

Biological and Phytochemical Screening of *Fumaria indica* extract on Chemically Induced Hepatocellular Carcinoma with Reference to Biochemical Parameters

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Received: 19th Jan, 19; Revised: 28th Feb, 19; Accepted: 20th Mar, 19; Available Online: 25th Jun, 2019

ABSTRACT

Liver disease or liver cancer is the sixth most common cancer and the third leading cause of cancer mortality in the world. Hepatitis viral infection, food additives, alcohol, fungal toxins (aflatoxins), toxic industrial chemicals, air and water pollutants are the major risk factors of liver cancer. Moreover, due to high tolerance of liver, HCC is seldom detected at an early stage and once detected treatment faces a poor prognosis in most cases. *Fumaria indica* possesses hepatoprotective activity as evidenced by the significant and dose dependent restoring the activities of entire liver cancer marker enzymes, diminution in tumor incidence, decrease in lipid peroxidation (LPO) and increase in the level of antioxidant enzymes (GSH, CAT, SOD, GPx and GST) through scavenging of free radicals, or by enhancing the activity of antioxidant, which then detoxify free radicals. These factors protect cells from ROS damage in NDEA and CCl₄-induced hepatocarcinogenesis. Histopathological observations of liver tissues too correlated with the biochemical observations. Thus, present investigation suggested that the *Fumaria indica* would exert a chemoprotective effect by reversing the oxidant-antioxidant imbalance during hepatocarcinogenesis induced by NDEA and CCl₄. Besides *Fumaria indica* is very much effective in preventing NDEA-induced multistage hepatocarcinogenesis possibly through antioxidant and antigenotoxic nature, which was confirmed by various liver injury and biochemical tumour markers enzymes. The hepatoprotective activity of a *Fumaria indica* of 50 % ethanolic extract was studied using rats. The animals received a single intraperitoneal injection of N-nitrosodiethylamine 200mg/kg body wt followed by subcutaneous injection of CCl₄ in a dose of 3 ml/kg body wt. *Fumaria indica* extract dose dependently and significantly the increase in serum hepatic enzyme levels after NDEA & CCl₄ treatment compared to the toxin control group. The results of this study confirmed the antioxidant and hepatoprotective activity of the *Fumaria indica* extract against carbon tetrachloride & N-nitrosodiethylamine induced hepatotoxicity in rats. In addition to this, studies on molecular aspect of hepatoprotective therapy will give mechanistic information in hepatoprotective therapy and also critical balance should be there between the animal model and clinical research. The hepatoprotective properties of *Fumaria indica* should provide useful information in the possible application in hepatic liver disease.

Keywords: Hepatocarcinogenesis, Antigenotoxic, Hepatoprotective, Carbon tetrachloride, N-nitrosodiethylamine, Hepatocellular carcinoma

INTRODUCTION

Herbal drugs are well known to play an essential role in the treatment of various hepatic disorders. *Fumaria indica* is one such herb widely used in many traditionally known medical systems commonly practiced in India and elsewhere. It is commonly known as Parpata/Suksmapatra in Sanskrit, Pitpapra or "Shahtrah" in Hindi "fumitory, earth smoke, beggary, fumus, vapor, fumittery or wax dolls" in English (Orhan et al., 2010). *Fumaria indica* (Hausskn.) Pugsley (Fumariaceae) is a small, scandent, branched, annual herb growing wild in plains and lower hills (Chopra et al., 2002). Traditionally, the plant has been used as anthelmintic, anti-dyspeptic,

anti-periodic, blood purifier, cholagogue, diuretic, diaphoretic, laxative, sedative, stomachic, tonic (Usmanghani et al., 1997), and has also been considered to be useful for treatment of abdominal cramps (Duke et al., 2002), diarrhea, fever (Haq & Hussain 1993), jaundice, leprosy and syphilis (Nadkarni et al., 1976), blood disorders and tuberculosis (Singh et al., 2010). Hepatocellular carcinoma (HCC) or liver cancer is the sixth most common cancer and the third leading cause of cancer mortality in the world (Ferlay et al., 2010). The burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as increasingly an adoption of cancer-

Table 1: Procedure for SGOT substrate.

Tube No.	Sodium pyruvate (ml)	Substrate (ml)	Water (ml)	SGOT units
1	0.5	0.5	0.2	0
2	0.4	0.6	0.2	22
3	0.3	0.7	0.2	55
4	0.2	0.8	0.2	95
5	0.1	0.9	0.2	150
6	0.0	1.0	0.2	215
Solutions	Test (ml)	Control (ml)	Blank (ml)	Standard (ml)
Substrate	1.0	1.0	1.0	1.0
Keep for 5 min in boiling water bath at 37°C				
Serum	0.2	-	-	-
Incubate at 37°C for 60 min				
Sodium pyruvate	-	-	-	0.2
Dinitrophenyl hydrazine	1.0	1.0	1.0	1.0
Serum	-	0.2	-	-
Allow to stand for 20 min at room temperature				
Sodium hydroxide	10.0	10.0	10.0	10.0

Table 2: Preparation of working solutions.

Tube No.		1	2	3	4	5
Enzyme activity (units/ml)		0	28	57	97	100
Reagent	1: Buffered alanine, pH 7.4	0.5	0.45	0.4	0.35	0.3
	MI	-	0.05	0.1	0.15	0.2
Reagent	4: Working pyruvate standard, 2mM	0.1	0.1	0.1	0.1	0.1
	MI	0.5	0.5	0.5	0.5	0.5
Purified	Water					
	MI					
Reagent	2: DNPH colour reagent					
	MI					
Mix well and allow to stand at room temperature for 20 min						
Solution I	MI	5.0	5.0	5.0	5.0	5.0

Table 3: test procedure reagents.

Reagent 1: Buffered alanine, pH 7.4	0.25 ml
Incubate at 37°C for 5 min	
Serum	0.05 ml
Mix well and incubate at 37°C for 30 min	
Reagent 2: DNPH colour reagent	0.25 ml
Mix well and allow to stand at room temperature for 20 min	
Solution I	2.5 ml

associated lifestyle choices including smoking, physical inactivity, and “westernized” diets. Liver cancer in men/women is the fifth/seventh most frequently diagnosed cancer worldwide but the second/sixth most frequent cause of cancer death. An estimated 748,300 new liver cancer cases and 695,900 cancer deaths occurred worldwide in 2008 (Jemal et al., 2011). Hepatitis viral infection, food additives, alcohol, fungal toxins (aflatoxins), toxic industrial chemicals, air and water pollutants are the major risk factors of liver cancer (Paraskevi and Ronald, 2006). Human liver is the major site in the body that metabolizes ingested material. Moreover, due to high tolerance of liver, HCC is seldom

detected at an early stage and once detected treatment faces a poor prognosis in most cases (Singh et al., 2009).

Review of plant

Fumaria indica

Botanical name: *Fumaria indica* Pugsely

Family: Fumariaceae

Part Used: Whole Plant

Chemical constituents

The whole plant contains Benzyl isoquinoline alkaloid - fumarizine, pthalide isoquinoline alkaloid - papraine, secopthalide isoquinoline alkaloid - papracine, paprafumine, paprarine and papraline, alkaloid - oxyhydrastinine, noroxyhydrastinine, fumaramine, stylophine, fumaritine, cryptopine, raddeanine and oxocoptisine, spirobeanzyl isoquinoline alkaloids - papracinine and paprazine [Rastogi et al., (1970-79); Chopra et al., (1985); Kakkar et al., (1965-1981); Atta-ur-Rahman et al., (1992 and 1995)].

Other parts of the plant show:

Aerial parts: Papracine, paprazine, sitosterol, stigmasterol, campesterol.

Root: Protopine, octacosanol, alkaloid-narcumine, narlumidine, adlumidine, orsanguinartine.

Table 4: Determination of serum alkaline phosphatase.

	Blank	Standard	Control	Test
Working buffered substrate	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Distilled Water	1.5 ml	1.5 ml	1.5 ml	1.5 ml
Mix well and incubate for 3 min at 37° C				
Serum	-	-	-	0.05 ml
Phenol standard, 10 mg%	-	0.05 ml	-	-
Mix well and incubate for 15 min at 37° C				
Chromogen reagent	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Serum	-	-	0.05 ml	-

Table 5: Reagents for total protein assay.

	Blank (B)	Standard (S)	Test (T)
Biuret Reagent	1 ml	1 ml	1 ml
Distilled water	2 ml	2 ml	2 ml
Protein Standard	-	0.5 ml	-
Serum	-	-	0.05 ml

Table 6: Reagents for albumin assay.

	Blank (B)	Standard (S)	Test (T)
Biuret Reagent	1 ml	1 ml	1 ml
Distilled water	2 ml	2 ml	2 ml
Protein Standard	-	0.01 ml	-
Serum	-	-	0.01 ml

Table 7: Reagents for total cholesterol.

	Blank (B)	Standard (S)	Test (T)
Cholesterol Reagent 1	5.0 ml	5.0 ml	5.0 ml
Distilled Water	0.025 ml	-	-
Standard (3)	-	0.025 ml	-
Serum/ Plasma	-	-	-

Table 8: Reagents for glucose estimation.

	Blank	Standard	Test
Serum	-	-	20 uL
Glucose standard	-	20 uL	-
Working glucose standard	1.5 ml	1.5 ml	1.5 ml
Mixed well and incubated at 37° C for 10 min			
Deionised water	1.5 ml	1.5 ml	1.5 ml

Leaves & stem: Narlumicine, protopine, narlumidine, non-acosanol, alcohol, n-alkanes.

Seed: Fumariline, dihydrocoptisine, tetrahydro coptisine, bicuculine, oxysanguinarine, narceimicine

Medicinal Properties & Uses

The plant is reported to be diuretic, diaphoretic and laxative. It is used in fever and influenza. It is also used to purify blood and in obstruction of liver. The plant is used as one of the component of many Ayurvedic, Unani and liver formulations (Rao and Mishra, 1998). The plant has reputation as anthelmintic, anti-dyspeptic, cholagogue, stomachic, sedative and tonic along with it is also considered useful to treat abdominal cramps, diarrhoea, fever, jaundice, leprosy and syphilis [John et al., (1981); Anonymous et al., (1956); Haq et al., (1993); and Nadkarni et al., (1976)].

Chemical Review

Pandey et al., (1971) isolated seven isoquinolone alkaloid such as protopine, tetrahydro coptisine, fumariline, bicuculine, narlumidine, fumarilicine and narceimine from the alcoholic extract of whole plant of *Fumaria indica*.

Satish and Bhakuni, (1972) isolated protopine, quaternary salt of protopine, nonacosanol and sitosterol from the stem and leaves of *Fumaria indica*.

Pandey et al., (1974) reported that protopine content of the seeds is about double that of whole plant, the yield of tetrahydro coptisine is 50 times more in seeds than in whole plant.

