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Original research article

In Vitro Antioxidant and Hepatoprotective Activity of Flavonoids and Flavonoid Fractions of *Scoparia Dulcis* L.

Abhishek Sharma, Priya Jain, Manmeet Singh Saluja

Department of Pharmacy, Sunrise University, Alwar, Rajasthan-301026

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Address for Correspondence: Abhishek Sharma
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Abstract:
Scoparia dulcis L. is widely used in the traditional system of medicine for treating liver ailments. In the present study the flavonoids and flavonoid fractions isolated from 9:1 Ethyl acetate and methanol (EAM) extract of *Scoparia dulcis* L. were tested for their in vitro 1, 1- Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. Selected samples from the assay were further tested for their in vitro hepatoprotective activity against CCl_4 induced hepatotoxicity in freshly isolated rat hepatocytes. In the in vitro antioxidant study, fractions 2, 4, and 9 and EAM extract show the DPPH radical scavenging activity. The phytochemical screening of all these fractions shows the presence of flavonoids. In the in vitro hepatoprotective study all these fractions and the EAM extract significantly prevent the CCl_4 induced changes in the aspartate aspartate amino transferase, alanine amino transferase and alkaline phosphatase levels ($p < 0.05$). The above results are comparable with the standard, silymarin. The results of the study indicate that, the EAM extract of *Scoparia dulcis* L. possesses potential hepatoprotective activity and this may be attributed to its free radical scavenging potential, which in turn may be attributed to the presence of flavonoids.
Key words: Scoparia dulcis L.; Hepatoprotective activity; Carbon tetrachloride

Introduction

The liver is a versatile organ which is responsible for the metabolism of chemicals and for the regulation of internal chemical environment. Hepatotoxicity may be caused by thousands of synthetic chemicals, drugs, bacteria, fungi, plants and animal toxicants. These agents cause liver damage either by themselves or by getting converted to toxic metabolites (Victor, 1952; Valmane, et al., 1974; Carlo, 1979; Gupta, 1983). The most common hepatic disorders are viral hepatitis, non-alcoholic fatty liver disease (NAFLD), drug-induced hepatotoxicity, and alcoholic fatty liver disease. In India, it is reported that about 1% of the population is infected with hepatitis C and 2 - 4% with hepatitis B virus. NAFLD is the most prevalent liver disease, affecting up to 24% of patients in the general population and up to 74% of those with obesity. The prevalence of this disease is likely to continue to rise, paralleling the increasing global prevalence of diabetes and obesity (Furquan, 2007). Although, liver has a tremendous ability for regeneration, acute liver

illnesses often leads to serious chronic sequelae such as, chronic hepatitis, cirrhosis and even carcinoma.

Herbal drugs play a major role in the treatment of hepatic disorders. A number of medicinal plants and their formulations are widely used for the treatment of these disorders. Scoparia dulcis L (family: Scrophulariaceae) is a glabrous under shrub with small white flowers commonly found on the waste lands and fallow fields. This plant is widely used in the traditional system of medicine for treating liver ailments (Kirtikar and Basu, 1988; Yoganarasimhan, 1996; Parrotta, 2001). Phytochemical screening carried out by earlier workers on the plant has revealed the presence of of 10 different types of flavonoids; apigenin, scutellarein, luteolin, luteolin 7 glucoside, vicenin-2, imarin, vitexin, isovitexin, scutellarein methyl ester (Ramesh et al., 1979).

In the present study an effort has been made to isolate the active compounds and the fractions containing flavonoids and to screen them for their in vitro hepatoprotective activity against CCl4 induced toxicity in freshly solated rat hepatocytes.

Materials and Methods:

Drugs and chemicals

Silymarin was a gift sample from Micro Labs, Hosur, India. Aspartate amino transferase (ASAT) and alanine amino transferase (ALAT), alkaline phosphatase (ALP) and total protein (TP) kits were from RANDOX Laboratories Ltd. United Kingdom. Collagenase, insulin and dexamethasone were from Sigma chemical Co, St Louis, USA. Ham's F 12 was from Hi-Media laboratories, Mumbai. All other chemicals and reagents used were of analytical grade.

