PRELIMINARY PHARMACOLOGICAL STUDY OF THE PTEROCARPANS MACCKIAN AND TRIFOLIRHIZIN ISOLATED FROM THE ROOTS OF *ONONIS VAGINALIS*

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ABSTRACT

Preliminary pharmacological study was conducted for the pterocarpans macckian (Mac) and trifolirhizin (Trif). The two compounds isolated from *Ononis vaginalis* were tested for their hepatoprotective effects against CCl_4 induced hepatotoxicity in rats. Activity was accessed by measuring liver enzymes and NP-SH groups. Estrogenic activity was expressed as increase in uterine weight of young female rats. Rat paw edema as a model of acute inflammation induced in Wistar rats using carragenan was used to evaluate the anti-inflammatory activity. Percentage inhibition of the aggregation induced by adenosine diphosphate (ADP) to platelets-rich plasma (PRP) obtained from rats was used as measure for antiplatelet aggregation effect.

The aglycone Mac was only more active than the glycoside Trif in the anti-inflammatory assay. It resulted in 65.7% reduction in carregeenan induced rat paw edema compares with 79.8% reduction by indomethacin at the same molar concentration. The activity of Trif was about half of that of silymarin in reducing the elevated levels of liver enzymes at the same molar concentration. About 10 fold molar concentration of Trif produced about half fold increase in uterine weight produced by 17β-estradiol.

Keywords: Hepatoprotective; estrogenic; anti-inflammatory; antiplatelet aggregation.

INTRODUCTION

Pterocarpans are the second largest group of natural isoflavonoids. They have been mainly found in members of the family Leguminosae (Fabaceae) (Jiménez-González *et al.*, 2008). Although pterocarpans are present in plants under normal conditions, however, their concentration dramatically increased following fungal or bacterial infections (Soby *et al.*, 1996; Keen and Kennedy, 1974). In the last decade several examples of pterocarpans with antitumor and antimitotic activities have been reported (Chaudhuri *et al.*, 1995; Maurich *et al.*, 2006; Militão *et al.*, 2006; Militão *et al.*, 2007). Other biological activities reported for pterocarpans include, anti-HIV (Engler *et al.*, 1993; Engler *et al.*, 1996; Schaefer *et al.*, 1993), anti-inflammatory (Selvam *et al.*, 2004) and antiparasitic (Chanphen *et al.*, 1998; Salem *et al.*, 2006) activities. Polyphenolic complexes from *Maackia amurensis* significantly reduced the hepatotoxicity induced by tetrachloromethane. The complex was rich in isoflavonoids including macckian (Saratikov *et al.*, 2005). Several classes of phytoestrogens were identified including: isoflavonoids, coumestrol and lignans (Whitten and Naftolin, 1991). Phytoestrogens can be used for the management of menopausal symptoms with few side effects (Glazier and Bowman, 2001). They are also used for prevention and treatment of diseases such as osteoporosis and breast cancer (Lindner, 1976). Several flavonoids exhibited anti-inflammatory activity most likely through inhibition the cyclo-oxygenase and/or the 5-lipoxygenase pathways of arachidonate metabolism (Harborne and Williams**,** 2000). Several reports indicated that flavonoids and flavonoids containing herbs have potential anti-platelet activity (Harborne and Williams**,** 2000; Chavez *et al.*, 2006). The inhibition of platelet function represents a promising approach for the prevention of thrombosis. A number of drugs have been developed and evaluated for their effects in preventing thrombosis or its recurrence (Stein and Fuster, 1989; MacMahon and Sharpe, 1991).

The roots of *Ononis vaginalis* are rich source of the two pterocarpans: macckian (Mac) and trifolirhizin (Trif) (Abdel-Kader, 2001). The yields of both compounds enable to carry out preliminary pharmacological study for some expected activities reported for pterocarpans or other flavonoid derivatives. Hepatoprotective, estrogenic, anti-inflammatory and antiplatelet aggregation were selected for the present work.