Pandey et al., (1979) isolated three alkaloids fumariline, 8 - methoxy dihydro sanguinarine and oxysanguinarine from *Fumaria indica*.

Tripathi et al., (1988) isolated seco-phalidi isoquinoline alkaloid narceimine from *Fumaria indica* seeds.

Pharmacological Review

Pandey et al., (1971) reported smooth muscles relaxant and hydrocholeretic effect of *Fumaria indica* is due to protopine which is present as major alkaloid in the plant. As a smooth muscles relaxant protopine was found to be slightly weaker than papaverine.

Rao and Mishra, (1997) reported that whole plant of *Fumaria indica* has hepatoprotective activities against carbontetrachloride, paracetamol and rifampicin-induced hepatotoxicities in albino rats. The petroleum ether extract against carbontetrachloride, total aqueous extract against paracetamol and methanolic extract against rifampicin - induced hepatotoxicities showed similar reductions in the elevated levels of some of the serum biochemical parameters in a manner similar to that of silymarin indicating its potential as a hepatoprotective agent.

Rao and Mishra, (1998) isolated monomethyl fumarate from methanolic extract of the whole plant of *Fumaria indica*, it was characterized and screened for its

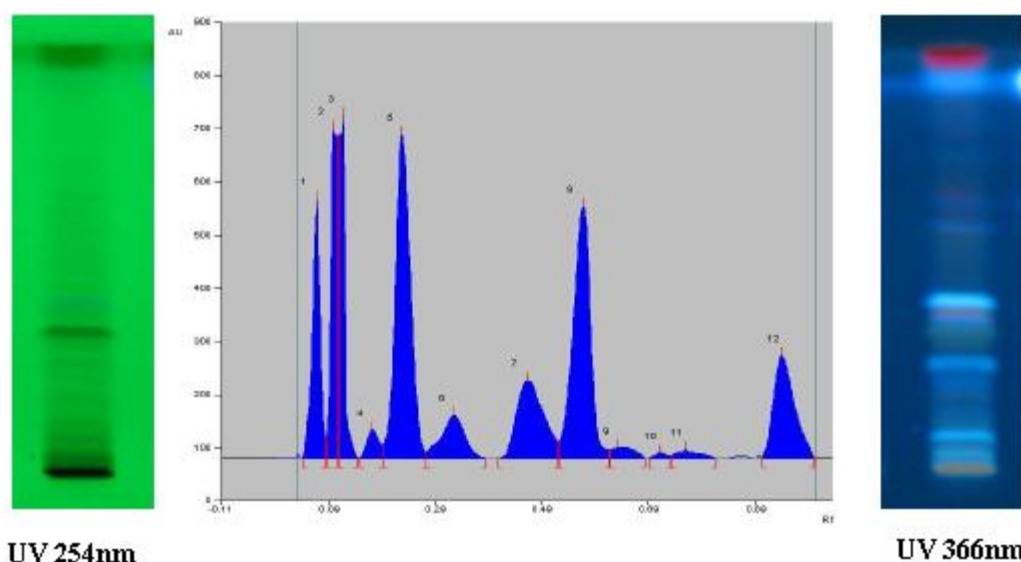


Figure: HPTLC finger print profile and densitometric scanning of 50% ethanolic extract of *Fumaria indica* whole plant at 254 nm. Solvent system: Methanol: chloroform (80: 20)

Preparation of 50% EtOH extract of *Fumaria indica*

S.No	Botanical Name	Family	Part Used	Solvent used for extracti on	% yield (Extr act)
1	<i>Fumaria indica</i>	Fumariaceae	Whole Plant	50% EtOH	9.85 %

Table: Rf values and the relative percentages of each spot in HPTLC.

Peaks	Rf	% Area in spot
1	0.04	9.68
2	0.08	9.62
3	0.10	10.21
4	0.15	1.61
5	0.19	22.56
6	0.27	4.59
7	0.40	9.60
8	0.52	20.02
9	0.61	1.15
10	0.69	0.28
11	0.73	0.78
12	0.90	9.88

antihepatotoxic activity in albino rats. The compounds showed significant antihepatotoxic activity against thioacetamide in vitro, and against hepatotoxicities induced by carbon tetrachloride, paracetamol and rifampicine in vivo to an extent almost similar to that of silymarine, a known antihepatotoxic agent.

Nimbkar and Juvekar, (2000) studied on hepatoprotective activity against anti-tubercular drug induced hepatotoxicity in Albino rats. Ethanolic extract of *Fumaria indica* showed normalization of biochemical parameters alanine and aspartate aminotransferase, alkaline phosphatase. Cholesterol levels were found to be slightly raised. Plasma levels of rifampicin were found to drop in *Fumaria indica* treated group as compared to anti-tubercular drug treated group.

Gilani et al., (2005) reported that crude extract of *Fumaria indica* whole plant (Fi.Cr) & its fractions were studied in vitro for spasmogenic & spasmolytic effects to rationalize some of the traditional uses. Fi.Cr (1.0- 5.0 mg/ml.) caused a moderate degree of atropine sensitive spasmogenic effect in guinea pig ileum. These findings indicate that the presence of cholinergic and CCB (calcium channel blocked) constituents in Fi.Cr may explain the respective traditional use of the *Fumaria indica* in constipation and diarrhoea.

MATERIALS AND METHODS

Collection and identification of the plant materials

The whole plant of *Fumaria indica* Pugsley (Fumariaceae) were collected from Botanical Garden of N.B.R.I. (National Botanical Research Institute), Lucknow, India. The plant material was identified and authenticated taxonomically by Dr. Priyanka Agnihotri, Scientist, Plant Diversity, Systematics & Herbarium Division, National Botanical Research Institute (CSIR), Lucknow, India. The herbarium, (LWG-81), was preserved at the department for future reference.

Animals Used

Wistar albino rats (150-170 g) and Swiss albino mice (25-30 g) were kept in the departmental animal house of Azad Institute of Pharmacy and Research, Lucknow, India. The animals were housed separately in polypropylene cage at temperature of $23 \pm 2^\circ\text{C}$ and 50-60% relative humidity, with a 12 h light/dark cycle respectively, for one week before and during the commencement of experiment. Animals were allowed to access standard rodent pellet diet (Dayal animal feed, Barabankhi, U.P., India) and drinking water. Food was withdrawn 18-24 h before the experiment, though water was allowed *ad libitum* and allocated to different experimental groups. All experimental procedures involving animals were conducted in accordance with the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). The study protocols

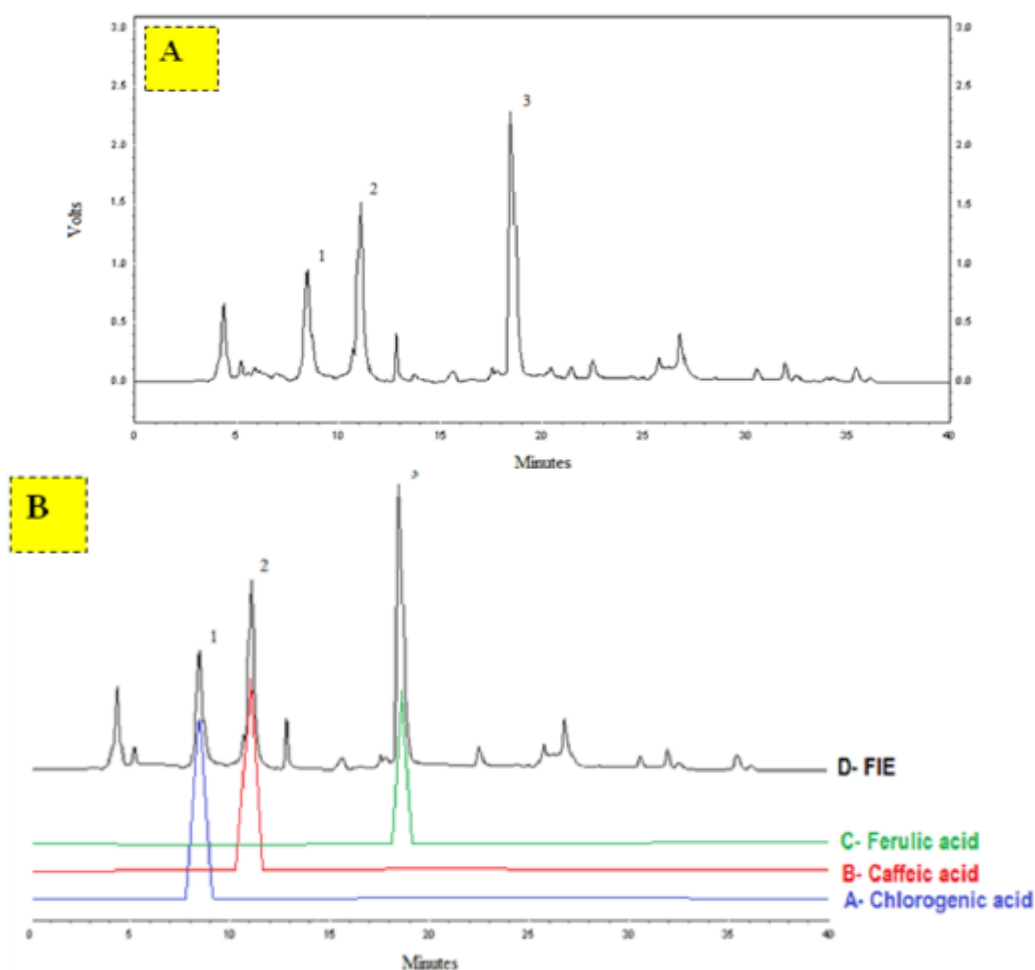


Figure: A- HPLC chromatograms of *Fumaria indica* extract (FIE).

B - Chromatographic overlay and identification of compounds were obtained by comparison of peak (254 nm) of the sample with that of standards, chlorogenic acid (1, Rt = 8.49), caffeic acid (2, Rt = 11.17) and ferulic acid (3, Rt = 18.53).

Table: Effect of 50% ethanolic extract of *Fumaria indica* on body weight of rats in subacute toxicity

Treatment/ Dose	Body weight (g)				
	Initial	1 week	2 week	3 week	4 week
Control	148.7±11.7	157.5±11.8	166.2±12.3	173.4±13.3	182.8±14.8
<i>Fumaria indica</i> 200 mg/kg	154.1±13.1	162.2±13.2	171.4±13.5	179.1±14.2	189.5±15.7
<i>Fumaria indica</i> 400 mg/kg	149.5±11.6	156.9±12.0	165.8±12.6	173.1±13.4	183.4±14.5

Values are expressed as Mean \pm SEM of 10 rats in each group

The increase in body weight of extract treated groups are not significantly different from control group at the end of study (28 days).

were approved by Institutional Animal Ethics Committee (IAEC) of Azad Institute of Pharmacy and Research, Lucknow, India (Reg. No. 1146/PO/ERe/08/CPCSEA).

