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EFFECT OF LIGHT INTENSITY ON SOME SECONDARY METABOLITES OF *ARTEMISIA ABROTANUM* L. BY TISSUE CULTURE TECHNIQUE.

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ABSTRACT

The effect of light intensity on plant growth *of Artemisia abrotanu*m L. was investigated *in vitro*. Plantlets of *Artemisia abrotanu*m L. with leaves of 1 cm. long were cultured on suitable medium under different light intensities of 300, 600, 1500, 3000, and 6000 lux, the environment for all growth *in vitro* was maintained at 25° ± 2°C, with a 16-h photoperiod. The number of shoots, roots, length of shoots and roots in 21 days of culture of plantlets, the collected data were analyzed by one way ANOVA followed by Tuky's HSD test values represent the mean ±SD of eight replicates and all experiments were repeated three times, mean difference is significant at the 0.05 level, the best result were found at 3000 lux and then 1500 lux on the other hand the least *in vitro* growth were in 300 lux. Preliminary phytochemical screening of *in vivo* and *in vitro* for *Artemisia abrotanum* L. showed that both samples were contains volatile oil, Carbohydrates and/or glycosides , tannins, flavonoid**s**, sterols and/or triterpenes, resins, while the *in vivo* sample contain traces of Alkaloid. But both samples free from Saponins, Iridoids, Cardiac glycosides and Anthraquinone glycosides. The total polyphenols and flavonoid**s** were investigated from obtained results *in vivo* plant sample showed the highest content of polyphenols (271.6mg/100g.d.w) and flavonoids (84.63 mg/100g.d.w) while the lowest content was recorded with the sample extract in 300 lux light intensity condition was (54.90 mg/100g.d.w) and (21.33 mg/100g.d.w).

KEYWORDS: *Artemisia abrotanu*m L., Light intensity. Phenolic compounds, Flavonoids and Secondary metabolites.

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] It helps in multiplying superior genotypes several times faster than normal one.[3] Family *asteraceae* is characterized by extreme bitterness of all parts of the plant.[4,5,6] Its cultivation has expanded from its center of origin (China) to Nigeria in response to the call by the World Health Organization for the use of Artemisinin-Combination Therapies (ACT) for treating malaria fever.[7] Three common derivatives found in Artemisia include Artesunate, Artemether and Artemisinin.[8, 9, 10] Genus Artemisia is the largest genus comprising 400 species widely distributed in South Africa and South America, and 34 species are found in India.[11] Some of them are source *of* volatile oil, almost all species consists of sequiterpene lactones.[12] Species *Artemisia* wide spread through out the world, are one of the most popular plants in Chinese traditional preparations and are frequently used for the treatment of diseases such as malaria, hepatitis, cancer, inflammation and infections of fungi, bacteria and viruses.[13] Some *Artemisia* species are used as stomachic, stimulant, flavoring, anthelminthic, antibacterial, antiinflammatory, antispasmodic, carminative.[14] *Artemisia abrotanum* L., belong to the Artemisia genus and it is used in traditional medicine for the treatment of a variety of illnesses.[15] It has also been used against cancer, cough, fever and tumors[16,17,18] *Artemisia abrotanum* L. has been screened for various pharmacological activities such as anti-inflammatory, expectorant and spasmolytic.[19] Plant tissue culture is an innovative technique for enhanced production of valuable drugs from medicinal plants.[20] It helps in multiplying superior genotypes several times

faster than normal one.[21] *In vitro* micro propagation and organogenesis of various Artemisia species have been previously established by using several explants in order to produce large number of plants, such as *A. annua*.[22, 23] *In vitro* micro propagation technique provides many advantages over conventional propagation methods.[24] There is physical and chemicals factors affecting on the micropropogation of the plant through tissue culture.[25] Light is more complicated physical factor was affecting on plant growth and development day length, intensity of light and source are known to effect on plant morphogenesis.[26] However, the induction of differentiation in cell and tissue may necessitate illumination.[27] Light effects may be subdivided into photoperiod, light intensity and wave length.[28] Optimum light for cultured tissue in stage I (Establishment aseptic culture) or stage II (Multiplication of propagule) was around 1000 Lux. However, light intensity between 3000 and 10.000 Lux was required for stage III, (Preparation of reestablishment of plants in soil). The higher light intensities improved the survival of plants which transferred to the soil, light intensity has shown an effected grown type in the culture.[29] *In vitro* plant growth light intensity play a significant role not only on dry weight accumulation but also on plant height, leaf number, leaf shape and leaf area.[30] The Plants are rich sources of phenolic, which are molecules that formed as secondary products and can act as antioxidants to prevent heart disease.[31,32.33] Reduce inflammation.[34,35,36] Lower the incidence of cancers.[37,38,39,40] And diabetes[41,42] as well as reduce rates of mutagenesis in human cells[43,44,45] Available reports tend to show that secondary metabolites of phenolic nature including flavonoids are responsible for the variety of pharmacological activities.[46,47]

