Global Veterinaria 4 (2): 149-155, 2010
ISSN 1992-6197

The Inhibitory Effects of Plant Methanolic Extract of *Datura stramonium* L. and Leaf Explant Callus Against Bacteria and Fungi

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Abstract: This study aimed to investigate the effects of methanolic extract from root, stem and leaf of *Datura stramonium* L. on the vegetative and generative phases of the growth process of four bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus epidermidis* and *Bacillus subtilis)* and four fungi strains *(Fusarium semithectum*, *Fusarium colmorum, Ceratocystis ulmi* and *Rhizoctoina solani*). In this research, methanol extract from flower, seed and leaf explant callus was also used. Liquid culture, pour plate, punch, direct drop and disk paper methods were used. The result showed that the methanol extract from green leaf explant callus had inhibitory effects on the growth of *B. subtilis* (ATCC KX1-A1 15561) and *S. epidermidis* (ATCC 0-1-B29997) with inhibition zones of 22 and 23mm, respectively. An inhibition effect of the methanol extract from clear callus on the growth of *E. coli* (ATCC W1485 25645) was observed with (inhibition zone of 17mm). An effect of the methanol extract from organogenesis callus on the growth of *C. ulmi* (ATCC 32731) was observed. The inhibition effect of the methanol extract of green callus on the growth of *F. semithectum* (ATCC 11599) was observed (Inhibition zones 17mm). An effect of the methanol extract from green callus on the growth of *F. colmorum* (ATCC 15620) was observed. Inhibition effect of the methanolic extract from stem and leaf in the vegetative phase on the growth of *B. subtilis* was observed (analysis of punch method). An inhibition effect of the methanolic extract from leaf and stem in the vegetative phase (inhibition zones 21 and 21 mm, respectively), from stem and root in the generative phase (with inhibition zone 20 mm) and from mature seed (inhibition zones 17 mm) on the growth of *B. subtilis* (inhibition zone 21mm) was observed (direct drop method). An effect of the methanolic extract from mature seed on the growth of *P. aeruginosa* (ATCC PA103-29260) (inhibition zone 21mm) was observed (direct drop method). An inhibitory effect of the methanolic extract from root in the vegetative phase and flower on the growth of *R. solani* was observed (inhibition zones 20 and 16 mm, respectively). The results showed that the active compound was atropine alkaloid. It was 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.

Key words: Atropine · Bacteria · Callus · *Datura stramonium* · Extract · Fungi · Plant methanolic

INTRODUCTION

The higher plants are among the most prominent natural resources. They provide nutrients, fiber, wood and many chemical compounds such as oils, flavonoids, paints and medicinal compounds such as alkaloids [1]. The cell, tissue and organ cultures lead to changes in the production of chemical compounds, since the botanical organs and cells have the required capacity for the production of different secondary metabolites [2]. The differentiation of *Datura stramonium* L. transformed roots results in the decrease of tropane alkaloids synthesis capacity, which shows a predominant change in physiologic condition. The nitrogen metabolism in

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Datura stramonium L. transformed roots culture (alkaloids productive system) and the culture of the differentiated suspension resulted from these roots was studied by Fliniaux [3]. In both types of cultures similar ranges of amino acids were studied. The prominent difference in the compression of nitrogen-filled compounds was reported. In differentiated roots culture, the observed peaks were related to secondary metabolites such as tropine. However, in differentiated explants; the peaks were not observed [3]. The plants of *Solanaceae*, produce many active biological alkaloids including nicotine and tropane alkaloids [4]. Among *Solanaceae* plants, *Datura stramonium* L. is highly regarded by the workers, since it has a great resource of tropane alkaloids [5,6]. Botanical alkaloids are one of the important botanical products and form the major part of medicinal compounds [7]. Tropane alkaloids (i.e. atropine, hyoscyamine and scopolamine) are generally found in *Hyosyamous*, *Datura stramonium* L. [8,9] and *Belladonna* [5].

