

Screening of Antimicrobial Activities of the Endophytic Fungi Isolated from *Sesbania grandiflora* (L.) Pers.

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ABSTRACT

The purpose of this research was to study the antimicrobial activities of the endophytic fungi from *Sesbania grandiflora* (L.) Pers. The endophytic fungi were isolated from branches and leaves of *Sesbania grandiflora* (L.) Pers., and sixty nine isolates were obtained. All isolates were screened for antibacterial and antifungal activities. The indicator organisms were 4 bacteria including *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, two yeasts, namely, *Candida albicans* and *Cryptococcus neoformans*, and 6 molds including *Rhizopus* spp., *Mucor* spp., *Penicillium* spp., *Aspergillus* spp., *Curvularia* spp., and *Alternaria* spp.. It was found that 28 and 16 isolates showed antibacterial activity against gram positive and gram negative bacteria, respectively. Also, 11 and 17 isolates showed antifungal activity against yeasts and hyaline non-septate hyphae, respectively. Besides, 13 and 65 isolates showed antifungal activity against hyaline septate and dematiaceous, respectively. There were 9 isolates that could inhibit bacteria, yeast, and molds. Macroscopic and microscopic examination of the fungal morphology revealed that most of the endophytic fungi (25 isolates) were hyaline septate hyphae. Only 3 isolates were hyaline non-septate hyphae. *Fusarium* spp. and *Acremonium* spp. were the predominate species among the isolated endophytic fungi. These results indicated that some endophytic fungi isolated from *Sesbania grandiflora* (L.) Pers. were potential sources of antimicrobial compounds against the tested bacteria, yeasts, and molds.

Keywords: Antifungal activity, Bioactive compounds, Co-evolution relations, Host plants.

INTRODUCTION

The need for new bioactive compounds used in medicine, industry, and agriculture has increased. Historically, many compounds have been isolated from the natural environment, particularly plants. Therefore, many of drugs available commercially are derived from plant-based chemicals. While plants have been a major source of new compounds for drug discovery, attention has more recently turned to endophytes as these microorganisms demonstrate great potential sources for new bioactive compounds (Strobel, 2003). Endophytic fungi are micro

fungi that colonize or live within the healthy plant tissues without producing any apparent symptoms or obvious negative effects to their hosts (Hirsch and Braun, 1992). They may benefit the host plant by producing bioactive substances to enhance plant growth and competitiveness of the host in nature. In addition, they produce novel antimicrobial secondary metabolites, which are now recognized as novel bioactive compounds (Carrol, 1988; Strobel *et al.*, 2004). Thailand is located in the tropics with great biodiversity of plant species and has a long tradition of herbal medicine (Inta *et al.*, 2008; Atjanasuppat *et al.*, 2009). Endophytes related to Thai medicinal plant

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and topics including their origins, biodiversity, endophyte-plant interactions, their role in ecology, and characterization of their secondary metabolites are of interest (Arnold, 2007; Saikkonen *et al.*, 2004). Therefore, the present study was undertaken to explore the endophytic mycoflora, isolated from Thai medicinal plants *Sesbania grandiflora* (L.) Pers., as they show great potential for obtaining useful and biologically active compounds. The genus *Sesbania* belong to the family Leguminosae and are commonly found in tropical regions of South East Asia, although they can be found in a wide range of different habitats. *Sesbania* spp. is used widely in Thai foods. With respect to medicinal uses, various species are still used to treat cold, fevers, and skin infection. However, there is still limited information available about the endophyte species of *Sesbania* spp.

The aims of this study were to isolate and identify endophytic fungi from the *Sesbania grandiflora* (L.) Pers., and study the antimicrobial secondary metabolites produced by these fungi.

MATERIALS AND METHODS

Collection of Plant Samples

Healthy leaves and stem were collected from *Sesbania grandiflora* (L.) Pers., in the forest area of Nakhon Nayok Province, Thailand. The fresh-cut ends of plant samples were cut by alcohol sterile scissor, wrapped with Parafilm M (3M Co. Ltd.), and placed in zip-lock plastic-bags and stored less than 72 hours prior to the isolation of endophytic fungi.