The objective of this study was determination the total polyphenols and flavonoids, which may find a relationship between the content of these biologically active constituents and the *in vitro* micro propagation of *A. abrotanum* L., under different light intensity the experiments of the present work were divided into two parts.

Part 1: *In vitro* micro propagation of the plant under investigation.

Part 2: Chemical analysis (phytochemical screening and spectrophotometric determination of total polyphenol and flavonoids of the plant under investigation).

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, during the period of 2013 – 2016.

2-All chemicals, solvents and reagents used were of analytical and pure grade.

3-Quercetin and Gallic Acid standers were purchased from Sigma chemical Co., St. Lewis, USA.

4-Plant Material: 4-1-The mother plant of *Artemisia abrotanum* L. which was cultivated in green house of Tissue Culture of (NODCAR) and it considered as *in vivo* sample as in photo (1)

Photo (1) *in vivo* stock mother plant.

Photo (2) *In vitro* plantlet of *A. abrotanum*.

4-2- And the well *in vitro* plants developed (shoots with roots) as in photo (2). Were washed thoroughly with running tap water to remove the traces of agar. Then transferred to air dried room and also in *vivo* plants until completely dry and then crushed with mortar then kept each sample in dark bottle and keep in refrigerator until phytochemical analysis.

2.1: *In vitro* micro propagation of *A. abrotanum* L., under different light intensity condition.

2.1.1 -Source of explants

Experiments were carried out on *Artemisia abrotanum* L. which was cultivated in Genetic Engineering and Research Institute (G.E.B.R.I), Sadat city University, Egypt and then cultivated in green house of Tissue Culture of (NODCAR). The mother plant were authenticated by prof. Dr. Abd EL Halim Abd El Motagaly Professor of Taxonomy, Department of Taxonomy &Flora, Agricultural Museum, Doky, Giza, Egypt. A voucher herbarium specimen had been deposited in the herbarium of Applied Research for Medicinal Plant Center (NODCAR).

2.1.2. Excision and maintenance of explants

Explants (shoot tips, auxiliary buds) were kept in anti-oxidant solution (100 mg/l ascorbic acid +100 mg/l citric acid) for 15 min, then were rinsed with a small amount of liquid soap and water for 15 minutes for assuring the removal of most external contamination, and rinsed again under running tap water for 30 minutes to remove all the remaining detergent, after that the explants were immersed in 20% (w/v) sodium hypochlorite (NaOCl) for 15 min (bleach or a disinfectant solution for household use can be used). Then the samples rinsed twice with distal water. After that the explants were dipped again in anti-oxidant solution (100 mg/l ascorbic acid +100 mg/l citric acid) for5min. Surface sterilization began under aseptic condition in laminar air flow cabinet .The explants were resized to 1.00 to 1.50cm long and inoculated on to the culture medium.

2.1.3 Culture medium

All the media used in this study were based on woody plant medium [46]. Supplemented with3% of sucrose .The pH of the media was adjusted to 5.8 and fortified with combination of growth plant hormones 6-benzylamino purine (BAP) 0.5-mg/L + αNaphthalene acetic acid (NAA) 0.1mg /L to study their response on multiplication of shoots and rooting under different light intensity in 300, 600, 1500, 3000, and 6000 lux.

2.1.4 -Initiation

The axillary buds and shoot tips explants of *Artemisia abrotanum L.* were inoculated on the culture medium under the aseptic condition. All these cultures were incubated under the temperature of 25±20C and different fluorescent light intensity from, 300, 600, 1500, 3000 and 6000 lux-.The cultures were maintained with the photoperiod regime of 16 hr. light and 8 hr. dark. The number of shoots, roots, length of shoots and roots in 21 days of culture were recorded based on the periodical observation. The cultures were observed on daily basis and number of shoots, roots and length of shoots, roots were recorded. The collected data were analyzed by one way ANOVA followed by Tuky's HSD test values represent the mean ±SD of eight replicates and all experiments were repeated three times, mean difference is significant at the 0.05 level.

