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Isolation and Pharmacological Screening of 8-*O*-Acetyl Harpagide from *Ajuga bracteosa* Wall

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8-*O*-Acetyl harpagide was isolated and characterized from *Ajuga bracteosa* Wall, a species indigenous to Pakistan. Pharmacological screening of the compound for antibacterial, antifungal, antispasmodic, cardiotonic and antipyretic activities was carried out. The compound was found effective against a number of human pathogenic bacteria and fungi. Antispasmodic and cardiotonic effects elicited by the compound were also found. The compound also exhibited antipyretic activity when administered in the higher doses.

Key words: *Ajuga bracteosa* wall, 8-*O*-Acetyl harpagide, Pharmacological screening.

Introduction

Ajuga bracteosa Wall is a perennial herb, distributed in Western Himalayas from Kashmir to Nepal ascending to an altitude of 7000 ft. (Manjunath 1948; Gupta 1968). The leaves are reported to be diuretic, stimulant and used as a substitute for cinchona (Manjunath 1948; Chopra *et al* 1956). The plant is also reported to possess cardiostimulant action in animals and anticancer activity in rats and mice. (Patel *et al* 1962; Dhar *et al* 1968). A number of saturated and unsaturated acids (Bhakuni and Kaul 1961) and unsaturated ketones (Bhakuni *et al* 1990), insect antifeedant diterpenes and insecticidal diterpenes (Kubo *et al* 1976; 1980 and 1982) have been isolated from aerial parts of *A. bracteosa*. Because of pharmacological importance of the plant species, studies were undertaken for the isolation of iridoid glycosides from *A. bracteosa wall*, a species indigenous to Pakistan and different pharmacological activities of 8-*O*-acetyl harpagide have been carried out.

Experimental

Materials. Plant material (with roots) was collected from village Nilgram, Swat in August 1990 and was identified against voucher specimen deposited at herbarium of PCSIR Laboratories Complex, Peshawar. All the solvents and chemicals used were of BDH analytical grade. Sabouraud Dextrose Agar, DMSO, Adrenaline hydrochloride, Miconazole, Ketoconazole and Acetylcholine were obtained from Fluorochem Ltd. and Aldrich Chemical Co. Ltd. For biological assay culture media were obtained from HEJ Research Institute of Chemistry, University of Karachi. For pharmacological work, experimental animals were obtained from the PCSIR animal house. Ampicillin and acetylsalicylic acid were purchased from the local chemist shop.

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For TLC precoated silica gel G60, F254 plates (0.2 mm thick, Merck) were used. Melting point was determined using electrothermal apparatus and uncorrected. The IR Spectra were recorded in KBr disc on Schimadzu IR 460 Spectrophotometer. Mass spectra were recorded on varian MAT 311-A Mass Spectrophotometer at 70 eV. ^{1}H-NMR were recorded on Bruker AM-300 NMR Spectrophotometer in DMSO-d_6 at 300 MHz.

Method. The air dried powdered plant material (150 g) was extracted successively with hexane, chloroform and ethyl acetate. The ethyl acetate was concentrated *in vacuo* and then cooled to room temperature. Precipitate formation occurred. The crude precipitated material was filtered and then suspended in ethyl acetate with stirring and shaking. Drop wise addition of methanol along with heating resulted in dissolution of the solid. The solution was allowed to stay at room temperature for crystallization. The crystals were filtered and re-crystallization procedure was repeated a couple of times to obtain pure white flaky crystals with m. p. 143-144°C. The compound was identified as 8-*O*-acetyl harpagide. The final yield was 1.9 g (1.27%).

Fungicidal bioassay. The assay was conducted in accordance with the method of Brass *et al* (1979). During the assay, Miconazole and ketoconazole were used as standard reagents. Different concentrations of 8-*O*-acetyl harpagide, miconazole and ketoconazole ranging from 100 µg/ml to 250 µg/ml were prepared in DMSO. These solutions were further diluted with

distilled water. Test and standard solutions were added to media contained in the test tubes. A control determination was also conducted under similar conditions. The test tubes holding varying concentrations of test and standard were inoculated with a 5 mm diameter disk of inoculum obtained from 7 days old cultures of fungi. The tubes were incubated at 25-29°C for 7 days. Evaluation was performed by linear length (mm) and growth inhibitions.

Bactericidal bioassy. The investigations were performed according to the method of Carren *et al* (1987). During the assay ampicillin trihydrate was used as a standard drug. Different concentrations of 8-*O*-acetyl harpagide and ampicillin ranging from 100 µg/100 µl to 200 µg/100 µl were prepared. Cultures 24 h-old possessing about 10^4 - 10^6 CFU was layered on the surface of Mueller Hinton agar plates. With the help of a sterile metallic borer, wells were dug in the media. Test and standard solutions were placed in their respective wells. The plates were incubated at 37°C for 24 h and zones of inhibitions measured.

Antipyretic assay. The method of Teotino *et al* (1963) was applied for antipyretic studies. During the assay, acetylsalicylic acid was used as a standard. Young rabbits of either sex weighing between 1.5-1.8 kg of inbred Himalayan Strain were divided into three groups. The animals were housed under uniform environmental conditions and maintained on a standard diet consisting of green fodder and vegetables. All animals were administered a subcutaneous injection of yeast (2.5 ml/kg, 12%). Rectal temperature of hyperthermic animals were recorded prior and after 16 h of injection. Test animals received 200-500 mg/kg doses of 8-*O*-acetylharpagide and the other group received 150 mg/kg of the standard drug. The control group was given only the vehicle 1% carboxymethyl cellulose. The temperature of each group of animals were recorded at intervals of 90, 180 and 270 min. after treatment.