Isolation of Fungal Endophytes

Samples were cleaned under running tap water for 5 minutes and then air-dried. Before surface sterilization, the cleaned stems were cut into 5-cm long pieces. Leaves and limb fragments were sterilized

by immersion in 70% ethanol for 1 minute, 5% sodium hypochlorite solution for 5 minutes, and sterile distilled water for 1 minute twice (Chomcheon *et al.*, 2006). The surface-sterilized leaves and stems were cut into small pieces about 0.5×0.5 cm² using a sterile blade and placed on sterile half strength potato dextrose agar plates. The plates were incubated at room temperature for 24–72 hours. The hyphal tip of endophytic fungus growing out from the plant tissue was cut by a sterile pasture pipette and transferred to a sterile half strength potato dextrose agar plate. After incubation at room temperature for 7-14 days, colony morphology of each endophytic fungi was determined. Culture purity was obtained by several times sub-culturing.

Bacterial and Fungal Strains

All endophytic fungal isolates were screened for antibacterial and antifungal activities. The indicator bacteria included *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. Also, two indicator yeasts, namely, *Candida albicans* and *Cryptococcus neoformans*, and 6 molds including *Rhizopus* spp., *Mucor* spp., *Aspergillus* spp., *Alternaria* spp., *Curvularia* spp., and *Penicillium* spp, obtained from Faculty of Medical Technology, Rangsit University, were used in the study.

Preparation of Bacterial and Yeast Culture

Each bacterial strain was grown in blood agar plate at 37°C for 24 hours to obtain a freshly grown pure culture. Several bacterial colonies were suspended in 0.85% NaCl. The suspensions were mixed for 15 seconds to ensure homogeneity. The turbidity of bacterial suspension was diluted to match the turbidity of a 0.5 McFarland standard by

spectrophotometry ($OD = 0.08-0.1$ at 625 nm) corresponding to 1×10^8 CFU ml^{-1} . Yeasts were cultured on potato dextrose agar plate at 37°C for 24 hours in the case of *C. albicans*, and at room temperature for 48 hours in the case of *C. neoformans*. Inocula were prepared as mentioned above and measured $OD = 0.12-0.15$ at 530 nm corresponding to $1-5 \times 10^6$ CFU ml^{-1} . A sterile cotton swab was used to apply each bacterial and yeast inocula onto the entire surface of the Mueller Hinton agar plate.

Agar Diffusion Assay

Antimicrobial activities of endophytic fungi were determined by dual-culture agar diffusion assay as described in the Clinical and Laboratory Standards Institute (CLSI) M7-A8, M27-A3, and M38A method for bacteria and yeast. Briefly, endophytic fungal isolates were grown on potato dextrose agar at room temperature for 7, 14, 21, and 28 days. Then, they were cut into a small agar block using a sterile cork borer (7-mm diameter) and placed on inoculated test plate. The test plates were incubated at 37°C for 24 hours for bacteria and *C. albicans* and for 48 hours at room temperature in the case of *C. neoformans*. The inhibition zone around the endophytic agar disk indicated antimicrobial activities of endophytic fungi. Antibacterial or antifungal activity was determined by measuring zone of inhibition produce by endophytic fungi against pathogenic bacteria. The inhibition zones of more than 9 mm diameter were considered positive.

Preparation of Mold Culture and Antagonistic Assay

Each mold strain was grown on potato dextrose agar at room temperature for 1-5 day to obtain a freshly grown pure culture until the colony approximated 1-2 cm. Then endophytic fungal isolates were cut into a small agar block using a sterile cork borer

(7-mm diameter). Endophytic fungal agar block was placed on inoculated test plate at about 2 cm distances in which both endophyte and indicator mold were inoculated in the same media plate. The plates were kept at room temperature for 1-5 days and daily changes were monitored. Antifungal activity was indicated by mycelia growth of the test fungus in the direction of an antagonist colony from the endophyte growth radius. No mycelia growth of indicator fungus was considered positive.

Macroscopic and Microscopic Study of Fungal Morphology

If the selected endophytic fungi showed an interesting potential antimicrobial activity, the characteristics of each isolate were determined using colony morphology, rate of growth, type of hyphae, and types of reproductive structure (Ellis *et al.*, 2007).

