DOI:10.5138/09750185.2402

AR JOURNALS ADVANCED RESEARCH

International Journal of Phytomedicine
2020;12(3):046-053

SHORT COMMUNICATION

In vitro hepatoprotective potential of whole plant of *Fumaria indica*(Haussk.) Pugsley and an isolated alkaloid Protopine

Anagha A Rajopadhye[1*] and Anuradha S Upadhye[2]

Abstract

Fumaria indica (Haussk.) Pugsley, Fumariaceae [syn. F. vaillantii Loisel.] is an important medicinal plant known as 'Fumitory'. Ethnobotanical and ayurvedic literature reports that the plant is used in treatment of liver diseases as well as diverse pharmacological activities. In present work, in vitro hepatoprotective effects of ethanol extract of *F. indica* (FUP) and alkaloid, protopine (PRT) on carbon tetrachloride induced oxidative stress has been demonstrated. An isoquinoline alkaloid, protopine was isolated from ethanol extract of F. indica and characterized by spectral data. Carbon tetrachloride (CCl_4) and ethanol has been used as a hepatotoxin. Cytotoxicity was estimated by quantitating the release of lactate dehydrogenase (LDH) in culture medium along with antioxidant enzymes namely superoxide dismutase, catalase and glutathione reductase. HPLC profile of FUP and PRT was developed using water: methanol (7:3) as a mobile phase. CCl_4 and ethanol induces 5.5 and 4 times more release of LDH from the liver cells and twice the amount of lipid peroxidation as compared to the cells from untreated liver tissue. These LDH and lipid perioxidation activities were reduced significantly in dose dependent manner after addition of FUP and PRT (at doses 0.5 % FUP and at does 0.025, 0.05 % PRT; $p < 0.001$). The activity of antioxidant enzymes was found to be elevated in CCl_4/ethanol treated cells. However, after addition of FUP/PRT along with cytotoxicant the activities were lowered significantly. The peak of PRT has been detected in FUP at retention times 1.670. sBased on these studies it may be precluded that protopine from F. indica, as a possible therapeutic for preventing oxidative stress in vitro by boosting the antioxidant capacity of the liver.

Keywords: Fumaria indica; Ethanol extract; Protopine; Liver slice culture model; In vitro Hepatoprotective

Introduction

Fumaria indica (Haussk.) Pugsley, Fumariaceae [syn. F. vaillantii Loisel.] is an important medicinal plant known as 'Fumitory'. The plant is a small herb growing as weed in wheat fields after harvest. It has dissected leaves and pink flowers in racemes. It is a major constituent of many common Ayurvedic, Unani medicinal preparations and marketed polyherbal liver formulations [1, 2]. The ethnobotanical and Ayurvedic literature reports the whole plant used as diaphoretic, diuretic, aperients, laxative, alterative, anthelmintic and in obstructions of the liver [3, 4]. Most of the earlier investigations on hepatoprotective effects of F. indica have been carried out intact animals using different in vivo animal models [5–7]. In present study to understand probable mechanism of action, we have used liver slice culture system to quantify the cytotoxic effects of carbon tetrachloride (CCl_4) and ethanol in terms of the release of lactate dehydrogenase (LDH) by the cells into the medium. Also the cellular levels of

*Correspondence: anaghaar@gmail.com

[1]Sinhgad College of Science,, S.No. 9/1/5 off West. 9/2/4, O Bypass Highway, Ambegaon,, Pune, 411041, Maharashtra, India

Full list of author information is available at the end of the article.

Received: 29 Feb 2020, Accepted:09 Aug 2020

the antioxidant enzymes (AOEs), superoxide dismutase, catalase and glutathione reductase along with lipid peroxidation were determined.

Material and Methods

Plant material

The herbs were collected from wheat fields of Junnar, Pune (Maharashtra, India) during winter season of 2013-2014. The plant sample was identified, authenticated and deposited in crude drug repository of Agharkar Research Institute, Pune 411 004; vide voucher specimen number WP-058.

