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IN VITRO CULTURE AND EVALUATION SOME PHYTOCHEMICAL COMPOUNDS OF *ERIOCEPHALUS AFRICANUS* L. PLANT.

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ABSTRACT

The effect of some factors on growth and development of *Eriocephalus africanus* L. plant *in vitro*, showing that B5 basal medium gave the best results in most of growth measurements when culturing the shoot tips explants. 2.0 mg/l BAP recorded the highest values in survival percentage, shootlets number /cluster and shooter strength 93.33%, 16.50 and 4.50, respectively. Use the high level from GA3 4.0 mg/l in medium was more effect in the elongation of shootlets. In rooting stage the moderate concentration from IBA 0.5 mg/l was more effect at increased roots number/explant and root length to 8.67 and 5.78cm, respectively. Planting media containing Peatmoss, Sand and Vermiculite interaction at equal volume 1:1:1 v/v was recorded the best results in acclimatization of rooted plants. The phenolic composition from plants of *E. africanus* L. *in vitro* produced and *in vivo* grown was qualitatively and quantitatively analyzed by using High Performance Liquid Chromatography HPLC which identified compounds namely Gallic acid, Chlorogenic acid, Caffeic acid, Catechin, Quercetin and Kaempferol from *E. africanus*. The antioxidant activity by using DDPH Free radical scavenging were investigated *in vitro* produced plants and *in vivo* grown plants at outside the greenhouse (mother plants) the obtained 81.01% and 78.58 mg %, respectively.

KEYWORDS: *Eriocephalus africanus*, *in vitro* and *in vivo*, Acclimatization, antioxidant activity, Phenolic and Flavonoids and HPLC.

1- INTRODUCTION

Natural products of plant and animal origin offer a vast resource of newer medicinal agents with potential in clinical use some of this are believed to promote positive health and maintain organic resistance against infecting by re-establishing the body´s equilibrium and conditioning the body tissues.[1] Plant tissue culture is an innovative technique for enhanced production of valuable in drugs from medicinal plants.[2] *In vitro* propagation by tissue culture provided rapid propagation for selected genotypes of medicinal plants. This is due to rejuvenation which is only possible *in vitro*.[3] The genus *Eriocephalus*, native from South Africa and naturalized in the Mediterranean region, is a very large and diversified member of the family Asteraceae, comprises about 32 species.[4,5] *Eriocephalus africanus* L. is commonly known as wild or African rosemary.[6] It is the only species that has been introduced and cultivated in Egypt as an ornamental and nice smelling shrub. It is a small fast growing evergreen shrub, with green-grey foliage and snow white flowers of a distinctive fragrance that give rise to cottony seeds.[6,7] Infusions of the plant are used as diuretic and diaphoretic, as well as to treat gastrointestinal disorders, asthma, coughs, fever and painful conditions. Moreover, this plant has also been traditionally used as medicine for the treatment of inflammation and dermal complications.[7,8] Five broad classes of plant growth regulators are important in plant tissue culture: the auxins, cytokinins, gibberellins, abscisic acid and thidiazuron.[9,10] Plant growth regulators are one of the most important factors affecting cell growth, differentiation and metabolite formation in plant cell and tissue cultures.[11,12] The objective of this study was shoot formation from shoot tip explants *in vitro*, acclimatization and evaluation of antioxidant activity, total phenolic compounds and flavonoid contents of *Eriocephalus africanus* L.

2- MATERIAL AND METHODS

1- This work was carried out in Applied Research Center of Medicinal Plants (Tissue Culture and Phytochemistry Lab.), National Organization for Drug Control and Research (NODCAR) Giza, Egypt and tissue culture lab of Horticulture Department,

Faculty of Agriculture, Cairo University, Giza, Egypt, during the period of 2013 – 2016.

2- All chemicals, solvents and reagents used were of analytical and pure grade.
3- All standers were purchased from Sigma chemical Co., St. Lewis, USA.

Plant Material

The plant was kindly identified by Dr. Mohamed EL Jabali, head of Herbarium, Orman Botanical Garden and Ministry of Agriculture. Cairo, Egypt. A voucher herbarium specimen had been deposited in the herbarium of Applied Research for Medicinal Plant Center (NODCAR).

Terminal cuttings from mother stock plants (Six months old) were taken from field of the Applied Research Center of Medicinal Plants (ARCMP) and planted in controlled greenhouse at 27±1°C during 2-3 months and then placed *in vivo* outside of greenhouse (mother plant) which it considered as *in vivo* sample as in photo (1) and the well *in vitro* plants developed (shoots with roots) as in photo (3).

Photo (1) *In vivo* stock mother plants Photo (2) Flowers of *E.africanus* L Photo (3) *In vitro* plantlets of *E.africanus* L.