RESULTS AND DISCUSSION

Sesbania grandiflora (L.) Pers., is not only a native economic tree, but also its bark, leaves, and flowers can be used in traditional medicine for treatment of cold, fever, stomach disorder, diarrhea, and jaundice and as skin cleanser. Traditionally, the bark is used as astringent and utilized for the treatment of smallpox, ulcers in the mouth and alimentary canal, in infantile disorders of stomach, and scabies (Vipin, *et al.*, 2011). The active compounds from the leaves are considered to be excellent sources of vitamin C, and calcium. Pectin and saponin are also found in the leave of this plant. The leaves are used as aperient, diuretic, and tonic in the form of poultice and they are applied to bruises (Devdatta *et al.*, 1954). Banjong *et al.* (2008) report that chloroform and hexane crude extract from *Sesbania grandiflora* (L.) Pers. leaf and flower can inhibit *E. coli* and *Salmonella* spp. Therefore, endophytic fungi that live inside this plant may have the same properties as



its host, by the horizontal genetic transfer mechanism. Isolation of endophytic fungi by surface sterilization technique can ensure that all the fungi retrieved are true endophytic fungi, since sodium hypochlorite and 70% ethanol already kill external fungi.

In this study, a total of 69 endophytic fungi were isolated from *Sesbania grandiflora* (L.) Pers. Most of endophytic fungi (62 isolates, 89.86%) were isolated from stem and only 10.14% (7 isolates) were isolated from the leaf. The exact genus of 28 isolates that showed potent broad spectrum antimicrobial activity was identified based on macroscopic colony and spore or conidia

characteristic (Ellis *et al.*, 2007), as shown in Table 1. Most of endophytic fungi were *Acremonium* spp. (42.86%), and *Fusarium* spp. (35.71%). However, *Phaeoacremonium* spp. (10.71%), *Phomopsis* spp. (3.57%), *Paecilomyces* spp. (3.57%), and *Cladosporium* spp. (3.57%) were also found (Table 1).

All endophytic fungi were preliminary screened for antimicrobial activities against 4 pathogenic bacteria and 2 pathogenic yeasts by using a modified agar disk diffusion assay. Antagonistic activity test was used for screening of antifungal activity against 6 molds. The results showed that 23

Table 1. Endophytic fungi isolated from *Sesbania grandiflora* (L.) Pers. showing interesting antimicrobial activity.

Isolate	Source	Growth rate	Name of fungi
2	Stem	Fast growth ^a	<i>Fusarium</i> spp.
3	Stem	Fast growth	<i>Fusarium</i> spp.
4	Stem	Slow growth ^b	<i>Fusarium</i> spp.
9	Stem	Slow growth	<i>Phaeoacremonium</i> spp.
13	Stem	Slow growth	<i>Fusarium</i> spp.
14	Leave	Fast growth	<i>Acremonium</i> spp.
17	Stem	Slow growth	<i>Acremonium</i> spp.
20	Stem	Slow growth	<i>Acremonium</i> spp.
22	Stem	Fast growth	<i>Fusarium</i> spp.
23	Stem	Slow growth	<i>Fusarium</i> spp.
25	Stem	Fast growth	<i>Fusarium</i> spp.
27	Leave	Fast growth	<i>Fusarium</i> spp.
28	Stem	Fast growth	<i>Acremonium</i> spp.
30	Stem	Fast growth	<i>Acremonium</i> spp.
37	Stem	Slow growth	<i>Fusarium</i> spp.
38	Stem	Fast growth	<i>Phaeoacremonium</i> spp.
39	Stem	Fast growth	<i>Phomopsis</i> spp.
43	Stem	Slow growth	<i>Acremonium</i> spp.
48	Stem	Fast growth	<i>Acremonium</i> spp.
49	Stem	Fast growth	<i>Acremonium</i> spp.
50	Leave	Fast growth	<i>Acremonium</i> spp.
51	Stem	Slow growth	<i>Acremonium</i> spp.
52	Stem	Slow growth	<i>Acremonium</i> spp.
53	Stem	Slow growth	<i>Paecilomyces</i> spp.
54	Stem	Slow growth	<i>Phaeoacremonium</i> spp.
59	Stem	Fast growth	<i>Cladosporium</i> spp.
63	Stem	Fast growth	<i>Acremonium</i> spp.
69	Leave	Fast growth	<i>Fusarium</i> spp.

