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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 16 (19): 984-990, 2013
ISSN 1028-8880 / DOI: 10.3923/pjbs.2013.984.990

In vitro Antibacterial Activity and Phytochemical Analysis of White Henbane Treated By Phytohormones

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Abstract: This study reported the effect of interaction between cytokinins and auxins to enhance accumulation of alkaloids in White henbane (*Hyoscyamus albus* L.). Plants of this specie were grown under controlled conditions and treated with plant-hormones: Auxins by: 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 3-Indole Acetic Acid (IAA), Cytokinins by: Kinetin (K) and Benzyl Amino Purine (BAP), at 0-10 and 20 mg L^{-1} rates isolated and interacted. The results showed that treatment of 2, 4-D and K at the highest applied rates 20 mg L^{-1} increased the accumulation threefold rate estimated to 2.321% in the root plant part and 1.702% in the aerial plant part with the same plant-hormones but dosage of (20×10 mg L^{-1}) in order. The TLC for alkaloid extracts shows that *H. albus* L. contains 6 alkaloids. In this study, it was concluded that the treatment with interaction of (K×2,4-D) (20×20 mg L^{-1}) gives the highest percent of alkaloids in the root and shoot parts compared to plant-hormones separated. The *in vitro* antibacterial activity was determined on microorganisms: *Pseudomonas stutzeri*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*. And performed by disc diffusion assay. Respectively, ethanol extracts showed no inhibitory effect on the microorganisms, however alkaloid extracts of *H. albus* L. of the same treatments with plant-hormones of shoot and root parts showed antibacterial activity against microorganisms that were tested. The results obtained in the present study suggest that alkaloid of *H. albus* L. can be used in treating diseases caused by the test organisms.

Key words: *Hyoscyamus albus* L., alkaloids, plant-hormones, antibacterial activity

INTRODUCTION

Aromatic and medicinal plants are known to produce certain bioactive molecules which react with other organisms in the environment, inhibiting bacterial or fungal growth (antimicrobial activity) (Chopra *et al*., 1992; Bruneton, 1995). The substances that can inhibit pathogens and have little toxicity to host cells are considered candidates for developing new antimicrobial drugs (Yano *et al*. 2006). Tropane group is one of the most important medicinally and pharmaceutically secondary plant metabolites (Robbers *et al*., 1996), alkaloids such as Hyoscyamine and scopolamine are among the oldest drugs in medicine (Garland and Barr, 2001), the latter being amore valuable drug due to its higher pharmacological activities and fewer side-effects (Oksman-Caldentey, 2007). These affect the parasympathetic nervous system and are used in ophthalmology, anæsthesia and treatment of cardiac and gastrointestinal diseases (Tyker *et al*., 1988). The characteristics of the plants that inhibit microorganisms and are important for human health have been researched in laboratories since 1926 (Erdogrul, 2002). These compounds are produced mainly by *H. albus* L. (one of Solanaceae family) in tiny quantities (Bruneton, 1995). Several studies have shown that this shrub is able to accumulated high amount of tropane alkaloids (Hibi *et al*., 1992) and these alkaloids showed antimicrobial activity to microorganisms (Larhsini *et al*., 2001). The objective in this study led us to study how to improve products in *H. albus* L., which grows widely in many areas of eastern Algeria and Mediterranean countries; known the effect of interaction between plant hormones applications on the rate of alkaloids accumulation and investigate antibacterial activities of *H. albus* L. extracts after treatment with plant-hormones when they were tested *in vitro* against four bacterial species *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas stutzeri* and *Klebsiella pneumonia*.

MATERIAL AND METHODS

Collection of plant material: *H. albus* L. was collected in Jun from MILA town situated in East of Algeria. The

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seeds of *H. albus* treated with fungicide, were germinated in March, in pot containing a mixture of soil and peat in a 5:1 ratio (Mazliak, 1982) and let to grow under shelter with periodic irrigation at field capacity. When the plants reached 8 cm height, they were transferred to pots containing the same soil mixture at a density 1 plant pot^{-1}. About 15 days after being transplanted, plants were irrigated periodically at half field capacity. The moderate stress has been shown, in previous studies (Davies, 1990; Dodds and Roberts, 1995).

