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Antifungal activity of bioactive constituents and bark extracts of *Rhododendron arboreum*

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Abstract

Rhododendron arboreum various fractions of bark as well as the isolated compounds were investigated against pathogenic fungi to provide evidence for the folkloric uses reported in the traditional system. Various solvent fractions and compounds were tested using agar well diffusion method at concentrations of 50, 25 and 12.5 mg/mL, the maximum inhibition recorded against fungi 17-32, 15-27 and 10-24 mm for methanol extract, 16-28, 14-26 and 10-22 mm to ethyl acetate extract, 17-21, 8-18 and 10-16 to chloroform extract and 8-15 and 8-12 mm to *n*-hexane extract respectively. Maximum inhibition at 10, 5 and 2.5 mg/mL of isolated triterpenoids was recorded against such as fungi 28-44, 25-42 and 20-40 mm for 3 β -acetoxyurs-11,12-epoxy-13 β ,28-olide, 23-44, 20-42 and 15-40 mm for betulin and 17-40, 15-37 and 13-34 for lupeol respectively. It is concluded from the present study that the antifungal activity of the extracts may be due to the presence of 3 β -acetoxyurs-11,12-epoxy-13 β ,28-olide, betulin, lupeol and taraxerol.

Introduction

Rhododendron arboreum belongs to the Ericaceae family. The genus *Rhododendron* consists of more than 1000 species (Kurashige et al., 2001) which are distributed throughout the world (Cakir et al., 2005), mostly concentrated in China, India, Pakistan, Malaysia and Nepal (Nisar et al., 2011). In Pakistan, *R. arboreum* can be found at Hazara division in Seran valley, Jammu and Kashmir (Cakir et al., 2005). It occurs at an altitude ranging from 1700-3400 m and reach to a height of 15 m (Brinkhaus et al., 2005). The genus has been reported to be effective as astringent, diuretic, choleric, antispasmodic, chronic eczema, diarrhea, dysentery, anti-irritable bowel syndrome therapy (Brinkhaus et al., 2005; Matin et al., 2001). A review of the literature revealed that the flowers of *R. arboreum* showed anti-diabetic, anti-hyperlipidemic, anti-inflammatory and

anti-nociceptive activities and an active compound (Hyperine) (Sahu et al., 2011; Verma et al., 2013; Verma et al., 2013). Phytochemical screening of *R. arboreum* revealed the presence of alkaloids, flavonoids, steroids, glycosides, tannins and saponins etc (Kiruba et al., 2011). Similarly the leaves of this plant showed a good hepatoprotective activity in rats (Verma et al., 2011a). The methanolic extract of the flowers had potent anti-glycation potential in rats and has potent anti-oxidant property (Mani et al., 2008; Verma et al., 2011a). In the present research work the various solvent fractions and isolated constituents were screened for antifungal activities.

Materials and Methods

Collection of plant material



Plant material was collected from Seran valley Khyber Pakhtoonkhwa, Pakistan, in February 2011, authenticated by Taxonomist at Department of Botany, University of Peshawar. The aerial parts of the plant were air dried under shade for two weeks.

Preparation of plant extracts

The dried plant material (bark) was pulverized by a mechanical grinder, sieved through 40 mesh, extracted with *n*-hexane, chloroform, ethyl acetate and methanol using Soxhlet apparatus, concentrated, dried under reduced pressure and used residue-free semi-solid masses for experiments (Verma et al., 2011b).

Isolation of constituents

The ethyl acetate fraction of the bark was subjected to column chromatography. Elution of the column with *n*-hexane: ethyl acetate (95:5) gave a triterpenoid, taraxerol, crystallized as colorless needles (m.p. 270-271°C) from acetone (yield 132 mg). Further elution of the column with increasing polarity with *n*-hexane: ethyl acetate (70:30) gave another triterpenoid, Betulin, amorphous solid (m.p. 250°C), yield 80 mg. Further elution of the column with *n*-hexane: ethyl acetate (6:4) gave an amorphous compound 3 β -acetoxyurs-11,12-epoxy-13 β ,28-olide (287°C), yield was 20 mg. Elution of the column with methanol: chloro-form (1:99) yield an amorphous compound lupeol (m.p. 213°C), yield was 20 mg. The structures of these compounds were confirmed by comparing the spectroscopic data with that of the literature.

