EFFECT OF DIFFERENT PLANT GROWTH REGULATORS ON PLANT REGENERATION AND CALLUS INDUCTION OF *PLUMBAGO AURICULATA* AND SOME SECONDARY METABOLITES CONTENT

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ABSTRACT

Plumbago becomes one of the essential medicinal, and decorative plants belonging to the family plumbaginaceae due to the present of plumbagin a secondary metabolite. In this study, the nodal segments of shoots of ***Plumbago auriculata*** were used as an explant in establishing *in vitro* cultures. *In vitro* callus initiation and shoot regeneration was studied using mixtures of auxins, antiauxins (p-Chlorophenoxyisobutyric acid and 2,3,5-triiodobenzoic acid), and cytokinins. The medium comprising one mg/l PCIB and 0.5 mg/l BA produced the most significant outcomes in the case of callus induction. However, the best shooting number was given (11.18) with the same combination after eight weeks. According to the data, the leaves extract had the highest level of polyphenols (742.6 mg/100g.d. w) and flavonoids (563.9 mg/100g.d. w).

Keywords: *Plumbago auriculata*; Cullus induction; Shoot induction; Anti auxin; Secondary metabolites;

Introduction

As consumer need for plant-derived products has expanded, unscientific extraction of certain species' natural habitats has forced the majority of them to the edge of extinction. (**Segarra-Moragues et al; 2005; Huang 2011**). Throughout the world, the genus ***Plumbago*** contains numerous medicinally significant species. There is a knowledge gap about some species within the genus, presenting a potential for novel research. This review establishes the utility of ***Plumbago auriculata*** as a valuable medicinal herb and the extent to which it is harmful. This species is used extensively worldwide, while little research has been undertaken in South Africa, where it is indigenous **(Singh et al; 2018**).

The ***Plumbago auriculata*** plant was used in ancient times as a traditional medicine and treatment for some human diseases and their elimination. It has high medical value because it contains active substances. Therapeutic capabilities have been discovered in all plant parts. The leaves and roots of this plant were revealed to hold neuroprotective, anti-atherosclerotic, cardiotoxic, and hepatoprotective effects **(Deshpande et al., 2014).** Research shows that the leaves and roots of the ***P. auriculata*** plant are used in the treatment of rheumatism, fractures, edema, skin lesions, warts, headaches, piles, diarrhea, malaria and as emetics **(Elgorashi et al., 2003; Chen and Gao., 2013).**

The strategy of the culture of medicinal tissue plants has been demonstrated to increase the quantity of high-value active compounds, and, in some cases, to produce new compounds that are used in the treatment of diseases.

Therefore, tissue culture is one of the techniques that is used very widely in the field of producing active compounds used in industry from plants and medicinal herbs. The progress of cell culture technology for the production of medicinal compounds enables the production of a varied variety of medications, including terpenoids, steroids, saponins, flavonoids, phenolics, alkaloids, fatty acids, oligosaccharides, amino acids, fructan, and inulin. Bioprocesses approaches are used to generate secondary metabolites from plant species (**Karuppusamy, 2009**).

Due to its many properties, including the easy handling of environmental conditions, the lack of impacts from others tissues or microbial interactions, and quick growth, callus cultures have been employed for research the biochemistry of plant species (**Guirgis *et al*., 2007**).

Material and Methods

This research was conducted at the Genetic Engineering and Biotechnology Research Institute of the University of Sadat City.

Plant material and sterilization

Plants of ***Plumbago auriculata*** were grown in the Genetic Engineering and Biotechnology Institute Garden (Egypt), and stem nodes of ***P. auriculata*** were collected from January 2018 to July 2020.

Explants of stem nodes from field-grown plants were employed to form callus cultures and regenerate shoots. Stem nodes were washed thoroughly with current tap water. Stem explants were surface sterilized with Clorox 50% (2.5% Sodium hypochlorite) for 20 min. Subsequently, explants were rinsed three times for five minutes each time with sterile distilled water. Additionally, the single node should be chopped into smaller pieces (0.8:1cm).

