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Callus Induction and Antimicrobial Activities of Callus and Intact Plant Extracts of *Datura stramonium* L.

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Abstract: ***Antimicrobial activities of callus and intact plant leaves of Datura stramonium L(Solanaceae) was investigated against Bacillus subtilus , Staphylococcus aureus , Escherichia coli ,Pseudomonas aeruginosa ,Candida albicans and Aspergillus niger by using agar diffusion method. In order to induce callus, leaf explants from D. stramonium were cultured on Murashige &Skoog (MS) medium supplemented with different types and concentrations of growth regulators. The explants of D. stramonium initiated callus very rapidly after three weeks when grown in(MS) medium supplemented with 2.0 mg/l 2,4-D,while the other concentrations showed less rate of callus formation. MS medium supplemented with combination of 0.05mg/l 2,4-D + (0.025mg/l) kinetin showed faster rate of callus initiation when compared with medium containing 0.05mg/l 2,4-D alone. Leaves of D. stramonium, and their derived callus were extracted by petroleum ether and methanol. Petroleum ether extracts for leaves of intact plant and their callus showed no activity against all tested organisms, while the methanolic extract of leaves intact plant showed highly inhibition zone(IZ) against Bacillus subtilis ,Staphylococcus aureus, Escherichia coli ,Pseudomonus aeruginosa ,Aspergilus niger, while the methanolic extract of leaf derived callus showed activity against Bacillus subtilus and Candida albicans. The antibacterial activity of Penicillin and Gentamicin were compared with the antibacterial activity of the tested extracts. D. stramonium leaf methanolic extracts showed more efficacy activity against Bacillus subtillus than Gentamicin at 50µg/ml. Phytochemical screening for both plants and callus extracts indicated the presence of secondary metabolites such as alkaloids, flavonoids and tannins***

Keywords: *Datura stramonium*, Callus Induction, Antibacterial activity, antifungal activity, phytochemical analysis.

1. Introduction

Datura species can be found throughout the world, except the colder or Arctic regions, it grows wild in all of the worlds warm and moderate region and it is very distributed in the temperate and tropical areas [18].

Iranbakhsh [5] investigated the effects of methanolic extracts from root, stem and leaf of *D. stramonium* (L.) on the vegetative and generative phases of growth process of four bacterial strains and four fungi strains. Methanol extract from flower, seed and leaf explants callus of *D.stramonium* was also used. The results showed that the active compound was atropine alkaloid. They concluded that organogenator callus extract has more inhibition effect on the growth of fungi in comparison to the other extracts. It seems that the effective antimicrobial ingredient goes back to alkaloids and is related to tissue and organ differentiation directly.

Johnson [6] studied and compared the antimicrobial potential of leaves, inter-nodal segments, leaves and inter-nodal segments derived calli of *Alternanthera sessilis*(L.) against four bacterial pathogens by the agar diffusion assay. The antibacterial effect of the leaves, inter-nodal, leaves and inter-nodal segments derived calli of *A. sessilis* was evaluated against *Proteus vulgaris, Streptococcus pyogenes, Bacillus subtilis and Salmonella typhi.* The ethanolic extracts of auxins on callus formation in this plant and they Showed that auxin in the form of 2,4-D had no callus formation in tomato. Other related study by Mungole [12], revealed that in vitro callus induction and shoot regeneration of *Physalis minima* L. (Solanaceae) by using different types of growth of leaves and leaves derived calli were more effective against the selected bacteria than other solvents.

Talreja [17] screened ethanol extracts prepared from the flowers and callus of *Moringa oleifera*, for their antimicrobial activity against some bacterial and fungal pathogenic strains by paper disc method. The tested G+ve bacterial strains were *Bacillus subtilis* and *Staphylococcus aureus*, G-ve bacterial strains were *Escherichia coli, Klebsiella pneumoniae* and fungal pathogen was *Candida albicans*. Among the flowers and unorganized tissue tested, the ethanol extract of callus exhibited higher antimicrobial activity when compared to the floral extract.