MATERIALS AND METHODS

Plant materials

Ononis vaginalis Vahl. Symb. (Fabaceae) (voucher MSA2) was described earlier (Abdel-Kader, 2001). The plants were recollected in 2007 and identified by comparison with voucher deposited in the Department of Pharmacognosy, College of Pharmacy, Alexandria University.

Extraction and Isolation

The dried powdered roots (5kg) were extracted with 95 %

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ethanol (9 L). The concentrated extract (300 mL) was diluted with 300 mL water and extracted with light petrol (3 x 400mL), ether (3 x 500 mL), ethyl acetate (3 x 400 mL), and butanol (3 x 300 mL). The ether soluble fraction afforded macckian (Mac) and trifolirhizin (Trif). Characterization of the two compounds was described in details earlier (Abdel-Kader, 2001).

Animals and Chemicals

Wistar albino rats (150-200g) of either sex roughly the same age (8-10 weeks) were obtained from the Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh and were used for hepatoprotective, anti-inflammatory and anti-platelets aggregation activity assays. Young female rats (about 120 gm body weight) were obtained from the same source and were used for estrogenic activity study. The animals were housed under constant temperature (22 ± 2°C), humidity (55%) and light/dark conditions (12/12 h). They were provided with Purina chow and free access to drinking water *ad libitum* (Abdel-Kader and Alqasoumi, 2008). All the experiments were carried out according to ethics of Helsinki declaration as approved by World Health Association (WHA) for manipulation of experimental animals. Silymarin, 17β-estradiol, indomethacin, prostacyclin and adenosine diphosphate (ADP) were purchased from Sigma Chemical Company, USA.

Hepatoprotective activity

Wistar rats were divided into five groups with six animals each. *Group I* was kept as a control group. *Groups II, III, IV* and *V* received 0.125 ml of carbon tetrachloride (CCl_4) in liquid paraffin (1:1) per 100 g body weight intraperitoneally. *Group II* received only CCl_4. *Group III* was administered silymarin at a dose of 10 mg/kg p.o. (20.7µmol/kg). Groups *IV* and *V* were treated with 5 and 7.5 mg/kg (20.7µmol/kg) of the Mac and Trif respectively. Drug treatment was started 5 days prior to CCl_4 administration and continued till the end of the experiment. After 48 h, following CCl_4 administration the animals were sacrificed using ether anesthesia. Blood samples were collected by heart puncture and the serum was separated for evaluating the biochemical parameters.

Determination of the enzyme levels

The biochemical parameters such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP) and total bilirubin were estimated by reported methods (Edwards and Bouchier, 1991). The enzyme activities were measured using diagnostic strips (Reflotron®, ROCHE) and were read on a Reflotron® Plus instrument (ROCHE).

Estimation of NP-SH

Non-protein sulfhydryl groups (NP-SH) were quantified according to the Sedlak and Lindsay method (Sedlak and Lindsay, 1968). The liver was cooled in a beaker immersed in an ice bath. 200 mg of liver were homogenized in 8 mL of 0.02 *M* ethylenediamine-tetraacetic acid (EDTA). Aliquots of 5 mL of the homogenate were mixed in 15 mL test tubes with 4 mL of distilled water and 1 mL of 50% trichloroacetic acid (TCA). The tubes were shaken intermittently for 10-15 min and centrifuged for 15 min at approximately 3000 rpm to precipitate the protein. Two mL of the supernatant were mixed with 4 mL of 0.4 *M* Tris buffer, pH 8.9 and 0.1 mL of 0.01 *M* 5, 5`-dithio-bis-(2-nitrobenzoic acid) (DTNB) added and the sample was shaken. The absorbance was measured spectrophotometrically within 5 min of addition of DTNB at 412 nm against a reagent blank with no homogenate.