Tissue culture prepare

One type 1-1.25 cm in length shoot tip segments of explants source was used, explants kept in antioxidant solution 100 mg/l ascorbic acid + 100 mg/l citric acid + 100 mg/l poly vinyl pyrrolidone PVP for one hour and washed several time by tap water, then rinsed with a small amount of liquid soap 5% for 5 minutes to remove the assuring of most external contamination, and rinsed again under running tap water for 30 minutes to remove all the remaining detergent, after that the sterilization began under aseptic condition. This procedure and all the steps of the sterilization were done under complete aseptic condition in the laminar air flow. Explants were immersed in 95% ethanol for 2 sec. surface sterilization was in 15 ml Clorox (0.7875% NaOCl) + 85 ml distilled water for 15 minutes. After surface sterilization, approximately 2 mm was removed from cut ends of the explants and they were thrice washed with sterile distilled water for 10 min duration each. Explants were then kept for 10 min in 100 mg/l ascorbic acid + 100 mg/l citric acid + 100 mg/l poly vinyl pyrrolidone (PVP) solution. The sterile explants were planted in sterile jars 350 ml containing 40 ml of B5 basal medium supplemented with 30 gm/l sucrose and solidified by 5.0 gm/l agar. The essential chemicals used for preparing the media were stock solution. The pH value was adjusted to 5.7 -5.8 by adding suitable amount of 0.1 N HCI and 0.1 N KOH by using the pH meter prior to autoclaving at 1.3 kg/cm^2 for 20 minutes.

The work designing

1 – *In vitro* micropropagation

A- Effect of media type (MS, White's and B5) on survival percentage and growth of shoot tip explants. Shoot tip explants were cultured on different media type[13,14,15], individually to study their effect on survival percentage and growth (starting stage). Data were recorded after 6 weeks incubation period.

B- Effect of cytokinin benzyl amino purine (BAP) on the growth and development of shoots. The shoots were obtained from shoot tip explants which grown on B5 medium full salt strength in starting stage and were cutting into nodes stem containing two axillary buds (0.50-0.75 cm) and recultured on different concentrations of BAP by rates (0.0, 0.25, 0.50, 1.0, 2.0 and 4.0 mg/l) individually. Data were recorded after 6 weeks incubation period.

C- Effect of gibberellin (GA3) on the elongation of shootlets. The clusters (group from shootlets) were obtained from explants which grown on B5 medium full salt strength supplemented with the best concentration of cytokinin benzyl amino purine (BAP) at last experiment and recultured on B5 basal medium supplemented with different concentrations of gibberellin (GA3) by rates 0.0, 0.25, 0.50, 1.0, 2.0 and 4.0 mg/l) individually. Data were recorded after 6 weeks incubation period.

D- Effect of auxin Indole-3-butyric acid (IBA) on root formation of shoots *in vitro*. The plantlets were obtained from explants grown on B5 medium supplemented with the best concentration of GA3 in multiplication stage and recultured on different concentrations of auxin (IBA) by

rates 0.0, 0.25, 0.50, 1.00 and 2.0 mg/l individually was added to B5 basal medium+0.15% active charcoal. Data were recorded after 6 weeks incubation period.

E- Effect of growing media; peatmoss, sand and vermiculite on plants acclimatization. Harmony plants were obtained from planting on B5 medium supplemented with the best concentration from auxin in rooting stage were transferred to greenhouse in May month and they left inside jars at greenhouse for 10-14 days. Before culturing the plants were washed from agar in running water and soaked in 2 gm/l solution of fungicide (Benlate, 0.1%) for 5 min after that it planting in plastic containers (6 cm diameter and 6 cm height) which contain different mixture of soil as follow: Peatmoss, Sand and Vermiculite individually by equal volume from Peatmoss: Sand (1:1 v/v), Peatmoss: Vermiculite (1:1 v/v), Sand: Vermiculite (1:1 v/v) and Peatmoss: Sand: Vermiculite (1:1:1 v/v). Data were recorded after three months incubation period in greenhouse.

Growth measurement

Survival %, Shoots number/explant, Shoot length cm/explant, Leaf number/explant, Plant strength, Rooting %, Roots number/explant and Root length cm/explant. Plant strength was estimated (as score) and presented as follow according the method described by Pattino[16], (a) negative growth result = 1 (b) below average growth = 2 (c) average growth = 3 (d) above average growth = 4 (e) excellent growth = 5.

2 – Phytochemical analysis

1.1. Qualitatively and quantitatively determination of phenolic constituents *in vitro* and *in vivo* of *Eriocephalus africanus* L. By using HPLC.