Culture media

Explants were cultured using the MS formula, including mineral salts, myo-inositol, vitamins, and glycine betaine. **(Murashige and Skoog, 1962)** 3.0 percent sucrose and seven g/L agar were added. Before agar administration and autoclaving for 20 min at 121°C (0.1. MPa), the pH was maintained to 5.7 using 1N KOH or HCl. In a growth chamber, the samples were incubated at 21°C with a 16h photoperiod and 8h dark (30 E m-2s-1, Philips TL 33 led).

The impact of various plant growth regulators (PGR) on the development of calluses and the proliferation of shoots:

Fragments with an area of 1.0 cm were cut off from the stem around the node. The impact of growth hormones such as 2,3,5-triiodobenzoic acid (TIBA) and antiauxin p-Chlorophenoxyisobutyric acid (PCIB), auxin 2, 4-Dichlorophenoxyacetic acid (2.4-D), and 1-Naphthaleneacetic acid (NAA) was evaluated in conjunction with 6-Benzylaminopurine (BAP) or Benzyl adenine (BA) to evaluate callus and shoot growth derived from inoculated explants. Kinetin (Kn) was used individually in the concentration of 3mg/l. Concentrations of anti-auxin with cytokinin were used for (PCIB) (1mg/l PCIB), (1mg/l PCIB + 0.5 mg/l BA), (3mg/l PCIB) and (3mg/l PCIB + 0.5 mg/l BA) and for (TIBA) the concentrations were (1 mg/l TIBA), (1 mg/l TIBA + 0.5 mg/l BA), (3 mg/l TIBA) and (3mg/l TIBA + 0.5mg/l BA), and for auxin was used with cytokinin in concentrations (1mg/l 2.4 D + 1.5ml/l NAA), (0.5mg/l 2.4 D + 0.5 ml/l BA), (2mg/l BAP + 0.5ml/l NAA).Callus was collected and weighted after eight weeks, then exposed to completely dry and finally weighted.

Phytochemical screening.

Extraction of callus crude extracts

A known quantity of one-month fresh callus was harvested, and oven-dried at 600 degrees Celsius to a constant weight. The callus was coarsely powdered and extracted with methanol for 8 hours using a Soxhlet extractor apparatus and solvent was removed by distillation.

Determination of the total phenolic content

Total polyphenols in vivo and *in vitro* specimens of ***Plumbago auriculate*** were determined using a spectrophotometer. Total phenolic content of the extracts was evaluated by a colorimetric method utilizing Folin-Ciocalteu reagent. 1 ml of extract were dissolved in 2 ml of methanol, 500µL aliquots of extract were mixed with 2.5 mL Folin–Ciocalteu reagent (diluted ten-fold) and 2.5 mL (75 g/L) sodium carbonate. The tubes were vortexed for 10 sec and allowed to stand for 2 hr at 25 ^{o}C. After incubation at 25 °C for 2 hr, absorbance was measured at 765 nm against reagent blank. Total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per gm (**Sembiring EN, et al., 2018**), (**V. L. Singleton and J. A. Rossi, 1965**) and (**Ribarova, and Atanassova, 2005**)**.**

Determination of total flavonoids content:

Total flavonoids were determined spectrophotometrically *in vivo* and *in vitro* specimens of ***Plumbago auriculata***. Modified AlCl3 calorimetric method was used, 1 ml of extract were dissolved in 2 ml of methanol in a 10 ml of volumetric flask. 5% NaNO3, 5% NaOH and 7% AlCl3 solution were prepared by using water in a 25 ml of volumetric flask. 200 microliters of extract were taken in a sealed glass vial and added 75 µl of 5% NaNO3 and left for 5 min at room temperature. Later on, 1.25 ml of AlCl3 and 0.5 ml NaOH were added to each vial. Then it was sonicated and incubated for 5 min at room temperature. After incubation, the absorbance of all working solution and standard solution was measured against methanol blank at 510 nm. The flavonoids content of extracts was estimated by using the quercetin standard calibration curve and the obtained results of flavonoids were expressed as microgram of quercetin equivalent (Qu) per 1 g of dry extract **(Sembiring EN, et al., 2018),** (**V. L. Singleton and J. A. Rossi, 1965**) and **(Chang, et al., 2018)**.

Statistical analysis

In the tissue culture experiments, Each treatment included 40-50 jars (5 cm height) containing three explants per jar. In the graphs, it was shown means ± SE. To determine the significance of differences between the means, the student t-test was performed.