Estrogenic Effect

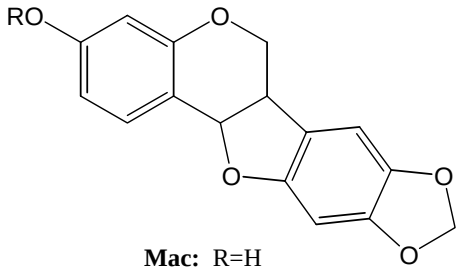
Young female Wistar rats were divided into 4 groups (N=4). *Group I* was kept as a control group. *Group II* received 17β-estradiol (1mg/kg) while *Groups III* and *IV* were administered Mac and Trif respectively at 20 mg/kg body weight. All tested compounds were administered intraperitoneally as a suspension in 0.25% aqueous sodium carboxy methyl cellulose. Twenty four hours after treatment all animals were anaesthetized with ether and the lower abdomens opened and the two joined uterine horn of each rat were removed carefully in their intact shape and blotted dry on a filter paper (Whatmann No-2) and weighted. The ratios of the weights of the uteri in control and treated animals to the whole animal weight were also calculated (Zamaraeva *et al*., 1999).

Anti-inflammatory activity

Rat paw edema as a model of acute inflammation induced in Wistar rats using 1% aqueous carragenan was used to evaluate the anti-inflammatory activity (Winter *et al*., 1962). The animals were divided into four groups 6 animals each. The first group served as negative control (0.5 ml of 1% carregeenan) while the animals in the second group were treated with a suspension of 1/mmol/kg indomethacin (4 mg/kg) in 0.25% sodium carboxy methyl cellulose one hour before carragenan injection via intraperitoneal route (positive control). Groups *III* and *IV* were treated similarly with 1/mmol/kg Mac (2.8 mg/kg) and Trif (4.5 mg/kg) respectively. Edema was induced by injecting 0.5 ml carragenan solution into the rat hind paws. The volume of the rat paws before, 3 hours after injection of carragenan were measured using a Hydro-Plethysmograph (Model 7150, Ug0, Basile, Haly). Results are expressed as % inhibition of edema (protection against inflammation) 3 hours after carragenan injection in comparison with the control group.

Antiplatelet aggregation effect

Wistar rats were anaesthetized with ether. Blood was collected from the rat via cardiac puncture in trisodium



Mac: R=H
Trif: R=Glc

Table 1: Effects of Mac and Trif on serum biochemical parameters.

Treatment (n=6)	Dose mg/kg (Orally)	Biochemical Parameters							
		SGOT (units/l)		SGPT (units/l)		ALP (units/l)		Bilirubin (mg/dl)	
		Mean ± S.E.	% Decrease	Mean ± S.E.	% Decrease	Mean ± S.E.	% Decrease	Mean ± S.E.	% Decrease
Normal (control)	Normal saline	90.06 ± 10.19		35.53 ± 6.57		381.00 ± 28.31		0.52 ± 0.06	
CCl_4 only (toxicity control)	1.25 ml/kg	387.66[a] ± 18.04***		340.33[a] ± 25.90***		919.16[a] ± 26.05***		3.82[a] ± 0.25***	
Silymarin + CCl_4	10	175.33[b] ± 21.75***	54.77	113.88[b] ± 19.50***	66.53	419.33[b] ± 31.58***	54.37	1.07[b] ± 0.18***	71.98
Mac + CCl_4	5	314.33[b] ± 23.68*	18.91	253.00[b] ± 37.01	25.66	693.31[b] ± 24.12***	24.57	3.44[b] ± 0.17	9.94
Trif + CCl_4	7.5	275.66[b] ± 27.00**	28.89	222.66[b] ± 23.98**	34.57	632.33[b] ± 31.45**	31.20	2.77[b] ± 0.15**	27.48

*p<0.05; **p<0.01; ***p<0.001, [a] as compared with the normal saline (control) group; [b] as compared with the CCl_4 only group.