The phenolic composition from plants of *E. africanus* L. *in vitro* produced and *in vivo* grown was qualitatively and quantitatively analyzed by using High Performance Liquid Chromatography HPLC. Phenolic acids and flavonoids were determined in the hydrolyzed-extract adopting the procedure described by Mattila et. al,[17] The analysis was performed using an Hewlett Packard HPLC system (Agilent, USA) constructed of a quaternary HP 1260 pump and HP 1260 series multiple wave length detector. Separation was done by a Thermo column, C18 and C8, 5µm (4.6 x 250 mm), Germany. Isocratic elution was employed using a mixture of acetonitrile-1.5% acetic acid (40: 60 v/v) as mobile phase for the flavonoids and (20: 80 v/v) for the phenolic acids. The flow rate of the mobile phase was 1 ml/min and the injection volumes were 20 µl of the standards and sample extracts. Detection was carried out by setting the UV detector at 270 nm for the phenolic acids and Catechin while the flavonoids were measured at 330 nm. Stock standards and samples were analyzed in triplicate. Concentration of 100 mg/ml sample solution was used while Gallic acid and Kaemperol concentrations of 0.005 to 0.1 mg/ml, Chlorogenic 0.4 to 4.0 mg/ml, Caffeic acids and Quercetin concentrations of 0.01 to 0.2 mg/ml, Catachin 1.0 to 8.0 mg/ml were used to obtain a calibration curve.

1.2. Determination of antioxidant activity *in vitro* and *in vivo* of *Eriocephalus africanus* L.

Free radical scavenging capacity was measured using the DPPH assay, according to Mensor et. al,[18] method but at different concentrations ranged 0.8, 0.16 and 0.032 mg/ml. DPPH (0.3mM, 1.0 ml) in methanol was added to 2.5 ml solution of the extract or standard and allowed to stand at room temperature in a dark chamber for 30 min. and the decrease in absorbance was spectrophotometrically measured at 515 nm. (UV-spectrophotometer BOECO S-20 Germany) The control sample was carried out at the same conditions but we will add 2.5 ml methanol instead of sample. All determinations were carried out in triplicate. The absorbance (Abs.) of each sample was converted to percentage antioxidant activity (AA %) or inhibition percentage of DPPH activity (IP %) using the formula:

AA% = 100 – [(Abs. of sample – Abs. of blank) × 100] / Abs. of control.

Statistical analysis

Data of all experiments were statistically analyzed by one way randomized blocks of variance (ANOVA) using Costat 6311Win and the mean values were compared using the L.S.D method at 5% level of significance.[19]

3- RESULTS AND DISCUSSION

1– *In vitro* micropropagation

A- Effect of media type (MS, White's and B5) on survival percentage and growth of shoot tip explants.

The presented data in **Table (1)** showed that, the highest value of survival percentage (100%) had been recorded when shoot tip explants cultured on B5 medium followed by MS medium where recorded (88.46%) and White's medium recorded (80.77%). The maximum of significant response in shoots number/explant, shoot length cm/explant and leaf number/explant were achieved in B5 medium (5.38, 2.75 cm and 8.56, respectively) while White's medium was recorded the lowest values (1.77, 1.86 cm and 7.11, respectively). B5 medium seemed to be the most suitable medium for tissue culture of *Echinacea purpurea*.[20] 3/4 B5 salt strength had significant effect on the growth and development on shoot tips of *Artemisia dracunculus* L.[21]

Table (1): Effect of media type on survival percentage and growth of shoot tip explants after 6 weeks from culturing *in vitro*.

Type of media	Survival %	Shoots number /explant	Shoot length (cm)	Leaf number /explants
MS	88.46	5.01	2.14	7.72
White's	80.77	1.77	1.86	7.11
B5	100	5.38	2.75	8.56
L.S.D at 5%	-	**3.00**	**0.2623**	**0.5630**

B- Effect of cytokinin benzyl amino purine (BAP) on the growth and development of shoots.

The effect of cytokinin benzyl amino purine BAP on growth response were showed in **Table (2)** B5 medium supplemented with the high concentration from BAP 2.0 and 4.0 mg/l gave the highest value of survival percentage 93.33% for each, whereas B5 (control) and B5 medium supplemented with the low concentration from BAP 0.25 mg/l gave the lowest value 66.67%. Shootlets number/cluster and shootlet strength was significantly affected by using the concentration of 2.0 mg/l BAP (16.50 and 4.50, respectively), whereas the B5 (control) gave the smallest number (1.70) and the shootlet strength recorded smallest value (3.00) with increase the BAP concentration from 0.25 to 1.0 mg/l. B5 (control) without any growth regulators recorded the highest values for shootlet length and leaf number (1.94 cm and 9.22, respectively), while the high concentration of BAP 4.0 mg/l recorded the lowest values (0.32 cm and 4.97, respectively). Cytokinins are often used to stimulate growth and development; kinetin and BAP being in common use. They usually promote cell division, especially if added together with an auxin. In higher concentrations (1-10 mg I-I) they can induce adventitious shoot formation, but root formation is generally inhibited. They promote axillary shoot formation by decreasing apical dominance and they retard ageing.[22,23]

Table (2): Effect of different concentrations of cytokinin (BAP) on the growth and development of shootlets cultured on B5 medium.