Phytochemical studies

Preparation of 50% EtOH extract of *Fumaria indica*

The freshly collected plant materials of *Fumaria indica* (4 kg) were washed with tap water to remove dirt and were shade dried. The dried materials were powdered and passed through a 10-mesh sieve. The coarse powdered materials (1 kg) were extracted with petroleum ether thrice to remove the fatty material and further marcs were exhaustively macerated thrice with ethanol (50%, v/v) by

cold percolation method at room temperature. The extracts were separated by filtration and concentrated at $40 \pm 1^\circ\text{C}$ on rotavapour (Buchi, USA) and then dried in lyophilizer (Labconco, USA) under reduced pressure. The yield for *Fumaria indica* extract (FIE) 9.85% (w/w). The extract obtained was subjected to preliminary phytochemical analysis, toxicological and pharmacological investigations.

Preliminary phytochemical screening

Ethanolic extract (50%) of *Fumaria indica* was subjected to qualitative tests for the identification of various active constituents viz. carbohydrate, glycoside, alkaloid, amino

Table: Effect of *Fumaria indica* extract on urine analysis of rats in subacute toxicity.

Treatment/ Dose	Volume	SP, gravity	Protein	Glucose	Ketone	Blood
Control	4.97±0.76	1.015±0.005	NIL	NIL	NIL	NIL
<i>Fumaria indica</i> 200 mg/kg	5.25±0.86	1.014±0.003	NIL	NIL	NIL	NIL
<i>Fumaria indica</i> 400 mg/kg	5.13±0.47	1.016±0.006	NIL	NIL	NIL	NIL

Values are expressed as Mean SEM of 10 rats in each group

The values observed in urine parameters in extract treated groups are not significantly different from control group at the end of study (28 days).

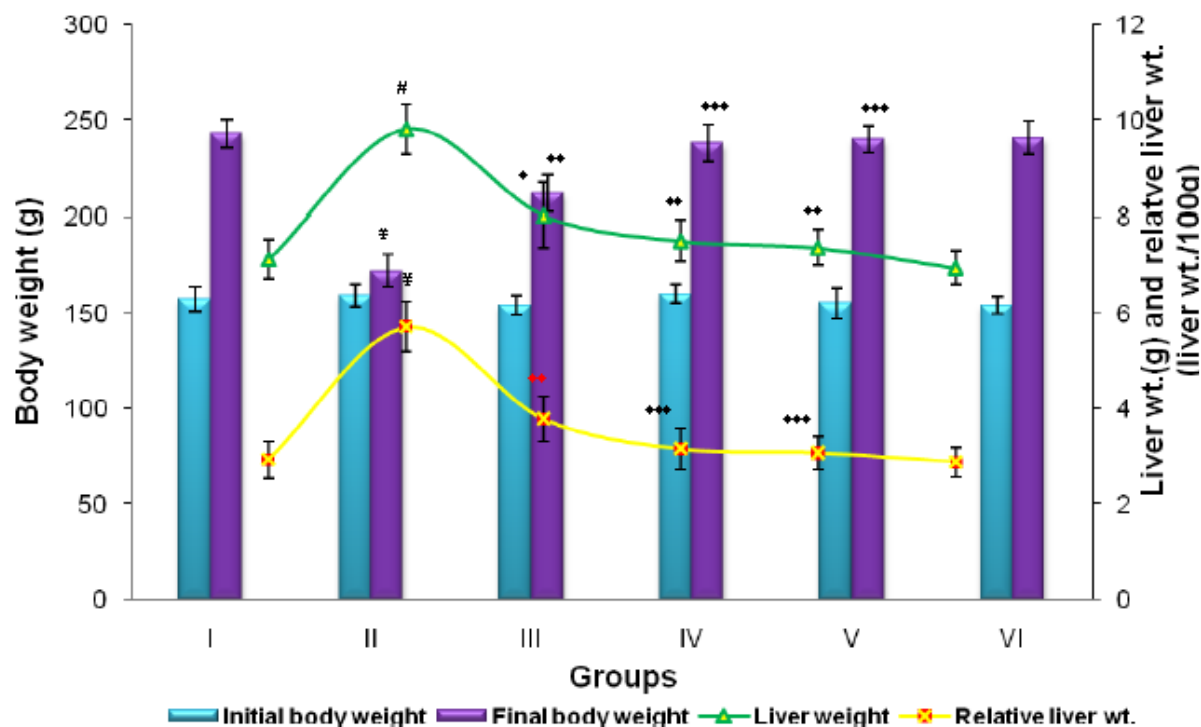


Figure: Effect of *Fumaria indica* extract on body weight, liver weight and relative liver weight of control and NDEA induced HCC in rats. Values are expressed as mean \pm SEM of 6 rats in each group. P values : # < 0.01 ¥ < 0.001 compared with respective control group I.P values : ♦ < 0.05, ♦♦ < 0.01 and ♦♦♦ < 0.001 compared with group II (NDEA + CC14).

acids, flavanoids, fixed oil, tannins, gum and mucilage, phytosterols etc. according to Khandelwal, (2008).

Preliminary phytochemical fingerprint (HPTLC) analysis of 50% ethanolic extracts of Fumaria indica.

Quantification and Documentation

The plates were visualized under UV 254 and Visible and Video documented Desaga video documentation unit. The plates were scanned densitometrically using CAMAG TLC scanner 3. CAMAG TLC scanner 3 was used for the quantification. Densitometry is *in situ* instrumental measurement of visible; the scanner converts the band on the layer in to a chromatogram consisting of peaks similar in appearance to HPLC. The portion of the scanned peaks on the recorder chart is related to Rf values of the spots on the layer and the peak height or area is related to the concentration of the substance on the spot.

Procedure

Finely powdered drug (5 g) was refluxed with 25 ml of methanol on a water bath for 25 min consecutively three times, filtered and the solvent was removed under reduced pressure. Extract (25 mg) was dissolved in 20 ml of methanol and 10 ml of different extracts was applied on pre-coated Silica gel 60 F254 plates with 0.2mm thickness (Merck) using automatic applicator (CAMAG Linomat IV). The plates were then run using different solvent system such as chloroform : methanol (80:20) for *Fumaria indica* in a CAMAG twin trough chamber up to a distance of about 9 cm, dry it and scan.

Visualization and scanning

The plates were visualized under UV 254 and visible. If required spray the plate with anisaldehyde -sulfuric acid and heat at 110 °C for 10 min. The Rf values were recorded and color of the resolved bands and video documented using Desaga video documentation unit. The plates were scanned densitometrically using CAMAG

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Values are expressed as Mean SEM of 10 rats in each group. The values observed in urine parameters in extract treated groups are not significantly different from control group at the end of study (28 days).

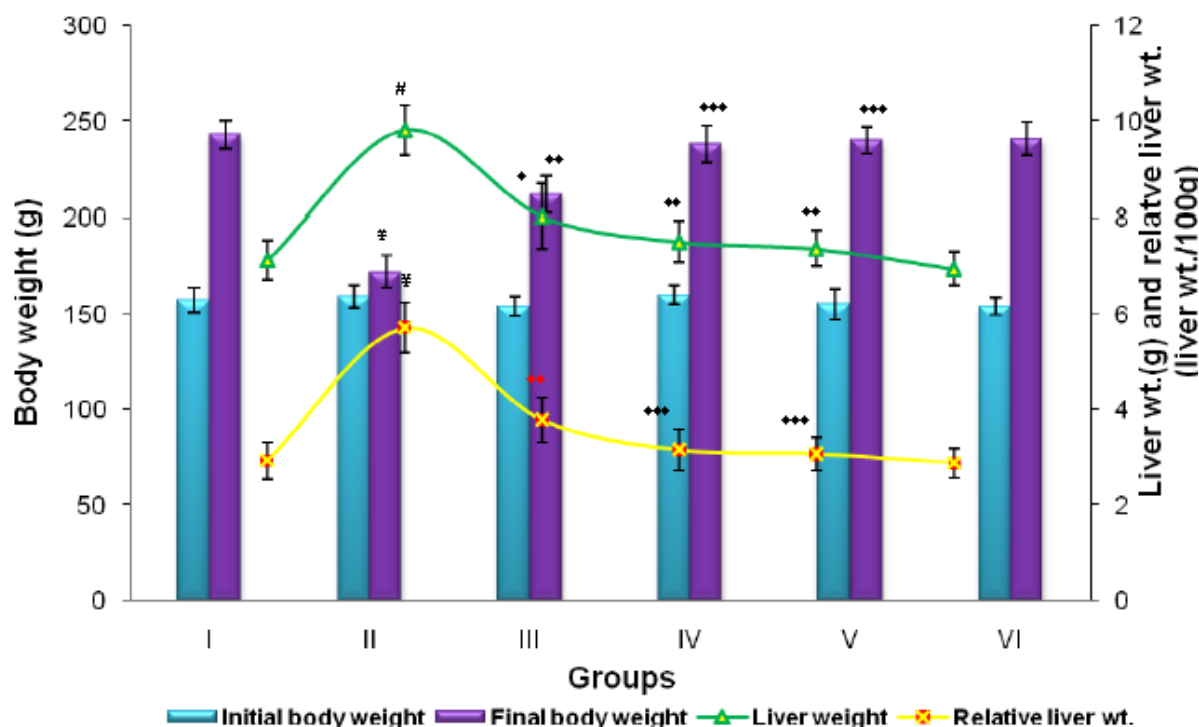


Figure: Effect of *Fumaria indica* extract on body weight, liver weight and relative liver weight of control and NDEA induced HCC in rats. Values are expressed as mean \pm SEM of 6 rats in each group. P values : # < 0.01 ¥ < 0.001 compared with respective control group I. P values : ♦ < 0.05, ♦♦ < 0.01 and ♦♦♦ < 0.001 compared with group II (NDEA + CC14).

TLC scanner at 254 nm wavelength.

HPLC analysis of *Fumaria indica* extract

Sample preparation

For the HPLC analysis, *Fumaria indica* extract was dissolved in HPLC grade MeOH (1mg/ml) and 10 μ L of it was injected into the HPLC apparatus.

Chromatographic conditions

Separation was achieved with a two pump linear gradient program for pump A (Water containing 1% acetic acid) and pump B (Acetonitrile). Initially started with a gradient of 18% B changing to 32% in 15.0 min and finally to 50% in 40 min. The flow rate was 1.0 ml/min. The standard compounds such as chlorogenic acid, caffeic acid and ferulic acid were run for comparative detection and optimization. All the samples were injected in triplicate and results (mg/g dry wt.) were obtained by comparison of peak areas (254 nm) of the samples with that of standards.