^a Endophytic fungi growth fills the culture plate within 5-7 days, ^b Endophytic fungi growth fills the culture plate in more than 7 days.

According to the modified agar disk diffusion assay, the results are in agreement with previous workers', who have reported that plant-derived natural products often show better activity against gram positive bacteria (Ebrahimi *et al.*, 2010). The possible reason may be due to the difference of cell wall composition. Gram negative bacteria cell wall is made up of lipopolysaccharide and protein and it covers a very few thinner layers of peptidoglycan as compared to gram positive bacteria. Therefore, this makes gram negative bacteria more tolerance to antibacterial compound from natural products than gram positive bacteria (Wyrzykiewicz *et al.*, 2005). Radji *et al.* (2011) screened extracts from 24 endophytes from *Garcinia mangostana*. The results showed that more than half of the active isolates inhibited

Table 2. Percentage of susceptible test bacteria and fungi to endophytic fungi.

No. of endophytic fungus isolates	Indicator microorganisms											
	Bacteria				Yeast				Mold			
	<i>S.aureus</i>	<i>B.subtilis</i>	<i>E.coli</i>	<i>P.aeruginosa</i>	<i>C.albicans</i>	<i>C.neoformans</i>	<i>Rhizopus</i> spp.	<i>Mucor</i> spp.	<i>Penicilliu</i> <i>m</i> spp.	<i>Aspergillus</i> spp.	<i>Cucularia</i> spp.	<i>Alternaria</i> spp.
Antimicrobi al activity (n= 69) (%)	23 (33.33)	22 (31.88)	5 (7.24)	13 (18.84)	9 (13.04)	7 (10.14)	11 (15.94)	14 (20.29)	5 (7.24)	8 (11.59)	64 (94.20)	54 (78.26)

**Table 3** Antibacterial and antifungal activity of some endophytic fungi isolated from *Sesbania grandiflora* (L.) Pers. determined by agar diffusion assay ^a.

Isolated	Antimicrobial activity					
	Bacteria				Yeast	
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>E.coli</i>	<i>P. aeruginosa</i>	<i>C.albicans</i>	<i>C. neoformans</i>
2	+	-	-	+	-	-
3	-	-	-	+	-	-
4	-	+	-	-	-	-
9	+	+	-	-	+	-
13	+	+	+	+	-	-
14	+	+	-	-	+	+
17	-	+	-	-	-	-
20	+	+	-	-	-	-
22	+	+	-	-	-	-
23	+	+	-	+	-	-
25	-	+	+	-	-	-
27	+	-	+	-	-	-
28	-	+	-	-	+	-
30	+	+	-	+	-	+
37	+	+	-	+	-	-
38	+	+	+	+	+	+
39	+	+	-	+	+	-
43	-	-	-	+	- ¹	-
46	+	+	-	-	-	-
48	+	+	-	+	+	+
49	+	+	-	-	+	+
50	+	+	-	+	+	+
51	+	+	+	-	-	-
52	+	-	-	-	-	-
53	+	+	-	-	-	-
54	+	+	-	+	-	-
59	+	-	-	-	-	+
61	+	-	-	+	-	-
63	+	-	-	-	-	-
69	-	+	-	-	-	-

^a += Produced inhibition zone, -= Not produced inhibition zone.

gram-positive bacteria better than gram-negative bacteria. Strobel *et al.* (1997, 1999) reported that the secondary metabolite from *Acremonium sp.* isolated from *Taxus baccata* and *Cryptosporiopsis cf. quercina* isolated from *Tripterigeum wilfordii* had shown antifungal activity against *C. albicans* and *C. neoformans*.