Results and Discussion

Callus induction

The antiauxin PGRs PCIB and TIBA, auxin 2, 4-D, and BAP were utilized in conjunction to investigate callus and shoot growth from inoculation ***Plumbago auriculata*** explants in this research. Individually, Kinetin (kin) was utilized at a dosage of 3 mg/l. After 14 days of growth on MS medium supplemented with 1.0 mg/l PCIB and 0.5 mg/l BA, callus development was detected from stem explants cultivated on various PGR. Intriguingly, adding 1.0 mg/l PCIB to the medium resulted in callus production but at a lower weight than when BA was administered (Figure 1). Otherwise, the addition of only TIBA to the media produced lower callus and shooting

numbers. Meanwhile, as shown in Fig. 1 & 2, all the growth regulators used to initiate callus induction and shoot regeneration are very close to the hormone-free media used as control (M.S only without treatment) except PCIB, which significantly gives the highest value.

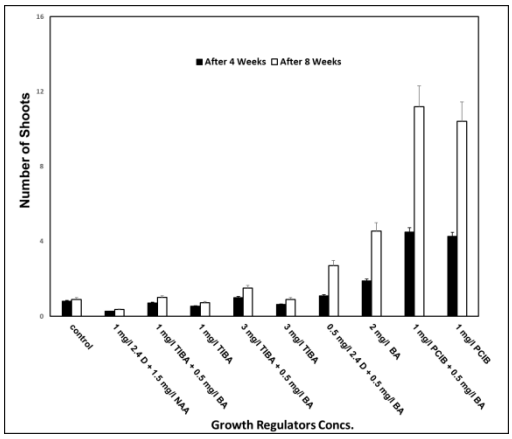

Figure 1 (A)

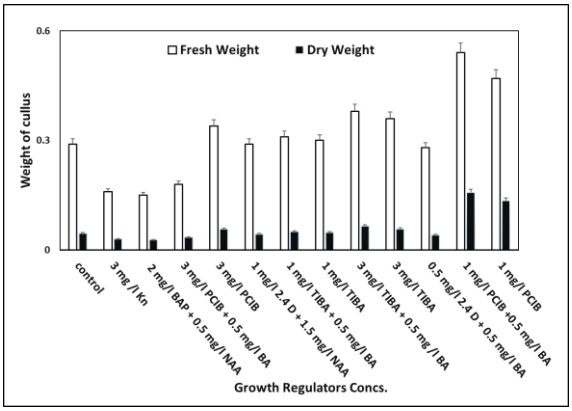

Figure 1 (B)

Fig. 1. Effect of different plant growth regulators on (A) Mean shoot numbers formed after 4 and 8 weeks of ***Plumbago auriculat***. (B) Callus fresh and dry weight after 8 weeks of *Plumbago auriculata.* Bars are standard errors.

Fig. 2. (A) *Induction of* callus on explants after 8 weeks from culture on M.S media supplement with (1.0 mg/l PCIB and 0.5 mg/l BA). (B) Effect of PCIB on new-shoot formation in stem explants of ***Plumbago auriculata*** produced *in vitro*. (C) Multiplication on explant after 8 weeks on M.S media supplement with 3 mg/l TIBA.

It is widely established that the auxin: cytokinin ratio is critical for regeneration (**Skoog and Miller, 1957**). The ***Plumbago*** species tendency to rebound, even if there is little or no auxin in the medium **(Deshpande et al., 2014).** It is possible that the callus has a lot of endogenous auxin activity. In growth regulator experiments with a variety of crop species, PCIB has shown antiauxin action (**Frenkel and Haard, 1973; Jacobs and Hertel, 1978; Quattrocchio et al., 1986; Trebitsh and Riov, 1987**). As a result, PCIB may aid in the reduction of roots and the enhancement of callus shoot regeneration in some plants.