Spasmolytic studies. The studies were carried out by the method of Barlow and Khan (1959). Acetylcholine chloride was used as a standard. Three to five centimeters long terminal portion of ileum was taken from guinea-pig weighing 300-500 g, killed freshly by a blow on head. The isolated ileum was suspended in aerated Tyrode solution maintained at 34-35°C in an organ bath of 20 ml capacity. Different concentrations of test compound and standard ranging from 100-1000 µg/ml were prepared from stock solution of 10 mg/ml concentration. After 30 min rest, doses of standard solutions were added to the bath to obtain uniform amplitude of contraction which was recorded on a smoked Kymograph by a frontal writing level with 5-6 fold magnification and 3-4 g tension on the tissue. The spasmogens were allowed to act for 20 sec. Various concentations of aqueous solutions of test compound on the spasmogen induced contraction was measured as percent reduction of contraction.

*Cardiotonic studies.*Cardiotonic activity was determined by the method of Circosta *et al* (1984) on isolated heart taken from a freshly slaughtered rabbit. The heart was removed with a small portion of the ascending aorta. The aorta was cannulated and the heart was perfused with oxygenated Ringer-Locke solution at 37°C under constant pressure by the Langendorff method. Adrenaline hydrochloride was used as a standard. Test and standard solutions (100 µg/ml to 1000 µg/ml) were prepared. The rate and amplitude of contraction of the heart were registered by a starling's heart level on a smoked Kymograph. The test compound of different concentrations was injected slowly into the perfusion fluid and percentage change in the amplitude of contraction and its duration measured.

Toxicity study. Toxicity was determined by the intraperitoneal route in 10 adult albino rats of either sex and 15-20 g in weight. Control animals were administered only the vehicle. The animals were observed for 5-6 h after dosage for toxic symptoms. Mortality during the next 24 h was recorded. The approximate LD_{50} was estimated by Brine Shrimps bioassay method and the maximum tolerated dose was also recorded.

Results and Discussion

8-*O*-Acetyl harpagide was isolated from *Ajuga bracteosa* wall, a species indigenous to Pakistan. The compound was characterised and identified as 8-*O*-acetyl harpagide by comparison of its UV, IR, ^{1}H-NMR, ^{13}C-NMR and HRMS data with the standard and close similarity was revealed. The results were consistent and were found to be identical with the data reported in the literature (Chung and Yoo 1985).

The results of antifungal assay are listed in Table 1. As evident, the test compound displayed variable activity against the organism tested. It was fairly effective against *Penicillium notatum* and *Zutarium rotatum* and moderately active against *Curvularia lunata* and *Remera solani.* However, inhibitiory effects were not recorded agasinst, *Aspergillus niger, Candida albicans, Microsporum canis* and *Fusarium solani.*

The antibacterial activity of 8-*O*-acetyl harpagide was carried out by agar well diffusion method at different concentrations. The results of the assay are listed in Table 2. As evident, the compound is only active against *Bacillus anthracis,* while inactive against *Staphylococcus aureus, Streptococcus agalactiae, Staphylococcus epidermidis, Aeromonas sobriae, Shigella boydii, Vibrio choleriae, Corynebacterium diphtheriae, Salmonella typhi* (gram-ve), *Pseudomonas aeruginosa* (gram-ve).

Table 1
The fungicidal activity of 8-*O*-acetyl harpagide

Name of organism	Concentration of test compound (µg/ml)			Zone of inhibition in mm	
	100	150	200	250	
Penicillium notatum	-	-	+	+ +	+ +
Aspergillus niger	-	-	-	-	
Curvularia lunata	-	-	-	+	+
Zutarium rotatum	-	-	+	+ +	+ +
Candida albicans	-	-	-	-	-
Microsporum canis	-	-	-	-	-
Fusarium solani	-	-	-	-	-
Remera solani	-	-	-	+	+

Key: + + + Highly sensitive (Inhibition zone 12-15 mm); + + Fairly sensitive (Inhibition zone 9-12 mm); + Slightly sensitive (Inhibition zone 6-9 mm); - Not sensitive.

Table 2
The antibacerial activity of 8-*O*-acetyl harpagide

Name of microorganism	Zone of inhibition in mm at 100 µg - 200 µg/100 µl of DMSO	
	100 µg/100 µl	200 µg/100 µl
Staphlococcus aureus	-	-
Streptococcus agalactiae	-	-
Staphylococcus epidermidis	-	-
Bacillus anthracis	100 mm	11 mm
Aeromonas sobriae	-	-
Shigella boydii	-	-
Vibrio choleriae	-	-
Corynebacterium	-	-
diphtheriae	-	-
Salmonella typhi (gram-ve)	-	-
Pseudomonas aeruginosa (gram-ve)	-	-

Key: - No antibacterial growth.

Table 3
Toxicity grading of compound by brine shrimp bioassay

Concentration of test solution	100 µg	10 µg	1 µg	Control
Alive	10	10	10	10
	10	10	10	
	9	10	10	
Death	1	0	0	0

LD50 > 1000 µg/µl

Antipyretic evaluation of the test compound exhibited antipyretic effects only when treatment was conducted using larger doses of the compound (500 mg/kg). Smaller doses of the compound could not produce the desired effects as elicited by the standard drug acetylsalicylic acid.

8-*O*-Acetyl harpagide exhibited antispasmodic activity on isolated ileum as well as cardiotonic effects on isolated rabbit heart in 100 µg/ml test doses.

Toxicity testing of the test compounds are listed in Table 3. As clear from the results mortality has not been observed in the experimental animals.

Therefore, it is concluded that 8-*O*-acetylharpagide, isolated from *A. bracteosa* wall was found active against a variety of microorganisms. The product also displayed therapeutic effects observed through biological assays. Injurious or toxic effects due to the compound were not detected. Therefore, the product was graded safe for therapeutic purposes.

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