cultures can be an essential source of access to new secondary metabolites not found in plants in the irnatural environments (Lystvan *et al.*, 2018 and Adil *et al.*, 2019). The main objectives of this study are to stimulate callus induction and growth from internodal explants of yellow oleander, evaluate the antioxidant activity of the callus (*in vitro*) and natural plant leaves (*in vivo*) extracts, and determine the active phytochemicals in callus and leaves extracts using GC/MS technique.

Materials and Methods

The experiments of this study were accomplished in the plant tissue culture laboratory, Al-Mussaib Technical College and laboratories of the Environment and Water Research Center - Ministry of Science and Technology.

Callus induction experiment

Internodes (3 cm long) were taken from healthy branches of the yellow oleander (*T.neriifolia* Juss) plant, where they were used as explants and were washed thoroughly with running water and liquid soap, then they were moved to the laminar air-flow cabinet wherein sterilized with ethanol (70%) for 40 seconds and then immersed in a solution of sodium hypochlorite (NaOCl, 3%, v/v) for 15 minutes, then washed with autoclaved distilled water. Then, the damaged ends of the explants were removed and cut into 1.5 cm piecesand transplanted to the medium of MS (Murashige and Skoog, 1962) prepared by dissolving of4.43 g of the powdered medium (HI Media, India) in a liter of distilled water and equipped with 30g.l^{-1} sucrose, agar 7g.l^{-1} and growth regulators 2,4-Dichlorophenoxy acetic acid (2,4-D)at 0.0, 4.52, 9.04, 13.57 and 18.09µM along with benzyl adenine (BA) at 0.0, 0.44, 0.89, 1.33 and 1.77µM. The pH of medium was adjusted to 5.7 ± 0.1 using 0.1 N from NaOH or HCl, then the medium was heated to dissolve the agar on a magnetic stirrer and was distributed by 15 mL per glass container (15*2.5 cm) and autoclaved at 121 ºC under pressure of 1.04 kg.cm^{-1} for 20 minutes. Cultures were incubated in the growth room at 25 ± 3ºC and photoperiod of 16/8 h to induce callus. Ten replicates were used for each treatment. The percentage of callus induction, fresh and dry weight of callus was calculated after five weeks of culture to determine the best combination of the growth regulators for proliferation and multiplication of callus.

Evaluation of the free radical scavenging activity of callus and leaves extracts

Callus has been propagated on the optimal combination of growth regulators from previous experiment to obtain appropriate amounts of callus. Healthy leaves were collected from the natural plants of yellow oleander. All the samples (callus and leaves) were dried in the air and crushed using a pestle. About 500 mg of callus and leaves powder were weighed separately andmixed with 10 mlof methanol (50%). The mixture was placed on a vibrator at 25 rpm and 25-30 ºC for 24h, then sonicated for 40 min. Then, the mixture was centrifuged at 6500 rpm for 10 minutes. The supernatant was filtered and collected in the Eppendorf tubes, then diluted the extract to 10 mg. mL^{-1} as final concentration, and stored in the refrigerator at 4 ºC for subsequent analysis (Khan *et al.*, 2013).

The activity of free radicle scavenging of callus and leaves extracts on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was tested according to the method of Goupy *et al.* (2003). This test is based on the use of the dark violet DPPH radical, which turns yellow when scavenging by antioxidant compounds, resulting in reduced absorption at a wavelength of 517 nm. Fifty microliters of several concentrations of extracts of callus and leaves samples (50, 100, 200, 300 and 400 µg.$µL^{-1}$) were incubated with 1250 µL of DPPH solution at a concentration of 4 mg.50 mL^{-1} methanol. After 30 min of incubation in the dark, the absorbance was measured at 517nm. The results were expressed as the quantities needed to reduce 50% of initial con-

centration (IC_{50}) DPPH and were calculated using quercetin and gallic acid as standards. Three replicates were used for each treatment and the experiment repeated twice. The percentage of discoloration of the DPPH radical was calculated according to the following equation:

Percentage of DPPH free radical scavenging activity(%) = (1 – AE/AD) × 100

Where: AE is the absorbance of DPPH solution in the presence of actual concentration of extract or standard. AD is the absorbance of DPPH solution alone.