Toxicological studies

Acute toxicity study

Acute oral toxicity of the 50% aqueous ethanolic extract of *Fumaria indica* was evaluated in Swiss albino mice of either sex (25–30 g), as per OECD guideline (Organization for Economic Co-operation and Development, Guideline-423, adopted on 17th December, 2001) with slight modifications. Twenty four animals were equally divided into four groups (n = 6) as per sex for each plant extract. The extract was administered in 0.3% carboxy-methyl cellulose (CMC) suspension at doses of 50, 300 and 2000 mg/kg, whereas the control group received the CMC suspension only. Food or water was withheld for a further 1-2 hours after drug administration. Mice were observed for the initial 4h after the administrations, and then once daily during the following days. The behavioural changes observed for were: hyperactivity, ataxia, tremors, convulsions,

salivation, diarrhoea, lethargy, sleep and coma. Total observation period for eventual mortality was 14 days. One-tenth and one-fifth of the maximum tolerated dose of

the extract (2000 mg/kg) tested for acute toxicity was selected for the subacute toxicity study i.e., 200 and 400

Table: Effect of *Fumaria indica* extract on the development of nodules in the liver of control and NDEA induced HCC in rats.

Groups	Treatment	No of rats with nodules/ total no of animals	Nodules incidence (%)	Total no of nodules	Average no of nodules/ nodules bearing liver
I	Control	0/6	0.00	00	00
II	NDEA (200 mg/kg) + CCl ₄ (3 ml/kg)	6/6	100	43	7.2
III	FIE (200 mg/kg) + NDEA + CCl ₄	4/6	66.67	27	6.8
IV	FIE (400 mg/kg) + NDEA + CCl ₄	2/6	33.33	13	6.5
V	Silymarin (200 mg/kg) + NDEA + CCl ₄	2/6	33.33	09	4.5
VI	FIE alone (400 mg/kg)	0/6	0.00	00	00

Values are expressed as mean of 6 rats in each group

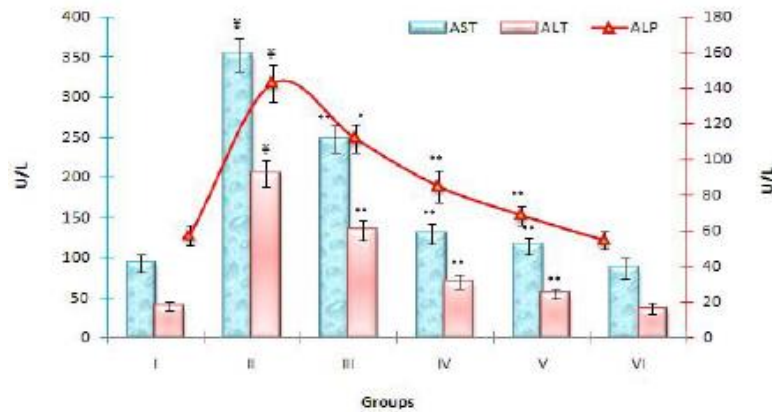


Figure: Effect of *Fumaria indica* extract on the activities of aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) in the serum of control and NDEA induced HCC in rats. Values are expressed as mean ± SEM of 6 rats in each group. P values : $\Psi < 0.001$ compared with respective control group I. P values : * < 0.01 , ** < 0.001 compared with group II (NDEA + CCl₄)

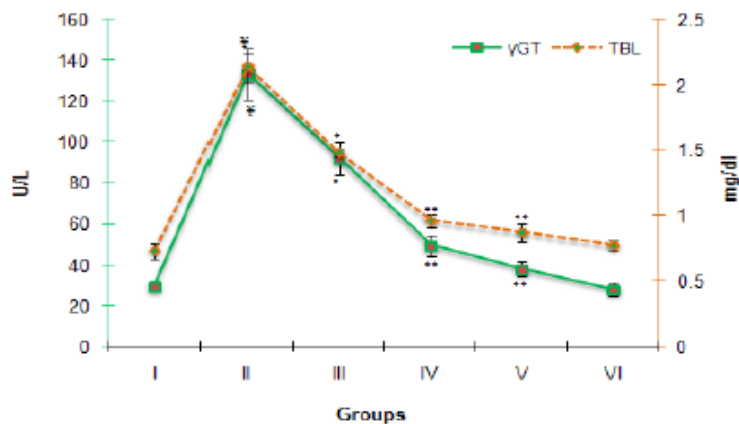


Figure: Effect of *Fumaria indica* extract on the activities of γ -glutamyl transferase (γ -GT) and Total bilirubin level (TBL) in the serum of control and NDEA induced HCC in rats. Values are expressed as mean ± SEM of 6 rats in each group. P values : $\Psi < 0.001$ compared with respective control group I. P values : * < 0.01 , ** < 0.001 compared with group II (NDEA + CCl₄).

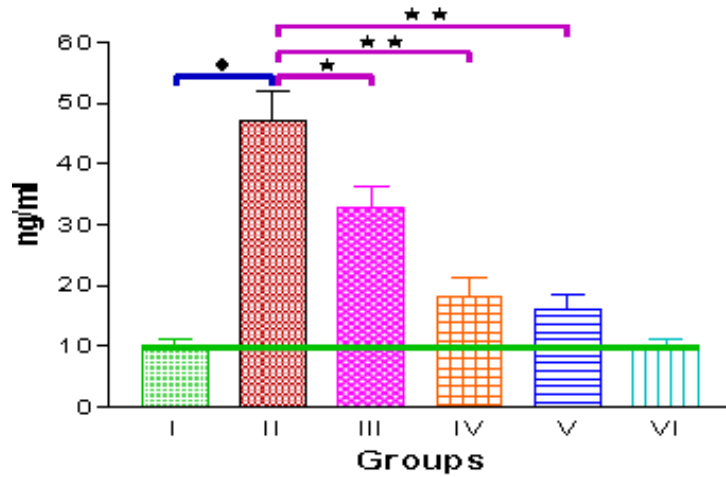


Figure: Effect of *Fumaria indica* extract on the levels of α -feto protein (AFP) in the serum of control and NDEA induced HCC in rats. Values are expressed as mean \pm SEM of 6 rats in each group. P values: <0.001 compared with respective control group I. P values: <0.01 and <0.001 compared with group II (NDEA + CCl₄).

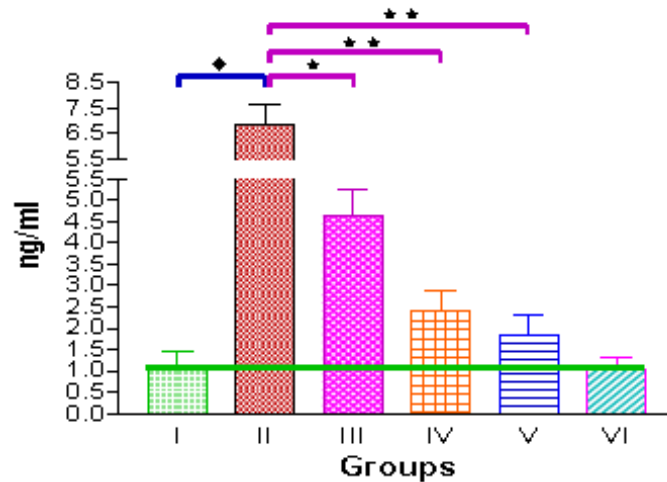


Figure: Effect of *Fumaria indica* extract on the levels of carcinoembryonic antigen (CEA) in the serum of control and NDEA induced HCC in rats. Values are expressed as mean \pm SEM of 6 rats in each group. P values: <0.001 compared with respective control group I. P values: <0.05 and <0.001 compared with group II (NDEA + CCl₄).

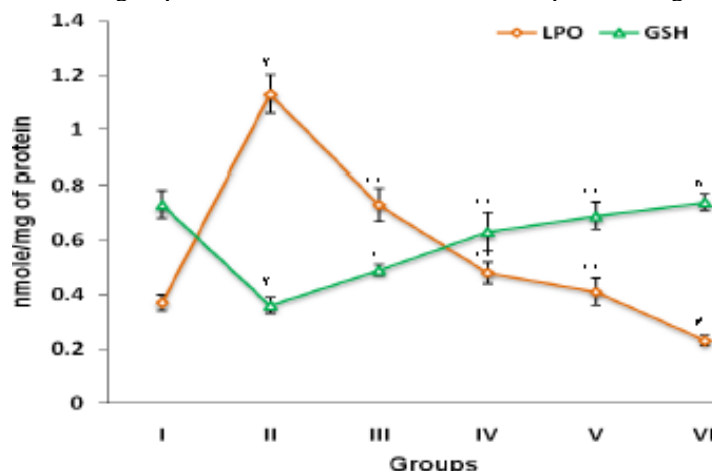


Figure: Effect of *Fumaria indica* extract on the levels of lipid peroxide and glutathione in the PMS of control and NDEA induced HCC in rats. Values are expressed as mean \pm SEM of 6 rats in each group. P values: $\text{¥}<0.001$ compared with respective control group I. P values: <0.05 and <0.001 compared with group II (NDEA + CCl₄). P values: $\text{\#}<0.05$ compared with respective control group I. n: non significant when compared to respective control group I.

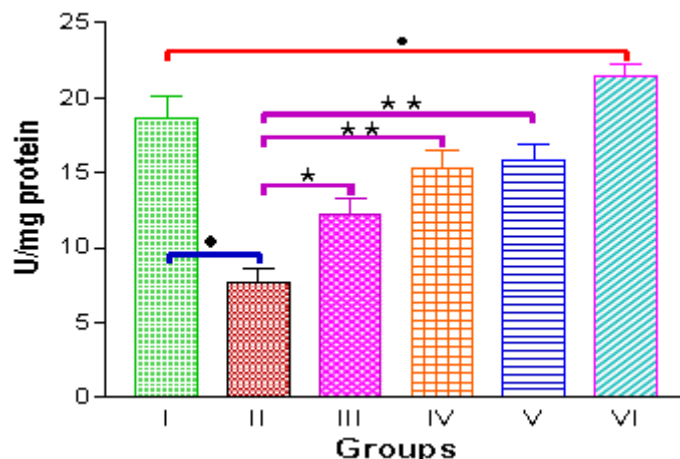


Figure:Effect of FIE on the antioxidant activities of SOD (50% inhibition of chromogen/min/mg protein) in the PMS of control and NDEA induced HCC in rats. Values are expressed as mean \pm SEM of 6 rats in each group. P values: <0.001 compared with respective control group I. P values: <0.01 and <0.001 compared with group II (NDEA + CCl₄). P values: •<0.05 compared with respective control group I.