Treatment (BAP mg/l)	Survival %	Shootlets number / cluster	Shootlet length cm	Leaf number / shotlet	Shootlet strength
B5 Control	66.67	1.70	1.94	9.22	3.40
0.25	66.67	6.70	1.23	8.30	3.00
0.50	80	9.33	0.90	6.42	3.00
1.0	83.33	9.83	0.53	5.96	3.00
2.0	93.33	16.50	0.74	7.11	4.50
4.0	93.33	14.00	0.32	4.97	4.00
L.S.D at 5%	-	**0.4710**	**0.1970**	**0.4450**	**0.3510**

C- Effect of Gibberellin (GA3) on the elongation of shootlets.

The presented data in **Table (3)** show that survival and rooting percentage were affected by using the concentrations of gibberellin GA3. The treatments of 0.25, 0.50 and 4.0 mg/l GA3 recorded the highest values with mean of survival percentage (100%) for each, while 1.0 mg/l GA3 recorded the least value (66.67%). Plantlets number, plantlet length cm and plantlet strength increased gradually with increase the GA3 concentrations from 0.25 to 4.0 mg/l where the high concentration of GA3 4.0 mg/l gave the highest significant values (12.50, 3.88cm and 3.84, respectively), whereas the low concentration of GA3 0.25 mg/l gave the lowest significant values (3.600.80cm and 2.22, respectively), at the same time B5 (control) without any growth regulators and the maximum concentration of GA3 4.0 mg/l recorded the biggest number of leaf (11.20 and 10.62, respectively), while the minimum concentration of GA3 0.25 mg/l recorded the smallest number (5.30). Also the data showed that increase the GA3 concentrations from 0.25 to 4.0 mg/l led to increased rooting percentage and root length where that the maximum concentration of GA3 4.0 mg/l recorded the highest values (83.33% and 1.90cm, respectively), whereas the lowest values (16.70% and 0.67cm, respectively) was recorded with the minimum concentration of GA3 0.25 mg/l. On other hand, the high concentration of GA3 4.0 mg/l recorded the biggest number of roots (6.33) while the low concentration of GA3 0.25 mg/l recorded the smallest number of roots (0.70). In micropropagation, auxins and cytokinins are by far the most important for regulating growth and morphogenesis[24], while gibberellins have been used in some instances to promote shoot elongation.[25]

Table (3): Effect of different concentrations of Gibberellin (GA3) on the elongation of shootlets cultured on B5 medium.

Treatment (GA3 mg/l)	Survival %	Plantlets number	Plantlet length (cm)	Leaf number	Plantlet strength	Rooting %	Roots number	Root length (cm)
B5 Control	85.71	6.20	0.80	11.20	2.92	50	1.20	0.67
0.25	100	3.60	1.00	5.30	2.22	16.70	0.70	0.67
0.50	100	4.10	1.20	6.70	2.30	33.33	3.00	1.14
1.0	66.67	4.70	1.81	8.60	2.33	37.50	3.00	1.15
2.0	83.33	7.40	2.63	9.86	3.45	70	2.83	1.57
4.0	100	12.50	3.88	10.62	3.84	83.33	6.33	1.90
L.S.D at 5%	-	**0.4792**	**0.1838**	**0.4654**	**0.3581**	-	**0.2547**	**0.3034**

D- Effect of auxin Indole-3-butyric acid (IBA) on root formation of shoots *in vitro* cultured on B5 medium.

Data of the effect IBA on the survival and rooting percentage are showed in **Table (4)**. The best survival and rooting percentage (100%) was recorded with B5 control without any growth regulators and the moderate concentrations (0.50 and 1.0 mg/l IBA) except B5 control without any growth regulators which recorded (82.86%) for rooting percentage but the maximum concentration of IBA at 2.0 mg/l recorded the lowest values for both survival, rooting percentage and root length (40% and 2.27cm/explant). While the concentration 0.50 mg/l IBA recorded the highest values of measurements; shoot length cm, roots number, root length cm/explant and plant strength (4.58 cm, 8.67, 5.78cm and 4.0, respectively) compared with B5 control without any growth regulators and minimum concentration of IBA at 0.25 mg/l which recorded the lowest values of plant strength (2.56 and 2.63, respectively) as well as the lowest value of shoots number/explant (1.91) was recorded with 0.5 mg/l IBA. The highest percentage of rooting explants (84.62±10.42) and mean number of roots (5.25 ± 1.25) of *Tulbaghia ludwigiana* at eight weeks was obtained in the presence of 2.5 µM IBA.[26] For rooting the regenerated shoots of *Holmskioldia sanguinea* one eighth strength MS medium supplemented with 2.0 mg/l IBA was the effective and economic medium.[27]

Table (4): Effect of different concentrations of auxin Indole – 3 - butyric acid (IBA) on the growth and development of *Eriocephalus africanus* L. cultured *in vitro* after 6 weeks from culturing.