Extraction

The whole plants were shade dried and coarsely powdered. The powder was successively extracted with petroleum ether (60-80^{o}C) and ethanol using Soxhlet apparatus. These extracts were concentrated at reduced temperature and pressure using rotary evaporator. Yield of ethanol extract (FUP) was 5.99 % and 13.79 % w/w respectively. In initial screening studies petroleum ether extract exhibited negligible hepatoprotective activity.

Isolation of Protopine and characterization

Protopine was isolated from whole plant of *F. indica* [8]. Freshly collected plant material was dried in shade and powered. The powdered material (500 g) was extracted with ethanol (1000 mL $\times$ 3) by cold percolation method. The extracts were filtered and the combined extract was concentrated in vacuo to give a dark green-brown gummy residue (16.6 g). The residue was dissolved in a mixture of water (300 mL) and concentrated hydrochloric acid (10 mL). The solution was kept in refrigerator (4$\pm$2ºC) for 3 days and filtered for removal of sediments. The acidic solution was extracted exhaustively with chloroform (300 mL $\times$ 5) to separate chloroform soluble hydrochloride. Chloroform extract was discarded. The filtrate was concentrated and repeatedly boiled with dilute hydrochloric acid (2 %, 30 mL) and the solution was extracted with ether to separate ether soluble portion. Ether extract was discarded and the acidic solution was then basified with excessive potassium hydroxide for precipitation. Precipitates were filtered off after standing for a while, washed with water and dissolved in a mixture of chloroform and methanol. Crude protopine (265 mg) was obtained by removal of solvent under reduced pressure. Repeated crystallization of the crude product by chloroform and methanol mixture was carried out to obtain pure protopine. The isolated compound was analyzed by IR, UV, and ^{1}H NMR and identified by comparison with the available spectral data [9].

HPLC analysis

Instrument : Agilent 1100 series quaternary HPLC with auto sampler

Column : Zorbax Eclipse, XDB, C_8, 4.6 mm x 150 mm, reverse phase

Detection : UV Detector at 254 nm

Mobile phase : Methanol: Water (20: 80)

Flow rate : 1 mL/min

Standard size injected : 0.25 mg/mL of methanol

Sample size injected : 1 mg/mL of methanol

Injection volume : 5 μL

Each sample was analyzed in triplicate and the peak of PRT was identified by comparison of their retention times (r_t).

Hepatoprotective Activity

Animals

To assess the hepatoprotective activity, adult albino mice (6–8 weeks old) of either sex breed in the animal house of Agharkar Research Institute, Pune- 411 004, were used for the preparation of liver slices. The approval for this work, using animals, was taken from the Institutional Animal Ethical Committee of Agharkar Research Institute, Pune- 411 004 (Registration Number: 101/199/CPCSEA).

Liver slice culture in vitro

Liver slice culture was maintained following the protocol developed by Wormser et al [10]. and Invittox protocol No. 42 [11]. The mice were dissected open after cervical dislocation, and liver lobes were removed and transferred to perwarmed Kred's Ringer Hepes (KRH) (2.5 mM Hepes, pH 7.4, 11 8mM Nacl, 2.85 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM KH_2PO_4, 1.18 mM $MgSO_4$, 5 mM β-hydroxy butarate and 4.0 mM glucose). The liver was cut into thin slices using sharp blade. The slices were weighed and the slice weighing between 4 and 6 mg was used for the experiment. Each experimental system contained 20–22 slices weighing together 100-120 mg. These slices were washed with 10 mL KRH medium, every 10 min over a period of 1 h. These were then pre-incubated for 60 min. in small plugged beakers containing 2 mL KRH on a shaker water bath at 37^{o}C. At the end of pre-incubation, the medium was replaced by 2 mL of fresh KRH and incubated for 2 h at 37^{o}C [12].

Experiment Design

These slices were further divided into different groups forming individual cultures for the further respective treatments.

FUP 0.25, 0.5, 1% and PRT 0.025, 0.05, 0.1% (suspended in 1 % w/v carboxymethylcellulose) were used in determining hepatoprotective activities.