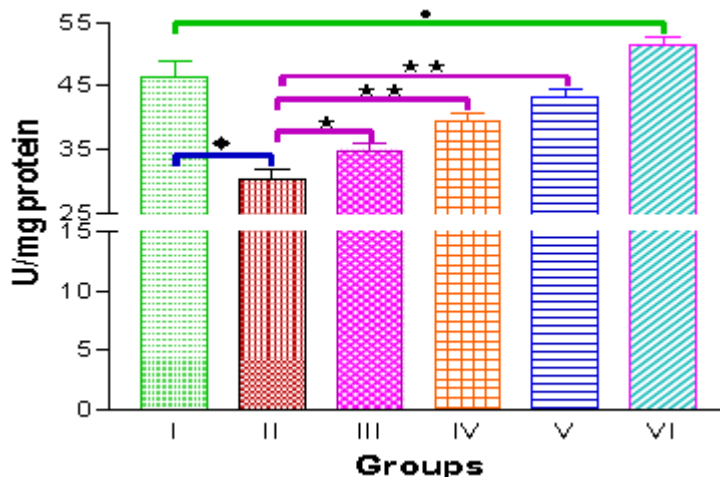


Figure:Effect of FIE on the antioxidant activities of CAT (μ mol H₂O₂ consumed/min/mg protein) in the PMS of control and NDEA induced HCC in rats. P values: <0.001 compared with respective control group I. P values: <0.05 and <0.001 compared with group II (NDEA + CCl₄). P values: •<0.05 compared with respective control group I.

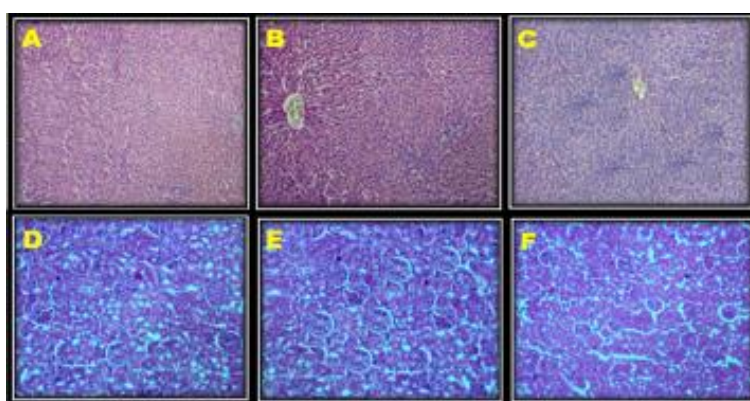


Figure: Histopathological picture of liver and kidney of control and experimental group of animals. (A) The section of liver from control animals revealed normal architecture and hepatic cells with granulated cytoplasm, (B) and (C) The section of liver from FIE 200 and 400 mg/kg exhibited normal architecture of hepatocytes indicating the safe nature of the extract. (D) The section of kidney from control animals showed normal size of glomeruli with normal tubules, (E) and (F) The section of kidney from FIE 200 and 400 mg/kg exhibited within the limits of normalcy.

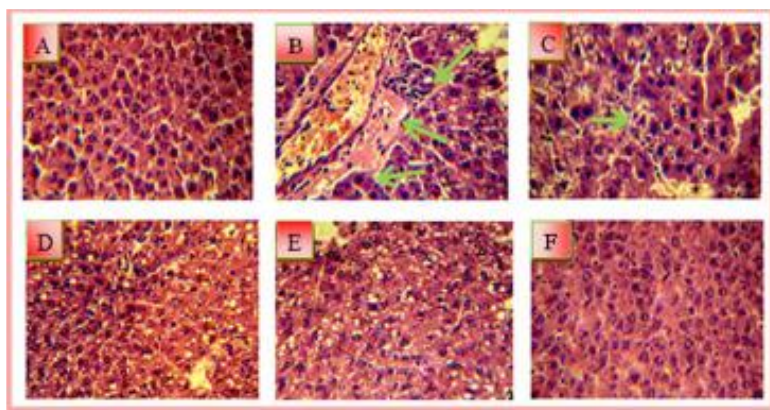


Figure: Histological study of liver tissue in control and experimental groups of rats. (A) Group I: Control animals revealed normal architecture (B) Group II: NDEA + CCl₄ induced carcinoma bearing animal showing neoplastic hepatocyte with inflammatory collection and loss of architecture. Neoplastic cells were larger than normal cells with larger hyperchromatic nuclei and hyaline globules (arrow) that represent proteins produced by the tumor cells (C) Group III: Administration of 50% ethanolic extract of FIE (200 mg/kg) on HCC bearing animal hepatocyte showing minimal inflammatory cell with few malignant hepatocytes. (D) Group IV: Administration of 50% ethanolic extract of FIE (400 mg/kg) on HCC bearing animals exhibited hepatocyte maintaining near normal architecture. (E) Group V: Administration of silymarin (200 mg/kg) on HCC bearing animals shows the structure close to proximity of normal hepatocytes. (F) Group VI: Administration of 50% ethanolic extract of FIE (400 mg/kg) alone exhibited normal architecture of hepatocytes indicating the non-toxic nature of the extract.

mg/kg.

Bio-chemical parameters investigated

Serum was analyzed for the following parameters as aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphate (ALP), total cholesterol (TC), total protein (TP) and albumin (ALB) glucose, creatinine, blood urea nitrogen (BUN), total bilirubin (TB).

Determination of Serum glutamic oxaloacetic transaminase (SGOT)/

aspartate transaminase (AST)

The SGOT activity was determined according to the method of Reitman and Frankel, (1957) method using SGOT.

Reagents

Phosphate buffer – pH 7.4, standard pyruvate (2 mM), α -keto glutarate-aspartic acid substrate for SGOT, 2,4-dinitrophenyl hydrazine, and 0.4 M sodium hydroxide.

Procedure

Substrate for SGOT: Place 29.2 mg of α -keto glutarate and 2.66 gm of aspartate in a beaker. Add 1M sodium hydroxide until it is completely dissolved. Adjust the pH to 7.4 with sodium hydroxide. Transfer quantitatively to 100 ml volumetric flask with phosphate buffer and dilute to the mark with buffer. Prepared a standard curve by set up a number of tubes as shown in the following table and added 1.0 ml of dinitrophenyl hydrazine solution to each tube.

Mix well and after 10 min. read the optical density (O.D) in a spectro photometer at 520 nm using green filter.

Plot the results against the concentration units for SGOT and connect the points by a smooth curve.

After 10 min. read the O.D. of test, control and standard against reagent blank at 520 nm using green filter. Mark the O.D. of test on the Y-axis of the standard curve and extrapolate it to the corresponding enzyme activity on X-axis.

Serum glutamic oxaloacetic transaminase was expressed as U/l.

Determination of Serum glutamate pyruvate transaminase (SGPT)/ alanine transaminase (ALT)

The SGPT level was estimated by Reitman and Frankel, (1957) method using SGPT.

Reagents

Reagent 1: Buffered alanine α -KG substrate, pH 7.4

Reagent 2: DNPH colour reagent

Reagent 3: Sodium hydroxide 4 N

Reagent 4: Working pyruvate standard, 2mM

Preparation of working solutions

Solution I: Dilute 1 ml of reagent 3 to 10 ml with purified water.

Procedure

Mix well by inversion. Allow to stand at room temperature for 10 min. and measure the O.D. of all the five tubes against purified water on a colorimeter using a green filter.

Test procedure

Mix well and allow to stand at room temperature for 10 min and observe the O.D against purified water on a colorimeter using a green filter. Mark the O.D. of test on the Y-axis of the standard curve and extrapolate it to the corresponding enzyme activity on X-axis.

Serum glutamate pyruvate transaminase (SGPT) was expressed as U/l

Determination of serum alkaline phosphatase (SALP)/ Alkaline phosphatase (ALP)

The alkaline phosphates level was estimated by King, (1965) method alkaline phosphatase.

Reagents

Reagents 1: Buffered substrate, pH 10.0

Reagents 2: Chromogen reagent

Reagent 3: Phenol standard, 10 mg%

Preparation of working solution

Reconstitute one vial of reagent 1, buffered substrate with 4.5 ml of distilled water.

Procedure

Mix well after the addition of each reagent and measure the O.D. of blank, test, standard and control at 510 nm against reagent blank.

Serum alkaline phosphatase (KA units): $O.D \text{ of test} - O.D \text{ of control} / O.D \text{ of standard} - O.D \text{ of blank} \times 10$

Alkaline phosphate was expressed as U/l.

Determination of serum γ glutamyl transferase (γ -GT) activity

Serum γ -GT activity was determined according to the method of Szasz, (1974).

Procedure

Reagents used were from Agape diagnostic kit. 1ml of the working reagents (reconstituted reagents tris buffer 182mM, pH 8.25 and L-gamma-glutamyl-3-carboxy-4-nitroanillide 2.97mM containing 85mm glycylglycine) was mixed with 0.1 ml serum. After 1 min, changes in absorbance were measured per minutes for 3 min at 405 nm using distilled water blank.

Serum GGT (U/L) = $(\Delta A / \text{min}) \times 1158$

Determination of serum bilirubin

The bilirubin level in serum was determined by Dangerfield and Finlayson, (1953).

Sulfanilic acid reacts with sodium nitrite to produce deoxidized sulfanilic acid. Total bilirubin couples with deoxidized sulfanilic acid in the presence of methylsulfoxide to produce azobilirubin which may be measured at 532-536 nm. In the absence of methyl sulfoxide, only direct (conjugated) bilirubin forms azobilirubin complex.

Reagents

Total bilirubin reagent, Sulfanilic acid, Dimethyl sulfoxide, Stabilizer.

Direct bilirubin reagent, Sulfanilic acid, Preservative.

Activator, Sodium nitrite.

Artificial standard – 10 mg/dl.

Procedure

Estimation of total bilirubin

To 1.0 ml total bilirubin reagent, 0.02 ml of activator and 0.1 ml of serum were added, mixed well and incubated for exactly 5 minutes at room temperature.

Sample blank was prepared by mixing 1.0 ml total bilirubin reagent with 0.1 ml of distilled water, mixed well and incubated for exactly 5 minutes at room temperature. The absorbance of each sample blank and test were measured at 532-546 nm against distilled water blank.

Total bilirubin and direct bilirubin level in serum was expressed as mg/dl.

Total protein (TP) and albumin (ALB)

Reagents

Biuret reagent

Buffered Dye reagent

Protein standard (6 gm %)

Albumin standard (4 gm %)

Procedure

Total protein assay

Pipette into three clean dry test tubes labeled blank (B), standard (S) and test (T).

Mixed well and incubated at 37°C for 10 min. Measured absorbance of standard and test and against blank on a photocolormeter with yellow-green filter or on a spectrophotometer at 555 nm.

Albumin assay

Pipette into three clean dry test tube labeled blank (B), standard (S) and test (T).

Mixed well and measured immediately absorbance of standard and against blank on photocolormeter with red filter or on a spectrophotometer at 630 nm.

Calculations

Total protein = $\text{Absorbance of test} / \text{Absorbance of sample} \times 6$

Albumin = $\text{Absorbance of test} / \text{Absorbance of sample} \times 4$

The total protein and albumin level were expressed as g/dl.