Gas Chromatography/Mass Spectrometry Analysis Conditions of Callus and Leaves Extracts

Callus and leaves extracts were analyzed using GC/MS apparatus (Agilent 19091S-33UI) equipped with National Institute of Standard and Technology (NIST) Library; column HP-5MS capillary column (cross bond 5% diphenyl-95% dimethyl polysiloxane); 30m×250 µm with a 0.25 µm film thickness; injection temperature: 290ºC; temperature of column: 4 ºC held to 2 min, rising 4 ºC.min^{-1}, then rising to 290 ºC and held for 5 min; mode of injection, split: split at ratio 1:20; injected volume: 5µL. Carrier gas was Helium (99.99%); acquisition mass range: 40-600 m.z^{-1}. The phytochemicals of the extracts were identified by comparing their retention indices with NIST Library.

Experimental design and statistical analysis

The current study experiments were designed as factorial experiments according to the completely randomized design and the results analyzed using the statistical analysis program of Gen Stat to investigate the effect of different treatments on the studied traits. Differences between means were compared using the least significant differenceusing analysis of variance (ANOVA) at the probability level of p d" 0.05 (Gen Stat, 2012).

Results and Discussion

Production of callus biomass

The results from Table 1 showed that most of the 2,4-D and BA combinations gave the best callogenes is response, which was mostly 100%, while the control treatment didn't give any response to callus formation.In addition, the fresh weight results showed that combinations of 4.52 µM 2.4-D + 1.33 µM BA and 13.57 µM 2.4-D + 0.89 µM BA were significantly superior, giving the highest means of 964 and 834 mg respectively.Furthermore, the highest dry weight means of 98 and 89 mg were recorded at the combinations 4.52 µM 2.4-D + 1.33 µM BAand 13.57 µM 2,4-D+ 1.33 µM BA, respectively. The means of these traits decreased as the concentration of growth regulators increased.Plant susceptibility of callus formation is increased when the culture medium is enriched with auxins and cytokinins, when appropriate combinations, promote cell division and cell growth with increasing the production of important or necessary contents to sustain division and growth such as amino acids and proteins,

Table 1. Effect of PGRs concentrations on the percentage of callogenes is (%), fresh weight(mg) and dry weight (mg) of callus of yellow oleander (*T. neriifolia*) plant after five weeks of culture on MS medium

PGR*(µM)	Callogenesis (%)	Fresh weight (mg)	Dry weight (mg)
2,4-D			
4.52	90.0^{ab}	450.8^{c}	66.5^{bcde}
9.04	92.5^{ab}	512.8^{c}	45.0^{ghij}
13.57	85.0^{b}	572.3^{bc}	57.3^{cde}
18.09	82.5^{bc}	510.5^{c}	48.3^{fgh}
BA			
0.44	72.5^{c}	336.8^{efg}	26.5^{k}
0.89	90.0^{ab}	448.3^{bc}	67.8^{bcde}
1.33	95.0^{ab}	503.3^{b}	71.3^{abc}
1.77	92.5^{ab}	358.0^{d}	51.5^{efgh}
2,4-D +BA			
4.52+0.44	70.0^{c}	263.0^{g}	45.0^{hijk}
4.52+0.89	90.0^{ab}	508.0^{cd}	89.0^{ab}
4.52+1.33	100.0^{a}	964.0^{a}	98.0^{a}
4.52+1.77	100.0^{a}	468.0^{de}	74.0^{bcdef}
9.04+0.44	80.0^{bc}	397.0^{defg}	41.0^{ijk}
9.04+0.89	90.0^{ab}	540.0^{c}	61.0^{efgh}
9.04+1.33	100.0^{a}	609.0^{bc}	80.0^{bc}
9.04+1.77	100.0^{a}	305.0^{fg}	38.0^{ijk}
13.57+0.44	70.0^{c}	362.0^{defg}	31.0^{k}
13.57+0.89	90.0^{ab}	834.0^{ab}	87.0^{ab}
13.57+1.33	90.0^{ab}	550.0^{c}	89.0^{ab}
13.57+1.77	90.0^{ab}	443.0^{def}	62.0^{defgh}
18.09+0.44	70.0^{c}	325.0^{efg}	29.0^{k}
18.09+0.44	90.0^{ab}	511.0^{c}	74.0^{bcdef}
18.09+0.44	90.0^{ab}	490.0^{d}	58.0^{fghi}
19.09+0.44	80.0^{bc}	516.0^{c}	72.0^{bcdefg}
MS0(control)	0.00^{d}	0.00^{h}	0.00^{l}

*PGR: plant growth regulators. Means followed by similar letters in the same column do not differ significantly from each other according to Duncan test at p≤0.05.

thus leading to increase the callus biomass by increasing fresh and dry weights, while, high concentrations of growth regulators maybe toxic to explants and thus lead to undesirable results (Chavan *et al.*, 2014 and Al-Jibouri *et al.*, 2016). The results of the callus experiment are performed to adoption of the combination 4.52 µM 2,4-D + 1.33 µMBA in the growth and multiplication of callus to obtain sufficient amounts of callus for subsequent experiments.