Group 1- CCl_4 induced cytotoxicity: set 1, control, slices incubated in KRH medium; set 2,slices incubated in 15.5 mM CCl_4; set 3 slices incubated in only 0.5 % FUP; set 4 slices incubated in only 0.05 % PRT; set 5, slices incubated in 15.5 mM CCl_4 + different concentration (0.1, 0.25, 0.5 %) of FUP; set 6, slices incubated in 15.5 mM CCl_4 + different concentration (0.01, 0.025, 0.05 %) of PRT; set 7 slices incubated in 15.5 mM CCl_4 + 10 mM ascorbic acid; set 8 slices incubated in only 10 mM ascorbic acid .

Group 2 - Ethanol induced cytotoxicity: set 1, control, slices incubated in KRH medium; set 2, slices incubated in 112 mM ethanol [13]; set 3 slices incubated in only 0.5 % FUP; set 4 slices incubated in only 0.05 % PRT; set 5 slices incubated in 112 mM ethanol + different concentration (0.1, 0.25, 0.5 %) of FUP; set 6 slices incubated in 112 mM ethanol + different concentration (0.01, 0.025, 0.05 %) of PRT; set 7 slices incubated in 112 mM ethanol + 10 mM ascorbic acid; set 8 slices incubated in only 10 mM ascorbic acid .

After the respective treatments all the cultures were incubated in constant temperature water bath at 37ºC for 2 hrs. At the end of incubation, each group of slices was homogenized in appropriate volume of chilled potassium phosphate buffer (100 mM, pH 7.8) in an ice bath to give a tissue concentration of 100 mg/mL. The culture medium was collected and used for estimation of lactate dehydrogenase (LDH), which was employed as a cytotoxicity marker. The homogenates were centrifuged at 10,000 rpm for 10 min at 4ºC and the supernatants assayed for LDH, catalase, peroxidase and superoxide dismutase. Ascorbic acid (AA) was used as standard.

Measurement of Lactate dehydrogenase activity and lipid peroxidation

Lactate dehydrogenase (LDH; EC 1.1.1.27) was estimated [14] and each unit of enzyme was calculated as 1 μmol of NAD reduced per minute. Enzyme units in the medium and in tissue homogenate were estimated and percent release of enzyme from liver slices was calculated as the ratio of LDH activity found in the supernatant to the total LDH (supernatant + homogenate) activity [10, 14]. Lipid peroxidation was estimated in terms of thiobarbituric acid reactive substances (TBARS) [15].

Measurement of antioxidant enzymes and proteins

Superoxide dismutase (SOD; EC 1.15.1.1) was assayed spectrophotometric method [16]. One unit of enzyme was defined as the amount of enzyme causing 50% reduction in formazan formation under specified conditions. Catalase (CAT; EC 1.11.1.6) assay was carried out according to the method of Aebi [17]. One unit of was defined as that amount of the enzyme that converts 1μmol CCl_4 to water in 1 min. Glutathione reductase (GR; EC 1.6.4.2) activity was measured [18] and one unit of was defined as that of the enzyme required to oxidize 1μmol of Nicotinamide adenine dinucleotide phosphate (NADPH) reduced to Nicotinamide adenine dinucleotide phosphate (NADP) per minute. Protein in the tissue homogenate was estimated according to the method described by Bradford [19].

Statistical A nalysis

The results of treatment effects were analyzed using one-way ANOVA test (Graph pad Prism 4) and p-values < 0.001 were considered as very significant and p-values < 0.05 were considered as significant.

Results

Characterization of Protopin and HPLC Analysis

Protopine - The corresponding molecular formula and molecular weight was $C_{20}H_{19}NO_5$ and 353 respectively. ^{1}H NMR (400 MHz, $CDCl_3$): d 1.1-1.6 (8H, m, C_7H, C_{10}H, C_{11}H, C_{12}H), 1.91 (3H, s, C_{20}H), 5.94 (1H, s, C_{18}H), 5.96 (1H, s, C_{19}H), 6.66 (1H, s, C_4H), 6.67 (1H, d, J=7.6 Hz,C_{14}H), 6.70 (1H, d, J=7.6 Hz, C_{15}H), 6.89 (1H, s, C_1H). IR (Nujol): n_{max} 2900 (CH strecching), 1720 (C=O), 1560, 1450 (aromatic) cm^{-1}.