Datura stramonium L. is a one-year-old herbal plant with a height of 30 to 80 cm. This plant sometimes grows over one meter in height [10]. Tropane alkaloids play an important role in medicinal and defensive industries [9]. Scopolamine, with the chemical formula of $C_{17}H_{21}NO_4$, is like a colorless crystal. Hyoscyamine ($C_{17}H_{23}NO_3$) is a counterclockwise optical isomer, which easily gets rusmic and changes to atropine. Atropine is a tropic and tropenole ester acid. Besides Datura, atropine is found in *Belladonna* and *Hyosyamous*. Tropane alkaloids have a profound impact on the eyes, nervous system, heart, blood circulation and body secretions. They are anti-cholinergic and antispasmodic and are widely applied in medical sciences [5]. The application of *Datura* in a small amount dries the throat up, makes some difficulties in swallowing the food, widens the pupil, speeds up the blood circulation, increases the body temperature and decreases the pain. The excessive application results in death in a state of bemusement and staring; and manifests itself in a state of suffocation [10].

The aim of this study was to determine the antimicrobial properties of Datura plant extract, its probable role in the biologic control and the dosage of the active ingredients.

MATERIALS AND METHODS

The natural Datura was found in the 12th kilometer of Rasht-Fouman Highway (North of Iran) and was identified in Farabi botanical garden (Tehran, Iran) in 2003. The seed was provided by the Seed Fund of Iranian Forests and Pastureland Research Institute. The microorganisms of the research were the bacteria of Escherichia coli (ATCC W1485 25645),

Pseudomonas aeruginosa (ATCC PA103-29260),
Bacillus subtilis (ATCC KX1-A1 15561),
Staphylococcus epidermidis (ATCC 0-1-B29997),

and the four fungous strains including

Rhizoctonia solani (ATCC 16118),
Certocystis ulmi (ATCC 32731),
Fusarium semitectum (ATCC 11599),
Fusarium colmorum (ATCC 15620)

The Method of Producing Methanolic Extract: The different plant organs and also the calluses obtained from the leaf explants were drained in 60 degrees Centigrade in an incubator for two days. Then, each was separately grinded by a blender. Three grams of different parts of grinded powders are separately poured in the containers that include 100 ml of methanol. In order to prevent the solvent evaporation, the containers are covered with Para film and foil. Next, they are kept in the fridge for 24 hours. After being sifted through the 0.22-micrometer Millipore filter, the solution is sanitized and used to study the microbial effects [6,11,12]. The microbial cultural matrix includes the nutrient broth cultural matrix and Mueller-Hinton agar matrix for the bacteria culture, malt broth matrix and Sabouraud dextrose agar for fungi culture.

Methods of Studying Antibacterial Effects: First of all, a suspension was made out of the bacteria. Mueller-Hinton broth matrix was poured into the test tubes equal to the number of bacteria and was then sanitized. Next, through a swap sanitization, some of the bacteria were transferred from the Petri dish to the test tubes that contained the Mueller-Hinton broth matrix and eventually the suspension was provided. In the next 24 hours, the tubes were held in the incubator with the degree of 37 degrees Centigrade to let the bacteria grow. After that, the tube murkiness was measured by the McFarland witnessed murkiness. The murkier the microbial suspension is, the weaker it would be. When the suspension is provided, it is evenly spread all over the Mueller-Hinton broth cultural matrix by a swap suspension in three directions and with a 60-degree-rotation each time on the cultural matrix in the Petri dish. It is all done in sanitization conditions. The antibacterial effect of the methanolic extract, which was provided from different parts, was studied by the following methods: minimum inhibitory concentration (MIC), well, pour plate, disk paper and direct drop.

Minimum Inhibitory Concentration (MIC) Method: 100 micro-liters of the microbial suspension from the 24-hour bacteria culture were added to the tubes numbered 1 to 9. The tubes were put in the incubator with the degree of 37 degrees C for 24 hours. Then, the relevant results were recorded.