Treatment (IBA mg/l)	Survival %	Shoot length cm/explant	Root %	Roots number /explant	Root length cm/explants	Plant strength
B5 Control	100	3.27	82.86	3.78	5.08	2.56
0.25	80	2.98	70	5.75	3.51	2.63
0.5	100	5.35	100	8.67	5.78	4.00
1.0	100	4.29	100	5.33	4.76	2.87
2.0	40	4.58	40	5.76	2.27	3.30
LSD at 0.05	-	**0.2750**	-	**0.4035**	**0.4601**	**0.2317**

E - Effect of growing media; peatmoss, sand and vermiculite on plants acclimatization.

The effect of growing media (peatmoss, sand and vermiculite) on plants acclimatization are presented in **Table (5)**. The soil medium which consist of peat: sand: vermiculite by equal volume (1:1:1 v/v), peat: sand (1:1 v/v) and sand: vermiculite (1:1 v/v) gave the best results in the all measurements (survival %, shoot length, root length and plant strength), the soil medium from both the peat and sand individually no significant effect in the all measurements and the recorded values were zero. The data also indicated that the treatments of peat: sand: vermiculite (1:1:1 v/v) and peat: sand (1:1 v/v) recorded the highest values of all measurements; survival percentage (85.71% and 78.57%, respectively), shoot length cm/explant (9.83 and 9.66cm, respectively), root length cm/explant (8.60 and 5.90cm, respectively) and plant strength/explant (4.50 and 4.0, respectively). While the treatment vermiculite individually recorded the lowest values of survival percentage and shoot length cm/explant (53.57% and 5.72cm, respectively) and the treatment peat: vermiculite (1:1 v/v) recorded the lowest values of root length cm and plant strength/explant (3.10cm and 1.20, respectively). The rooted shootlets of *Philodendron domesticum* were established in trays containing peatmoss, sand and vermiculite (1:1:1 v/v).[28] The maximum survival rate of rooted shoots of *Echinacea spp* was recorded after transplantation of them to a small plastic pots containing moist sand: peatmoss: vermiculite (1:1:1 v/v) under 30% of day light and 25˚C.[29]

Table (5): Effect of different types of transplanting media on the growth and development of *Eriocephalus africanus* L. after 8 weeks from culturing in greenhouse.

Treatments	Survival %	Shoot length cm/explant	Root length cm/explant	Plant strength
Peatmoss	0.0	0.0	0.0	0.0
Sand	0.0	0.0	0.0	0.0
Vermiculite	53.57	5.72	4.80	2.33
P : S (1:1 v/v)	78.57	9.66	5.90	4.00
P : V (1:1 v/v)	63.10	7.20	3.10	1.20
S : V (1:1 v/v)	71.43	8.93	5.30	3.94
P : S : V (1:1:1 v/v)	85.71	9.83	8.60	4.50
L.S.D. at 5%	-	**0.7527**	**0.4544**	**0.4430**

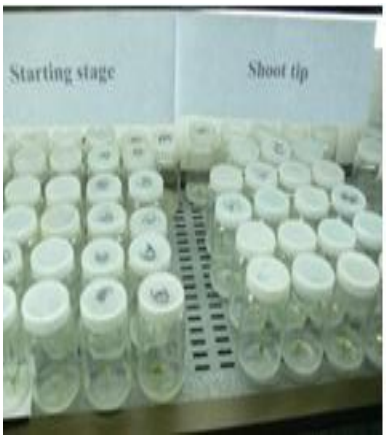
Mother plants in the field (Six months old)

Cutting from mother plants and planted in greenhouse

One month

Tow months

Three months



A- Starting stage

B&C- Multiplication stage

D- Rooting stage

E- Acclimatization stage Photo.

(4) Growth and development of *Eriocephalus africanus* L.

2 – Phytochemical analysis

***2.1.* Qualitatively and quantitatively determination of phenolic constituents *in vitro* and *in vivo* samples of *Eriocephalus africanus* L.**

The results of HPLC analysis of the hydrolyzed methanol extract *in vitro* and *in vivo* at outside the greenhouse samples of *E. africanus* L. **Tables (6 & 7)** and **Figures. (1 & 2 & 6 & 7)** allowed the identification of three phenolic acids and three flavonoid aglycones of the hydrolyzed methanol extract *in vitro* and *in vivo* samples of *E. africanus* L. major identified phenolic acids was chlorogenic acid and major identified flavonoid aglycones was catechin. Also *in vitro* produced plants showed the high content of phenolic acids and flavonoids that was identified. It is noteworthy to mention that this is the first report on containment the plants produced *in vitro* of *Eriocephalus africanus* L. on the phenolic acids and flavonoids.