Amiri [1] investigated the in vitro propagation and plant regeneration of *D. stramonium* and found that the presence of 2, 4-D is necessary for callus induction from leaf explants of this plant since the treatments lacking of 2,4-D failed in callus formation or produced too little callus. They also found that the low concentrations of kinetin (1≥ mg/l) in combination with 2,4-D can expedite callus initiation and proliferation from leaf explants. It was revealed that, the presence of kinetin can enhance the effect of 2, 4-D on callus formation from this type of explants. Brasileiro [2] studied the callus formation and plant regeneration in tomato *(Lycopersicon esculentum*), they reported that the presence of cytokinins encourages the effect regulators and the best result in term of percentage response of callus induction (90%) from apical leaf was obtained from 0.4 mg/l of 2,4-D.

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Khalilsaraie [8] studied antimicrobial activity of aqueous leaf and leaf callus extracts of *Tinospora cordifolia* against G+ve and G- ve bacteria. The callus extract has shown inhibition of both Gram positive and Gram negative organisms while leaf extract has shown inhibition of only Gram negative bacteria indicating the significant activity of callus extract than leaves. The phytochemical contents of callus and that of leaf extract were found to be different. The presence of additional compounds in callus extract may attribute to its activity against Gram positive organisms.

Christhudas [3] identified promising antimicrobial metabolite from *Datura stramonium* similar to what producing by Streptomycin strain. Physiological, biochemical and 16S rRNA studies strongly suggested that this isolate belonged to Streptomycin spp and has ability to produce enzymes such as amylase, lipase and catalase.

2. Materials and Methods

2.1 Plant Materials

The mature seeds of *Datura stramonium* were collected from Shambat, faculty of Agriculture, university of Khartoum and from Al Kadaro area north of Khartoum.

2.2 Microorganisms

The standard microorganisms used in this study were the following: *Bacillus subtilus* (NCTC 8236*), Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853*), Aspergillus niger* (ATCC 9763), *Candida albicans* (ATCC 7596). The test organisms were obtained from the Department of Microbiology, Medicinal and Aromatic Plants Research Institute, Khartoum, Sudan.

2.3 Reference drugs

Reference drugs used in this study were Ampicillin (10 µg/disc) and Gentamicin (10 µg/disc) sensitivity discs from Himedia, and Gentamicin 50 µg/ml from SPIC, China.

2.4 Seed surface sterilization and germination

Seeds of *D. stramonium* were firstly soaked in conc. H_2SO_4 for 40 sec. and then rinsed in tap water many times then the surface sterilized by soaking in 15 % Clorox (0.5 % free chlorine) with 2 drops of Tween-20 for 10 min, and rinsed 3-5 times in sterile distilled water .The sterilize seeds of *D. strmonium* were cultured in germination medium MS [13] basal medium.

2.5 Callus induction

The leaves were used as explants for *D. stramonium* in this study. MS medium was used. Two types of auxin (2,4-D and NAA) were used separately at different concentrations (0.0 as control, 0.05, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0) mg/l to assess their effects on callus induction for explants of *D. stramonium*. To compare the effect of the presence of cytokines in callus induction medium, 2 different concentration of kinetin (0.025 and 0.05 mg/l) were used in combination with (0.05 mg/l) 2, 4-D in Datura leaf explants.

Each of the sterilized explants was cut into 2-3 mm pieces using sterile scalpel. Four pieces were inoculated in each vial containing sterile culture medium (MS medium) with different concentrations and combination of growth regulators. Cultures were incubated for 8 weeks in the dark at 25±20 C, and data were recorded every two weeks.

2.6 Preparation of plant and callus crude extract

Each of the coarsely powdered plant material was exhaustively extracted for 4 hours with petroleum ether in Soxhlet apparatus. The petroleum ether extract was filtered with a filter paper and evaporated under reduced pressure at 30 0C using a rotatory evaporator apparatus (Rota-vap). The extracted plant material was air dried and repacked again and extracted with methyl alcohol. The methanolic extract was filtered with a filter paper and evaporated under reduced pressure at 650C using Rot-evap.

Crude extract of callus was prepared by similar way except the callus was dried at first by freeze drying using freeze dryer and then powdered and extracted with two different solvents, petroleum ether and methanol in Soxhlet apparatus.

2.7 Preparation of bacterial suspension

One ml aliquots of 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37 ^{0}C for 24 hours. The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce a suspension containing about (108 -109) colony forming units per ml (CFU./ml). The suspension was stored in the refrigerator at 40 C until used. The average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique [11].