Plant-hormones: auxins: 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 3-Indole Acetic Acid (IAA) and cytokinins: Kinetin (K) and Benzyl Amino Purine (BAP), were applied in April at three rates 0-10-20 mg L^{-1} isolated and interacted [auxins X cytokinins] in a three factorial randomized complete block design with three replications. At the end of the following stages (Jun), shoots and roots samples were hand harvested, air and oven dried and then finely ground to be used for chemical and antibacterial analysis.

Preparation of ethanol extracts: Shoot and root of *H. albus* L. of treatments: Witness, K×2,4-D 20×20 mg L^{-1}, IAA×2,4-D 20×20 mg L^{-1}, IAA×BAP 20×20 mg L^{-1}, BAP×2,4-D 20×20 mg L^{-1}, BAP×2,4-D 20×20 mg L^{-1}, K×IAA 20×20 mg L^{-1} were dried and powdered under sterile conditions, then 10 g of each treatment were extracted with 150 mL of ethanol 70% by maceration for 72 h. The extracts were filtered using Whatman filter paper (No. 1) and then concentrated in vacuo at 40°C using a Rotary evaporator. The residues obtained were stored in a freezer until further antibacterial tests. The same procedure used for all treatments to preparation of ethanolic extracts to be used in estimation of the accumulated alkaloids.

Preparation of alkaloid extracts: Estimation of accumulated alkaloids of all treatments was performed according to the procedures described by Pelletier and Fodor (1970).

Chromatography analysis: The various extracts, obtained from the different treatments were subjected to identify the different types of alkaloids and to know the effect of treatment with plant-hormones on alkaloids type (Trease and Evans, 1978; Stahl, 1969).

Antibacterial activity assay: The disc-diffusion assay (Kim *et al*., 1995) was used to determine the growth inhibition caused by plant extracts against the following bacterial strains: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas stutzeri* and *Klebsiella pneumonia*. Filter paper discs (Whatman No. 3, 6 mm diameter) were sterilized by autoclaving. Ten microliter of the five lightening (13/6, 5/3, 5/1, 625/0, 812 mg mL^{-1}) plant extracts sited above were applied $filter^{-1}$ paper disc. Each extract was tested in triplicate, with a penicillin disc as reference or positive control. Negative controls were prepared using the same solvent employed to dissolve the plant extracts (ethanol 70%). The plates were evaluated after incubation at 27°C for 24 h after which the zones of inhibition around each disc were measured. The ratio of the inhibition zone (mm) produced by the plant extracts and the inhibition zone around the penicillin reference (mm) was used to express antibacterial activity (Djipa *et al*., 2000).

Statistical analysis: Data obtained from the measured variables in this study were subjected to statistical analysis of variance (ANOVA) using STATITICA (version 6) package at $p<0.01$ significant level.

RESULTS AND DISCUSSION

Photochemical analysis of *H. albus* L.

Estimation percent of total alkaloids percent

Effect of plant-hormones (K, 2, 4-D, IAA and BAP) speared on percent of total alkaloids accumulated in *H. albus* L.: Analysis of variance revealed a significant treatment effect for the percent of alkaloids produced by shoot and root plant parts. Treatment with IAA (20 mg L^{-1}) produced the highest percent of alkaloid 2.153% in the root part of plant while the best value of 1.103% was observed in the shoot part of treatment with K (20 mg L^{-1}). The check produced the low value of 1.115% by the treatment with K (10 mg L^{-1}) in the root part and 0.743% of treatment with IAA (10 mg L^{-1}). From the comparison results between witness or chick and who were treated with plant-hormones (IAA, 2, 4-D, BAP, K) at 10 and 20 mg L^{-1}, we can conclude that the treatment with plant hormones speared increases the accumulation of tropic alkaloids witch is differed with plant hormone rates (Fig. 1).

It was reported that favorable increase in growth and alkaloid yield with *Hyoscyamus muticus* L. treated with kinetin (Trease and Evans, 1996), when increased concentration of auxin stimulated growth of root culture and inhibited alkaloids biosynthesis in *Hyoscyamus niger* L. (Van Rensburg *et al*., 1993) and the IAA is the predominant auxin and is important for plant growth and development (Oetiker and Bacher, 1997).