Micro-organisms

Test organisms of 5 fungi were procured from American Type Culture Collection (ATCC). They were maintained on nutrient agar (Hi-Media) broths. The formula (g/L): Beef extract: 10 g; peptone: 10 g; sodium chloride: 5 mg; agar: 20 g; and distilled water: 100 mL; pH: 7.4 \pm 0.2. MH agar (38 g) was weighed and dissolved with 1000 ml of distilled water and adjusted to pH 7.3 \pm 0.2, sterilized by autoclaving at 121°C for 15 min at 15 psi pressure and used for sensitivity tests. A few colonies of the fungal strains selected for study were picked from the agar broths, inoculated into 4 mL each of peptone-water in the test tubes and incubated for 2-4 hours to produce suspensions, then diluted, if necessary, with saline to a density that was visually equivalent to that of standard prepared by adding 0.5 mL of 1% barium chloride to 99.5 mL of 1% sulfuric acid. These suspensions were used for seeding. The plant extracts and isolated compounds were dissolved with DMSO.

Agar-well diffusion method

Antifungal activity was determined by agar-well diffusion method (Quiroga et al., 2001) with modifications according to the present experimental

conditions. Different concentrations of the extracts (50, 25 and 12.5 mg/mL) and isolated compounds (10, 5 and 2.5 mg/mL) were prepared by 2-fold dilution method and tested.

Determination of antifungal activity

All the stock cultures were maintained in sabouraud dextrose agar. Inoculums for *Candida albicans* were prepared by spread plating of 24 hours old culture grown in sabouraud broth. For *Aspergillus niger*, 10⁴ spore/mL of fungi was uniformly distributed on the surface of SDA plates with the help of sterile cotton swab. For the dermatophytes, inoculation was done by taking a piece of fungal colony on a sterile cotton swab and gently swabbing on the surface uniformly. The plates were allowed to dry at room temperature. Subsequently, 6 mm diameter wells were bored in the agar of each plate. Different concentrations of the solvent extracts and isolated compounds were added into the wells using micropipettes and allowed for diffusion. The plates were incubated at similar temperature of 28°C but a different time period is required for each fungus depends on the incubation time required for a visible growth. The solvent without extracts served as negative control. Standard antibiotics were used as positive controls (Quiroga et al., 2001).

Recording of antifungal activity of incubation

In case of fungi the zone of inhibition (mm) was recorded after required incubation time. The experiment was repeated thrice and the average values were calculated.

Results

The antifungal effect of the various solvent fractions is presented in Table I. The crude methanolic extract demonstrated a dose-dependant fungicidal effect against all tested fungi. The mean zone of inhibition observed with methanolic extract (50 mg/mL) against *A. niger*, *C. albican*, *C. flavus*, *F. solani*, *M. canis* and *D. glaberata* was 25 \pm 1.2, 29 \pm 1.0, 28 \pm 2.0, 17 \pm 1.4, 28 \pm 1.6, 32 \pm 1.7 mm respectively. The antifungal action of remaining lower concentrations was lesser than the mentioned effect. When ethyl acetate was tested against these fungi a similar to methanolic effect observations were recorded. At the tested concentration of 50 mg/mL, ethyl acetate fraction exhibited 22 \pm 1.9, 28 \pm 1.0, 25 \pm 1.3, 16 \pm 1.2, 24 \pm 1.1 and 28 \pm 1.0 mm zone of inhibition against *A. niger*, *C. albican*, *C. flavus*, *F. solani*, *M. canis* and *D. glaberata* respectively. The chloroform fraction produced antifungal effect which was lesser than ethyl acetate and methanolic extract, while the *n*-hexane fraction failed to produced outstanding fungicidal effect.