From the data in **Figures. (3, 4 & 5)** the standard calibration curves of phenolic acids as Gallic acid is a linear straight line expressed by the equation y = 33358x – 92.342, R^2 =0.9989, Chlorogenic acid y = 10506x + 371.32, R^2 =0.9969 and Caffeic acid y = 29419x + 26.687, R^2 =0.9992, where x was represented the absorbance of HPLC and y was the phenolic acid concentration. The presented data in **Table (6)** & **Figures. (1&2)** show that the sample extract from *in vitro* produced plants showed that the highest content of Gallic acid, Chlorogenic acid and Caffeic acid (0.030, 3.600 and 0.054 mg/100 mg d.w, respectively) while the lowest content recorded (0.023, 1.077 and 0.035 mg/100 mg d.w, respectively) with the sample extract from *in vivo* grown plants. And when we identified the total phenolic acids we found that it reached to 3.665 mg/100 mg in the sample extract from *in vitro* produced plants compared with sample extract from *in vivo* grown plants which was reached to 1.135 mg/100 mg.

Table (6). Phenolic constituents identified by HPLC in the hydrolyzed extracts of the all plant parts *in vitro* and aerial parts *in vivo* of *Eriocephalus africanus* L.

Rt *	Constituents	Concentration % (mg/100 mg d.w)	
		In vitro	***In vivo***
3.2	Gallic acid	0.030	0.023
3.5	Chlorogenic acid	3.600	1.077
5.3	Caffeic acid	0.054	0.035
	Total identified phenolic acids	**3.665**	**1.135**
Rt * : **Retention time**			

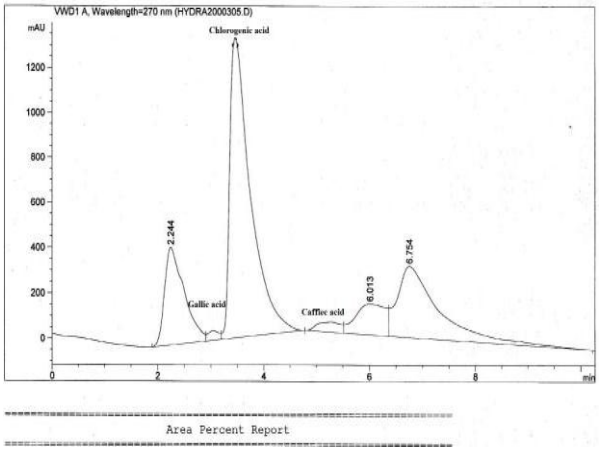


Fig. (1): HPLC chromatogram of Phenolic compounds in extract *in vitro* sample.

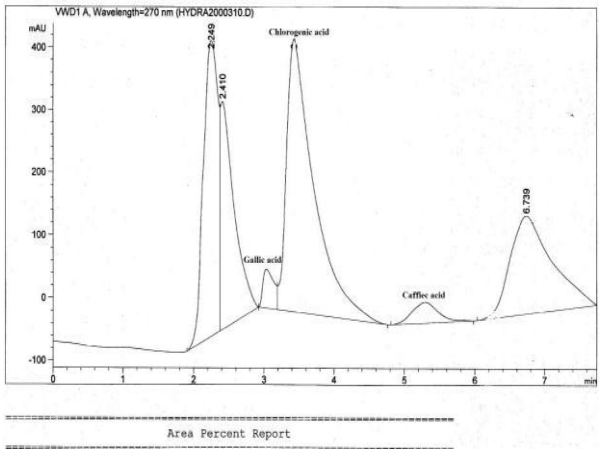


Figure. (2): HPLC chromatogram of Phenolic compounds in extract *in vivo* sample.

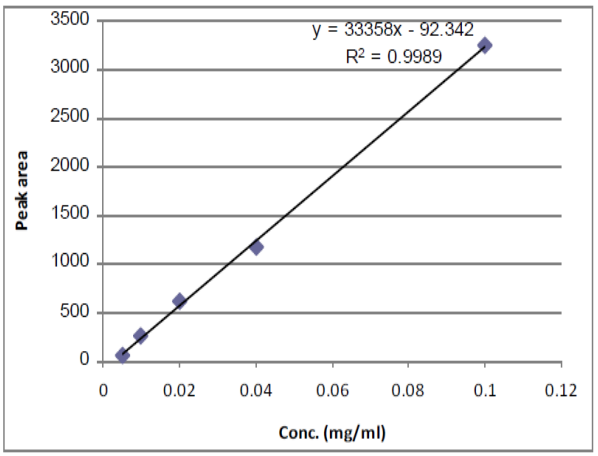


Figure. (3): Standard calibration curve of Gallic acid by HPLC.

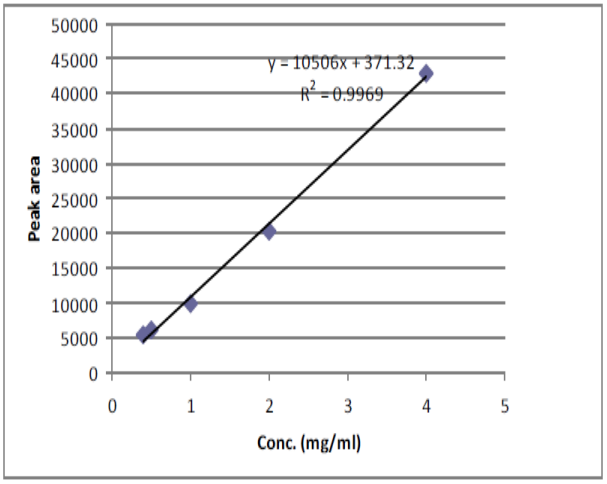


Figure. (4): Standard calibration curve of Chlorogenic acid by HPLC.