2.2: Chemical analysis (phytochemical screening and spectrophotometric determination of total polyphenols and flavonoids of the plant under investigation.

2.2.1 -Phytochemical screening.

The extract of the aerial parts of *Artemisia abrotanum* L. *in vivo* and *in vitro* subjected to preliminary phytochemical screening was carried out on their extract using the standard screening method.[47,48,49,50] Steam distillation test for volatile substances, The molish's test and fehling's test were carried out for carbohydrate. Foam test for saponins[51,52], and Dragendorff's reagents for Alkaloids and/ or nitrogenous base. Aq. Sodium hydroxide test, conc. Sulphuric acid and Shinoda's tests were carried out for flavonoids, Ferric chloride solution for Tannins, Antharaquinones and Cardiac glycosides tests.

2.2.2 Spectrophotometric determination of the total polyphenols *in vivo* and *in vitro* samples of *Artemisia abrotanum* L.

The total content of phenolic compounds is determined by using the Folin Ciocalteu reagent[53,54], Calibration curve was prepared by mixing ethanolic solution of Gallic acid (1 ml; 0.01 to 0.09 mg/ml) with 5 ml Folin Ciocalteu reagent (diluted tenfold) and sodium carbonate (4 ml, 0.7 M). We measured absorbance at 765 nm and drew the calibration curve. One milliliter of ethanolic extract (0.05 mg/ml) was also mixed with the reagents above and after 2 h the absorbance was measured to determine total plant phenolic contents. All determinations were carried out in triplicate. The total content of phenolic compounds in the extract in Gallic acid equivalent (Gallic Acid Equivalent) was calculated by the following formula:

$Y=0.0076x - 0.0015$, $r^2 =0.9968$.

T = C.V/M Where: T: total content of phenolic compounds, milligram per gram extract, in Gallic Acid equivalent. Equivalent. C: the concentration of Gallic acid established from the calibration curve, milligram per milliliter. V: the volume of extract, milliliter. M: the weight of ethanolic plant extract in gram.

2.2.3 Spectrophotometric determination of the Total flavonoids *in vivo* and *in vitro* samples of *Artemisia abrotanum* L.

Aluminum chloride colorimetric method is used for flavonoids determination Two milliliters of 2% AlCl3 in ethanol is added to 2 ml of the test sample. The UV absorption is measured at 420 nm after 1 h at room temperature. Concentration of 0.05 mg/ml sample solution is used while Quercetin concentrations of 0.01 to 0.09 mg/ml are used to obtain a calibration curve. Determinations were performed in triplicates. Total flavonoid contents were obtained from the regression equation of the calibration curve of Quercetin ($Y= 0.0103+0.0102$, $r^2= 0.9966$).

3. RESULTES AND DISCUSSION

3.1 Effect of light intensity on the Micro-propagation of *Artemisia abrotanum* L.

There are positive responses to increasing light intensities as in photo (1) at ranges from 300 to 3000lux, ANOVAAs. As observed that the strength values is ranging from lowest value 2 to highest 5. The group subjected to 300 lux has a 41.7% score of 4 and 4.2% score of 5, 600 lux has a 54.2% score of 4, 1500 lux has a 75.0% score of 4 and 12.5% score of 5; 87.5% score 4 and above, 3000 lux has a 100% score 4 and above, 3000 lux has a 54.2% score 4 and above dropped to the same strength as 600lux as it explained in fig (3).