Preparation of PDM extract The flavonoid extract of *Scoparia dulcis* L. was prepared using Ethyl acetate and methanol as reported earlier (Manirudin and Jasmin, 1990). Authenticated (voucher no. BUB12005) whole plant was collected from the campus of Manipal College of Pharmacy, Udupi, India. The whole fresh plant was dried under shade at room temperature for seven days and reduced to coarse powder (sieve No.10/40). This powder was used for the preparation of Ethyl acetate and methanol (EAM) extract by soxhlet extraction method. The dried powder (125 g) was extracted three times with 2.5 liters of DM at 50 - 55 °C for 24 h. The extract obtained was concentrated at 50 ° C for 12 h.

Isolation of flavonoids and flavonoid fractions from EAM extract

PDM extract (25 gm) was mixed with 25 gm of silica gel (60 to 120 mesh size). The column was packed with silica gel (60 to 120 mesh size) ten times to the weight of dry crude extract using petroleum ether by wet packing method. The adsorbed crude drug was subjected to the prepared column. The column was eluted initially with Ethyl acetate: methanol. 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and 0:100), in a gradient manner. A total 14 fractions were obtained in different polarities after a silica gel column chromatography of Ethylactate: Methanol (9:1) fraction using ethyl acetate and methanol as solvents. Then Fraction3 selected for further isolation by subcolumn because show they show highest phenolic and flavonoid content. Among the 8 subfractions obtained from fraction 3 using chloroform, methanol and formic acid (4:1:1) as solvents.

1, 1-Diphenyl-2-picrylhydrazyl [DPPH] scavenging assay

The assay was carried out in 96 well micro titre plates. The extract (100 µl) and the standard (100 µl) were serially diluted with double distilled water. DPPH (100 mM, 100 µl) solution was added to all the wells except the blank and incubated at room temperature for 20 min. The absorbance was measured at 490 nm using ELISA reader (BIORAD - 550).

In vitro hepatoprotective activity of EAM extract and its fractions

The in vitro hepatoprotective activity of the PDM extract and its selected fractions were carried out using freshly isolated rat hepatocytes. The rat liver cells were isolated as per the modified procedure of Seglen (1976). A

male Wistar rat (250 g) was given insulin (500 IU/animal, i.p.) and anaesthetized with ketamine and xylazine injection (ketamine 83 mg/ml, xylazine 16.7 mg/ml, dose 0.75 ml/kg, i.p.). The abdomen of the rat was cleaned with 70% alcohol and cut opened to expose the portal vein. The portal vein was isolated and a cannula was inserted and secured with the help of a silk thread. The inferior vein cava was cut open before starting the perfusion. The perfusion was started with 100 ml warm (37 °C) calcium free HEPES buffer containing 1% bovine serum albumin at the flow rate of 10 ml/ min, then with 50 ml of TPVG (Trypsin, Phosphate buffer saline, Versine, Glucose), followed by calcium free HEPES buffer containing 0.075% collagenase and 4 mM calcium chloride.

After perfusion, the liver lobes were removed and transferred into a sterile conical flask containing calcium free HEPES buffer, the tissue was dispersed gently and the cell suspension was stirred with the help of a magnetic stirrer for 5 min to release the hepatocytes into the solution. The cell suspension was filtered through nylon mesh (250 µ) and the filtrate was centrifuged at 1000 rpm for 15 min. The resulting cell pellet was gently re-suspended in calcium free HEPES buffer. This washing procedure was repeated three times. The cell viability was determined by the trypan blue dye exclusion method. The isolated hepatocytes were cultured in Ham's F12 medium, supplemented with 10% new born calf serum, antibiotic, 10-6 M dexamethasone and 10-8 bovine insulin. The cell suspension was incubated at 37 °C in a humidified incubator under 5% CO2.

CCl4 induced heaptoxicity assay was carried out, after an incubation for 24 h, the hepatocytes were exposed to the fresh medium containing CCl4 (1%) along with or without various concentrations of test samples and standard, silymarin (n = 6). After 60 min of CCL4 challenge the media was analyzed for aspartate amino transferase (ASAT), alanine amino transferase (ALAT), alkaline phosphate (ALP) and total proteins (TP) using commercial kits in Merck auto analyser.