Compared to other work with ***P. auriculata*** **(Deshpande et al., 2014**, **Chen and Gao 2013),** one-year-old ***P. auriculata*** plants were micro propagated using nodal and leaf tissue. They concluded that using young nodal explants is ideal since leaves have a strong differentiation potential and a restricted ability for regeneration. **(Deshpande et al.,2014), *P. auriculata*** elevated mass callus production in vitro when various growth hormones were used, including indoleacetic acid (IAA), 1-Naphthaleneacetic acid (NAA), and Indole-3-butyric acid (IBA). IAA is a simple molecule (auxin) that aids in the developing and proliferation of plants **(Davies, 2004).** IBA is the most widely used economically for plant reproduction because it promotes undesired root growth and is frequently more resistant to degradation in vivo than IAA **(Davies, 2004).** NAA is a synthesized plant hormone often used to increase the synthesis of cellulose fiber in agriculture, horticultural processes, and tissue culture (**Davies, 2004)**. However, in big concentrations, it is harmful to the plant. The growth hormone NAA caused more callus production in all plantlets; however, the mixture of hormones IBA and NAA produced the highest amount of leaf explant callus development. Configurations of BAP and NAA resulted in the best callus formation from explants of stem and shoot apex. BAP is a cytokinin synthesized in the laboratory that is utilized in tissue culture to stimulate cell division, bud development, and stem branch expansion (**Zhang et al., 2005; Madhavam et al., 2009**). Vegetative propagation is also possible by placing 15 cm long stem cuttings in polybags and treating them with IAA and IBA to encourage root development (**Joy et al., 1998; Lakshmanan et al., 2016**).

The total phenol constituent was measured as gallic acid equivalent (GAE). The total flavonoid constituent was quantified as quercetin equivalent (QE) *in vitro* using the standard calibration curve and the absorbencies determined for each specimen. The results showed that different plant growth regulators were marked by decreased secondary metabolites (total phenols & total flavonoids) as compared to either control callus (untreated) or leaves of plants (Table 1). Moreover, the contents of both total phenolics and flavonoids in the stems of ***Plumbago auriculata*** were significantly lower than in all other samples. It is clear that the leaves extract showed the highest total content of phenols (742.6 mg/100 g.d.w) and the total content of flavonoids (563.9 mg/100 g.d.w).

Polyphenolic substances found in natural plant products, including phenolic acids, tannins, anthocyanins, and flavonoids, have been beneficial in metabolic disorder treatments such as diabetes, obesity hypercholesterolemia, and overweight (**Martin and Appel, 2010**). Numerous studies on natural products have been conducted to determine their biological characteristics, including antioxidant and anti-lipase activity (**Dzomba and Musekiwa, 2014**).

Consequently, Flavonoids have become an essential component in a wide range of nutraceutical, pharmacological, therapeutic, and cosmetic uses. This is due to their ability to control critical cellular enzyme activity as well as their anti-oxidative, anti-inflammatory, anti-mutagenic, in the treatment of Alzheimer's disease (AD), and anti-carcinogenic capabilities. Research on flavonoids has gotten a boost with the revelation of a low cardiovascular mortality rate as well as the prevention of coronary heart disease (CHD). **(Panche *et al*., 2016**).

Table 1. Total phenolics and flavonoids *in vivo* and *in vitro* of methanol extracts of *Plumbago auriculata* callus after plant growth regulator treatments.

Treatments	Total Phenolics mg/100g Sample	Total Flavonoids mg/100g Sample
Control	519.2±11.8	327.3±23.4
1 mg/l PCIB + 0.5 mg/l BA	483.1±14.5	264.7±33.4
1 mg/l PCIB	394.5±32.5	215.8±42.4
3 mg/l PCIB + 0.5 mg/l BA	381.4±21.5	197.2±13.2
3 mg/l PCIB	456.3±17.4	248.5±22.5
1 mg/l TIBA + 0.5 mg/l BA	147.6±33.5	96.4±23.2
1 mg/l TIBA	448±31.2	239.6±26.5
3 mg/l TIBA + 0.5 mg/l BA	406.9±27.5	218.3±33.5
3 mg/l TIBA	234.1±12.5	153±18.7
1 mg/l 2.4 D + 1.5 mg/l NAA	415.7±22.5	278.2±22.5
0.5 mg/l 2.4 D + 0.5 mg/l BA	98.2±11.7	61.3±10.3
3 mg /l Kn	389.5±29.9	217.4±17.4
2 mg/l BAP + 0.5 mg/l NAA	432.3±39.7	265.7±22.4
Stems	187.1±14.5	94.1±12.3
Leaves	742.6±74.4	563.9±26.9

Values are the mean (±S.E.) of three replicates

REFERENCES

Chang, C. C., Yang, M. H., Wen, H. M., & Chern, J. C. (2002). Estimation of total flavonoid content in propolis by two complementary colometric methods. *Journal of food and drug analysis*, *10*(3), 3. cancer cells and human blood/serum compatibility testing. *Therapeutic delivery*, *4*(10), 1247-1259.