Free radical scavenging activity of callus and leaves extracts usingthe DPPH method

The DPPH free radical scavenging activity is one of the widely used methods for evaluating the scavenging activity of phenolic compounds and plant extracts. This test uses DPPH, a stable radical with dark-purple color that changes to yellow when it is treated with antioxidant compounds that give it an electron or proton (Goupy *et al.*, 2003). The results showed that the standard compounds, callus and leaves extracts have the DPPH free radical scavenging activity in a way that is directly proportional to the increase in concentration (Fig. 1). The highest free radical scavenging activity means were recorded for standard compounds quercetin and gallic acid, callus and leaves extracts, which reached 90.2%, 89.2%, 94% and 87.6%, respectively at the highest concentration of 400µg.ml^{-1}. In addition, the results of the present study showed that the appropriate concentration of callus and leaves extracts, which scavenge 50% of DPPH were 68 and 97 µg.mL^{-1}, respectively. It is evident that the callus and leaves extracts contain secondary compounds possess the antioxidant activity and have a significant role in sustaining callus tissues and natural plant from damage by oxidative stress.

By comparing the DPPH free radical scavenging activity of callus and leaf extracts (Table 3), there was a significant superiority of the callus extract concentrations compared to the leaves extract with the exception of the concentration of 50 µg.l^{-1}. This may indicate that the callus contains more antioxidant compounds than the leaves. Besides, the presence of growth regulators in the growth medium of callus with appropriate conditions of temperature and light which was not only for the growth and multiplication of callus but also as practical elicitors for the biosynthesis of active secondary compounds in callus tissues (Khan *et al.*, 2018 and Ali *et al.*, 2019).