UV λ_{max} (MeOH) nm (log ε): 293 (3.28).

Under optimized conditions HPLC with C18 column and UV detector at 254 nm using isocratic mixture of methanol and water as mobile phase gave well resolved symmetric peak for protopin. The peak of PRT has been detected retention times 1.670 in FUP. Figure 1 shows the HPLC profile of FUP and PRT.

Release of LDH in liver slice culture

Release of LDH in the liver slice culture medium was used as cytotoxicity marker. FUP and PRT were found to be non-toxic in dose range 0.1-0.5 % and 0.01-0.05 % respectively. Liver slices from both sets treated with the dose 0.5 % FUP and 0.1 % PRT showed that the release of LDH percentage was found to be similar to control untreated slices. In all further experiments doses of 0.1-0.5% FUP and 0.01-0.05 % PRT were used.

Liver slices released 5.5 and 4 times more LDH into the medium in the presence of ethanol and CCl_4 respectively. After addition of FUP and PRT along with these cytotoxicants in the medium, the amount of LDH released was reduced significant in dose dependent manner (at doses 0.5 % FUP and at does

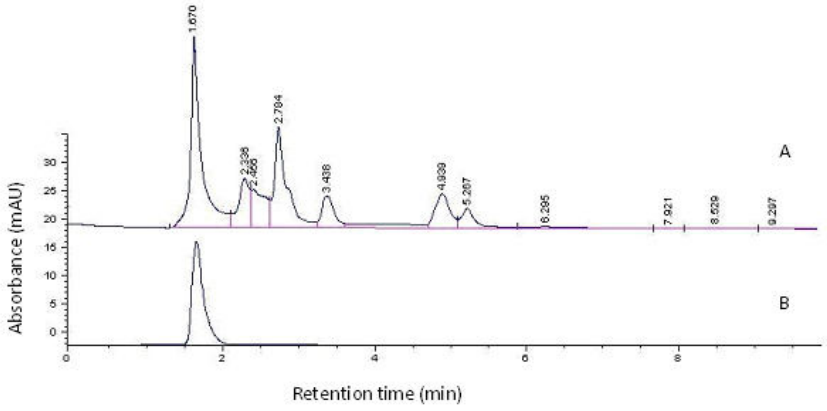

Figure 1 HPLC chromatogram of F. indica ethanol extract and protopine. A:Ethanol extract; B: Protopine

0.025, 0.05 % PRT; $p < 0.001$). The activity was comparable with ascorbic acid used as standard (Table 1 and 2).

Lipid peroxidation of liver tissue under different condition

CCl_4 and ethanol are known to generate oxidative stress in cells, which can be measured from the extent of lipid peroxidation in liver tissue. The lipid peroxidation levels were assessed by TBARS assay and measured in terms of thiobarbituric acid reactive substances and expressed as μmol of malondialdehyde formed/100 mg tissue. The amount of lipid peroxidation increased two folds in CCl_4 (3.9$\pm$0.34) and ethanol(4.1$\pm$0.78) treated liver cells compared to respective control. Liver slice medium treated with FUP or PRT along cytotoxicants were significantly reduced the extent of lipid peroxidation levels (Figure 2 and 3).

Time course of lipid peroxidation was assessed in the presence of cytotoxic agents alone and together with FUP or PRT. Cytotoxicants treated liver slices shows increase in lipid peroxidation parallel with the increase in LDH release. However, in presence of FUP or PRT along with cytotoxic agents the lipid peroxidation and LDH release, returned to the control levels (Figure 2 and 3). Since lipid peroxidation is caused by free radicals, FUP and PRT may be reducing the generated free radicals substantially.