Well Method: In some parts of the matrix on which the bacteria were cultured, some wells with the diameter of 3 millimeters appeared. Therefore, about 150 micro-liters of extract was poured by a sampler and the Petri dishes were kept in the fridge for 2 hours until the extract properly permeated the cultural matrix around the wells. It was repeated three times (every two hours). A well was also considered for the methanol solvent. Then, the Petri dishes were carefully put in a 37-Centigrade incubator for 24 hours. When the bacteria grew in the proper time, the impact of extracts and solvent on the bacteria growth was studied and recorded.

Pour Plate Method: Before the cultural matrix completely cooled down and was covered (40 °C), one milliliter of the methanolic extract was added to the 9 millimeters of the cultural matrix. Then, the Petri dishes were slowly shaken in different directions until the extract properly and evenly spread over the cultural matrix. When the cultural matrix was covered, the bacteria were cultured over them and the samples were kept in 37-Centigrade incubator for 24 hours. The same phases were applied for the 1 milliliter of methanol as witnessed.

Disk Paper Method: Under Laminar Airflow, 150 microliters of the extracts and the methanol solvent was poured on the blank disks (for three successive times, 50 microliters each time). The blank disks had been sanitized with autoclave. When the solvent was evaporated in the proper time, the disks, which now contain the extracts, were put on certain spots of the cultural matrix on which the bacteria were previously cultured. The Petri dishes were put in an incubator at a temperature of 37 degrees Celsius for 24 hours. Later, the results were recorded.

Direct Drop Method: 150 microliters from each extract is directly dropped on single spots of the cultural matrix surface. The drops were left to permeate the matrix. Finally, The Petri dishes were put in an incubator at a temperature of 37 degrees Celsius for 24 hours. Later, the results were recorded.

Methods of Studying Anti-Fungous Effects: The analysis of the antifungous effects of methanolic extract was done similarly to the methods applied in the study of antibacterial effects, so they were not re-mentioned to avoid the redundancy. To provide the calluses obtained from the leaf explants, Iranbakhsh *et al.* method was applied [6,9].

RESULTS

The Antimicrobial Characteristics of Different Parts of *Datura stramonium* L

The Impact of Different Parts Extract of Datura on Four Bacterial Strains: The results of studying from the methanolic impact of different Datura parts extracts on the four bacterial strains in the Well Method is summarized in Table 1. It was clear that, at the vegetative stage, the methanolic extracts of stem and leaf have an inhibitory impact on *Bacillus subtilis* bacteria (Fig. 1).

The results of studying from the methanolic impact of different Datura parts extracts on the four bacterial strains in the Direct drop Method could be observed in Table 2. The methanolic extracts of stem and leaf at the vegetative stage, the methanolic extracts of stem, leaf and root at the generative stage and also the seed have a positive effect on the *bacillus subtilis*. The most effective antibacterial impact is attributed to the stem and leaf at the vegetative stage of plant life. Nevertheless, the seed was effective on *Pseudomonas aeruginosa* bacteria.

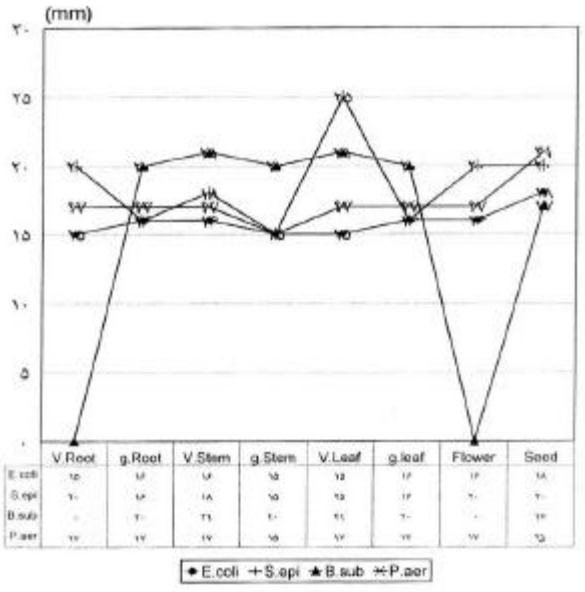

Fig. 1: The antibacterial effects of different *Datura* parts on the four bacterial strains (Inhibitory Zone)