Table I

Antifungal activity of various solvent fractions of *Rhododendron arboretum*

	<i>n</i> -Hexane (mg/mL)			Chloroform (mg/mL)			Ethyl acetate (mg/mL)			Methanol (mg/mL)			Micnazole
	50	25	12.5	50	25	12.5	50	25	12.5	50	25	12.5	
<i>A. niger</i> (10549)	7 (1.3)	-	-	18 (1.0)	13 (1.1)	10 (1.3)	22 (1.9)	18 (1.2)	15 (1.6)	25 (1.2)	23 (1.9)	20 (1.4)	28 (1.0)
<i>C. albacan</i> (2091)	15 (1.0)	12 (1.1)	8 (1.2)	20 (1.9)	18 (1.9)	12 (1.7)	28 (1.0)	26 (1.0)	22 (1.0)	29 (1.0)	27 (1.0)	24 (1.9)	45 (0.0)
<i>C. flavus</i> (32611)	14 (1.1)	8 (1.6)	-	21 (2.0)	19 (1.2)	12 (1.8)	25 (1.3)	21 (1.8)	18 (1.3)	28 (2.0)	25 (1.1)	23 (1.0)	40 (0.8)
<i>F. solani</i> (11712)	7 (1.9)	-	-	12 (1.1)	8 (2.1)	-	16 (1.2)	14 (1.2)	10 (1.6)	17 (1.4)	15 (1.8)	10 (1.4)	25 (0.1)
<i>M. canis</i> (11622)	8 (2.0)	-	-	17 (1.0)	14 (1.9)	10 (1.2)	24 (1.1)	20 (1.4)	18 (2.0)	28 (1.6)	22 (1.7)	19 (1.7)	20 (0.1)
<i>D. glaberata</i> (90030)	12 (1.0)	8 (1.3)	-	20 (1.1)	18 (2.9)	16 (2.0)	28 (1.0)	25 (2.0)	22 (1.5)	32 (1.7)	27 (2.0)	24 (2.0)	40 (0.3)

Table II

Antifungal effects of isolated compounds of *Rhododendron arboretum*

Microorganisms, ATCC No	Taraxerol (mg/mL)			Betulin (mg/mL)			Lupeol (mg/mL)			3β-acetoxyurs-11, 12-epoxy-13β, 28-olide (mg/mL)			Micnazole
	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5	
<i>A. niger</i> (10549)	8 (2.3)	-	-	39 (4.2)	35 (2.0)	30 (2.0)	36 (3.0)	35 (4.0)	33 (1.3)	44 (2.0)	42 (1.9)	39 (1.5)	28 (1.0)
<i>C. albacan</i> (2091)	15 (3.3)	-	-	44 (3.3)	42 (3.9)	40 (1.8)	40 (2.0)	36 (1.9)	28 (2.9)	41 (4.3)	35 (2.0)	32 (2.0)	45 (0.0)
<i>C. flavus</i> (32611)	7 (1.7)	-	-	43 (1.7)	41 (1.7)	38 (3.4)	38 (2.7)	37 (5.9)	34 (2.3)	42 (2.4)	41 (1.9)	40 (1.9)	40 (0.8)
<i>F. solani</i> (11712)	-	-	-	23 (1.3)	20 (3.0)	15 (3.9)	28 (1.8)	25 (1.8)	20 (1.9)	32 (143)	31 (2.3)	30 (3.1)	25 (0.1)
<i>M. canis</i> (11622)	12 (2.0)	11 (1.3)	7 (1.9)	35 (1.9)	32 (1.4)	27 (1.8)	17 (4.0)	15 (1.1)	13 (1.4)	28 (1.1)	23 (1.3)	20 (1.8)	20 (0.1)
<i>D. glaberata</i> (90030)	9 (3.7)	-	-	40 (1.8)	37 (1.9)	33 (3.9)	32 (4.0)	28 (2.0)	25 (1.3)	39 (2.0)	37 (1.9)	35 (1.3)	40 (0.3)

Various isolated constituents exhibited variable degree of antifungal effect as presented in Table II. The outstanding fungicidal action was noticed with 3β-acetoxyurs-11, 12-epoxy-13β, 28-olide against *A. niger*, *C. albacan*, *C. flavus*, *F. solani*, *M. canis* and *D. glaberata* with mean zone of inhibition 44 ± 2.0 , 41 ± 4.3 , 42 ± 2.4 , 32 ± 143 , 28 ± 1.1 and 39 ± 2.0 mm respectively. The antifungal effect at the tested concentration of 10 mg/mL was almost similar to standard antifungal drug (micnazole). The antifungal effect at lower concentrations was also significant. A significant fungicidal activity was noticed with lupeol (10, 5 and 2.5 mg/mL), the maximum activity was observed at 10 and 5 mg/mL. the mean zone of inhibition at tested concentration of 10 mg/mL of Lupeol against *A. niger*, *C. albacan*, *C. flavus*, *F. solani*, *M. canis* and *D. glaberata* was 36 ± 3.0 , 40 ± 2.0 , 38 ± 2.7 , 28 ± 1.8 , 17 ± 4.0 and 32 ± 4.0 mm respectively. It was very interesting that the fungicidal activity of lupeol was comparable with micnazole.