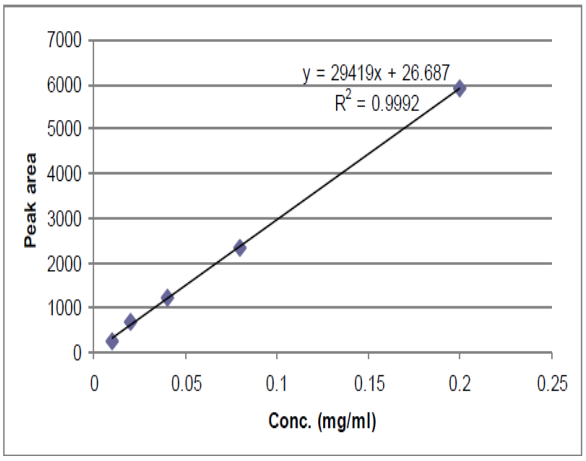


Figure. (5): Standard calibration curve of Caffeic acid by HPLC.

2.1.2. Quantification of flavonoids constituents *in vitro* and *in vivo* of *Eriocephalus africanus* L.

From the data in **Figures. (8, 9&10)** the standard calibration curves of flavonoids contents as catechin is a linear straight line expressed by the equation y = 46224x +3616.1, R^2 =0.9879, Quercetin y = 16569x - 10.292, R^2 =0.9986 and Kaempferol y = 14444x - 27.501, R^2 =0.998, where x was represented the absorbance of HPLC and y was the flavonoid concentration. The presented data in **Table (7)** & **Figures. (6&7)** show that the sample extract from *in vitro* produced plants showed the highest content of Catechin (5.095 mg/100 mg d.w) and lowest content of Quercetin and Kaempferol (0.013 and 0.010 mg/100 mg d.w). While the sample extract from *in vivo* grown plants recorded the lowest content of Catechin (1.655 mg/100 mg d.w) and recorded the highest content of Quercetin and Kaempferol (0.070 and 0.062 mg/100 mg d.w) but it was observed that the high amount of total flavonoids reached to 5.118 mg/100 mg in sample extract from *in vitro* produced plants compared with sample extract from *in vivo* grown plants which reached to 1.787 mg/100 mg d.w Dawoud et. Al.[30]

Table (7). Flavonoid constituents identified by HPLC in the hydrolyzed extracts of the all plant *in vitro* and aerial parts *in vivo* of *Eriocephalus africanus* L.

Rt *	Constituents	Concentration % (mg/100 mg d.w)	
		In vitro	*In vivo*
2.3	Catechin	5.095	1.655
4.3	Quercetin	0.013	0.070
6.5	Kaempferol	0.010	0.062
	Total identified flavonoids	**5.118**	**1.787**
Rt *: Retention time.			

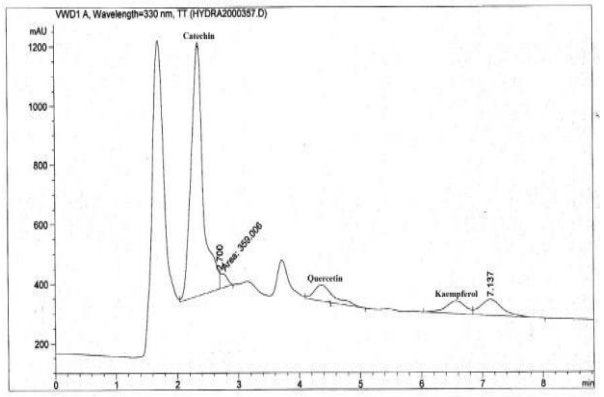


Figure. (6): HPLC chromatogram of Flavonoid compounds in extract *in vitro* sample.

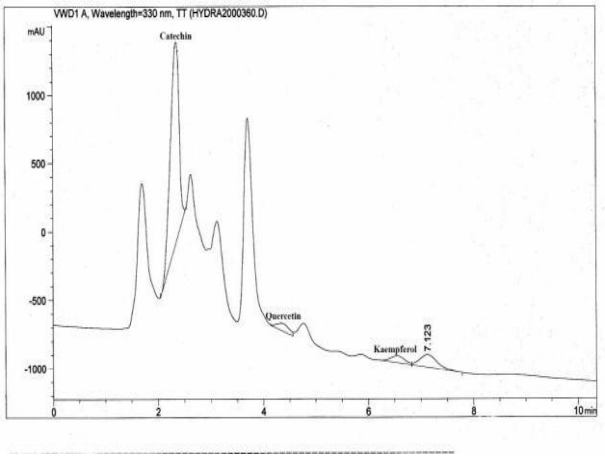


Figure. (7): HPLC chromatogram of Flavonoid compounds in extract *in vivo* sample.