Table 1: Impact of Different Parts Extract of Datura on four Bacterial Strains (Well Method)

		Zone diameter (mm)							
		Root		Stem		Leaf			
Seed	Flower	Generative Stage	Vegetative Stage	Generative Stage	Vegetative Stage	Generative Stage	Vegetative Stage	Methanol Solvent	Bacterial Strains
-	-	9	-	-	-	-	-	9	*E. coli* (ATCC W1485 25645)
9	-	7	-	7	7	9	17	8	*S. epi* (ATCC 0-1-B29997)
-	-	8	-	9	10	10	13	10	*B. sub* (ATCC KX1-A1 15561)
-	-	-	-	-	-	-	-	10	*P. aer* (ATCC PA103-29260)

Table 2: Impact of Different Parts Extract of Datura on four Bacterial Strains (Direct Drop Method)

		Zone Diameter (mm)							
		Root		Stem		Leaf			
Seed	Flower	Generative Stage	Vegetative Stage	Generative Stage	Vegetative Stage	Generative Stage	Vegetative Stage	Methanol Solvent	Bacterial Strains
18	16	16	15	15	16	16	15	18	*E. coli* (ATCC W1485 25645)
20	20	16	20	15	18	16	25	20	*S. epi* (ATCC 0-1-B29997)
17	-	20	-	20	21	20	21	15	*B. sub* (ATCC KX1-A1 15561)
21	17	17	17	15	17	17	17	17	*P. aer* (ATCC PA103-29260)

Table 3: Impact of Different *Datura* Parts Extract on Four Fungal Strains (Direct Drop Method)

		Zone Diameter (mm)							
		Root		Stem		Leaf			
Seed	Flower	Generative Stage	Vegetative Stage	Generative Stage	Vegetative Stage	Generative Stage	Vegetative Stage	Methanol Solvent	Fungus Strains
11*	20*	10	16*	-	10	11	8	12	*C. ulmi* (ATCC 32731)
-	15	-	-	-	20	-	-	-	*R. sol* (A.T.C.C 16118)
6	-	6	-	8	6	10	5	10	*F. sem* (ATCC 11599)
-	-	-	-	-	-	-	-	-	*F. col* (ATCC 15620)

* Results showed that Different Datura Parts Extract is fungistatic

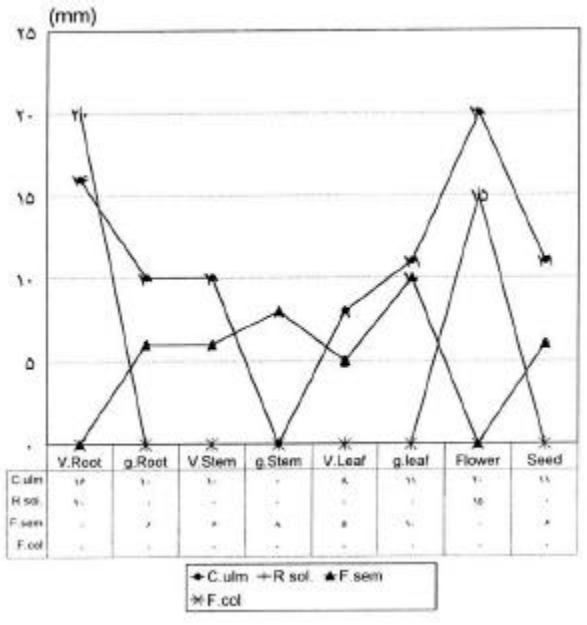

Fig. 2: The antifungous effects of different *Datura* parts on the four fungous strains (Inhibitory Zone)

Impact of Different *Datura* Parts Extract on Four Fungous Strains: The results of studying the methanolic impact of different Datura parts extracts on the four fungous strains in the Direct Drop Method could be observed in Table 3. The methanolic extract of the vegetative root and the flower of Datura show an effective response to *Rhizoctonia solani* fungus (Fig 2).