Phytochemical analysis of PDM extract and its active fractions

The PDM extract and its active fractions were analyzed for the presence of phytochemicals by using standard methods (Tyler et al., 1981).

Results

Preparation of EAM extract

The soxhlet extraction of 500 g of the plant material with 9:1 Ethyl acetate and methanol, yielded 35 g of greenish brown residue (yield 7% w/w).

Isolation of flavonoids and flavonoid fractions from EAM extract

A total 14 fractions were obtained in different polarities after a silica gel column chromatography of Ethylactate: Methanol (9:1) fraction using ethyl acetate and methanol as solvents. Among the 8 subfractions obtained from fraction 3 using chloroform, methanol and formic acid (4:1:1) as solvents.

1,1- Diphenyl-2- Picryl Hydrazyl (DPPH) radical scavenging assay

The results of DPPH assay of ethanolic extract and its fractions and subfractions are given in. Among all the tested compounds ethyl acetate: methanol (9:1) fraction showed an highest DPPH free radical scavenging activity (IC_{50} 11.3 ± 3.1 µg /ml), followed by ethanolic extract (IC_{50} 167.4 ± 6.0 µg /ml), ethyl acetate: methanol (9:1) fraction (IC_{50} 47.9 ± 2.9 µg /ml), fraction 3 (IC_{50} 147.6 ± 0.5 µg /ml), fraction 3_7 (IC_{50} 132.4 ± 0.7 µg /ml), fraction 3_8 (IC_{50} 76.3 ± 0.3 µg /ml). The standard vitamin C showed an IC_{50} value of 31.2 ± 0.4 µg/ml.

***In vitro* antioxidant activity of ethanolic extract, Its fractions, subfractions and single compound**

Name of the sample	IC_{50} in µl
	DPPH assay
Ethanol Extract	167.4 ± 6.0
Ethyl acetate:Methanol (9:1) fraction	47.9 ± 2.9
Fraction 3	147.6 ± 0.5
SF 7	132.4 ± 0.7
SF 8	76.3 ± 0.2
Vitamin C	31.2 ± 0.4

Values are mean ± SD, n=3.

Note: In the DPPH and NO scavenging assays, the IC_{50} refers to concentration of the test sample required to scavenge 50% of DPPH or NO radicals. In reducing power assay the IC_{50} refers to concentration of the test sample required to increase the reducing power by 50%.

***In vitro* hepatoprotective activity of selected fractions and its subfractions**

The results of the *in vitro* hepatoprotective activity of the selected fractions and its subfractions. All the fractions and subfractions at the tested concentrations of 125, 63 and 31 µg/ml showed significant dose dependent protection against CCl_4 induced elevation in the ASAT, ALAT and ALP ($P<0.05$). But, they showed no significant effect on CCl_4 induced changes in TP levels. Standard, silymarin at concentration of 20 µg/ml significantly prevented the CCl_4 induced elevation in the ASAT, ALAT and ALP ($P<0.05$). Silymarin also showed no significant effect on TP levels.

Effect of selected fractions and its sub fractions on CCl_4 intoxicated freshly isolated rat hepatocytes