2.8 Preparation of fungal suspension

Fungal cultures were maintained on Sabouraud dextrose agar, incubated at 25^0 C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in 100 ml of sterile normal saline, and the suspension was stored in the refrigerator until used.

5.9 *In vitro* testing of extracts for antimicrobial activity

a)Testing for antibacterial activity

The cup-plate-agar diffusion method [7] was adopted. Negative controls involving the addition of the respective solvents instead of extracts were carried out separately. After incubation the diameters of the resultant growth inhibition zones were measured. Mean values were tabulated.

b)Testing for antifungal activity

The same method as for bacteria was adopted. Instead of nutrient agar, Sabouraud dextrose agar was used.

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c) Preliminary phytochemical screening

The extracts of leaves of intact plant and callus were evaluated for the presence of different phytochemicals to ascertain the presence of metabolites such as tannins, alkaloids and flavonoids by using the methods described by [19] [16], [4] and [10], with some minor modifications.

3. Results and Discussion

3.1 Callus Induction

The callus was successfully induced by different concentrations of 2, 4-D and combinations with kinetin (0.025 and 0.05 mg/l) (Table 1 & Fig. 1). Among all this concentrations, 2.0 mg/l of 2, 4-D was found to be the most effective auxin concentration to induce callus from *D. stramonium* leaf explants. Combination of 0.05 mg/l 2,4-D + 0.025 mg/l of kinetin was more effective in callus induction in comparison to 0.05 mg/l 2,4-D alone (Fig. 2). These results agree with Amiri [1], who studied in vitro propagation and plant regeneration of *D. stramonium* and found that the presence of 2, 4-D is necessary for callus induction from leaf explants of *D. stramonium* since the treatments lacking 2,4-D failed to induce callus or produced too little callus. They also found that the low concentrations of kinetin (1≥ mg/l) in combination with 2, 4-D can expedite callus initiation and proliferation from leaf explants. It was revealed that, the presence of kinetin can enhance the effect of 2, 4-D on callus formation from this type of explants. Brasileiro [2] studied the callus formation and plant regeneration in tomato *(Lycopersicon esculentum*), they reported that the presence of cytokinins encourages the effect of auxins on callus formation in this plant, otherwise against our results, they showed that auxin in the form of 2,4-D had no effect on callus formation in tomato. Other related study by Mungole [12] showed that the in vitro callus induction and shoot regeneration for *Physalis minima* L. (Solanaceae) by using different types of growth regulators gave best result as 90% rate from apical leaf was obtained from 0.4 mg/l of 2,4-D.

Table 1: Effects of different concentrations of 2, 4-D, and combinations of kinetin and 2,4-D on MS medium on the induction of callus of *Datura stramonium* leaf explants.

Growth regulator mg/l		Rate of callus formation	Callus color
2,4-D	kinetin		
0.0	-	-	-
0.05	-	+	C
0.05	0.025	++	LCB
0.05	0.05	+	C
0.5	-	+	LCB
1.0	-	+	LCB
2.0	-	+++	LCB
3.0	-	++	C
4.0	-	++	LCB
5.0	-	++	LCB
6.0	-	++	LCB
7.0	-	++	C

C=Creamy, LCB= Light Ceramic Brown
- = no callus, + = slow rate of callus formation, ++ = medium rate of callus formation, +++ = fast rate of callus formation



Figure 1: Callus induced from *Datura stramonium* leaf explants with different concentrations of 2,4-D, and combinations of kinetin and 2,4-D on MS medium.

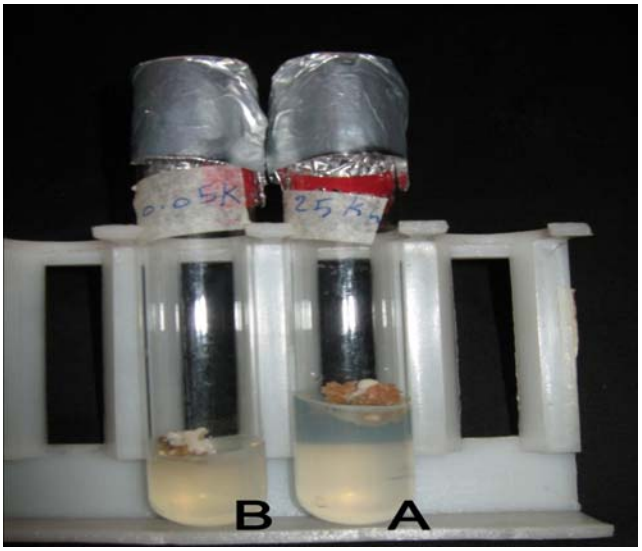


Figure 2: Callus induced from *Datura stramonium* leaf explants with combination of 2,4-D and kinetin
A= 0.05 mg/l 2, 4-D + 0.025 mg/l kinetin
B= 0.05 mg/l 2, 4-D + 0.05 mg/l kinetin