Some differences in antimicrobial activity by endophytic fungi cultured in different periods of time were noticed: the antimicrobial activities were significantly increased when incubation period was

increased to 28 days. This could be due to the fact that the active compounds were chemically modified by the fungi themselves or the environmental changes such as dissolved oxygen and pH of the culture. It is well realized that culture time can affect the presence or absence of secondary metabolite (Strobel *et al.*, 1996). This suggested that culture time could affect bioactivities expressed by endophytic fungi in term of occurrence of secondary metabolites and/or the amount of these substances. Therefore, study of the culture duration time is required

in order to improve the production of antimicrobial substances from endophytic fungi.

There were few endophytic fungi that had the ability to inhibit indicator non-septate hyphae and septate hyphae. Only 11 (15.94%) and 14 (20.29%) isolates could inhibit *Rhizopus* spp. and *Mucor* spp., respectively, while 8 (11.59%) isolates inhibited both *Rhizopus* spp. and *Mucor* spp. Most of them showed wide inhibition zone against the tested molds from the first day of co-culture time. Five (7.24%) and 8 (11.59%) isolates displayed antifungal activities against *Penicillium* spp., and *Aspergillus* spp.,

respectively (Table 4). On the other hand, it seemed that endophytic fungi showed the best antifungal activities against indicator dematiaceous fungi, since 65 (94.20%) and 54 (78.26%) isolates could inhibit *Curvularia* spp. and *Alternaria* spp., respectively. In addition, 49 isolates (71.01%) inhibited both *Curvularia* spp. and *Alternaria* spp. It can be concluded that 17, 13, and 65 isolates showed antifungal activity against the indicator non-septate hyphae, septate hyphae, and dematiaceous fungi, respectively. Interestingly, antifungal activities of endophytic fungi against indicator septate hyphae and dematiaceous

Table 4. Antifungal activity by some positive endophytic fungi isolated from *Sesbania grandiflora* (L.) Pers. by antagonistic assay ^a.

Isolated	Antifungal activity			
	<i>Rhizopus</i> spp.	<i>Mucor</i> spp.	<i>Penicillium</i> spp.	<i>Aspergillus</i> spp.
2	+	+	-	+
14	-	+	-	+
17	+	+	-	+
20	-	+	-	-
27	-	-	-	+
30	+	+	-	-
31	-	-	-	+
33	-	+	-	-
36	-	+	-	-
38	-	+	-	-
39	+	+	-	-
40	+	-	-	-
41	-	+	-	-
48	-	-	-	+
49	+	+	-	-
50	+	-	-	+
51	-	-	-	-
53	-	-	+	-
54	+	-	-	-
55	-	-	+	-
59	+	+	-	+
61	+	+	-	-
62	-	-	+	-
63	+	+	-	-
65	-	-	+	-
69	-	-	+	-

^a += Produced inhibition zone, -= Not produced inhibition zone.

Note: 65 isolates of endophytic fungi could inhibit *Curvularia* spp., except isolates 29, 31, 46, and 60. 54 isolates of endophytic fungi could inhibit *Alternaria* spp., except isolates 1, 2, 12, 15, 22, 23, 24, 28, 31, 36, 41, 58, 61, and 66.



fungi predominated from 3-5 days of the culture time. It may be mentioned that endophytic fungi isolate number 2, 17, and 59 had a broad spectrum anti fungal activity against more than 5 indicator molds.

The antagonistic test suggested that the extent of antagonistic ability increased as the endophytic fungal colonies matured. The significant inhibition in growth without direct contact of mycelia suggests that the prevailing antagonisms may be due to the production of inhibitory substances by the fungi or due to the competition for nutrients or both (Nourozian *et al.*, 2006). However, the mechanisms of inhibition in colony growth of the tested fungi were not addressed in our studies. In addition, the inhibition zone of the tested fungi to endophytic fungi was noticed in the early days, indicating that the antagonistic ability became active in early stage of co-culture time. Interestingly, most of endophytic fungi isolated in this study, have anti-fungal ability against dematiaceous fungi than fungi in other groups. This result was correlated with a previous work that demonstrated that 5 endophytic fungi isolates obtained from 5 Iranian native medicinal plants displayed antifungal activity against at least one of three saprophytic fungi (*Aspergillus niger*, *Penicillium* spp. and *Alternaria* spp.) (Ebrahimia *et al.*, 2010).