Antimicrobial Characteristics of Different Calluses Extracts

Impact of Different Calluses Extract on Four Bacterial Strains: As you can observe in the Fig 3 and Table 4, the methanolic extracts of green and organogenesis calluses meaningfully affect the *Bacillus subtilis* and *Staphylococcus* epidermis bacteria, which are both Gram-positive. The crystalline and half-crystalline callus extract has not been effective on *bacillus subtilis*. The crystalline callus extract had a minor effect on *Pseudomonas aeruginosa*. The half-crystalline callus had a positive inhibitory effect on *Staphylococcus epidermis*. The crystalline callus extract was effective on *Escherichia coli*.

Table 4: Impact of Different Parts Extract of Datura on four Bacterial Strains

Zone Diameter (mm)					
Methanol (Control)	Organogenator Callus Extract	Green Callus Extract	Half-crystalline Callus Extract	Crystalline Callus Extract	Bacterial Strains
12	12	8	12	17	*E. coli* (ATCC W1485 25645)
12	20	22	17	17	*S. epi* (ATCC 0-1-B29997)
-	22	23	-	-	*B. sub* (ATCC KX1-A1 15561)
15	12	15	12	17	*P. aer* (ATCC PA103-29260)

Table 5: Impact of Different *Datura* Parts Extract on Four Fungal Strains

Zone Diameter (mm)					
Methanol (Control)	Organogenator Callus Extract	Green Callus Extract	Half-crystalline Callus Extract	Crystalline Callus Extract	Fungus Strains
18	25	20	15	20	*C. ulmi* (ATCC 32731)
-	-	-	-	-	*R. sol* (ATCC 16118)
12	17	17	12	15	*F. sem* (ATCC 11599)
-	-	8	-	-	*F. col* (ATCC 15620)

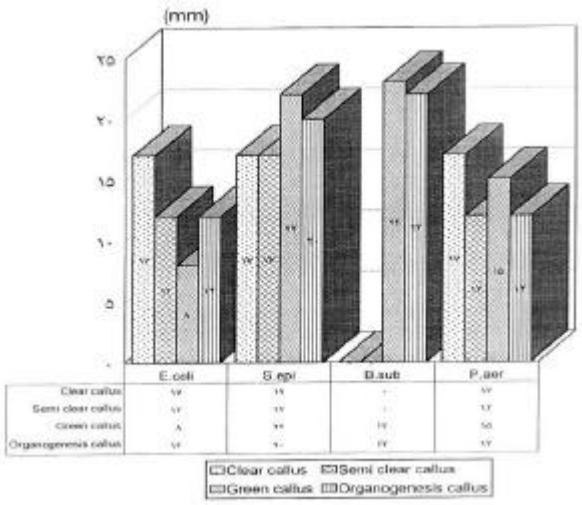

Fig. 3: The antibacterial effects of different calluses methanolic extracts of *Datura* leaf explants on the four bacterial strains (Inhibitory Zone)

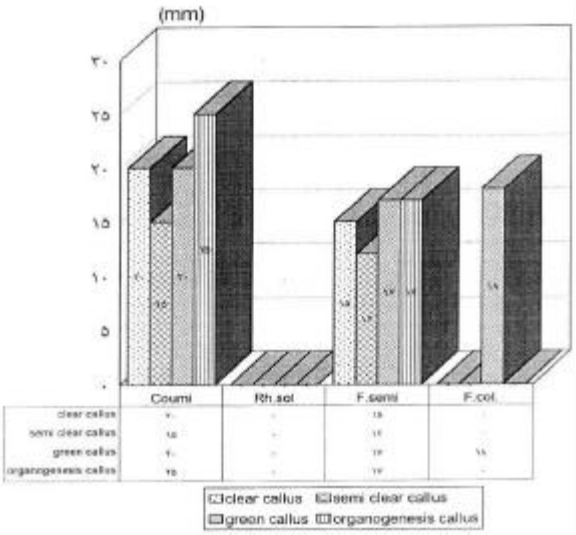

Fig. 4: The antibacterial effects of different calluses methanolic extracts of *Datura* leaf explants on the four fungous strains (Inhibitory Zone).