Antioxidant enzymes (AOEs) activities in liver slice culture under different conditions

Both CCl_4 andethanol induces oxidative stress in the cells by generation of ROS. Activities of all three AOEs were checked in liver slice culture treated with CCl_4 or ethanol alone, with FUP or PRT, and alone FUP, PRT. The activities of antioxidant enzymes SOD, CAT, GR were found to be increased significantly in liver slices treated with ethanol / CCl_4 (Table 2 and 3). In case of SOD, the activity increased 2.3 and 2.5 times in the culture medium treated with CCl_4 and ethanol respectively. CAT showed 3.5 and 2.7-times more activity when treated with CCl_4 and ethanol respectively. GR activity increased 2 times more when either treated with CCl_4 or ethanol. When both FUP and PRT were added along with the toxicants in the medium, the activity of three antioxidant enzymes were reduced substantially comparable to that of untreated cells, especially at the higher concentration used. Ascorbic acid, used as standard, also showed reduced antioxidant enzymes activities when added along with the toxicants.

.

Cytotoxicity was assessed in terms of % lactate dehydrogenase (LDH) released, and the response to oxidative stress was measured in terms of antioxidant enzymes SOD, superoxide dismutase; CAT, Catalase; GR, Glutathione reductase activity. Ascorbic acid was used as a standard. Values represent means of at least three experiments and their standard deviation.

Significantly differ compared with respective CCl4 treated group, p < 0 05

* Significantly differ compared with respective CCl_4 treated group, $p < 0.001$, (one- way Anova).

Cytotoxicity was assessed in terms of % lactate dehydrogenase (LDH) released, and the response to oxidative stress was measured in terms of antioxidant enzymes SOD, superoxide dismutase; CAT, Catalase; GR, Glutathione reductase activity. Ascorbic acid was used as a standard. Values represent means of at least three experiments and their standard deviation.

Significantly differ compared with respective CCl4 treated group, p < 0 05

* Significantly differ compared with respective CCl_4 treated group, $p < 0.001$, (one- way Anova).

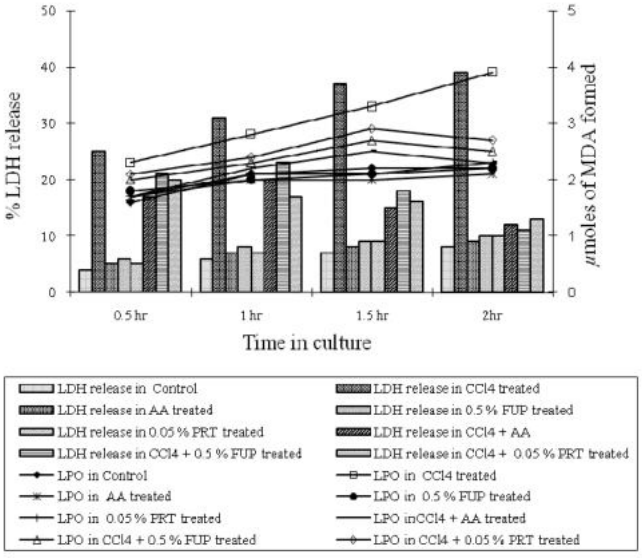

Figure 2 Time course of % LDH release and extent of lipid peroxidation in liver slice tissue in presence of CCl_4 /along with FUP or PRT.

Values are mean± SEM of five experiments. Values are mean ± SEM of five experiments. Lactatedehydrogenase, LDH; lipid perioxidation, LPO; Carbon tetrachloride, CCl4;ascorbic acid, AA;