Chen, Y., & Gao, S. (2013). Preliminary report of PGR's influence to multiple shoots induction and plant regeneration on Plumbago auriculata.

Davies, P. J. (Ed.). (2004). *Plant hormones: biosynthesis, signal transduction, action!* Springer Science & Business Media.

Dzomba, P., & Musekiwa, C. (2014). Antiobesity and antioxidant activity of dietary flavonoids from dioscorea steriscus tubers.

Elgorashi, E. E., Taylor, J. L., Maes, A., van Staden, J., De Kimpe, N., & Verschaeve, L. (2003). Screening of medicinal plants used in South African traditional medicine for genotoxic effects. *Toxicology letters*, *143*(2), 195-207.

Frenkel, C., & Dyck, R. (1973). Auxin inhibition of ripening in Bartlett pears. *Plant Physiology*, *51*(1), 6-9.

Frenkel, C., & Haard, N. F. (1973). Initiation of ripening in Bartlett pear with an antiauxin α (p-chlorophenoxy) isobutyric acid. *Plant physiology*, *52*(4), 380-384.

Grunenfelder, L. (2005). Physiological studies of light-induced greening in fresh market potatoes.

Guirgis, A. A., Abd El-Kawi, M. A., Abbas, H. N., Araffa, A. M., & Maksoud, A. I. (2007). High rosmarinic acid content in induced mutants and in in vitro elicited sweet basil (Ocimum basilicum L.) callus. *Asian Journal of Plant Sciences*.

Huang, H. (2011). Plant diversity and conservation in China: planning a strategic bioresource for a sustainable future. *Botanical Journal of the Linnean Society*, *166*(3), 282-300.

Jacobs, M., & Hertel, R. (1978). Auxin binding to subcellular fractions from Cucurbita hypocotyls: in vitro evidence for an auxin transport carrier. *Planta*, *142*(1), 1-10.

Joy, P.P., Thomas, J., Mathew, S. and, Skaria, B.P. (1998). Medicinal Plants. Tropical Horticulture **2**: 1-211.

Karuppusamy, S. (2009). A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ and cell cultures. *Journal of Medicinal Plants Research*, *3*(13), 1222-1239.

Kim, S. K., Kim, Y. M., Hong, M. J., & Rhee, H. I. (2005). Studies on the inhibitory effect of Eugenia aromaticum extract on pancreatic lipase. *Journal of Applied Biological Chemistry*, *48*(2), 84-88.

Lakshmanan, G., Bupesh, G., Vignesh, A., Sathiyaseelan, A., & Murugesan, K. (2016). Micropropagation and anticancer activity of methanolic extract of Plumbago auriculata Lam. *International Journal of Advanced Biotechnology and Research*, *4*, 2001-2011.

Madhavan, V., Hema Prem Kumar, B., Murali, A., & Yoganarasimhan, S. N. (2009). Antifertility activity of Drosera burmannii. *Pharmaceutical Biology*, *47*(2), 128-131.

Martin, K. R., & Appel, C. L. (2009). Polyphenols as dietary supplements: A double-edged sword. *Nutrition and Dietary Supplements*, *2*, 1-12.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, *15*(3), 473-497.

Panche, A. N., Diwan, A. D., & Chandra, S. R. (2016). Flavonoids: an overview. *Journal of nutritional science*, *5*, e47. https://doi.org/10.1017/jns.2016.41

Quattrocchio, F., Benvenuto, E., Tavazza, R., Cuozzo, L., & Ancora, G. (1986). A study on the possible role of auxin in potato «hairy root» tissues. *Journal of plant physiology*, *123*(2), 143-149.

Ribarova, F., & Atanassova, M. (2005). Total phenolics and flavonoids in Bulgarian fruits and vegetables. *Journal of the university of chemical technology and metallurgy*, *40*(3), 255-260.