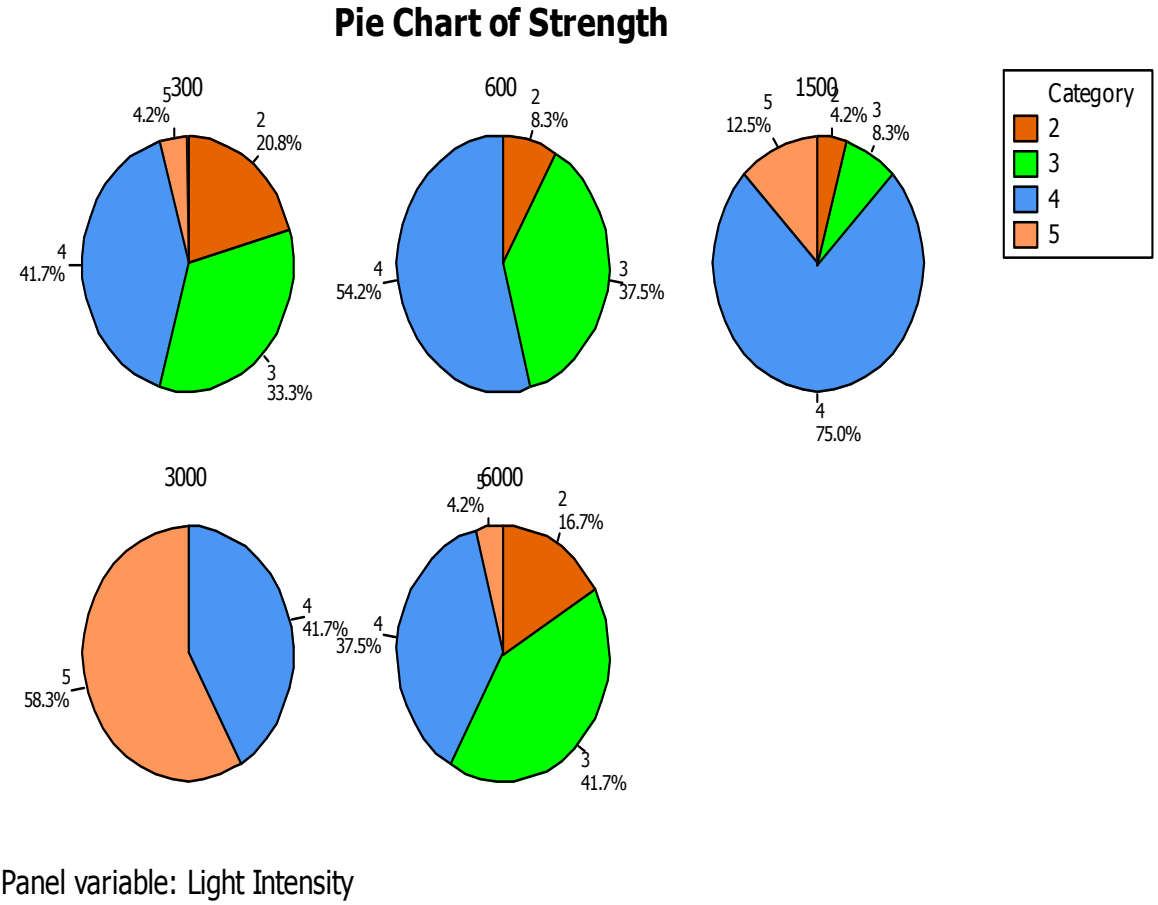

Fig (1) Pia chart strenght explain the effect of different light intensity on the strength of the *in vitro* growth of *Artemisia abrotanum* L,

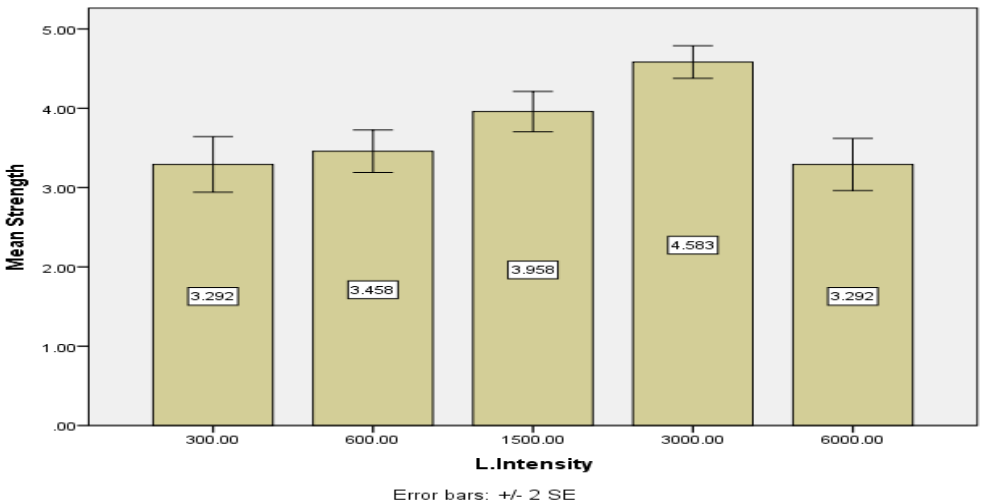

Fig (2) Simple Bar Charts (Displaying Error Bar ±2SE) of Responses (Y-Axis) to Different Light Intensities (X-axis).