Impact of Different Calluses Extract on Four Fungous Strains: As it is observed in Fig 4 and Table 5, the organogenesis callus extract has been effective on *Certocystis ulmi*. None of the extracts was effective on *Rhizoctonia solani*. The green and organogenesis extract was effective on *Fusarium semitectum*. Only the green callus extract could largely show the inhibitory effect on *Fusarium culmorum*. Consequently, the organogenesis callus extract showed to have the highest inhibitory effect on fungi.

DISCUSSION

The results of studies on the antimicrobial effects of different Datura parts, i.e. root, stem and leaf at the vegetative and generative stages, flower and seed were illustrated. In liquid culture method, none of the aquatic extracts applied in the consistency showed the inhibitory effect. In pour plate method, none of the aquatic and methanolic extracts of different *Datura* parts showed the inhibitory effect on bacterial strains. In Well Method,

the inhibition zone could be observed for none of the aquatic extracts and only the methanolic extracts of leaf and stem in the vegetative phase had the antibacterial effect on *Bacillus subtilis*. In direct drop method, none of the methanolic extracts had an effect on the *E. coli*. On *S. epidermis*, the vegetative leaf had the best response and the inhibition zone was observed. On *B. subtilis*, the vegetative leaf and the vegetative stem showed the best inhibition zone and after them stood the leaf in the generative phase and root and stem in the generative phase. The flower extract proved to have no effect, either. On *P. aeruginosa*, none of the extracts showed the inhibition zone. In disk paper method, none of the disks, which had the extracts of different Datura parts, made the inhibition zone around them. However, the antibiotic disks of penicillin, ampicillin, vancomycin and cefalexine had the most effect on *Bacillus subtilis*. Vancomycin was effective on *Staphylococcus epidermis*. These antibiotics showed no effect on *E. coli* and *Pseudomonas aeruginosa*.

Apparently, the effective chemical substance that has the bactericidal role does not dissolve in water and methanol is its solvent. A little of tropane alkaloids consistency would possibly make the extracts ineffective against bacteria. Nevertheless, the high sensitivity of *Bacillus subtilis* and *Staphylococcus epidermis* leads this low consistency to make the inhibition zone around the point of extracts impact on the bacterial strains. On the other hand, it was found out that direct drop method could be the best operative method about this matter (small amount of effective substance) to get the best possible response from the least possible amount. The other result is that the effective substance's inhibitory effect on Gram-positive bacteria is more than Gram-negative bacteria. In their studies of the antimicrobial effects of two henbane species, Majd, Chalabian [15] stated that the microbial characteristics of the hyoscyamine are stronger than that of the scopolamine.

Alkofahi *et al.* [25] studied the antibacterial and antifungous effect of *Hyoscyamus reticulatus* ethanol extract on eight microorganisms and declared that the ethanol extract of the examined plant has an effective antimicrobial effect. In this research, it was found out that *Bacillus subtilis* and *Staphylococcus epidermis* took the most effect from the methanol extracts, which shows a meaningful difference from other bacteria. These results are in sympathy with Majd, Mehrabian's report [1,14], who stated that *Staphylococcus epidermis* is sensitive to the alkaloids of *Glaucium corniculantum* and *Glaucium flavum* plants. However, the results of these studies differ from the report of Cabo *et al.* [15] against *G. flavum*, who believed the extract obtained from the root has a more antimicrobial effect.

The impact on four fungous strains proved that none of the Pour Plate, Well, or Disk Paper Methods were effective and the best response was observed in Direct Drop Method. Regarding the *Certocystis ulmi* fungus, the most effective extracts were successively the flower extract and that of the root in the vegetative phase. The static fungus has been the effect of root in the vegetative phase, but the fungus grew again with the passage of time. The other extracts showed less effect than methanol solvent. On *Rhizoctonia solani* fungus, the stem methanol extract in the vegetative phase and the flower had the most effective response and the other extracts and solvents had no effect at all. None of the extracts had a meaningful effect on the fungous strain of *Fusarium*.