It is remarked that there were 9 isolates (9, 14, 28, 38, 39, 48, 49, 50, and 59) that had a broad spectrum anti microbial activity against bacteria, yeast, and mold. Altogether, 19 isolates (2, 3, 4, 13, 17, 20, 22, 23, 25, 27, 30, 37, 43, 51, 52, 53, 54, 58, 63, and 69) had a broad spectrum anti microbial activity against only bacteria and mold. Totally, 28 isolates should be further studied.

CONCLUSIONS

In conclusion, the potential bioactive compounds produced by endophytic fungi isolated from Thai traditional medicinal plants are demonstrated. This study

suggested that some fungal isolates might produce different bioactive compound(s) when cultivated in semi-solid media in different time periods. Before conducting large-scale fermentation in liquid medium for isolation and purification of bioactive compounds, small-scale cultivation in semi-solid medium should be done under primary screening by agar diffusion or antagonistic assay. In this work, endophytic fungi were shown to be a promising source of new natural bioactive compounds against some indicator microorganism. Moreover, *Sesbania grandiflora* (L.) Pers. showed as a good candidate for the isolation of active endophytic fungi. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) against the tested pathogens should be further determined. The structures of bioactive compounds synthesized by these endophytic fungi should be determined and their relationship with the secondary metabolite production in the host plant should be studied. Benefits to the host plant from antagonism towards pathogenic microorganism could be speculated. The diversity of antagonistic properties of the endophytic fungi is important for screening new antifungal agents. Investigations on the interactions of *Sesbania grandiflora* (L.) Pers. and its endophyte would be the next direction for future research.

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غربال کردن فعالیت های ضد میکروبی قارچ های درون رست جدا شده از سسبانيا
Sesbania grandiflora (L.) Pers.

پ. پوتانگ، ب. جانتراپانو کورن، ا. تانگمی، پ. سانتورنسیتیچاروان

چکیده

هدف این پژوهش بررسی فعالیت های ضد میکروبی قارچ های درون رست جدا شده از سسبانيا بود. شصت و نه (۶۹) جدایه از قارچ های درون رست از شاخه ها و برگهای *Sesbania grandiflora* (L.) Pers. به دست آمد. همه جدایه ها برای فعالیت های ضد باکتریایی و ضد قارچی غربال شدند. موجودات معرف عبارت بودند از ۴ باکتری شامل *Bacillus subtilis* ATCC 6633، *Staphylococcus aureus* ATCC 25923، *Escherichia coli* ATCC 25922 و *Pseudomonas aeruginosa* ATCC 27853، دو مخمر به نام *Rhizopus spp.*، *Mucor spp.*، و ۶ کپک شامل *Cryptococcus neoformans*، *Aspergillus spp.*، *Curvularia spp.*، *Penicillium spp.* و *Alternaria spp.*. نتایج نشان داد که از مجموع جدایه ها، تعداد ۲۸ جدایه فعالیت هایی ضد باکتری های گرم مثبت و ۱۶ جدایه فعالیت ضد گرم منفی داشتند. نیز، تعداد ۱۱ جدایه فعالیت ضد قارچی بر علیه مخمر ها و ۱۷ مورد علیه قارچ های ریشه ای بدون بند بی رنگ داشتند. همچنین، ۱۳ جدایه فعالیت هایی ضد قارچ های بی رنگ بند دار و ۶۵ جدایه ضد قارچ های تیره رنگ نشان دادند. نیز، ۹ جدایه ضد رشد باکتری، مخمر و کپک بودند. آزمون های ماکروسکوپی و میکروسکوپی ریخت شناسی قارچ ها نشان داد که بیشتر قارچ های درون رست (۲۵ جدایه) بی رنگ، بند دار و ریشه ای بودند. فقط سه جدایه بی رنگ، بدون بند و ریشه ای بودند. در میان قارچ های درون رست جدا شده در این پژوهش، گونه های چیره شامل *Fusarium spp.* و *Acremonium spp.* بودند. نتایج نشان داد که شماری از قارچ های درون رست جدا شده از سسبانيا منابع مستعدی برای مواد ضد میکروبی علیه باکتری ها، مخمر ها، و کپک های مطالعه شده بودند.