Table 2: Effect of Mac and Trif on the level of NP-SH groups in the liver of rat treated with CCl_4.

Treatment (n=6)	Dose mg/kg (Orally)	NP-SH		
		Mean ± S.E. (μmol/g wet weight tissue)	% Increase with reference to the content of CCl_4-treated liver	% decrease of NP-SH content to normal
Normal (control)	Normal saline	4.37 ± 0.67		
CCl_4 (toxicity control)	1.25 ml/kg	1.45 ± 0.58*		66.81
Silymarin + CCl_4	10	3.88 ± 0.17*	167.58	11.21
Mac + CCl_4	5	1.73 ± 0.30	19.31	60.41
Trif + CCl_4	7.5	2.46 ± 0.24*	69.65	43.70

*p<0.01 as compared with the CCl_4 only group.

citrate solution (3.6% w/v in water). The ratio of blood to citrate was 9:1. The blood was slowly mixed and centrifuged for 10 minutes at 1600 rpm. The platelets-rich plasma (PRP) was aspirated and distributed into siliconized glass cuvettes in aliquots of 0.50 ml. Aggregation of the platelets was induced using adenosine diphosphate (ADP) in a final concentration of (10 μM) and aggregometer known as Platelet Aggregation Profiler, model PAP-4 was used. Each cuvette was initially heated for 2 minutes at 37˚C and then a magnetic stirrer was added followed by the aggregating dose of ADP. Aggregation was allowed to proceed for 4 minutes (El-Tahir *et al*., 1990). The extent of aggregation was measured using aggregometer supplied by Bio/Data Corporation (PAP-4) Model (Horsham, PA, USA). To examine the influence of Mac and Trif the compounds were dissolved in platelets-poor plasma (PPP). The solutions were then added to PRP and stirred for 5 minutes before the addition of ADP. The aggregations produced were then calculated as percentage change relative to ADP-induced aggregation (Table 5). Prostacyclin (PGI2) was used as control at a concentration of 10 ng/ml.

Statistical analyses

For each set of experiments where two or more than two groups were compared, an analysis of variance (ANOVA) test was used to determine the significance of the differences. Differences between the controls and treated groups were compared for significance using student's *t*-test for non paired samples (Woolson, 1987). All the values shown are the mean ± SEM.

RESULTS

The results of all experiments are presented in tables 1-5 along with the negative and positive controls for each experiment. Data were expressed as the mean ± SEM. Significant difference between the control and the treated groups was performed using Student's *t*-test and *P* values. The difference in results was considered significant when *P* values are at least < 0.05.

DISCUSSION

Treatment of animals with the hepatotoxic agent CCl_4 resulted in significant increase of transaminases (SGOT and SGPT) and alkaline phosphate levels (ALP) due to hepatocytes damage (Zafar and Ali, 1998). Sever jaundice was reflected by increase level of serum bilirubin (Lin *et al.*, 1997) (table 1). Treatment of animals with silymarin at 20.7 μmol/kg dose resulted in significant decrease in the elevated levels of SGOT, SGPT, ALP and bilirubin (p<0.001) (table 1). Silymarin act as an antioxidant by scavenging prooxidant free radicals and by increasing the intracellular concentration of GSH. It also exhibits a regulatory action of cellular membrane permeability and increase in its stability against xenobiotics injury (Dehmlow *et al.*, 1996; Saller *et al.*, 2007). Silymarin treatment also resulted in good recovery of the NP-SH groups. The reduction in these groups was significantly limited to 11.21% (p<0.01) (table 2). Treatment of animals with Mac and Trif (20.7μmol/kg) prior to CCl_4 revealed that the glycosidic form, Trif was more active than the aglycone Mac in reducing the elevated levels of the hepatic liver enzymes. Although the reduction was significant (p<0.01), the activity of Trif was about half that of silymarin (table 1) at the same molar concentration. Trif was less effective in restoring the NP-SH (table 2). The percentage reduction were 43.70% after treatment.