Total cholesterol

The total cholesterol level was estimated by Zlatkis, (1953).

Reagents

1. Cholesterol reagent

2. Precipitating reagent

3. Standard (200mg %)

Procedure

Total Cholesterol

Pipette into dry test tubes labeled blank (B), standard and test (T).

Mixed well and immediately place in a boiling water bath for exactly 60 seconds. Cooled immediately in running tap water and measured the absorbance of test (T), sample (S) against blank (B) on the photocolormeter with yellow green filter or on a spectrophotometer 560 nm within 15 min.

Calculations

Total cholesterol = $\text{Absorbance of test} / \text{Absorbance of sample} \times 200$

The cholesterol level was expressed as mg/dl in serum.

Estimation of serum creatinine

Creatinine is the catabolic product of creatinine phosphate, which is used by the skeletal muscle. The daily production depends on muscular mass and it is excreted out of the body entirely by the kidneys. Elevated levels are found in renal dysfunction, reduced renal blood flow (shock, dehydration, congestive heart failure) diabetes acromegaly. Decreased levels are found in muscular dystrophy.

Calculations

Creatinine in mg/dl = $\text{Absorbance of test} / \text{Absorbance of sample} \times 200$

Glucose estimation

The collected serum samples of different study group were subjected to the serum glucose level estimation by enzymatic GOD-POD method by using (Braham and Trinder, 1972)

Reagents

Reagent 1 Glucose reagent- Glucose oxidase, peroxidase, aminoantipyrine, buffer, stabilizers

Reagent 2 Glucose diluent- Diluent, phenol preservative

Reagent 3 Glucose standard (100 mg/ml) – Dextrose, benzoic acid

Working glucose reagent was prepared by transferring the contents of one vial of reagent 1 (glucose reagent) to a clean black coloured plastic bottle (provided in the kit) and reconstituted the content of each bottle with reagent 2 (diluent).

Procedure

Blood was mixed with 0.48 ml of freshly made trichloro acetic acid (TCA 4%) solution and centrifuged at 3000 rpm for 10 minutes. The supernatant of this was used for the estimation of blood glucose.

Preparation of the Blank, Standard and Tests were carried out as follows:

The solutions in tubes were mixed well and incubated for 15 minutes at 37°C. The

Mixed well and absorbance were measured at 505 nm against reagent blank.

Calculation

Glucose (mg/dl) = Absorbance of test/Absorbance of sample x 250

Estimation of Alfa- Fetoprotein (AFP)

AFP is a glycoprotein with a molecular weight of between 65,000 and 70,000 daltons including 4% of carbohydrate. During fetal development, AFP maintains high levels in the serum and drops to very low levels throughout the remainder of life. AFP is elevated in the malignant diseases of hepatocellular, testicular nonseminomatous origin, and occasionally of other endodermal origin.

AFP may be slightly elevated or persisted in the patients with large hepatic metastases or viral hepatitis. AFP measurement is widely accepted as tumor marker and for monitoring the therapeutic effectiveness of hepatocellular cancer and nonseminomatous testicular cancer.

Sample collection and storage

Serum

A Serum separator tube was used and samples were allowed to clot for two hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000 x g. Freshly prepared serum was assayed immediately or store samples in aliquot at -20 or -80°C for later use.

Plasma

Plasma was collected using EDTA or heparin as an anticoagulant. Samples were centrifuge for 15 minutes at 1000 x g at 2 – 8 °C within 30 minutes of collection. Serum was removed and assayed immediately or store samples in aliquot at -20 or -80°C for later use.

Reagent preparation

All kit components and samples were kept at room temperature (18-25°C) before use.

Standard: Standard was reconstituted with 1.0 ml of standard diluent, kept for 10 minutes at room temperature, shaken gently (not to foam). The concentration of the standard in the stock solution is 80 mg/ml. stock solution diluted to 20 mg/ml and the diluted standard serves as the high standard (20 mg/ml). Diluted standard was used to produce a dilution series. Each tube were thoroughly mixed before the next transfer. Seven points of diluted

standard such as 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.312 mg/ml were setup, and the last EP tubes with standard diluent is the blank as 0 ng/ml.

Assay Diluent A and Assay Diluent B

6 ml of assay diluent A or B were diluted, concentrated with 6 ml of deionized or distilled water to prepared 12 ml of assay diluents A or B.

Detection Reagent A and Detection Reagent B

The stock detection A and B were centrifuge before use. The working concentration with working assay diluent A or B were diluted, respectively (1:100).

Wash Solution

600 ml of wash solution was prepared as per instruction.

TMB substrate

The needed dose of the solution was aspirated with sterilized tips and the residual solution was not dumped into the vial again.

Assay procedure

Wells for diluted standard, blank and sample were determined. Seven wells for standard and 1 well for blank were prepared. 100 µl each of dilutions of standard, blank and samples was added into the appropriate wells. Covered with the plate sealer and incubate for 2 hours at 37 °C.

The liquid was removed from each well.

100 µl of detection reagent A working solution was added to each well and incubated for 1 hour at 37 °C after covering it with the Plate sealer.

The solution was aspirated and washed with 400 µl of 1x wash solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1~2 minutes. The remaining liquid was removed from all wells completely by snapping the plate onto absorbent paper. Repeated for 3 times. After the last wash, remaining wash buffer was removed by aspirating or decanting. plate was inverted and blotted with absorbent paper.

100 µl of detection reagent B working solution was added to each well. Incubated for 30 minutes at 37 °C after covering it with the plate sealer.

The aspiration/wash process was repeated for five times as in step 4.

90 µl of substrate solution was added to each well. Covered with a new plate sealer and incubated for 15 - 25 minutes at 37 °C. It was protected from light. The liquid turned blue by the addition of substrate solution.

50 µl of stock solution was added to each well. The liquid turned yellow by the addition of stock solution. The liquid was mixed by tapping the side of the plate. If color change does not appeared uniform, the plate was gently taped to ensure thorough mixing.

The drop of water was removed and fingerprinted on the bottom of the plate and it was confirmed there was no bubble on the surface of the liquid. Then, the microplate reader was run and measurement was conducted at 450 nm immediately.

Calculation

The standard curve was constructed by plotting the mean absorbance for each standard on the x-axis against the

concentration on the y-axis and the best fit curve was drawn through the points on the graph. The data was linearized by plotting the log of the AFP concentrations versus the log of the O.D. and the best fit line was determined by regression analysis.

Detection range

0.312-20 mg/ml. The standard curve concentrations used for the ELISA's were 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.312 mg/ml.

Estimation of Carcinoembryonic Antigen (CEA)

Carcinoembryonic antigen (CEA) is a cell-surface 200-kd glycoprotein. Increased levels of CEA are observed in patients with cancer of the lung, liver, pancreas, breast, colon, head or neck, bladder, cervix, and prostate. Elevated plasma levels are related to the stage and extent of the disease, the degree of differentiation of the tumor, and the site of metastasis. CEA is also found in normal tissue.

Sample collection and reagents preparation: Same as above mentioned.

Assay procedure

Wells for diluted standard, blank and sample were determined. Seven wells for standard and 1 well for blank were prepared. 100 µl each of dilutions of standard, blank and samples was added into the appropriate wells. Covered with the plate sealer and incubate for 2 hours at 37 °C.

The liquid was removed from each well.

100 µl of detection reagent A working solution was added to each well and incubated for 1 hour at 37 °C after covering it with the Plate sealer.

The solution was aspirated and washed with 400 µl of 1x wash solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1~2 minutes. The remaining liquid was removed from all wells completely by snapping the plate onto absorbent paper. Repeated for three times. After the last wash, remaining wash buffer was removed by aspirating or decanting. Plate was inverted and blotted with absorbent paper.

100 µl of detection reagent B working solution was added to each well. Incubated for 30 minutes at 37 °C after covering it with the plate sealer.

The aspiration/wash process was repeated for five times as in step 4.

90 µl of substrate solution was added to each well. Covered with a new plate sealer and incubated for 15 - 25 minutes at 37 °C. It was protected from light. The liquid turned blue by the addition of substrate solution.

50 µl of stock solution was added to each well. The liquid turned yellow by the addition of stock solution. The liquid was mixed by tapping the side of the plate. If color change does not appeared uniform, the plate was gently taped to ensure thorough mixing.

The drop of water was removed and fingerprinted on the bottom of the plate and it was confirmed there was no bubble on the surface of the liquid. Then, the microplate reader was run and measurement was conducted at 450 nm immediately.

Calculation

The standard curve was constructed by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and the best fit curve was drawn through the points on the graph. The data was linearized by plotting the log of the CEA concentrations versus the log of the O.D. and the best fit line was determined by regression analysis.

Detection range

0.156-10 mg/ml. The standard curve concentrations used for the ELISA's were 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.312 mg/ml, 0.156 mg/ml.

Histopathological studies

The kidney and liver were dissected out and were fixed in 10% formalin solution. Paraffin sections were made and stained with haematoxylin and eosine for detailed histopathology study

Pharmacological Studies

Experimental design and induction of hepatocellular carcinoma (HCC)

The experimental male Wistar rats were divided into six groups (for each plant), each group comprising of six animals (n = 6) for a study period of 20 weeks. Group I served as normal control and treated with 0.9% normal saline throughout the study. HCC was induced in groups II, III, IV and V with single intraperitoneal injection of NDEA in normal saline at a dose of 200 mg/kg body weight followed by weekly subcutaneous injections of CCl₄ (3 ml/kg/week) for 6 weeks, as the promoter of carcinogenic effect (Sundaresan and Subramanian, 2008). After administration of NDEA, test groups III and IV were administered orally 400 mg/kg TPE, in the form of aqueous suspension daily once a day throughout the study. Group V received silymarin, the known hepatoprotective and anti-hepatocellular carcinoma compound at a dose of 200 mg/kg (Ramakrishnan et al., 2006; Singh et al., 2009). The dose of FIE (400 mg/kg) was administered orally to rats of group. The experiment was terminated at the end of 20 weeks of experimental period, the body weight of each rat was taken before sacrifice. The overnight fasted animals were anaesthetized and sacrificed 48 h after the last dose of the drug. Blood was collected by retro-orbital plexus followed by heart puncture and allowed to clot before centrifugation at 2500 × g for 15 min at 4 °C to separate serum. The liver tissue was washed twice with ice cold saline, blotted, dried, observed for the presence of nodules and then weighed. The relative liver weight was calculated as the percentage ratio of liver weight to the body weight.

A small portion of the tissue was fixed in formalin for histological examination. The remaining tissues were stored at 20 °C for not more than 12 h before analysis (Singh et al., 2009).