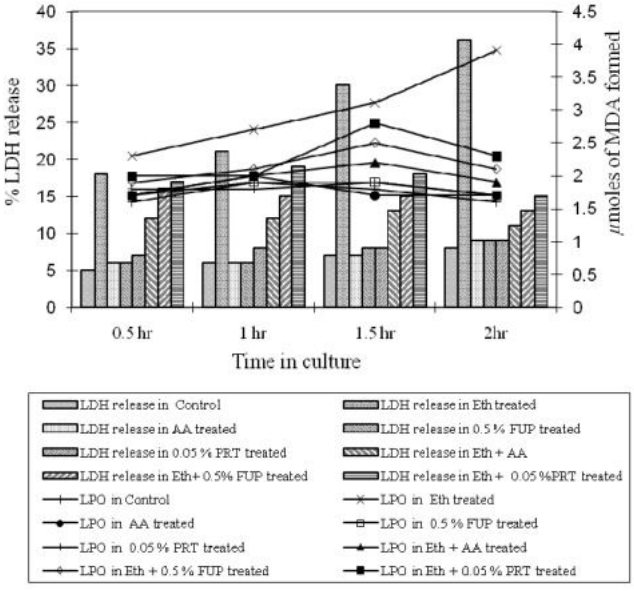

Figure 3 Time course of lipid peroxidation of liver slice tissue and LDH release in presence of ethanol/ along with FUP or PRT

Values are mean ± SEM of five experiments. Lactate dehydrogenase, LDH; lipid perioxidation, LPO; ethanol, Eth; ascorbic acid, AA;

Table 1 Effect of FUP and PRT on the levels of SOD, CAT and GR in liver slice culturein vitro against CCl_4 induced cytotoxicity.

Treatments	LDH Units/100 mg tissue wet wt.	SOD Units/100 mg tissue wet wt.	CAT Units/100 mg tissue wet wt.	GR Units/100 mg tissue wet wt.
Control	6.83±1.16	16.83±1.47	14.00±1.41	0.141±0.012
15.5 mM CCl_4	48.33±1.63	55.33±2.25	86.5±1.04	0.488±0.008
AA	7.83±1.16	16.5±2.14	14.33±1.75	0.152±0.007
FUE	6.98±2.09	17.5±0.98	14.0±1.94	0.143±0.013
PRT	7.89±0.81	17.56±1.41	14.83±1.67	0.144±0.002
CCl_4 + 0.1 % FUE	38.0±0.89^a	37.67±1.63^a	47.5±1.04^a	0.354±0.005^a
CCl_4 + 0.25 % FUE	32.33±1.21^a	33.33±0.81^a	44.67±0.81^a	0.321±0.007^a
CCl_4 + 0.5 % FUE	26.83±0.75*	28.67±1.03*	39.33±2.80*	0.268±0.008*
CCl_4 + 0.01 %PRT	25.83±0.75^a	29.83±0.75^a	33.5±1.37^a	0.272±0.008^a
CCl_4 + 0.025 % PRT	22.33±2.48*	25.5±0.54*	30.24±1.09*	0.236±0.008*
CCl_4 + 0.05 %PRT	17.83±1.21*	22.17±1.16*	25.74±2.28*	0.206±0.008*
CCl_4 + 50 mM AA	13.67±1.03*	15.33±1.63*	19.83±1.83*	0.189±0.018*

Table 2 Effect of FUP and PRT on the levels of SOD, CAT and GR in liver slice culturein vitro against ethanol induced cytotoxicity.

Treatments	LDH Units/100 mg tissue wet wt.	SOD Units/100 mg tissue wet wt.	CAT Units/100 mg tissue wet wt.	GR Units/100 mg tissue wet wt.
Control	7.56±1.19	17.5±1.37	14.67±2.16	0.134±0.019
112 mM Ethanol	46.50±1.87	53.5±1.04	73.83±1.47	0.662±0.013
AA	8.16±1.47	16.67±1.63	14.83±1.16	0.145±0.014
FUE	7.66±1.36	18.03±0.89	15.56±1.47	0.140±0.008
PRT	8.16±1.78	17.66±2.16	14.98±0.89	0.148±0.013
Ethanol + 0.1 % FUE	35.5±0.54^a	42.83±0.75^a	48.17±0.75^a	0.442±0.004^a
Ethanol + 0.25 % FUE	32.17±1.69^a	39.17±0.98^a	43.67±1.36^a	0.412±0.008^a
Ethanol + 0.5 % FUE	27.83±1.39*	36.00±0.89*	39.33±2.16*	0.36±0.014*
Ethanol + 0.01 % PRT	26.17±0.40^a	33.17±0.75^a	34.17±0.75^a	0.312±0.013^a
Ethanol + 0.025 % PRT	21.5±0.54*	29.33±0.81*	27.73±0.75*	0.26±0.01*
Ethanol + 0.05 %PRT	17.5±1.04*	21.00±0.75*	25.00±1.41*	0.231±0.008*
Ethanol + 50 mM AA	14.56±1.14*	13.17±0.75*	13.17±1.47*	0.168±0.017*