4. Antimicrobial Activity

The petroleum ether extracts for both leaf and leaf derived callus were found to be ineffective against all tested microorganisms. The methanol extracts of *D. stramonium* leaf show inhibition zone against both G+ve and G-ve bacteria, with maximum zone of inhibition (30 mm) against *B. subtilus* (Fig. 3 & 4), while the methanolic extracts of leaf derived callus show antibacterial activity only with *B. subtilus*. In agree with our results Iranbakhsh [5], found that methanolic extracts of leaf and leaf derived callus of *D. stramonium* have antibacterial effect on *B. subtilus*, and they found that the methanol might be the effective solvent for the chemical substance that have the bactericidal role. In general, the cell wall of G-ve bacteria, which is more complex than G+ve ones, act as a barrier and making them less susceptible to the antibacterial agents than G+ve bacteria. Kumar [9] found that ethanolic extracts of *Datura stramonium* leaf exhibited activity against *Ps. aeruginosa*

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this finding agree with the findings in this study, with exception that *D. stramonium* showed activity against *E.coli*. The methanolic extracts of leaf derived callus showed (14 mm) inhibition zone with *C. albicans* (Fig.5), while the methanolic extracts of *Datura stramonium* leaf showed inhibition against *A. niger*. This results agree with Saadabi[14] who investigated many different extracts of *D. stramonium* leaf and found that methanolic extracts of *D. stramonium* leaf show moderate inhibition against *A. niger*, but contrary to our result they found strong inhibition against *C. albicans*.

The comparison of results given in(Table 2 & Fig. 3) showed that the methanolic extract of *D. stramonium* showed activity higher than Gentamicin at 50 µg/ml against *Bacillus subtilus*, also methanolic extract of *D.* stramonium leaf showed activity against *E. coli*, *S. aureus* and *Ps. aeruginosa* while no activity was observed for Ampicillin at 10 µg/ against this bacteria. These results confirm the antimicrobial effect of *D. stramonium* methanolic extract towards *Bacillus subtilus*.