In this research, the methanol effect of crystalline, half-crystalline, green and organogenesis calluses on the four bacterial and the four fungous strains were studied. Direct Drop was the method applied.

The results show that the methanol extracts of green and organogenesis calluses has a meaningful effect on the *Bacillus subtilis* and the *Staphylococcus epidermis*, which are both Gram-positive. The crystalline and half-crystalline callus extracts were not effective on *bacillus subtilis*. However, the methanol extract of crystalline callus is effective on *E. coli*. It was also found out that the effective substance is the alkaloid of atropine, which is synthesized during the organ and tissue formation and tissular separation.

Iranbakhsh *et al.* [10,11] showed how different calluses, obtained from *Datura* leaf explants, are produced.

The results proved that the methanol extracts of crystalline, green and organogenesis calluses, which have tropane alkaloids, have inhibitory effect on *Certocystis ulmi*. The methanol extract of organogenesis callus has been more effective than other extracts. None of the extracts showed a meaningful effect on *Rhizoctonia solani* fungus. The methanol extracts of green and organogenesis calluses were effective on *Fusarium semitectum* fungus. Meanwhile, the crystalline callus extract showed the inhibitory effects. The effect of half-crystalline callus extract was similar to that of methanol solvent. On *Fusarium culmorum* fungus, only the green callus extract somehow showed effect and the other extracts were ineffective. The methanol extracts of green and organogenesis calluses showed an antimicrobial role on *Fusarium* fungus. Regarding the Fusarium culmorum, only the green callus extract somehow showed the microbiocidal effect.

Consequently, it can be said that compared with other extracts, the extract of organogenesis callus has the highest inhibitory effect. In other words, it has been more effective than other extracts and seems to be the antimicrobial effective substance in direct connection with the tissue and organ separation. The entity of this substance is the alkaloid of atropine (hyoscyamine rusmic isomer, $C_{17}H_{23}NO_3$, melting point: 114-118 °C, MW: 289.38 and 5.93 pKa). A study on the ready resources showed no similar researches regarding the discussed subject.

The current results were not in contrast with Majd, Chalapian [15] report, the findings of Mehrabian and Nourani [7] on two species of *Glaucium*, Majd and Arbabian [14] on the genus of *Vinca* and Peterson *et al.* [7] who reported the antifungous effect of alkaloids on three species of *Vinca* genus.

According to the results of these studies, it seems the effective substance, which has the microbiocidal role, does not dissolve in water and methanol is its solvent [8,9,12]. Furthermore, the low consistency of tropane alkaloids makes the extracts ineffective on bacteria and fungi. However, because of the high sensitivity of *Bacillus subtilis* and *Staphylococcus epidermis*, even despite the low consistency amounts of the effective substance, the inhabitation zone is observed in the point of impact between the extract and bacterial or fungous strains [10,12]. These results are in sympathy with the findings of Alishahi Nourani and Mehrabian [1]on two species of *Glaucium flavum*.

Majd, Arbabian [14] reported the antifungous effects of alkaloids on three species of *Vinca* genus. Our findings are in sympathy with their report [12]. According to the results and the low consistency of the effective substance, Direct Drop Method seems to be the best operative method. The other noteworthy point is that the inhibitory effect of effective substance on Gram-positive is more than on Gram-negative bacteria. Our results correspond to Majd and Chalabian [12]. Alkofahi *et al.* [5] studied the antibacterial and antifungous effect of Hyoscyamus reticulatus ethanol extract on eight microorganisms and declared that the ethanol extract of the examined plant has an effective antimicrobial effect.

In their studies upon the antimicrobial effects of two henbane species, Majd and Chalabian [5] stated that the microbial characteristics of the hyoscyamine are stronger than that of the scopolamine.

Cabo *et al.* [3] reported the antimicrobial effects of the extracts obtained from the root, stem, leaf and pericarp of Glaucium flavum and stated that the root extract has a more antimicrobial effect.

Peterson [7] observed the alkaloids antifungous effect of three species of vinca genus.

It was 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.

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