Effect of interaction between cytokinins (K and BAP) and auxins (2, 4-D and IAA) on percent of total alkaloids accumulated in *H. albus* L.: Analysis of variance showed

a significant interaction between cytokinins and auxins. The treatment by (K×2, 4-D) (20×20 mg L^{-1}) gives the highest percent of alkaloids in the root part with a percent mean value of 2.321% in the root part as compared with witness witch accumulated the amount of 0.873% (Fig. 2)

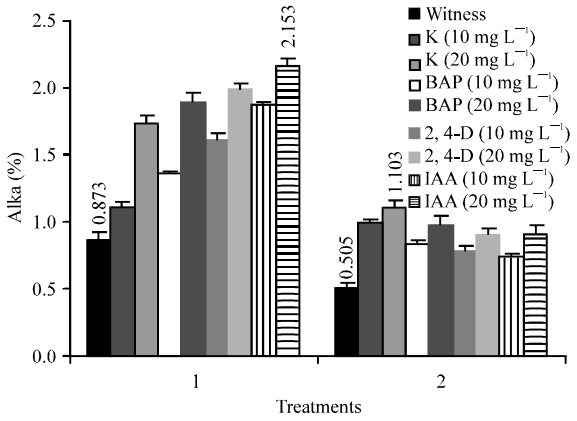

Fig. 1: Effect of plant-hormones (K, 2, 4-D, IAA and BAP) speared on percent of total alkaloids accumulated in *H. albus* L. (2) 4-Dichlorophenoxyacetic acid (2, 4-D), IAA: 3-indole acetic acid, K: kinetin, BAP: Benzyl amino purine

and 1.702% in shoot part with same plant hormones but dosage of (20×10 mg L^{-1}) as compared with witness witch gives 0.505% (Fig. 3).

These results indicated that alkaloids production is enhanced by interacted or combined application of cytokinins and auxins. This is in agreement with results of El-Bahr *et al.* (1997) who found that the better growth of callus culture of *H. muticus* L. and maximum alkaloid production was obtained with 1 mg L^{-1} of both 2, 4-D and K.

Value of 0.24% of alkaloids was measured in *H. albus* L. under natural conditions of Afghanistan (Pelt *et al*., 1967). This value is lower than that measured in witness of the present experiment witch lead to conclude that growth conditions may enhance too alkaloids accumulation in this species.

Auxins and cytokinins enhanced the production of ethylene by a factor 8-10 when sprayed on leaves and the ethylene favors accumulation of secondary products *in vitro* culture. Grewal *et al.* (1979) and Cary *et al.* (1995) mentioned that K in combined with IAA and NAA at 10^{-5} M/L produced alkaloid during the 4, 5 and 6th week and the rapidly dividing undifferentiated callus cultures of *H. muticus* L. are capable of producing alkaloids depended on hormonal level. From the previous mentioned data, it is concluded that the applied plant

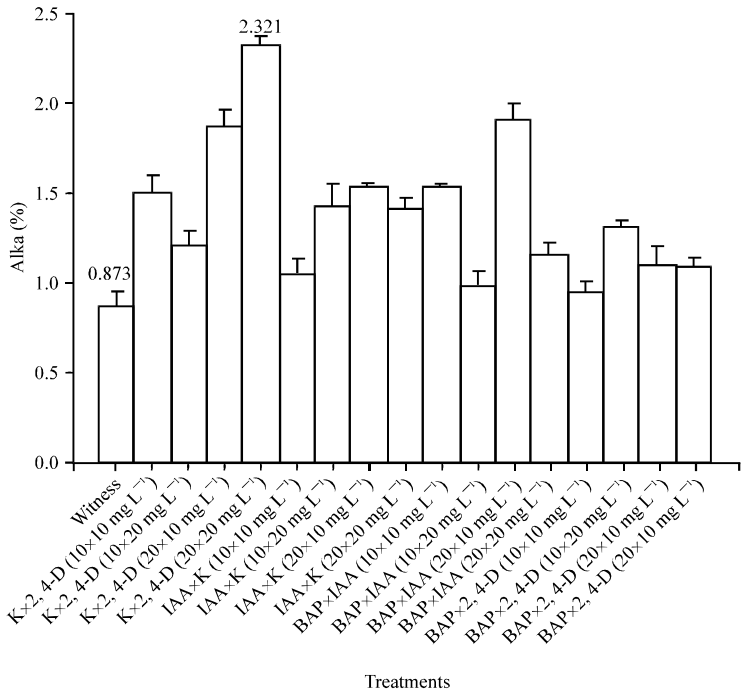

Fig. 2: Effect of interaction between cytokinins (K and BAP) and auxins (2, 4-D and IAA) on percent of root total alkaloids accumulated in *H. albus* L. 2: 4-Dichlorophenoxyacetic acid (2, 4-D), IAA: 3-indole acetic acid, K: Kinetin and BAP: Benzyl amino purine