Assessment of liver injury markers and liver tumor markers

The activities of biochemical parameters investigated in serum was, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), Total bilirubin level (TBL) and γ -glutamyl transferase (γ -GT) as well as quantitative estimation of serum hepatic tumor

markers - α -feto protein (AFP) and carcinoembryonic antigen (CEA) were based on ELISA using assay kits from USCN LIFE science and technology (Wuhan, China).

Assessment of antioxidant parameters

Preparation of PMS (post mitochondrial supernatant)

Hepatic tissues of rats were homogenized (10%) in phosphate buffer (pH 7.4) with a Potter Elvehjem glass homogenizer. The homogenate was centrifuged at $12,000 \times g$ for 20 minutes at 4°C to obtain post mitochondrial supernatant (PMS) and it was used for the estimation of the following parameters.

Assessment of lipid peroxidation (LPO)

A volume of the homogenate (0.2 ml) was transferred to a vial and was mixed with 0.2 ml of a 8.1% (w/v) sodium dodecyl sulphate solution, 1.5 ml of a 20% acetic acid solution (adjusted to pH 3.5 with NaOH) and 1.5 ml of a 0.8% (w/v) solution of thiobarbituric acid (TBA) and the final volume was adjusted to 4.0 ml with distilled water. Each vial was tightly capped and heated in a boiling water bath for 60 min. The vials were then cooled under running water. Equal volumes of tissue blank or test samples and 10% trichloroacetic acid were transferred into a centrifuge tube and centrifuged at $1000 \times g$ for 10 min. The absorbance of the supernatant fraction was measured at 532 nm (Beckman DU 650 spectrometer). Control experiment was processed using the same experimental procedure except the TBA solution was replaced with distilled water (Jamall and Smith, 1985). Malonyldialdehyde (MDA) is an end product of lipid peroxidation, which reacts with thiobarbituric acid to form pink chromogen thiobarbituric acid reactive substance. 1,1, 3, 3-tetraethoxypropane was used as standard for calibration of the curve and is expressed as n mole/mg protein.

Assessment of reduced glutathione (GSH)

Reduced glutathione (GSH) level was measured by the method of Ellman, (1959). The PMS of rat liver (720 μ l) and 5% TCA were mixed to precipitate the protein content of the supernatant. After centrifugation at $10,000 \times g$ for 5 min, the supernatant was taken. DTNB (5, 5'-dithio-bis (2-nitrobenzoic acid) Ellman's reagent was added to it and the absorbance was measured at 412 nm. A standard graph was drawn using different concentrations of a standard GSH solution. GSH contents were calculated in the PMS of rat liver and expressed as n mol/mg of protein.

Assessment of catalase (CAT)

Catalase activity was measured according to the method of Aebi, (1997). One unit of catalase was defined as the amount of enzyme required to decompose 1.0 μ M of hydrogen peroxide (H_2O_2) in 1 min. The reaction was initiated by the addition of freshly prepared 20 mM H_2O_2 (1.0 ml). The rate of decomposition of H_2O_2 was measured at 240 nm for 1 min, at 25°C . The enzyme activity was expressed as U/mg of protein.

Assessment of superoxide dismutase (SOD)

The activity of superoxide dismutase in the PMS of liver was assayed (Kakkar et al., 1984) based on the oxidation of epinephrine adrenochrome transition by enzyme. The

post-mitochondrial suspension of rat liver (0.5 ml) was diluted with distilled water (0.5 ml). To this, chilled ethanol (0.25 ml) and chloroform (0.15 ml) were added. The mixture was shaken for 1 min and centrifuged at $2000 \times g$ for 10min. The PMS (0.5 ml) was added with PBS buffer (pH 7.2; 1.5ml). The reaction initiated by the addition of epinephrine (0.4 ml) and change in optical density (O.D., min^{-1}) was measured at 470 nm. SOD activity expressed as U/mg of protein. Change in O.D. (min^{-1}) at 50% inhibition to adrenochrome transition by the enzyme was taken as one enzyme unit.

Assessment of Glutathione peroxidase (GPx)

Glutathione peroxidase activity was measured by the method described by Rotruck (1973). To 0.2 ml of buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.5 ml of PMS were added. To that mixture, 0.2 ml of glutathione solution and 0.1 ml of H_2O_2 were added. The contents were mixed well and incubated at 37°C for 10 minutes along with the control tubes containing the entire reagent but no enzyme. After 10 minutes, the reaction was arrested by the addition of 0.4 ml of 10% TCA. Then 0.2 ml of PMS was added to the control tubes and the tubes were centrifuged. To 0.5 ml of supernatant, 3.0 ml of sodium hydrogen phosphate and 1.0 ml of DTNB were added and the color developed was read at 412 nm immediately in spectrophotometer. Graded concentrations of the standard were also treated similarly. Glutathione peroxidase activity in liver homogenate was expressed as U/mg of protein.

Assessment of glutathione-S-transferase (GST)

The activity of glutathione-S-transferase was measured according to the method of Habig et al., (1974). The PMS of liver (0.1ml), PBS buffer (pH 7.4; 1.0 ml), double distilled water (1.7 ml), and 1-chloro-2,4-dinitrobenzene (CDNB; 0.1ml) reagent were taken in the screw cap tubes and incubated at 37°C for 15 min. the reaction was started by the addition of GSH (1.0 ml) was added and increase in optical density was recorded at 340 nm from 0 to 3.0 minutes. The reaction mixture without the enzyme was used as blank. The activity of GST was expressed as U/mg of protein.

Histopathological assessment

At the end of each scheduled the control as well as treated rats were sacrificed by using cervical dislocation. For histologic studies, the liver sections were made immediately from the liver of different groups of rats, fixed in 10% formalin, dehydrated in gradual ethanol (50–100%), cleared in xylene, and embedded in paraffin wax. Sections were cut at 4 μ m thick (Automatic tissue processor, Lipshaw) in a rotary microtome and the pathological changes were observed microscopically after staining with hematoxylin and eosin (H & E).

Statistical Analyses

The data were represented as mean \pm S.E.M. for six rats. Analysis of variance (ANOVA) test was followed by individual comparison by Newman-Keuls test using Prism Pad software (Version 3.0) for the determination of level of significance. The value of probability less than 5% ($P < 0.05$) was considered statically significant.

RESULTS

Results of Fumaria indica

Preliminary phytochemical studies

Extraction and Preliminary phytochemical screening of 50%EtOH extract of Fumaria indica

Fumaria indica was subjected to 50% ethanolic (EtOH) extraction and the percentage yield was observed as 9.85% (w/w) which is presented in table. In the preliminary phytochemical screenings of 50% EtOH extract of *Fumaria indica* has shown the positive tests for various phytoconstituents such as alkaloids, carbohydrates, glycosides, amino acids, flavonoids, terpenes, saponins and steroids.

HPTLC analysis of 50% EtOH extract of Fumaria indica

The preliminary HPTLC studies revealed that the solvent system chloroform : methanol (80:20) was ideal for the 50% ethanolic extract of *Fumaria indica* and plates were visualized under UV 254 and visible. The plates were sprayed with anisaldehyde -sulfuric acid and heated at 110°C for 10 min. The plates were scanned densitometrically using CAMAG TLC scanner at 254 nm wavelength. The R_f values and the relative percentage of area in each peak in the extract applied were also calculated and are presented in table.

HPLC analysis of Fumaria indica

HPLC analysis of FIE revealed the presence of chromatographic peaks consistent with the pattern showed by the marker components such as chlorogenic acid (1), caffeic acid (2) and ferulic acid (3). Quantitative HPLC analysis showed that chlorogenic acid (138.3 µg/g of FIE), caffeic acid (207.6 µg/g of FIE) and ferulic acid (415.3 µg/g of FIE) with retention times (R_t) of 8.49, 11.17 and 18.53 minutes respectively, were the main phenolic acids in FIE.

Pharmacological Studies

Effect of 50% ethanolic extract of Fumaria indica on body weight, liver weight and relative liver weight in control and NDEA + CCl₄-induced HCC (hepatocellular carcinoma) rats

The body weight (initial and final) and liver weight of control and experimental groups of animals. The final body weight of normal group I rats showed 243 ± 7.4 g which was significantly decreased to 172 ± 8.6 g (P < 0.001) compared to group II rats following NDEA + CCl₄ treatment. In FIE-treated group III (200 mg/kg) and IV (400 mg/kg) rats, the final body weights significantly increased 212 ± 9.8 (P < 0.01) and 238 ± 9.5 g (P < 0.001) when compared to the NDEA group II, respectively. NDEA treatment significantly increased the relative liver weight to (P < 0.001) 5.71 ± 0.52/100 g body weight when compared to the control (group I, 2.93 ± 0.38/100 g body weight). Administration of 200 and 400 mg/kg FIE significantly reduced (P < 0.01 and P < 0.001) the relative liver weight to 3.78 ± 0.47 and 3.15 ± 0.43/100 g body weight, respectively, compared to 5.71 ± 0.52/100 g in NDEA treatment. FIE treated group IV activity was less to standard silymarin-treated group V rats at the concentration used. The animals treated with FIE alone (400 mg/kg) showed no significant change in the body

weight and relative liver weight when compared to control group I.

Effect of 50% ethanolic extract of Fumaria indica on the development of liver nodules in control and NDEA + CCl₄- induced HCC rats

The total number of nodules, nodule incidence and average no of nodules per nodule bearing liver in the normal and experimental groups of animals. When rats were treated with NDEA (group II), 100% developed nodules in the liver were observed, whereas administration of FIE 200 mg/kg (group III) and 400 mg/kg (group IV) showed marked decrease in the number and multiplicity of the nodules as compared to group II rats. The incidence of nodule growth was reduced to 66.67% in group III and 33.33% in group IV, respectively. The nodule incidence of FIE treated group IV was less to standard silymarin treated group V rats. The occurrence of hepatic nodules was not observed in the normal group I and FIE alone treated group VI animals.

Effect of 50% ethanolic extract of Fumaria indica on liver injury and cancer markers in control and NDEA + CCl₄-induced HCC in rats

The effect of FIE on liver injury is shown, NDEA-treated group II rats showed increased serum AST (353.12 ± 20.23 U/l, P < 0.001), ALT (203.57 ± 16.68 U/l, P < 0.001), ALP (142.65 ± 10.12 U/l, P < 0.001), γGT (133.42 ± 14.43 U/l, P < 0.001) and TBL (2.14 ± 0.23 mg/dl, P < 0.001) compared to control group I rats (92.13 ± 11.06 U/l, 38.81 ± 5.11 U/l, 57.14 ± 5.77 U/l, 29.56 ± 4.37 U/l and 0.73 ± 0.09 mg/dl), respectively. The FIE treated groups III and IV rats at 200 and 400 mg/kg significantly decreased AST (247.63 ± 16.47 and 129.18 ± 12.67 U/l, P < 0.001), ALT (133.41 ± 12.22 and 68.81 ± 8.91 U/l, P < 0.001), ALP (111.56 ± 8.17 and 84.76 ± 8.63 U/l, P < 0.01 and P < 0.001), γGT (92.13 ± 9.82 and 49.48 ± 5.13 U/l, P < 0.01 and P < 0.001) and TBL (1.48 ± 0.16 and 0.96 ± 0.11 mg/dl, P < 0.01 and P < 0.001), respectively compared to group II animals. The levels of the tumor markers α-feto protein (AFP) and carcinoembryonic antigen (CEA). Their levels were found to be increased significantly (AFP, 47.23 ± 4.71 ng/ml; CEA, 6.81 ± 0.83 ng/ml, P < 0.001) in NDEA-induced rats whereas they were significantly decreased (AFP, 32.73 ± 3.42 and 18.31 ± 2.91 ng/ml, P < 0.01 and P < 0.001; CEA, 4.63 ± 0.61 and 2.39 ± 0.47 ng/ml, P < 0.01 and P < 0.001) on treatment with 200 and 400 mg/kg of FIE.