Discussion

The liver slice is a microcosm of the intact liver consisting of highly organized cellular community in which different cell types are subject to mutual contact. Such culture offers analysis of hepatotoxic events by measuring the release of LDH into the medium. Therefore, liver slice culture model is an in vitro technique that offers the advantages of in vivo as it provides desirable complexity of structurally and functionally intact cells [20]. It provides valuable approaches for screening of plant extracts/fractions for their hepatoprotective activity and elucidation of possible mechanism of actions.

In present work, we have studied the in vitro hepatoprotective activity of FUP and their isolated PRT. Oxidative stress was induced by adding cytotoxic agents CCl_4 or ethanol to the liver slice culture. Both pro-oxidants were highly toxic to the treated cells which increase LDH concentration in the medium as compare to control. On application of FUP or PRT, the cytotoxic effects were substantially lowered in dose dependent manner by decreasing LDH concentration probably through the reduction of oxidative stress. It is clear that the pre-treatment of FUP and PRT significantly reduces release of LDH from the liver cells and acts as a hepatoprotective agent.

Activities of antioxidant enzymes such as SOD, CAT, and GR were measured to assess the oxidative stress in the intact cells. Oxidative SOD and CAT are known to prevent damage by directly scavenging the harmful active oxygen species [21]. GR plays a role in recycling the oxidized glutathione to reduced glutathione, which acts as an antioxidant. The results showed that

the activities of SOD, CAT and GR significantly increased in cultures treated with CCl_4 and ethanol, indicating that the toxicity of these pro-oxidants led to an oxidative stress in the liver tissue and induced the activity of these antioxidant enzymes. When FUP and PRT were added along with these pro-oxidants, the activity of all three enzymes decreased to significant levels. FUP and PRT were showed comparably effective in vitro activity as ascorbic acid in terms of enzymatic assessing hepatoprotection

Conclusion

The results suggest protopine, an isoquinoline alkaloid from F. indica, may be a possible therapeutic for preventing hepatotoxicity in vitro caused by oxidative stress generated due to the pro-oxidants carbon tetrachlorideand ethanol. The hepatoprotective activity may be resultant due to free radical scavenging activity or boosting the antioxidant capacity of the liver.

Acknowledgments

The authors are greatly thankful to the Director and the Incharge, Botany group, Agharkar Research Institute, Pune, India for providing facilities of Animal House and HPTLC instrument. The authors are also thankful to Dr D.G. Naik, ex Scientist, Chemistry Group, Agharkar Research Institute, Pune for kind help in characterization phyto-constitute protopine. The second author is thankful to the Dr Magan P. Ghatule, Principal, Sinhgad College of Science, Pune, India for encouragement throughout the work.

Author details

[1]Sinhgad College of Science,, S.No. 9/1/5 off West. 9/2/4, O Bypass Highway, Ambegaon,, Pune, 411041, Maharashtra, India. [2]Plant Sciences Division, Ex-Scientist, Agharkar Research Institute, 411 004, Pune, Maharashtra, India.

References

[1] Handa SS, Sharma A, Chakraborti KK. Natural products and plants as liver protecting drugs. Fitoterapia. 1986;57:307–351.

[2] Handa SS, Sharma A, Chakraborti KK. Antihepatotoxic activity of some Indian herbal formulations as compared to Silymarin. Fitoterapia. 1991;62:229–229.

[3] Satyavati GV, Raina MK, Sharma M. Medicinal Plants of India. 1976;I.

