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ANTIFUNGAL ACTIVITY OF ETHANOLIC EXTRACT OF *PHLOMIS FRUTICOSA* L.

MARINA D. SOKOVIĆ[1], P. D. MARIN[2], and D. BRKIĆ[3]

[1]*Department of Plant Physiology, Mycological Laboratory, Institute for Biological Research "Siniša Stanković",*
[2]*Institute of Botany, Faculty of Biology,*
[3]*Institute of Medicinal Plant Research "Josif Pančić",* 11 000 Belgrade, Yugoslavia

Abstract - In the ethanolic extract of *Phlomis fruticosa* L. two flavonoid aglycones, luteolin and chrysoeriol were identified. Antifungal activity of the total ethanolic extract, extract hydrolyzed with β-glucosidase and that hydrolyzed with HCl against seven micromycetes: *Aspergillus niger, A. ochraceus, Penicillium ochrochloron, Cladosporium cladosporioides, Trichoderma viride, Fusarium tricinctum* and *Phomopsis helianthi* was tested. The ethanolic extract hydrolyzed with HCl possessed the greatest antifungal activity against all fungal species tested.

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INTRODUCTION

Phlomis fruticosa L. is distributed in the region of East Mediterranean, westward to Sardinia (Kyparissis *et al.* 1993). This species belongs to the family Lamiaceae, which includes a number of species with proved medicinal properties. Traditionally, an infusion of the leaves is used as a tonic drink (Tammaro and Xepapadakis 1986). In Italy the leaves are used as a poultice on wounds (Bucar *et al.* 1998; Tammaro 1984). Iridoids isolated from ethanolic extract of *Ph. fruticosa* expressed antiviral activity (Asilbekova *et al.* 1983). Ristić *et al.* (1999) reported that essential oil of *Ph. fruticosa* is very active against some micromycetes and bacteria. Ethanolic extract of *Ph. tuberosa* showed antibacterial and antifungal activities (Abdel Sattar *et al.* 1995) while *Ph. armeniaca* was show to possess cytotoxic and cytostatic activity (Saracoglu *et al.* 1995). *Phlomis* species contain alkaloids, iridoids and flavonoids (Hoppe 1946; Saracoglu *et al.* 1997). Luteolin, chrysoeriol, apigenin, naringenin and quercetin were the most frequent flavonoid aglycones found in these species (Kumar *et al.* 1985; Tomás *et al.* 1986; Sabbry *et al.* 1986; Tomás-Barberán *et al.* 1992). In the ethanolic extract of *Phlomis fruticosa* 4 flavonoid glycosides (chrysoeriol 7-glucoronide, chrysoeriol 7-rhamnosylglucoside, luteolin 7-rhamnosylglucoside and luteolin 7-glucu-ronide) were detected (Aziziian and Cutler 1982). Flavonoids are unique to plants and represent an essential part of their success in adapting to life as sedentary organisms living in diverse and varying surrounding. These compounds play important roles in coloration of flowers, fruits, seeds and leaves, as defense compounds and as signaling molecules in reproduction, pathogenesis and symbiosis (Shirley, 1996). They are widely known antioxidants that inhibit the oxidation of low-density lipoproteins and reduce thrombotic tendencies (Rauha *et al.* 2000). In recent years, they have been reivewed for their wide biological activities, focusing in particular on the potential therapeutic use of this class of compounds as antiinflamatory, antiallergic, antiviral, antimicrobial, anticancer or immunostimulant drugs (Weidenbröner *et al.* 1990; Martin *et al.* 1998).

According to literature, *Phlomis* species possess biological activity, hence the present study was performed with the aim to get more information on relationship between the molecular structure and fungicidal activity of ethanolic extract of *P. fruticosa* in which we identified 2 aglycones of flavonoids. In this work antifungal activity of the total ethanolic extract, ethanolic extract hydrolyzed with β-glucosidase and 2N HCl was analyzed.