Effect of 50% ethanolic extract of Fumaria indica on Lipid peroxidation (LPO) and levels of antioxidant enzymes in liver of control and NDEA + CCl₄-induced HCC in rats

The LPO levels in liver homogenate were found to be significantly increased in the NDEA-treated group II rats. The LPO value of control group I was 0.37 ± 0.03 U/mg protein which increased to 1.13 ± 0.07 U/mg protein (P < 0.001) (Table 5.16). Administration of FIE showed significant reduction in LPO as 0.73 ± 0.06 and 0.48 ± 0.04 U/mg protein, P < 0.001 and P < 0.001 at doses 200 and 400 mg/kg, respectively. NDEA treatment (group II) decreased the levels of hepatic GSH, CAT and SOD from

0.73 ± 0.05 to 0.36 ± 0.03 (P < 0.001), 46.56 ± 2.27 to 30.41 ± 1.53 (P < 0.001), and 18.58 ± 1.54 to 7.71 ± 0.83 U/mg protein (P < 0.001), respectively when compared to group I animals. However, FIE at 200 and 400 mg/kg significantly increased the levels of GSH, CAT and SOD from 0.49 ± 0.02 to 0.63 ± 0.07 (P < 0.05 to P < 0.001), 34.85 ± 1.13 to 39.43 ± 1.34 (P < 0.05 to P < 0.001) and 12.14 ± 1.13 to 15.31 ± 1.21 U/mg protein (P < 0.01 to P < 0.001), respectively when compared to group II rats. Levels of GPx and GST were significantly decreased in NDEA-induced HCC bearing rats (2.33 ± 0.26 and 410.83 ± 24.13 U/mg protein, P < 0.001, respectively) which is presented in Table. On the contrary, the FIE (200 and 400 mg/kg) also increased the level of GPx from 3.42 ± 0.23 to 4.49 ± 0.36 U/mg protein (P < 0.05 to P < 0.001) and GST from 547.76 ± 29.11 to 693.81 ± 32.12 U/mg protein in liver (P < 0.01 and P < 0.001) as compared to NDEA-treated group II rats, respectively. Silymarin at 200 mg/kg significantly reduced the elevated LPO level (0.41 ± 0.05 U/mg protein, P < 0.001), but increased the levels of GSH, CAT, SOD, GPx and GST [0.69 ± 0.05 (P < 0.001), 43.21 ± 1.23 (P < 0.001), 15.87 ± 1.06 (P < 0.001), 5.17 ± 0.35 (P < 0.001) and 703.62 ± 31.29 (P < 0.001) U/mg protein], respectively compared to NDEA treated group II rats.

Histopathological observations

The histopathological examinations support the results obtained from serum enzyme and tumor marker assays. Revealed the normal architecture (group I) and hepatic cells with granulated cytoplasm, small uniform nuclei and nucleolus. Group II NDEA-treated rats showed loss of architecture and neoplastic cells arranged in lobules separated by fibrous septa with inflammatory collection and small bile duct proliferation. Neoplastic cells were larger than normal cells with granular cytoplasm and larger hyperchromatic nuclei and hyaline globules (arrow) that represent proteins produced by the tumor cells. Architecture of liver sections of FIE treated (200 mg/kg) group III rats showed normal architecture with some hepatocytes and minimal inflammatory cell infiltration around the portal triads with few malignant hepatocytes., whereas FIE treated (400 mg/kg) group IV rats showed normal architecture with few preneoplastically transformed cells and hepatocytes maintaining near normal architecture which was almost comparable to the standard silymarin treated group V rats. Administration of 50% ethanolic extract of *Fumaria indica* (400 mg/kg) alone exhibited normal architecture of hepatocytes with granulated cytoplasm.

Effect of 50% ethanolic extract of Fumaria indica treatment alone

When FIE alone at a dose of 400 mg/kg was administered to the non-NDEA and CCl₄-treated rats (Group VI), no significant changes were observed in any of the body weight, liver weight, relative liver weight and liver marker enzymes while gentle significant changes (P < 0.05) were observed in LPO, SOD, CAT and GPx when compared to normal control group I rats. This indicates that *Fumaria indica* extract contributes to exert

antioxidant defense mechanism by metabolizing lipidperoxides and scavenging endogenous peroxides as well as its depicting then on-toxic nature of the plant extract. At this dose level, there were no significant changes in the histological observations depicting the non-toxic nature of FIE.

DISCUSSION

Hepatocellular carcinoma (HCC) one of the leading causes of death in developing countries. Hepatocellular carcinoma (HCC) is one of the most common cancers with poor prognosis. However, various exogenous and endogenous factors are known to affect the normal pattern of cell growth, by which cell becomes a cancerous. N-nitrosodiethylamine (NDEA) is the most important carcinogen among nitrosamines and primarily induces tumour of liver. The liver is one of the largest organs in the body. It plays an important role as a neutralizer of exo and endotoxins. Recent studies during the last few years have shown new hopes in the treatment of this dreadful diseases by measures aimed at the close relationship between free radical production and hepatic cancer (Prince et al., 2004). Among other functions it serves as an interface that processes absorbed nutrients into chemicals that are nontoxic for the organism and can safely be utilized by other tissues and organs. Indeed, curative treatment such as tumour resection and orthotopic liver transplantation are not feasible in advanced stages of Hepatocellular carcinoma (HCC). Therefore, searching for effective chemotherapeutic agents is important to improve the survival rate of patients with advanced or recurrent HCC.

In addition, HCC is well known for multi-drug resistance and its poor response to current chemotherapeutic agents (Geng et al., 2003). On the other hand, Ayurveda, an indigenous system of medicine has long been used for treating liver disorders based on traditional knowledge (De et al., 1993). However, traditional medicine, especially the herbal medicine plays a vital role in the management of various liver disorders. The relative importance of *Fumaria indica*(FIE) is considered useful in the treatment of various ailment as well as liver disorders and tumour. Hence, it is usually intensive to chemotherapeutic drugs currently used in clinical setting, and there is an urgent need for the evaluation of new active drugs against HCC. In the present investigation, biochemical parameters were observed in animal of hepatoma(NDEA and CCl₄) induced hepatocellular carcinoma in the rats. In the present study, 50% ethanolic extract of the plant tested showed significant improvement in biochemical parameters shows the potentiating the level of back to normal and also likely show the level of these enzymes near to the level when treated with the standard drug which indicates, the anti carcinogenic effects of plant in (NDEA and CCl₄) treated hepatoma rats. In groups treated with 50% ethanolic extract of the plant showed significant results, reducing the levels of these elevated levels in a dose dependent manner, indicates the restoring serum marker enzymes back to normal. To prove the anticancer activity of

Fumaria indica(FIE), histopathological studies were carried out. In the present investigation, noticeable changes were observed in the architecture of liver of cancer bearing animals. These indicates the presence of neoplastic conditions following NDEA and CCl₄ administration. In drug treated animals, the NDEA and CCl₄ damage was recovered due to anticancer potency of *Fumaria indica*(FIE). The regression of the tumours in liver may be due to the protective effect of *Fumaria indica*(FIE).

CONCLUSION

Hepatocellular carcinoma (HCC) is one of the common cancers in the world. The most important part is the evolution of experimental hepatocarcinogenesis and its importance as an animal model in treatment of disease relating to human efficacy. The promoter which is as important as carcinogen which boost the process of malignancy and decrease the latency period of occurrence of malignancy. NDEA and CCl₄ is been widely used as a study for chemical induced hepatocellular carcinoma and its mechanism of action is also well illustrated. Based on this, potentiation of plant for the treatment of dreaded disease like cancer may shows a ray of light for better protocol for further treatment of hepatocellular carcinoma. Recent studies on tumour inhibitory compounds of plant origin have yielded an impressive array of research on medicinal plant. In the acute toxicity study, FIE produced neither mortality nor changes in behavior in mice. In subacute toxicity study, FIE did not produce any significant difference in their body weight, food consumption and water intake, hematological and biochemical parameters in experimental groups of rats. Histopathological study revealed normal architecture of kidney and liver of FIE treated rats. These finding demonstrated that there is a wide margin of safety for the therapeutic use of *Fumaria indica*. Thus further corroborated the traditional use of these extract as an anti hepatocarcinogenic agent. The efficacy of *Fumaria indica* in experimental liver cancer described in the present investigation offer the potential for reaching on understanding of anticancer potency. The administration of *Fumaria indica* extract and Silymarin show the rehabilitating capability of extracts in respect with anticancer potency in comparison with the standard drug Silymarin. These finding suggested that the oral administration of *Fumaria indica* possesses antihepatocellular carcinoma activity as evidenced by the significant and dose dependent restoring the activities of entire liver cancer marker enzymes, diminution in tumor incidence, decrease in lipid peroxidation (LPO) and increase in the level of antioxidant enzymes (GSH, CAT, SOD, GPx and GST) through scavenging of free radicals, or by enhancing the activity of antioxidant, which then detoxify free radicals. These factors protect cells from ROS damage in NDEA and CCl₄-induced hepatocarcinogenesis. Histopathological observations of liver tissues too correlated with the biochemical observations. Thus, present investigation suggested that the *Fumaria indica* would exert a chemoprotective effect

by reversing the oxidant-antioxidant imbalance during hepatocarcinogenesis induced by NDEA and CCl₄. Besides *Fumaria indica* is very much effective in preventing NDEA-induced multistage hepatocarcinogenesis possibly through antioxidant and antigenotoxic nature, which was confirmed by various liver injury and biochemical tumour markers enzymes. These observations and description of mechanism of *Fumaria indica*, which interplay with cancer biology and pharmacology lead to rapid development in cancer treatment. In addition to this, studies on molecular aspect of cancer therapy will give mechanistic information in cancer therapy and also critical balance should be there between the animal model and clinical research. This holds great promise for future research in human beings. The anticancer properties of *Fumaria indica* should provide useful information in the possible application in cancer prevention and cancer therapy.

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