Betulin exhibited antifungal effect against tested fungi, which was equal or higher than standard antifungal drug. Betulin (10 mg/mL) showed 39 ± 4.2 , 44 ± 3.3 , 43 ± 1.7 , 23 ± 1.3 , 35 ± 1.9 and 40 ± 1.8 mm zone of inhibition against *A. niger*, *C. albacan*, *C. flavus*, *F. solani*, *M. canis* and *D. glaberata* respectively. Taraxerol showed non-significant antifungal effect.

Discussion

Fungi are microscopic eukaryotic organisms produce superficial, subcutaneous and systemic infections in animals and human beings (Sparagano and Foggett, 2009). Superficial and subcutaneous mycotic infections include dermatophytosis and candidiasis caused by various *dermatophytes* and *candida albican* while systemic mycotic infections include aspergillosis, cryptococcosis, histoplasmosis, sporotrichosis

etc (Guarner and Brandt, 2011). Antifungal activities of various solvent fractions like *n*-hexane, chloroform, ethyl acetate and methanol were tested against *A. niger*, *C. albican*, *C. flavus*, *F. solani*, *M. canis* and *D. glaberata* while miconazole was used as a standard. In the present study it is cleared that all the fractions of *R. arboreum* showed statistically significant antifungal activity at high concentration as shown in Table I. Other species of this genus also have reported good antimicrobial activity which is an evidence of the folk use of the genus in traditional medicine. The antimicrobial activity of the methanolic extracts of leaves and stem of *R. smirnovii* at 100, 250 and 500 $\mu\text{g}/\text{mL}$ have been reported (Çakir et al., 2005). The methanolic stem extract of *R. smirnovii* have shown no activity against the tested pathogens while the methanolic leaves extract have reported good activity at elevated concentration. The antifungal activity of *R. smirnovii* at 500 $\mu\text{g}/\text{mL}$ against *C. albican* already reported is 14.2 mm, which is low activity compared to the standard drug. In the present case all the fractions showed good to significant activity against *C. albican*

and other fungi like *A. niger*, *C. flavus*, *F. solani*, *M. canis* and *D. glaberata* while *n*-hexane fraction showed moderate activity against the prescribed fungal species.

Antifungal activities of various isolated compounds taraxerol, betulin, lupeol and 3 β -acetoxyurs-11, 12-epoxy-13 β , 28-olide as depicted in Figure 1, were tested against *A. niger*, *C. albican*, *C. flavus*, *F. solani*, *M. canis* and *D. glaberata* while miconazole was used as a standard. Among the pure compounds 3 β -acetoxyurs-11, 12-epoxy-13 β , 28-olide showed significant activity against the entire pathogenic fungi that is *C. albican*, *A. niger*, *C. flavus*, *F. solani*, *M. canis* and *D. glaberata* as in Table II. Similarly betuline showed good activity towards all the tested pathogens, while lupeol and taraxerol showed moderate and low activities respectively. As it is cleared that all these activities are concentration-dependent i.e. increase with increase in concentration. The high antifungal activities of 3 β -acetoxyurs-11, 12-epoxy-13 β , 28-olide and betulin may be due to the hydrophilic nature of these compounds, while in contrast the low antimicrobial activities of lupeol and taraxerol may be attributed to their