MATERIAL AND METHODS

Plant material

The leaves of *Phlomis fruticosa* were collected during flowering period, near Bar (Montenegro, Yugoslavia). The species was identified in the Laboratory of Taxonomy, Institute of Botany, Faculty of Biology, University of Belgrade, where the voucher specimens are deposited.

Preparation of leaf extract and isolation of flavonoids

30 *g* of powdered leaves were extracted for 2 min. with $CHCl_3$, the mixture was then filtered and the filtrate dried under vacuum. The residue was extracted with 150 mL of 70% ethanol, heated 1-2 min. in a water bath for the enzyme inactivation. After 24 *h* extract was filtered and dried under vacuum. A stock solution containing 1 *mg*/mL of dried evaporated extract in ethanol was used for investigating antifungal activity.

Paper chromatography

The concentrated etnanolic extract was spotted on the Wathman No.1 paper one dimensionally, in BAW and 15% HOAc. UV visible spots were circled and fumed with NH_3 vapor. Color changes and Rf values were calculated in different solvents (BAW and then in 15% HOAc). The spots were eluted with EtOH and subjected to UV spectroscopy using standard procedure (H a r b o r n e and T u r n e r 1984).

Enzymatic hydrolysis of the glycosides

0.13 *mg* (1 units) of β-glucosidase was added to the 1 *mg*/mL of ethanolic extract at room temperature. After 1 *h*, time needed for enzyme reaction, stock solution was used to investigate antifungal activity.

Acid hydrolysis of the glycosides

With the aim of obtaining aglycones (separated of glycosides) the total ethanolic extract was treated with 2N HCl at 100 °C for 120 min. After hydrolysis, the mixture was extracted with EtOAc (1:1). The upper EtOAc phase containing the aglycone moiety, and the lower aqueous layer containing sugars, were concentrated separately. The aglycones were subjected to 1-D chromatography using different solvent system, BAW = n-BuOH : HOAc : H_2O (4:1:5), CAW (2:1) = $CHCl_3$: HOAc : H_2O (30:5:2), 50% HOAc and forestal = HOAc : H_2O : HCl (30:10:3), against authentic markers (H a r b o r n e and T u r n e r 1984). Markers of aglycones, apigenin, luteolin, chrysoeriol and quercetin were used. In this way only O-glycosides were hydrolyzed (M a r k h a m 1982). Chromatograms were dried and observed under UV lamp. UV visible spots were circled and fumed with NH_3 vapor. Color changes and Rf values were calculated. The stock solution of 1 *mg*/mL of this hydrolyzed extract was used to test for antifungal activity.

High Performance Liquid Chromatography (HPLC)

- HPLC was performed in a Liquid chromatograph Hewlett Packard 1090 LC, with DAD detector. LiChrospher CART 250-4 HPLC-Cartridge column, protected by a RP-18 guard column (5 *μm*) 250 x 4 mm was used. The following solvents were employed: A, acetonitrile (Sigma Aldrich) and solvent B, 10 mL H_3PO_4 ("Zorka" Šabac) in 500 mL H_2O. Elution profile was scanned at a flow rate of 0.5 mL/min, 30% to 80% solvent A for 40 *min*. Apigenin 0.8 *mg*/5mL, chrysoieriol 1*mg*/5mL, luteolin 0.8*mg*/5mL and quercetin 0.5 *mg*/5mL served as standards. Volume of sample (hydrohylated extract) injected was 20 *μ*L (loop), UV detection at 254 *nm*, 286 *nm* and 360 *nm*.

Test for antifungal activity

The following fungi were: *Aspergillus niger* (ATCC 6275), *A. ochraceus* (ATCC 12066), *Penicillium ochrochloron* (ATCC 9112), *Cladosporium cladosporioides* (ATCC 13276), *Trichoderma viride* (IAM 5061), *Fusarium tricinctum* (CBS 514478) and *Phomopsis helianthi* (ATCC 201540). The organisms were taken from Mycotheca of the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research, Belgrade.