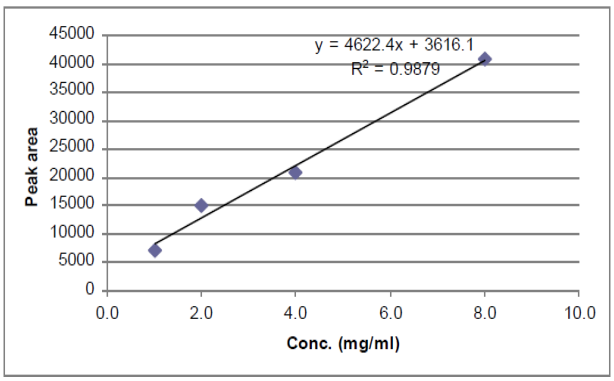


Figure. (8): Standard calibration curve of Catechin by HPLC.

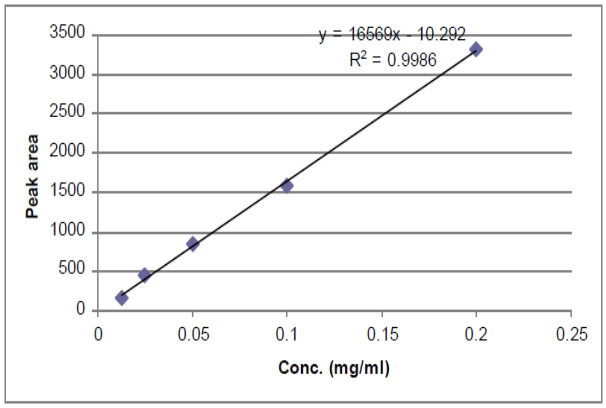


Figure. (9): Standard calibration curve of Quercetin by HPLC.

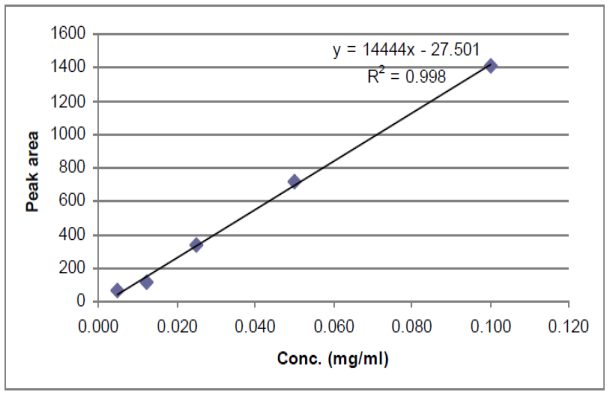


Figure. (10): Standard calibration curve of Kaempferol by HPLC.

2.2. Determination of antioxidant activity *in vitro* and *in vivo* samples of *Eriocephalus africanus* L. extracts using UV- spectrophotometer.

From the results in **Table (8)** & showed that, the antioxidant activity (free radical scavenging) were investigated *in vitro* produced plants was higher than *in vivo* grown plants at outside the greenhouse (mother plants) was 81.01% and 78.58% respectively. The antioxidant activity, total phenolic compounds and flavonoid contents of *Myrtus communis* L. parameters were 82%, 112 µg% and 125 µg %, respectively with the extract of *in vitro* leaves while the extract of *in vivo* leaves (mother plants) showed 75%,102 µg % and 114 µg %, respectively.[31]

Table (8): DPPH radical scavenging (antioxidant activity) in different extracts of the studied plants as percentage.

Name of extract	Free radical scavenging (antioxidant activity) %
In vitro – produced plant	81.01
In vivo – grown plant (mother plants)	78.58

3- CONCLUSION

The data obtained in this study indicate that, add 2.0 mg/l BAP to B5 basal medium was the most suitable to improve the growth and development of shoot tips explants *in vitro* for *E.africanus* L. also use 4.0 mg/l from GA3 in medium was more effect in the elongation of shootlets, at the same time B5 basal medium supplemented with 0.5 mg/l IBA led to increased roots number/explant and root length, on the other hand planting media containing peatmoss; sand and vermiculite interaction at equal volume 1:1:1 v/v was an important for *E.africanus* L. acclimatization as well as *E.africanus* L. The phenolic composition of hydroethanolic extracts from *E. africanus* was described here, by means of HPLC analysis.

Therefore the described compounds, namely Gallic acid, Chlorogenic acid, Caffeic acid, Catechin, Quercetin and Kaempferol were identified in *E. africanus*. Both extracts showed potent antioxidant capacities, as measured in DPPH scavenging, variations could be found in between the extracts. Correlation of biological activity with the quantitative data of phenolic compounds suggested that the phenolic composition of hydroethanolic extracts might be particularly associated with the antioxidant activity of the extracts occurring through the electron donation mechanism.

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