Segarra-Moragues, J. G., Palop-Esteban, M., González-Candelas, F., & Catalán, P. (2005). On the verge of extinction: genetics of the critically endangered Iberian plant species, Borderea chouardii (Dioscoreaceae) and implications for conservation management. *Molecular Ecology*, *14*(4), 969-982.

Sembiring, E. N., Elya, B., & Sauriasari, R. (2018). Phytochemical screening, total flavonoid and total phenolic content and antioxidant activity of different parts of Caesalpinia bonduc (L.) Roxb. *Pharmacognosy journal*, *10*(1).

Singh, K.(2017). *Structure, biology and chemistry of plumbago auriculata (plumbaginaceae)* (Doctoral dissertation).

Singh, K., Naidoo, Y., & Baijnath, H. (2018). A comprehensive review on the genus Plumbago with focus on Plumbago auriculata (Plumbaginaceae). *African Journal of Traditional, Complementary and Alternative Medicines*, *15*(1), 199-215.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture*, *16*(3), 144-158.

Skoog, F., & Miller, C. (1957). Chemical regulation of growth and organ formation in plant tissues cultured. In *Vitro Symp Soc Exp Biol*.

Sumsakul, W., Karbwang, J., & Na-Bangchang, K. (2016). Application of SPECT/CT imaging system and radiochemical analysis for investigation of blood kinetics and tissue distribution of radiolabeled plumbagin in healthy and Plasmodium berghei-infected mice. *Experimental parasitology*, *161*, 54-61.

Trebitsh, T., & Riov, J. (1987). Inhibition of activity of the ethylene-forming enzyme by α (p-chlorophenoxy) isobutyric acid. *Journal of Plant Growth Regulation*, *5*(4), 207-215.

Wang, C. C., Chiang, Y. M., Sung, S. C., Hsu, Y. L., Chang, J. K., & Kuo, P. L. (2008). Plumbagin induces cell cycle arrest and apoptosis through reactive oxygen species/c-Jun N-terminal kinase pathways in human melanoma A375. S2 cells. *Cancer letters*, *259*(1), 82-98.

Zhang, Z., Zhou, W., & Li, H. (2005). The role of GA, IAA and BAP in the regulation of in vitro shoot growth and microtuberization in potato. *Acta Physiologiae Plantarum*, *27*(3), 363-369.

تأثير منظمات نمو النبات المختلفة على تجديد النبات و تكوين الكالس في بلومباغو أوريكولاتا و بعض محتوى المركبا ت الثانوية

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البلمباجو

هو احد النباتات الطبيه التى تنتمى الى عائله (Plumbaginaceae) نظرا لوجود البلامباجين كاحد المركبات الثانويه فى النبات . فى هذا البحث تم استخدام عقده ساقيه واحده لنبات (البلمباجو) بطول من (١:٠.٨سم) وعقمت وزرعت فى بيئه (Murashige and Skoog (M.S) مضافا اليها تركيزات مختلفه من الاكسينات ومضادات الاكسينات والسيتو كينين (p-Chlorophenoxyisobutyric acid and 2,3,5-triiodobenzoic acid) TIBA ((PCIB -)وذلك لدراسه انتاج الكالس على اجزاء النباتيه المنزرعه. لوحظت افضل النتائج من حيث تكوين الكالس على البيئات المختلفه وايضا تم تكوين اعلى عدد افرع (١١.١٨) فرع فى بيئه 1mg/l PCIB + 0.5 mg/l BA وهى المعامله (١) بعد ٨ اسابيع من الزراعه . تم فحص اجمالى mg/l PCIB + 0.5 mg/l BA (مضافا اليها(M.S) البوليفينول والفلافونويد فى الورقه والساق والكالس الناتج من المعاملات المختلفه واظهرت الورقه اعلى محتوى من مركب البوليفينول(٧٤٢,٦ مجم / ١٠٠ جم وزن العينه) والفلافونويد (٥٦٣.٩ مجم / ١٠٠ جم وزن العينه)

الكلمات المفتاحية : بلامباجو; تكوين كالس; تكوين افرع ; مضاد الاكسين ; ومركبات ثانويه