The fungi were maintained on potato dextrose agar (PDA) and malt agar (MA), (B o o t h 1971). The cultures were stored at +4 °C and subcultured once a month. In order to investigate antifungal activity of essential oils and ethanolic extracts, the mycelial growth test with malt agar was used (I s h i i 1995). According to the same author the most commonly used method for investigation of antifungal activity is mycelial growth test on a culture medium supplemented with different concentrations of a fungicide examined. The minimum inhibitory concentration (MIC) of an investigated component necessary for a complete inhibition of mycelial growth of the fungal strain was determined. Different concentrations of total ethanolic extracts, extract hydrolyzed with β-glucosidase and that hydrolyzed with 2N HCl (1, 5, 10 and 20 *μg*/mL medium) were separately diluted in Petri dishes with malt agar (MA). All fungal species were tested in triplicate. Petri dishes with ethanol were used as a control. Ethanolic extracts were added into molten malt agar (MA) (+35 °C) and poured into Petri dishes. The tested fungi were inocu-

lated at the center of the plates. The plates were incubated for three weeks at room temperature, after that period the MIC was determined by measuring the fungal growth and comparing it with the control. No growth was considered as MIC values.

RESULTS AND DISCUSSION

Phlomis species contain, mainly, O-glycosides but C-glycosides (orientin, vicenin-2 and lucenin-2), were also, detected in some species (T o m á s - B a r b e r á n *et al.* 1992). According to the literature data, showing that plant extract which contain flavonids in glycosidic form possessed lower antifungal potential than hydroxilated or pure compounds (R a u h a *et al.* 2000), we decided to hydrolyze the total ethanolic extract with acid (HCl) and enzyme (β-glucosidase) to separate sugars from flavonoid aglycones.

According to literature, 4 flavonoid glycosides (chrysoeriol 7-glucuronide, chrysoeriol 7-rhamnosylglucoside, luteolin 7-rhamnosylglucoside and luteolin 7-glucuronide) were detected in *Phlomis fruticosa* (A z i z i a n *et al.* 1982). In extract hydrolysed with 2 N HCl of *Ph. fruticosa,* we identified 2 aglycones, two flavones (chrysoeriol and luteolin) by paper chromatography and HPLC.

The results on antifungal activity of total ethanolic extract of *Ph. fruticosa* are presented in Table 1. No antifungal activity was recorded when total ethanolic extract was employed in the concentration of 1 μg/mL. Total ethanolic extract showed only low fungistatic activity against *Aspergillus ochraceus, Cladosporium cladosporioides* and *Phomopsis helianthi* in the concentration of 5 μg/mL. In the concentration of 10 μg/mL the extract expressed fungistatic activity against *A. ochraceus, C. cladosporioides* and *P. helianthi,* with inhibition of 45.6, 58.5 and 48.7%, respectively. Concentration of 20 μg/mL of total ethanolic extract, completely inhibited the mycelial growth of *A. ochraceus, C. cladosporioides* and *P. helianthi.* This was taken to represent the MIC value for these three fungal species. Low fungistatic activity was obtained against *A. niger* and *F. tricinctum. P. ochrochloron* and *T. viride* were the most resistant to total ethanolic extract of *Ph. fruticosa.*

The ethanolic extract hydrolyzed with β-glucosidase expressed a greater antifungal activity against investigated micromycetes than total extract (Table 1). In the concentration of 1 μg/mL the extract showed fungistatic activity against *A. ochraceus, C. cladosporioides* and *P. helianthi,* with the inhibition of 25.5, 55.5 and 35.0%, respectively. The concentration of 5 μg/mL inhibited even more the same species (75.5, 80.5 and 75.0%). Total inhibition of mycelial growth of these species was achived by 10 μg/mL, which represents the MIC. The same concentration possessed only low fungistatic activity against *A. niger* (5.5%) and *F. tricinctum* (25.0%), while 20 μg/mL inhibited these species by 20.5 and 85.5%, respectively. This concentration possessed low fungistatic activity against *P. ochrochloron* (25.5%) and *T. viride* (15.5%). It can be seen that 5 μg/mL expressed a strong fungistatic activity, while 10 μg/mL led to a total inhibition of *A. ochraceus, C. cladosporioides* and *P. helianthi* (MIC).