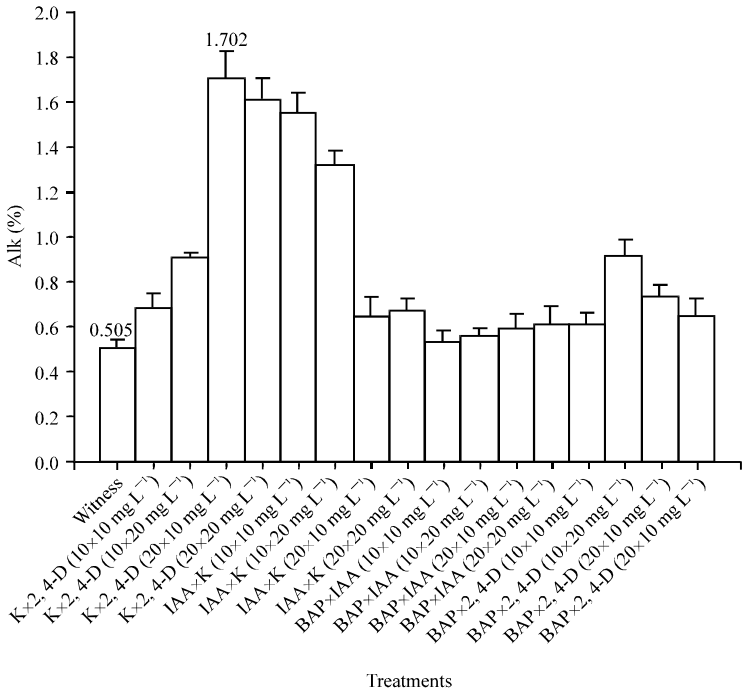

Fig. 3: Effect of interaction between cytokinins (K and BAP) and auxins (2, 4-D and IAA) on percent of shoot total alkaloids accumulated in *H. albus* L. 2: 4-Dichlorophenoxyacetic acid (2, 4-D), IAA: 3-indole acetic acid, K: kinetin and BAP: Benzyl amino purine

hormones (K, IAA, BAP, 2, 4-D) acted on the physiological processes of the plant, leading to an increase in nitrogenous products enhancing ions absorption and permitted more production of tropic alkaloids in *H. albus* L. reported by Wernicke *et al.* (1979).

TLC investigation of alkaloid extracts of the aerial and root parts of *H. albus* L.: Thin layer chromatographic screening of the alkaloid extract of witness and all treatment with 2, 4-D, K, IAA and BAP were carried out using silica gel chromatoplates and solvent systems witch was chloroform: methanol (9:1) (Isogai *et al*., 1990).

The developed plates were visualized by UV light [wave length 254 mm] and Dragendorff's spray reagent followed by spraying with 05% sodium nitrite (Bhojwani, 1990) when six spots were detected, then the rate of flow (R_f) of spots were measured. Three were known the: atropine (R_f = 0.21), belladonine (R_f = 0.00) and scopolamine (R_f = 0.56). The results of TLC investigation of all treatments showed that plant-hormones have not any effect on alkaloid types, when we have found that the *H. albus* L. contains 6 alkaloids witch are: The atropine, the scopolamine, the belladonine, the meteloidine, the tigloidine and the hyoscyamine (Pelt *et al*., 1967). Atropine and scopolamine were isolated as major alkaloids in *H. albus* L., the same that founded by Basu and Chand (1998).

Results of TLC investigation of witness and treatment with K×2, 4-D (20×20 mL L^{-1}) are illustrated in Table 1.

Antibacterial activity for the extracts of *H. albus* L.

Antibacterial activity of ethanolic extracts of the shoot and root parts of *H. albus* L.: The control disks injected with 10 μL of ethanol and the ethanol extracts of shoot and root *H. albus* L. of treatments: Witness, K×2, 4-D 20×20 mg L^{-1}, IAA×2, 4-D 20×20 mg L^{-1}, IAA×BAP 20×20 mg L^{-1}, BAP×2, 4-D 20×20 mg L^{-1}, BAP×2, 4-D 20×20 mg L^{-1}, K×IAA 20×20 mg L^{-1} showed no inhibitory effect against the microorganisms tested: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas stutzeri* and *Klebsiella pneumonia*. The same result was founded by Nostro *et al*. (2000) when they used ethanolic extracts of some medicinal plants.