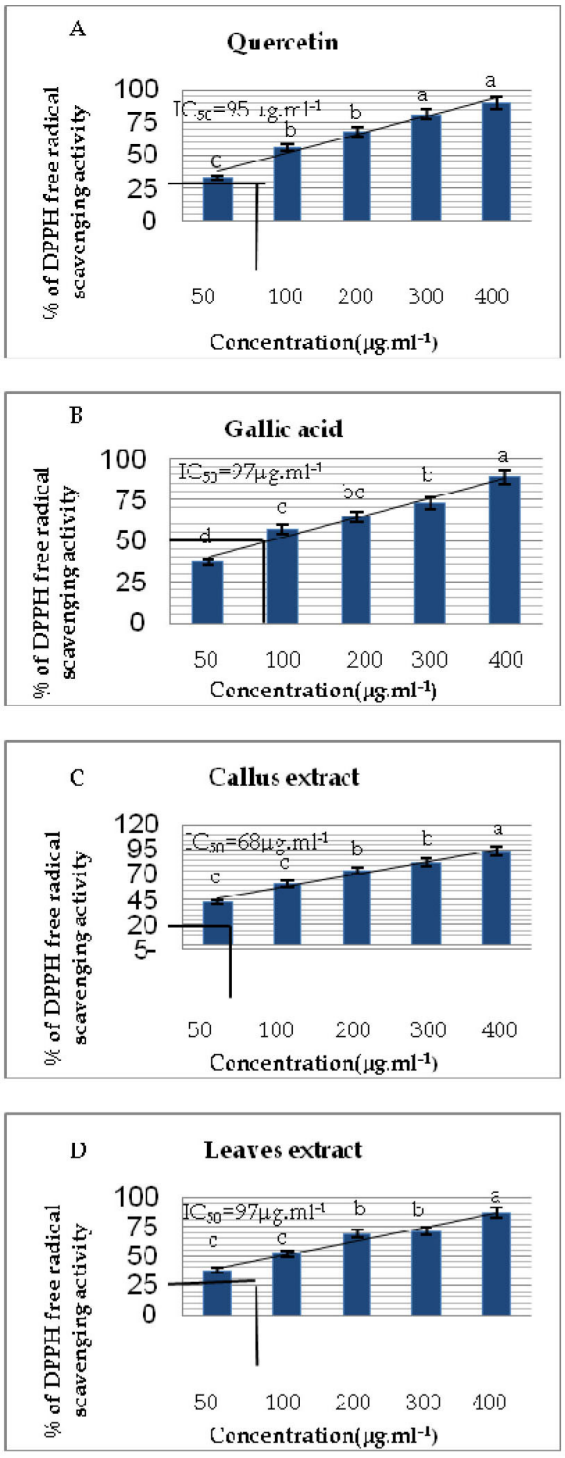

Fig. 1. Free radical scavenging activity using DPPH method of: (A) Quercetin, (B) Gallic acid, (C) Callus extract and (D) Leaves extract.

Secondary metabolites analyzed with GC/MS technique

The analysis of GC/MS of callus and leaves extracts revealed that there were 28 and 15 compounds respectively as shown in Table 4 and Fig. 2 with different retention times and relative contents. Among these compounds, 18 of them have antioxidant activity as well as their importance in treating many diseases. The most significant diagnosed compounds are vitamin E, Eicosane, Fucosterol, Oleic acid, Stigmasterol, β-Sitosterol, α and β-Amyrins. It is noticed that the compounds present in a callus extract are more than those in the leaves extract. Moreover, there were 3 unknown compounds that have not been diagnosed in the callus extract. The reason for increasing the content and composition of active compounds in the callus tissue compared to the natural plant may be due to the availability of appropriate conditions such as heat, light and nutrients in the culture medium, which increase produc-

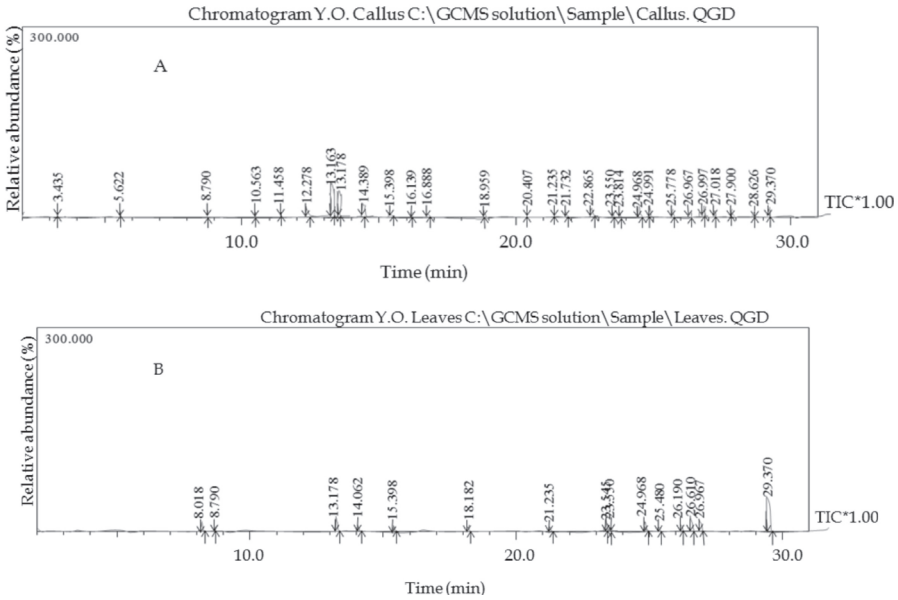

Fig. 2. The GC/MS chromatogram analysis of yellow oleander: (A) callus extract and (B)Leaves extract.

Table 2. Effect of different concentrations of quercetin, gallic acid, callus and leaves extracts on the percentage of DPPH free radical scavenging activity (%)

Concentration ($\mu g.ml^{-1}$)	Quercetin (%)	Gallic acid (%)	Callus extract (%)	Leaves extract (%)
50	33.0^{c}	37.2^{d}	40.3^{e}	38.0^{d}
100	56.3^{b}	57.2^{c}	61.2^{d}	51.7^{c}
200	68.6^{b}	65.0^{bc}	76.0^{c}	69.3^{b}
300	81.3^{a}	73.3^{b}	82.3^{b}	71.3^{b}
400	90.2^{a}	89.2^{a}	94.0^{a}	87.6^{a}

Means followed by similar letters in the same column do notdiffer significantly according to Duncan test at pd"0.05.