[4] Lindley JF, Medica. New Delhi, India: Ajay Book Service; 1981.

[5] Kurma SR, Mishra SH. Hepatoprotective activity of the whole plant of Fumaria indica. Indian Journalof Pharmaceutical Science. 1997;59:165–170.

[6] Rao KS, Mishra SH. Antihepatotoxic activity of monomethyl fumarate isolated from Fumaria indica. Journal of Ethnopharmacology. 1998;60:207–213.

[7] Rathi A, Srivastava AK, Shirwaikar A, Rawat AKS, Mehrotra S. Hepatoprotective potential of Fumaria indica Pugsley whole plant extracts, fractions and an isolated alkaloid protopine. Phytomedicine. 2008;15(6-7):470–477. Available from: https://dx.doi.org/10.1016/j.phymed.2007.11.010.

[8] Pandey VB, Dasguta B, Bhattacharya SK, Lal R, Das PK. Chemistry and pharmacology of the major alkaloid of Fumaria indica. Current Science. 1971;17:455–457.

[9] Tian YH, Kim HC, Cui1 JM, Kim YC. Hepatoprotective Constituents of Cudrania tricuspidata. Archives of Pharmacal Research. 2005;28:44–48.

[10] Wormser U, Zakine SB, Stivelband E, Eizen O, Nyska A. The liver slice system: A rapid in vitro acute toxicity test for primary screening of hepatotoxic agents. Toxicology in Vitro. 1990;4(6):783–789. Available from: https://dx.doi.org/10.1016/0887-2333(90)90049-y.

[11] Invittox Protocol No. 42. Liver slice hepatotoxicity screening system. . In: and others, editor. The ERGATT/FRAME Data Bank of in vitro techniques in toxicology. England: INVITTOX; 1992. .

[12] Sinha S, Dixit P, Bhargava S, Devasagayam TPA, Ghaskadbi S. Bark and Fruit Extracts ofGmelina arborea. Protect Liver Cells from Oxidative Stress. Pharmaceutical Biology. 2006;44(4):237–243. Available from: https://dx.doi.org/10.1080/13880200600713667.

[13] Renner K, Amberger A, Konwalinka G, Kofler R, Gnaiger EB. Changes of mitochondrial respiration, mitochondrial content and cell size after induction of apoptosis in leukemia cells. Biochimica et biophysica acta. 2003;1642:115–123.

[14] Wormser U, Ben-Zakine S. The liver slice system: An in vitro acute toxicity test for assessment of hepatotoxins and their antidotes. Toxicology in Vitro. 1990;4(4-5):449–451. Available from: https://dx.doi.org/10.1016/0887-2333(90)90098-e.

[15] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry. 1979;95(2):351–358. Available from: https://dx.doi.org/10.1016/0003-2697(79)90738-3.

[16] Beauchamp C, Fridovich I. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry. 1971;44(1):276–287. Available from: https://dx.doi.org/10.1016/0003-2697(71)90370-8.

[17] Aebi HE. Catalase. In: Bergmeyer HU, et al., editors. Methods of Enzymatic Analysis. vol. 3. Gmbh: Weinhein,

Verlagchemie; 1983. p. 277–282.

[18] Goldberg DM. Glutathione reductase. In: HU B, et al., editors. Methods of Enzymatic Analysis. vol. 3. . Gmbh: Weinhein, Verlagchemie; 1983. p. 277–277.

[19] Bradford MM. A rapid sensitive method for the quantitation of microgram quantities of protein utilising the principle of dye binding. Analytical Biochemistry. 1976;72:248–254.

[20] Frazier JM. In vitro toxicity testing: Application to Safety evaluation. New York: Marcel Dekker, Inc; 1992.

[21] Naik RS, Mujumdar AM, Ghaskadbi S. Protection of liver cells from ethanol cytotoxicity by curcumin in liver slice culture in vitro. Journal of Ethnopharmacology. 2004;95(1):31–37. Available from: https://dx.doi.org/10.1016/j.jep.2004.06.032.