Group	Treatment	Concentration	ASAT (u/l)	ALAT (u/l)	ALP (u/l)	TP (g/dl)
1	Normal	0.5% DMSO	14.0 ± 1.3	16.1 ± 4.0	173.4 ± 1.4	1.1 ± 0.05
2	Control	0.5% DMSO	74.3 ± 4.5	56 ± 5.8	116.4 ± 0.6	0.8 ± 0.01
3	Silymarin	20 µg/ml	24.1 ± 4.1*	18.6 ± 3.9*	103.4 ± 0.3*	0.9 ± 0.02
4	70% Ethnaol extract	125 µg/ml	22.0 ± 1.3	22.1 ± 4.0	126 ± 1.2	0.9 ± 0.05
		63 µg/ml	39.3 ± 4.5	26 ± 5.8	124 ± 0.9	0.8 ± 0.01
		31 µg/ml	43.1 ± 4.1*	31.6 ± 3.9*	141 ± 0.6*	0.9 ± 0.02
		16 µg/ml	59.0 ± 2.9*	37.2 ± 4.2*	176 ± 0.8*	0.8 ± 0.05
5	Ethyl acetate: Methanol (9:1) fraction	125 µg/ml	21.5 ± 2.5*	22.3 ± 2.1*	98.5 ± 0.6*	0.9 ± 0.05
		63 µg/ml	36.3 ± 2.6*	29.6 ± 2.3*	106.4 ± 0.9*	0.8 ± 0.06
		31 µg/ml	43.4 ± 2.5*	35.6 ± 3.5*	123.5 ± 0.8*	0.8 ± 0.02
		16 µg/ml	71.4 ± 3.5	48.1 ± 2.0	125.1 ± 1.0	0.8 ± 0.02
6	Fraction 3	125 µg/ml	24.5 ± 2.1*	32.6 ± 2.9*	108 ± 0.7*	0.9 ± 0.04
		63 µg/ml	38.6 ± 3.1*	36.6 ± 2.3*	124.6 ± 0.3*	0.8 ± 0.05
		31 µg/ml	59.9 ± 2.8*	48.3 ± 3.5*	114.6 ± 0.2*	0.8 ± 0.03
		16 µg/ml	67.4 ± 2.5	49.1 ± 3.0	94.1 ± 6.0	0.8 ± 0.05
7	Subfraction 7	125 µg/ml	22.0 ± 1.3	22.1 ± 4.0	126 ± 1.2	0.9 ± 0.05
		63 µg/ml	39.3 ± 4.5	26 ± 5.8	124 ± 0.9	0.8 ± 0.01
		31 µg/ml	43.1 ± 4.1*	31.6 ± 3.9*	141 ± 0.6*	0.9 ± 0.02

		16 µg/ml	59.0 ± 2.9*	37.2 ± 4.2*	176 ± 0.8*	0.8 ± 0.05
8	Subfraction 8	125 µg/ml	25.2 ± 5.6*	25.6 ± 3.6*	130 ± 0.4*	0.8 ± 0.06
		63 µg/ml	41.0 ± 1.3*	25.1 ± 4.0*	128 ± 1.2	0.9 ± 0.05
		31 µg/ml	53.3 ± 4.5*	28 ± 5.8*	125 ± 0.9	0.8 ± 0.01
		16 µg/ml	62.1 ± 4.1	32.6 ± 3.9	164 ± 0.6	0.9 ± 0.02

All the groups received 1% CCl_4 treatment, except group 1
Values are mean ± SEM, n=6, *: $P < 0.05$ when compared to group 2

Qualitative phytochemical screening of ethanolic extract, Its fractions, subfractions and single compound

	Flavonoids	**Phenolics**	**Steroids**	**Alkaloids**	**Glycosides**
Ethanolic extract	+	+	-	-	-
Ethyl acetate: Methanol (9:1) fraction	+	+	+	-	-
Fraction 3	+	+	-		
SF 7	+	-	+	-	
SF 8	+	+	+	-	

+: Present, -: Absent, SF: Subfraction

Discussions

In the present study, 14 different fractions and four pure compounds were obtained from the EAM extract by column chromatography. The in vitro antioxidant activity study of these isolated fractions and pure compounds confirmed that, only 70% ethanolic extract, Ethylacetate: methanol (9:1) extract, fractions 3 and subfraction 7, 8 have DPPH scavenging potential. Among these fractions showing DPPH scavenging activity, subfraction 8 shows the highest potency. In the in vitro hepatoprotective study, using freshly isolated rat hepatocytes, all the fractions (selected from the antioxidant study) and the PDM extract show significant protection against CCl4 induced changes in the ASAT, ALAT and ALP levels. Subfraction 8 also show the highest the highest activity against CCl4 induced damage, when compared to all other fractions and PDM extract. The preliminary phytochemical screening of the EAM extract, Ethyl acetate: methanol extract (9:1), fractions 3 and its Subfraction 7 & 8 show the presence of flavonoids.

The results of the present study EAM extract, Ethyl acetate: methanol extract (9:1), fractions 3 and its Subfraction 7 & 8 of this plant against CCl4 induced liver cirrhosis in rats and CCl4 induced acute liver injury in mice, respectively. (Praveen et al., 2008; Praveen et al., 2009)

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