Antibacterial activity of alkaloid extracts of the shoot and root parts of *H. albus* L.: Analysis of variance showed a significant antibacterial activity to microorganisms tested. The results shown in Table 2 of the pure alkaloids extracts

Table 1: TLC investigation of alkaloid extracts of *H. albus* L.

Color of spots	Alkaloids	No. of spots	Real RF	Extracts	Plant part
Orange	Belladonin	6	0.00	Witness	Aerial
Orange	Unknown		0.16		
Orange	Atropine		0.21		
Orange	Unknown		0.33		
Orange	Unknown		0.42		
Orange	Scopolamine		0.56		
Orange	Belladonin	6	0.00	2, 4-D×K (20×20 mg L^{-1})	Aerial
Orange	Unknown		0.16		
Orange	Atropine		0.21		
Orange	Unknown		0.33		
Orange	Unknown		0.42		
Orange	Scopolamine		0.56		
Orange	Belladonin	6	0.00	Witness	Root
Orange	Unknown		0.16		
Orange	Atropine		0.21		
Orange	Unknown		0.33		
Orange	Unknown		0.42		
Orange	Scopolamine		0.56		
Orange	Belladonin	6	0.00	2, 4-D×K (20×20 mg L^{-1})	Root
Orange	Unknown		0.16		
Orange	Atropine		0.21		
Orange	Unknown		0.33		
Orange	Unknown		0.42		
Orange	Scopolamine		0.56		

for the root system illustrated the existing of sensitivity of the microorganisms tested that showed inhibition diameters differ according to the type of microorganism, the extract dosages and treatment applied.

Alkaloid extracts of *H. albus* L. of treatments: Witness, K×2, 4-D 20×20 mg L^{-1}, IAA×2, 4-D 20×20 mg L^{-1}, IAA×BAP 20×20 mg L^{-1}, BAP×2, 4-D 20×20 mg L^{-1}, BAP×2, 4-D 20×20 mg L^{-1}, K×IAA 20×20 mg L^{-1} showed no inhibition zone to the microorganism *P. stutzeri*.

Extracts of all treatments of *H. albus* L. in concentration of 1.625 and 0.812 mg mL^{-1} showed also no inhibition zone to the microorganisms tested.

The highest average of inhibition zone has been recorded at *S. aureus* by the value 22 mm at the dosage of 13 mg mL^{-1} of the positive control penicillin and 14 mm at the shoot system of *H. albus* L. treated by K×2, 4-D 20×20 mg L^{-1} witch accumulate the highest percent of alkaloid.

Antibacterial activity of alkaloid extracts of root parts of *H. albus* L. treated by plant-hormones: The alkaloid root extracts of *H. albus* L. treated by interacted plant-hormones mentioned above had showed better antibacterial activity compared to the shoot extracts.

The inhibition zones of the microorganisms sensitive to alkaloid root extract were 4-18 mm, respectively

Table 2: Antibacterial activity of alkaloid extracts of the shoot parts of *H. albus* L.

	Inhibition zones (mm)			
	Microorganisms			
Plant extracts	*E. coli*	*K. pneumoniae*	*S. aureus*	*P. stutzeri*
Witness				
13	6.0	8.0	11.0	-
6.5	5.0	7.0	10.0	-
3.25	4.5	7.0	8.0	-
1.625	-	-	-	-
0.812	-	-	-	-
K×2, 4-D 20×20 (mg L^{-1})				
13	12.0	13.0	14.0	
-				
6.5	12.0	12.0	13.0	-
3.25	11.0	11.5	11.0	-
1.625	-	-	-	-
0.812	-	-	-	-
IAA×2, 4-D 20×20 (mg L^{-1})				
13	8.5	11.0	13.0	-
6.5	8.0	10.0	12.0	-
3.25	7.0	9.5	11.0	-
1.625	-	-	-	-
0.812	-	-	-	-
IAA×BAP 20×20 (mg L^{-1})				
13	9.0	11.0	13.0	-
6.5	8.5	10.0	13.0	-
3.25	7.0	10.0	12.0	-
1.625	-	-	-	-
0.812	-	-	-	-
BAP×2, 4-D 20×20 (mg L^{-1})				
13	7.5	9.5	11.0	-
6.5	7.0	8.5	10.0	-
3.25	6.5	8.0	9.0	-
1.625	-	-	-	-
0.812	-	-	-	-
K×IAA 20×20 (mg L^{-1})				
13	9.5	10.0	10.0	-
6.5	8.5	9.0	9.0	-
3.25	8.0	8.5	8.5	-
1.625	-	-	-	-
0.812	-	-	-	-
Penicillin				
13	20.0	18.0	22.0	19
6.5	18.0	18.0	20.0	18
3.25	17.0	18.0	20.0	17
1.625	17.0	17.0	19.0	17
0.812	15.0	16.0	18.0	16