Table 3. Effect of extract type on DPPH free radical scavenging activity (%)

Extract type Concentrations ($\mu g.ml^{-1}$)	50	100	200	300	400
Callus (%)	40.3^{a}	61.2^{a}	76.0^{a}	82.3^{a}	94.0^{a}
Leaves (%)	38.0^{a}	51.7^{b}	69.3^{b}	71.3^{b}	87.6^{b}

Means in the same column followed bysimilar letters do notdiffer significantlyaccording to Duncan test at pd"0.05.

tion of those compounds in the callus (Mohy *et al.*, 2009). It can be deduced from the investigations of this study the importance of yellow oleanderas medicinal plant as a result of containing many active compounds, which are characterized by different types, structures and their ability in the treatment ofvarious diseases. Several researchers also recorded the importance of callus cultures as one of the important biotechnologies in the production of many effective phytochemicals from small explants within a controlled area throughout the year and without damaging the natural plant environment to obtain these compounds (Lystvan *et al.*, 2018; Salim, 2018 and Ali *et al.*, 2019).

Table 4. Secondary metabolites of yellow oleandercallus and leaves extractsanalyzed with GC/MS

Seq.	R.T.*	Compound Name	Callus extract (%)	Leaves extract (%)
1	3.435	Aziridine,2-methyl-	0.412	-
2	5.622	Phthalic acid,di(8-chloroactyl) ester	0.190	
3	8.018	9,12-Octadecanoic acid (z,z)-methyl ester	-	7.246
4	8.790	Oleic acid	0.498	0.318
5	10.563	Methadone N-oxide	0.150	-
6	11.458	Cyclobarbital	0.159	-
7	12.278	l2-O1eanen-3-y1 acetate,(3.a1pha.)-	12.313	-
8	13.163	Urs-l2-ene-3-o1, acetate,(3.beta.)	18.641	-
9	13.178	.alpha.-Amyrin	15.636	12.365
10	14.062	Cyclopenteno[4,3-b] tetra hydro Furan	-	2.837
11	14.389	Benzo(b)naphtha(1,2-d) furan	10.715	-
12	15.398	trans-Geranylgeraniol	1.046	2.101
13	16.139	Bendazol	0.292	-
14	16.888	(4-Oxo-4H-quinazolin-3-yl)-acetic acid, methyl ester	0.070	-
15	18.182	beta-Phenyl-propiolophenone	-	2.611
16	18.959	Lup-20(29)-en-3-o1, acetate,(3.beta.)-	2.765	-
17	20.407	3-N-Nitroso-solanocapsine	0.324	-
18	21.235	2,2-Dimethy1 propionic acid	0.253	1.521
19	21.732	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	-
20	22.865	Eicosane	5.492	-
21	23.545	Tetradecanoic acid	-	8.921
22	23.550	Vitamin E	1.831	0.413
23	23.814	2-Ethylacridine	0.995	-
24	24.968	(+)-trans-3,4-Dimethyl-2-phenyltetrahydro-1,4-thiazine	4.019	7.953
25	24.991	Unknown	5.206	-
26	25.480	Campesterol	-	1.611
27	25.778	Unknown	1.968	-
28	26.190	Stigmasterol	-	4.077
29	26.610	Fucosterol	-	11.285
30	26.967	beta-Sitosterol	2.942	10.920
31	26.997	Pyrro1o[2,3-b]indo1e,1,2,3,3a,8,8a-hexahydro-5-methoxy-3a,8-dimethy1-	7.291	-
32	27.018	Unknown	1.061	-
33	27.900	Taraxasterol	2.544	-
34	28.626	Bisphenol,bis(tert-buty1dimethy1si1y1) ether	0.358	-
35	29.370	beta-Amyrin	1.852	25.821
Total			100.00	100.00

* R.T. : Retention time(minute).

Conclusion

The results of the present study showed that the combination of 4.52 µM2,4-D + 1.33µM BA was the most successful in the initiation and multiplication of the callus from yellow oleander internodes. The callus extract has significantly proved to be effective in antioxidant activity through scavenging of free radicals compared with leaves extract of natural plant. The GC/MS analysis of callus and leaves extracts showed the presence of different active compounds with adequate antioxidant capacity as well as the compounds present in callus extract were more than that of the leaves extract. This enhances the importance of callus cultures of yellow oleander plant in the production and manufacture of many economically and medically important compounds at high rates in short periods of time within limited controlled areas and without disturbing the ecological balance of natural plants.

Acknowledgement

The researcher would like to thank the employees of the Environment and Water Research Center-Ministry of Science and Technology for facilitating the task in conducting biochemical tests and GC/MS analysis of callus and leaves samples in their laboratories.