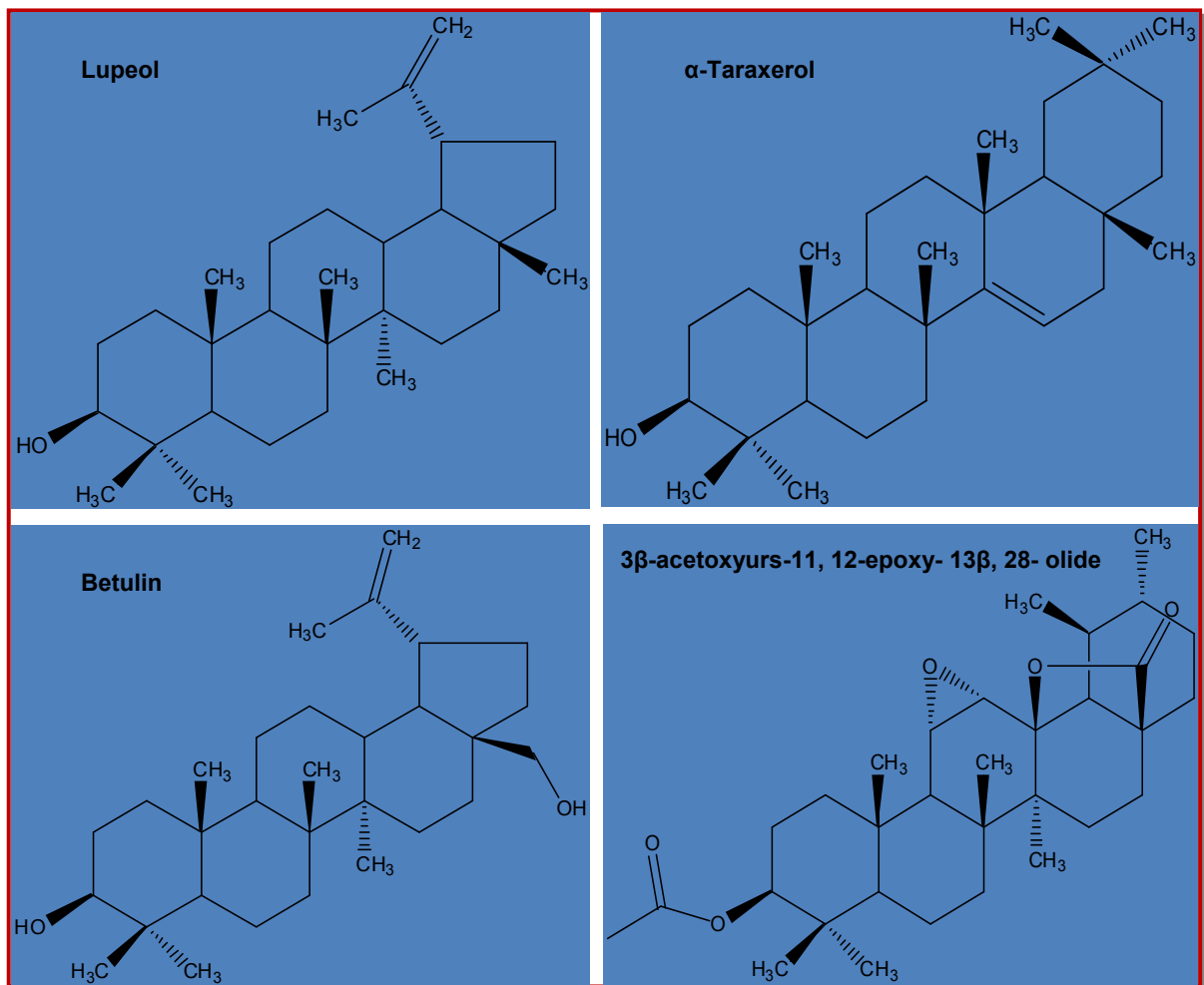


Figure 1: Chemical structure of isolated compounds from *Rhododendron arboretum*

hydrophobic nature. The potential of *R. arboreum* extracts against fungi may be explored to develop topical antifungal therapy to promote skin wound healing (Arora and Kaushik, 2003). Phenolics including tannins are used topically for care and repair of skin wounds. Tannins can be toxic to filamentous fungi, yeast and bacteria (Hamburger and Hostettmann, 1991). Thus, in the present study, the presence of phenolics and high content of tannins in the methanol extract may be responsible for better antifungal action.

In summary, the extracts and pure compounds isolated from *R. arboreum* showed potent antifungal activity against pathogenic fungi. The findings of the present study suggest the use of either extracts or pure compounds of *R. arboreum* in the development of drugs for the prevention of fungal infections.

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