-: No antibacterial activity

(Table 3). The highest inhibition zone 18mm was observed in treatment with K×2, 4-D 20×20 mg L^{-1} at dosage of 13 mg mL^{-1} against *S. aureus* compared to the positive control witch showed 22 mm against the same microorganism at dosage of 13 mg mL^{-1}.

This study indicate that ethanol and pure alkaloid extracts of shoot and root of *H. albus* L. treated by plant-hormones showed various antibacterial effects on the microorganisms listed above. But the ethanol showed no inhibitory effects on the same microorganisms.

Table 3: Antibacterial activity of alkaloid extracts of root parts of *H. albus* L. treated by plant-hormones

	Inhibition zones (mm)			
	Microorganisms			
Plant extracts	*E. coli*	*K. pneumoniae*	*S. aureus*	*P. stutzeri*
Witness				
13	7.0	8.0	8.5	8.5
6.5	6.0	7.5	8.0	8.0
3.25	5.5	6.0	8.0	7.0
1.625	4.0	5.0	5.0	6.0
0.812	4.0	5.0	4.0	6.0
K×2, 4-D 20×20 (mg L^{-1})				
13	16.0	15.0	18.0	14.0
6.5	16.0	14.0	16.0	12.0
3.25	15.0	13.5	14.0	11.0
1.625	14.0	11.0	13.0	8.0
0.812	10.0	11.0	10.0	8.0
IAA×K 20×20 (mg L^{-1})				
13	10.5	11.0	15.5	10.0
6.5	9.0	11.0	14.0	9.0
3.25	8.0	9.5	13.0	9.0
1.625	8.0	7.0	12.0	8.0
0.812	6.0	7.0	12.0	7.0
IAA×BAP 20×20 (mg L^{-1})				
13	12.0	14.0	16.0	13.0
6.5	11.5	13.0	14.0	12.0
3.25	9.0	12.0	13.0	12.0
1.625	8.0	10.0	12.0	11.0
0.812	8.0	9.0	12.0	10.0
BAP×2, 4-D 20×20 (mg L^{-1})				
13	10.5	12.5	14.0	12.0
6.5	9.0	11.5	13.0	11.0
3.25	7.5	8.0	12.0	11.0
1.625	7.0	7.0	12.0	10.0
0.812	7.0	7.0	10.0	8.0
K×IAA 20×20 (mg L^{-1})				
13	15.5	15.0	16.0	12.0
6.5	14.5	14.0	15.0	10.0
3.25	13.0	3.5	14.5	10.0
1.625	12.0	11.0	12.0	9.0
0.812	10.0	11.0	12.0	9.0
Penicillin				
13	20.0	18.0	22.0	19.0
6.5	18.0	18.0	20.0	18.0
3.25	17.0	18.0	20.0	17.0
1.625	17.0	17.0	19.0	17.0
0.812	15.0	16.0	18.0	16.0

CONCLUSION

This study indicated that treatment with plant-hormones: auxins and cytokinins at three rates 0-10-20 mg L^{-1} isolated and interacted [auxins×cytokinins] enhanced the alkaloids accumulation in *Hyoscyamus albus* L. When treatment by (K×2, 4-D) (20×20 mg L^{-1}) given the highest percent of alkaloids in the root part with a percent mean value of 2.321 and 1.702% in shoot part with same plant hormones but dosage of [20×10 mg L^{-1}] in order. As a result of TLC, *Hyoscyamus albus* L. contained 06 alkaloids. Alkaloidical extracts of root part of *Hyoscyamus albus* L. showed various antibacterial activities against the microorganisms: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas stutzeri* and *Klebsiella pneumonia*. According to our results *Hyoscyamus albus* L. could be used as raw material for phototherapy because of their antibacterial activities.

ACKNOWLEDGMENT

This study was founded by the University of Khenchela.

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