References

Abbasi, B.H., Khan, M.A. and Mahmood, T. 2010. Shoot regeneration and free-radical scavenging activity in *Silybummarianum* L. *Plant Cell Tiss Organ Cult.* 101 : 371-376.

Adil, M., Ren, X. andJeong, B.R. 2019. Light elicited growth, antioxidant enzymes activities and production of medicinal compounds in callus culture of *Cnidium officinale* Makino. *J Photochem Photobio B Biol.* 196 :1-7.

Ali, A., Mohammed, S., Khan, M.A., Raja, N.I., Arif, M., Kamil, A. and Mashwani, Z.R. 2019. Silver nanoparticles elicited *in vitro* callus cultures for accumulation of biomass and secondary metabolites in *Carallumatuber culata*. *Arti Cells Nanomed Biotech.* 47 (1) : 715-724.

Chavan, J.J., Gaikwad, N.B., Umdale, S.D., Kshirsagar, P.R., Bhat, K.V. and Yadav, S.R. 2014. Efficiency of direct and indirect shoot organogenesis, molecular profiling, secondary metabolite production and antioxidant activity of micropropagated *Ceropegiasantapaui*. *Plant Growth Reg* 72:1-15.

Eba, A.I. 2005. *Isolation and Characterization of Bioactive Compounds from Suriname and Madagascar Flora. ÉÉ A Synthetic Approach to Lucilactaceae.* A PhD in Chemistry Dissertation Submitted to the Faculty of the Virginia Polytechnic Institute and State University Blacksburg, Virginia. Pp. 1-2.

Gen Stat, 2012. SAS/Stat User Guide for Personal Computer, SAS Institute Inane Cary, N.C. USA.

Goupi, P., Dufour, C., Loonis, M. and Dangles, O. 2003. Quantitative kinetic analysis of hydrogen transfer from dietary polyphenols to the DPPH radical. *J Agric Food Chem.* 51 : 615-622.

Joshi, S.G. 2000. Family Apocynaceae. In: Medicinal Plants, 1st ed. New Delhi, Oxford and IBH Publishing Company Pvt. Ltd. Pp.40-51.

Khan, M.A., Abbasi, B.H. and Ahmed, N. 2013. Effects of light regimes on *in vitro* seed germination and silymarin content in *Silybummarianum*. *Indus. Crop Prod.* 46 : 105-110.

Khan, T., Abbasi, B.H. and Khan, M.A. 2018. The interplay between light, plant growth regulators and elicitors on growth and secondary metabolism in cell cultures of *Fagoniaindica*. *J Photochem Photobiol B.* 185 : 153-160.

Lystvan, K., Kumorkiewicz, A., Szneler, E. and Wybraniec, S. 2018. Study on betalains in *Celosia cristata* Linn. Callus culture and identification of new malonylated amaranthine. *J Agric Food Chem.* 66 : 3870-3879.

Mohy, A., Khan, Z., Ahmed, M., Kashmiri, M.A., Yasmin, S. and Mazhar, H. 2009. Chemotaxonomic significance of flavonoids in *Solanumnigrum* complex. Department of Botany GC University, Katchery Road, Pakistan.

Salim, S.A. 2018. *In vitro* induction of callus from different explants of *Terminaliaarjuna* (Roxb.) Wight and Arn. and detection of its active secondary metabolites using GC-MS analysis. *Plant Arch.* 18 (2) : 2519-2527.

Save, S.A., Lokhande, R.S. and Chowdhary, A.S. 2015. *The vetiaperuviana*: the good luck tree. *Inn Pharmaceut Pharm J.* 3 (3) : 586-606.

Shanmuga, P.R., Elavarsi, A. and Parbha, D.S. 2015. *In vitro* studies on the effect of precursors for the production of secondary metabolites in *Ipomeapes-caprea* (L.) Br. *Adv Res J Med Clinic Sci.* 1 (2) : 28-32.

Tiwari, P., Kumar, B., Kumar, M., Kaur, G. and Kaur, H. 2011. Phytochemical screening and extraction: a review. *Int Pharm Sci.* 1 : 98-106.

Visentin, V., Prevot, D., Marti, L. and Carpene, C. 2003. Inhibition of rat fat cell lipolysis by mono amine oxidase and semicarbazide-sensitive amine oxidase substrates. *Eur J Pharmacol.* 466 (3) : 235-243.