The potencies of phytoestrogens are estimated to be approximately 1000-fold weaker than that of 17β-estradiol (Jordan, 2004). Due to their structure sililarity with some phytoestrogens the estrogenic effect of the two compounds was evaluated (table 3). The effect was accessed by the reported method utilizing uterine weight as indicator for the activity (Zamaraeva *et al*., 1999). Again the glycosidic form, Trif, was about ten times more active than Mac (table 3). 17β-Estradiol was used as positive control at 1mg/kg dose. Approximately 10 fold molar concentration of Trif resulted in slightly more than half the increase in uterine weight by 17β-estradiol.

Edema, redness and pain are common symptoms of inflammation (Carol, 1986). Anti-inflammatory agents help in reducing all these symptoms. The anti-inflammatory activity was explored using rat paw edema as a model of acute inflammation induced by 1% aqueous

Table 3: Effect of Mac and Trif on uterine weights.

Treatment (n=4)	Dose in mg/kg	Weight of the uterus	Ratio of uterine weight/body weight	% increase in uterine weight
Control	-	0.410± 0.015	$3.34x10^{-3}$	-
17β-estradiol	1	1.118± 0.09*	$9.71x10^{-3}$	172.7± 9
Mac	20	0.44 ± 0.03	$3.61x10^{-3}$	9.1 ± 3.5
Trif	20	0.789 ± 0.06*	$6.44x10^{-3}$	93.0 ± 2.1

*p<0.05 as compared with the control group.

Table 4: Effect of Mac and Trif on carregeenan-induced rat paw edema.

Treatment N=6	Volume of paw (ml) after carregeenan mean ± SEM		Increase in paw volume after 3 hrs (ml) ± SEM[a]	% Protection
	Before treatment	3 Hrs after treatment		
Negative control	0.95 ± 0.1	1.95 ± 0.11	0.99 ± 0.17	-
indomethacin	0.97 ± 0.06	1.17 ± 0.04	0.20 ± 0.04**	79.8
Mac	0.90 ± 0.13	1.23 ± 0.04	0.34 ± 0.12*	65.7
Trif	0.91 ± 0.1	1.55 ± 0.09	0.64 ± 0.08	35.5

[a]SEM denotes the standard error of the mean. *p<0.01 as compared with the control group.
**p<0.001 as compared with the control group.

carragenan (Winter *et al.*, 1962). Anti-inflammatory agents will express their potential through reduction in the size of edema. In this assay Mac showed 65.7% inhibition of inflammation compared with 79.8% produced by the positive control indomethacin as calculated after 3hr of carragenan injection. Trif was less active and the 35.5% reduction in edema was statistically insignificant.

ADP is stored in dense bodies inside blood platelets and is released upon platelet activation. ADP interacts with a family of ADP receptors found on platelets (P2Y1, P2Y12 and P2X1), leading to further platelet activation (Murugappa and Kunapuli, 2006). Addition of ADP to PRP will cause 100% aggregation. Compounds possessing antiplatelet aggregation activity such as PGI_2 will prevent or reduce the effect of ADP. Trif was able to produce 54.2% inhibition in aggregation caused by ADP at 800 μg/mL. Mac was much less active. At 1400 μg/mL only 45.65% inhibition was obtained.

The above results showed that although the two compounds have the same skeleton, however, significant differences in their activities were observed. Mac was less active in all the assays except the anti-inflammatory assay. The mechanisms of actions are still to be explored. However, some of these activities may be related to the antioxidant potentials of the two compounds.

Table 5: Effect of Mac and Trif on rat platelets aggregation induced by ADP.

Treatment	Conc. (μg/mL)	% Inhibition of Aggregation
Mac	400	-
	700	25.4
	1400	45.6
Trif	400	37.5
	800	54.2
PGI_2	0.1	100

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