It was observed that there is a positive response to increasing light intensities at ranges from 300 to 3000lux, but at 600lux, there was a decrease in all responses except length of roots. This means that the optimum light intensity giving highest strength is 3000lux flowed by 1500lux.The effect of different light intensity (1500, 2000, 3000, 4000, 5000lux) on the *in vitro* plant growth of *Phaius tankervilliae* and *Vavda. coerulea* Griff. Causing the increase in the dry matter accumulation , plant height , leaf number, and leaf area development of these two species of plantlets were best at the intensity of 4000lux.[55]

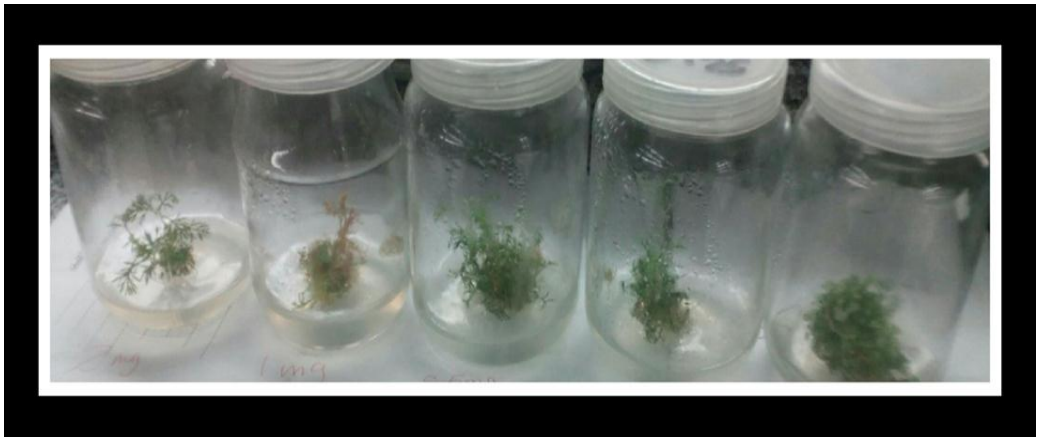

Photo (3) Effect of different light intensity *on in vitro* growth of *Artemisia abrotanum* L.

3.2.1 Results and Discussion of phytochemical screening

Preliminary phytochemical screening of *Artemisia abrotanum* L. showed that it contains volatile oil, Carbohydrates and/or glycosides, tannins, flavonoids, alkaloids, sterols and/or triterpenes, resins. It is free from Saponins, Iridoids, cardiac glycosides and anthraquinone glycosides as represented in **Table (1),** the phytochemical screening in ethanolic extract of *A. abrotanum* L. showed that presence of alkaloids, Terpenoids, Sterols,Tannins, Phenol and Flavonoids.[56]

Table (1): Results of preliminary phytochemical screening of *in vivo* & *in vitro* samples of *Artemisia abrotanum* L.

Constituents	*In vito*	*In vivo*
Steam volatile substance	+	+
Carbohydrates and/or glycosides	±	+
Tannins	+	++
Flavonoids	++	++
Saponins	-	±
Sterols and/or Triterpenes	++	++
Alkaloids and/or nitrogeneous bases	-	±
Anthraquinones	-	-
Cardiac glycosides	-	-
Resins	±	+

Present in large amount (++), present (+), traces (±), (-) absent

3.2.2 Results and Discussion of total polyphenols.

2-2- From the standard calibration curve, and the absorbencies obtained for each sample, the total phenolic content was calculated as Gallic acid equivalent (GAE) in the *in vivo* and *in vitro* plants of *Artemisia abrotanum* L., results are recorded in Table (2) & Fig. (3) The results showed that.

1-The *in vivo* plants showed the highest content of polyphenols (271.6 mg/100g.d.w), while the least content was recorded with the sample extract in 300lux light intensity (54.90 mg/100g.d.w).