Ethanolic extract hydrolyzed with 2N HCl showed the greatest antifungal activity (Table 1). In the concentration of 1 μg/mL this extract acted as a fungistatic against *A. ochraceus, C. cladosporioides* and *P. helianthii* with the inhibition of 75.5, 85.5 and 75.0%. The same species were sensitive to 5 μg/mL which represents the MIC value. Concentration of 10 μg/mL inhibited mycelial growth of *F. tricinctum,* while expressing only low fungistatic activity of 10.0% against *P. ochrochloron* and *T. viride.* At 20 μg/mL this extract showed a strong antifungal activity against *A. niger* (80.0%), *P. ochrochloron* (55.5%) and *T. viride* (45.5%). The most susceptible micromycetes to this extract were *A. ochraceus, C. cladosporioides* and *P. helianthii. P. ochrochloron* and *T. viride* were the most resistant species. Concentration of 1 μg/mL possessed a strong antifungal activity, while 5 μg/mL led to a total inhibition of the three micromycetes. The minimum inhibitory concentration of this extract was 5 μg/mL, in contrast to the results obtained with total ethanolic extract and that hydrolyzed with the enzyme, where 20 and 10 μg/mL, respectively, completely inhibited the same micromycetes.

According to some literature data concerning the antimicrobial activity of flavonoid glycosides no substantial effect could be detected (H e d i n and W a a g e 1986). Some flavonoids have shown antifungal activity only after hydrolysis with enzymes or acids (L u i j e.n i j k 1995).

If we compare the results on antifungal activity of total ethanolic extract, ethanolic extract hydrolyzed with β-glucosidase and that hydrolyzed with HCl, it can be seen that their antifungal potential increased in the following order: total ethanolic extract < extract hydrolyzed with β-glucosidase < extract hydrolyzed with HCl.

Under standard conditions β-glucosidase hydrolyses β-D-glucosides and also partly β-D-glucuronides and β-D-galactosides. It will not remove glucose from O-diglucosides such as rhamnoglucosides and (1-2) diglucosides. However, HCl hydrolyses flavonoid 3-O-glycosides and 7-O-rhamnosides completely for 2-6 min., flavone (and flavonol) 7- and 4'-O-glycosides, flavonoid 3,7 and 4'-O-glucuronides for 60-250 min. (M a r k h a m 1982).

Table 1. Antifungal activity of the total ethanolic extract of *Phlomis fruticosa* L. (a), the extract hydrlyzed with β-glucosidase (b) and that hydrolyzed with 2 N HCl (c).

Conc. of extract (μg/mL)	Percent of inhibition (x ± SE)						
	A. niger	*A. ochraceus*	*P. ochrochloron*	*C. cladosporioides*	*F. tricinctum*	*P. helianthi*	*T. viride*
1 a	0	0	0	0	0	0	0
b	0	25.5 ± 2.5	0	55.5 ± 3.5	0	35.0 ± 2.5	0
c	0	75.5 ± 2.5	0	85.5 ± 3.5	0	75.0 ± 2.5	0
5 a	0	23.5 ± 4.0	0	25.3 ± 3.0	0	30.0 ± 2.5	0
b	0	75.5 ± 4.5	0	80.5 ± 4.0	0	75.0 ± 2.0	0
x	7.0 ± 3.5	100[b]	0	100[b]	55.5 ± 2.5	100[b]	0
10 a	0	45.6 ± 3.6	0	58.5 ± 4.0	0	48.7 ± 3.5	0
b	5.5 ± 3.0	100[b]	0	100[b]	25.0 ± 2.5	100[b]	0
c	25.0 ± 3.5	100[b]	10.0 ± 3.5	100[b]	100[b]	100[b]	10.0 ± 4.0
20 a	8.9 ± 2.5	100[b]	0	100[b]	15.0 ± 2.5	100[b]	0
b	20.5 ± 2.5	100[b]	25.5 ± 2.5	100[b]	85.5 ± 2.5	100[b]	15.5 ± 3.5
c	80.0 ± 2.0	100[b]	55.5 ± 2.5	100[b]	100[b]	100[b]	45.5 ± 3.5