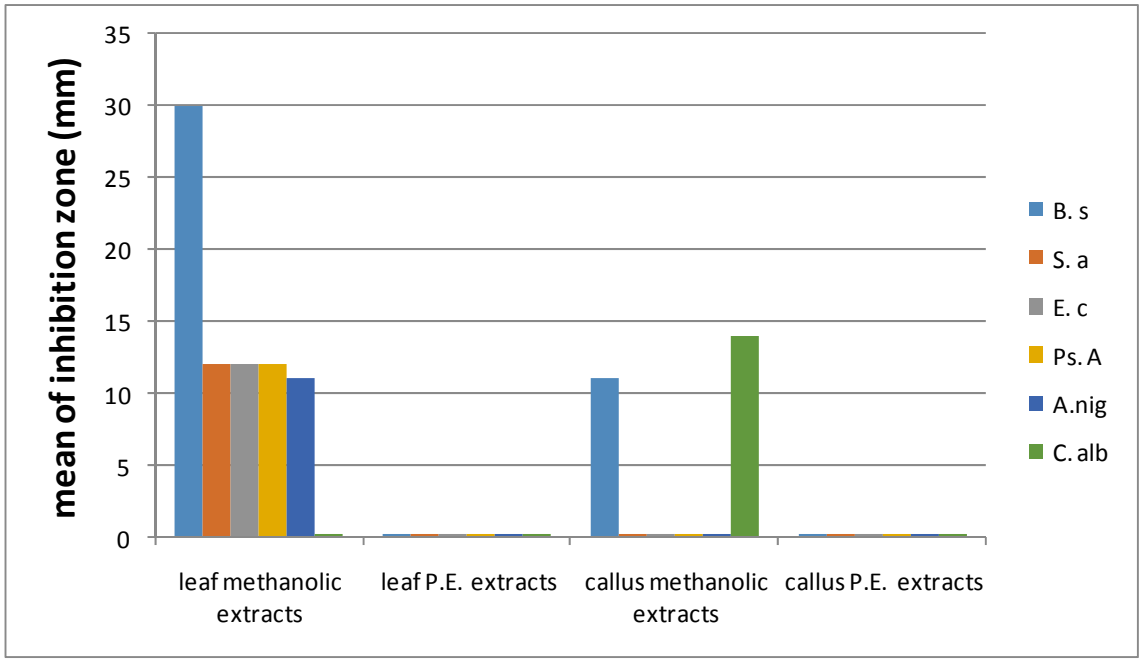


Figure 3: Histogram showing the average of inhibition zone (mm) of *Datura stramonium* leaf and leaf derived callus extracts against standard microorganisms.

B.s = *Bacillus subtilus*; S.a = *Staphylococcus aureus*; E.c = *Escherichia coli*
Ps.a = *Pseudomonas aeruginosa*; As.n = *Aspergillus niger;*
C. alb = *Candida albicans* P.E. = *Petroleum Ether*



Figure 4: *I.Z. by methanol extract of *Datura stramonium* leaf against Bacillus subtilus

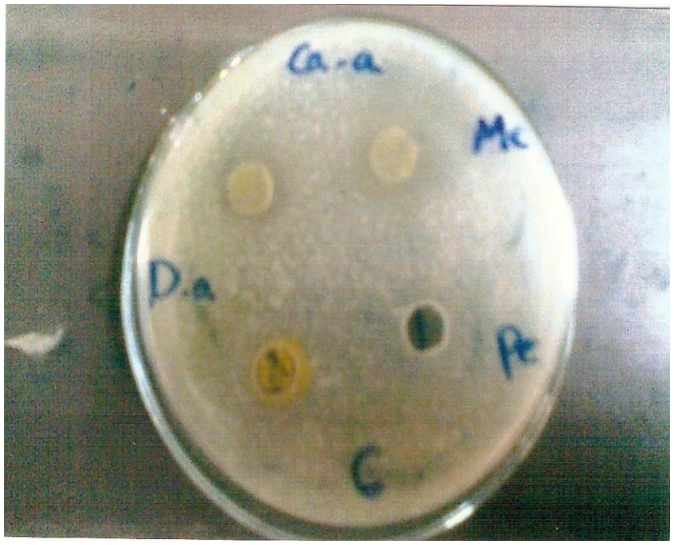


Figure 5: *I.Z. by methanolic extract of *Datura stramonium* leaf derivative callus against *Candida albicans*

*I.Z. = inhibition Zone

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Table 2: Antibacterial activity of reference drugs against standard microorganisms

Drugs	*M. D. I. Z. (mm)***			
	B.s	*S. a*	*E. c*	*Ps. a*
Ampicillin 10µg/disc	*	0.0	0.0	0.0
Gentamicin 10µg/disc	*	14	15	13
Genamycin 50 µg/ml	25	32	28	30

* = not tested with Ampicillin and Gentamycin at 10µg/disc.
B.s = Bacillus subtilus; S.a = Staphylococcus aureus; E.c = Escherichia coli
Ps.a = Pseudomonas aeruginosa
** M. D. I. Z. (mm) = Mean diameter of growth inhibition zone (mm).

5. Phytochemical Screening

Table (3) showed the presence of alkaloids, flavonoids and tannins on both leaf and callus extracts. These secondary metabolites could be responsible for the antimicrobial activity exhibited by many medicinal plants belonging to various families. These confirm the findings of Sharma and Shama [15]; they stated that antimicrobial activity was higher in naturally grown plant and the plants that rich with metabolite such as flavonoids, phytosterols and alkaloids develop great bactericidal and fungicidal activity.

Table 3: Preliminary screening for secondary metabolites of *Datura stramonium* leaf and leaf derivative callus

Test	*Callus*	*Leaves*	*Observation*
Alkaloids	positive	Positive	Turbidity and precipitate
Flavonoids	positive	Positive	Yellow color
Tannins	positive	Positive	Blue – green color

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