2-The highest content of polyphenols in the *in vitro* sample was observed in the *in vitro* plantlet under 6000 lux extract (131.30 mg/100g.d.w), respectively. Higher light intensity cause the production of excess phenolic compounds in *Zosteria mariana*.[57]

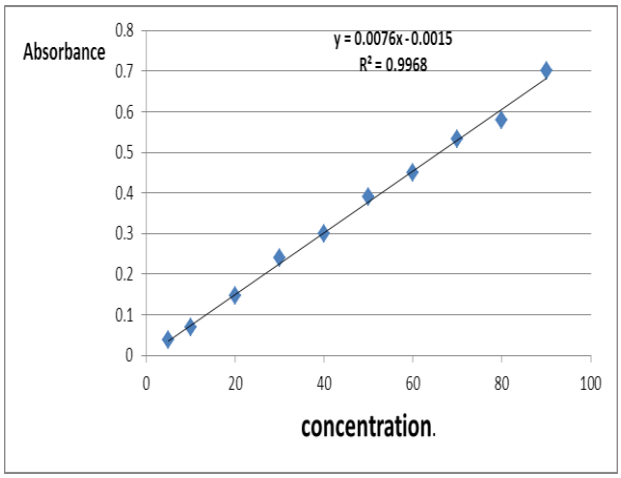

Fig. (3): The calibration curve of Gallic acid with different concentrations.

Table (2): Total phenolic compounds (Measured by Folin Ciocalteu Assay), flavonoids (Measured by $AlCl_3$) *in vivo* and *in vitro* of methanol extracts of the studied *Artemisia abrotanum* L., as percentage in extracts.

Sample		Total polyphenols % (mg/100g.d.w)	Total flavonoids % (mg/100g.d.w)
In vivo		271.6	84.63
In vitro	300 lux	54.90	21 .37
	600 lux	100.20	29.68
	1500 lux	112.03	34.19
	3000 lux	131.30	39.34
	6000 lux	106.70	47.58

3.2.3 Results and Discussion of total Flavonoids.

2-2- From the standard calibration curve, and the absorbencies obtained for each sample, the total flavonoids content was calculated as Quercetin

Equivalent (QE) in the *in vivo* and *in vitro* plants of *Artemisia abrotanum* L. Results are recorded in Table (2) and Fig.(4).

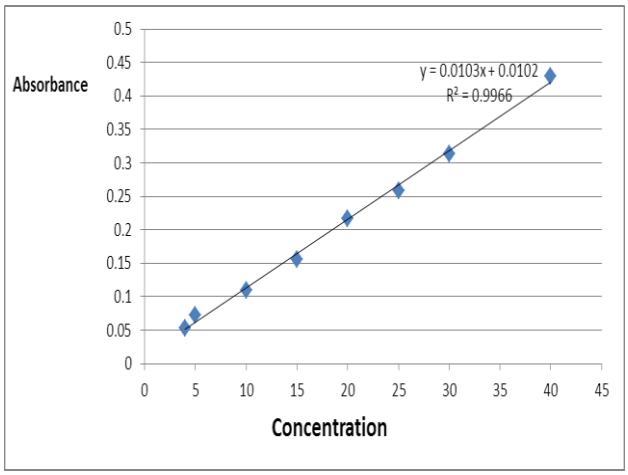

Fig. (4): The calibration curve of quercetin with different concentrations.

The results showed that

1. The *in vivo* plants showed the highest content of flavonoids was (84.63 mg/100g.d.w) but in the *in vitro* plantlet under 6000 lux extract sample was (47.58 mg/100g.d.w), while the least content was recorded with the sample extract in 300 lux light intensity (21.37 mg/100g.d.w).
2. The highest content of flavonoids in the *in vitro* sample was observed in the *in vitro* plantlet under 6000 lux extract, followed by the 3000 lux extract respectively.

4. CONCLUSION

From the present investigation, *Artemisia abrotanum* L. proved to have high therapeutic value by registering Flavonoids, polyphenols and Terpenoids which are of medicinal value and has their importance from the ancient times and light intensity play a significant role not only *in vitro* growth but also on accumulation of secondary metabolites. The preliminary screening of bioactive compounds had paved a way for further investigation of these compounds towards the usage in commercial application in future.

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