[b] Minimum inhibitory concentration (MIC)

Antifungal activity of total extract (although low comparing to others) may be due to the presence of some aglycones, unstable flavonoid glycosides, or some other bioactive secondary metabolites. This is in agreement with literature that plant extracts generally contain flavonoids in glycosidic form. This may be the reason why the plant extract did not produce as marked inhibition as some fractionated extracts or as many of the pure compounds. Definitely, flavonoid glycosides failed to show low activity against the most microorganisms (R a u h a *et al.* 2000).

Lower activity of extract hydrolyzed with β-glucosidase than HCl hydrolyzed extract can be explained by the fact that main flavonoids of *Phlomis fruticosa* are diglycosides (which are resistant to enzyme hydrolysis) and glucuronides which are partly hydrolyzed with β-glucosidase.

Growth of tested microorganisms responded differently to various components (ethanolic extract) and their concentrations, which indicated that various components may have different modes of action or that the metabolism of some microorganisms was able to better overcome the effect of the tested compounds or to adapt to it. So, that could be the other reason for a stronger antifungal activity of ethanolic extract hydrolyzed with HCl.

From the given results it may be concluded that all extracts investigated in this work possessed antifungal activity, the strongest one being observed when acid hydrolyzed extract was applied. Further studies could comprise to bioassay-guided fractionation to characterize active constituents and their use in pharmacy and medicine.

In additions, these results support traditional medicinal uses of the *Phlomis fruticosa* and reinforce the concept that the ethnobotanical approach to screening plants as potential sources of bioactive substances could be successful.

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АНТИФУНГАЛНА АКТИВНОСТ ЕТАНОЛНОГ ЕКСТРАКТА *PHLOMIS FRUTICOSA L.*

МАРИНА Д. СОКОВИЋ[1], П. Д. МАРИН[2] и Д. БРКИЋ[3]

[1]*Одељење за биљну физиологију, Миколошка лабораторија, Института за Биолошка истраживања "Синиша Станковић",* [2]*Институт за Ботанику, Биолошки факултет,* [3]*Институт за истраживања лековитог биља "Јосиф Панчић",* 11 000 Београд, Југославија

У етанолном екстракту врсте *Phlomis fruticosa* L. идентификована су два агликона флавоноида, лутеолин и хризоериол. Антифунгална активност укупног етанолногекстракта, етанолног екстракта хидролизованог β-глукозидазом и екстракта хидролизованог хлороводоничном киселином истраживана је на седам микромицета: *Aspergillus niger, A. ochraceus, Penicillium ochrochloron, Cladosporium cladosporioides, Trichoderma viride, Fusarium tricinctum* и *Phomopsis helianthi.* Најосетљивије микромицете су *P. helianthi* и *C. cladosporioides.* Поменуте врсте показале су најмању резистентност на сва три испитивана екстракта. Микромицете које су показале највећу резистентност на сва три испитивана екстракта су *P. ochrochloron* и *T. viride.* Укупни етанолни екстракт показао је најмањи антифунгални потенцијал. Етанолни екстакт хидролизован ензимом показује пораст антифунгалног потенцијала, док етанолни екстракт хидролизован хлороводоничном киселином поседује највећи антифунгални потенцијал.