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Original Article

ROLE OF *VICIA FABA* FRUIT EXTRACT AGAINST CYTOTOXICITY INDUCED BY ACETAMINOPHEN IN PRIMARY CULTURED RAT HEPATOCYTES

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ABSTRACT

Objective: In the present study, *Vicia faba* fruit extract was screened for their hepatoprotective activity against cytotoxicity induced by acetaminophen in primary cultured rat hepatocytes.

Methods: *Vicia faba* fruit, seed and leaf were subjected to assessment of its total phenolic content, reducing power, free radical scavenging activity, superoxide anion radical scavenging activity and lipid peroxidation. HPLC analysis, DNA damage protection and hepatoprotective activities of *V. faba* fruit extract were also analysed.

Results: Among the tested extracts, *V. faba* fruit extract exhibited highest total phenolic content (104.90 mg gallic acid equivalents (GAE)/g), reducing power (0.83 ascorbic acid equivalents (ASE)/ml), free radical scavenging activity (IC_{50}= 31.27 µg/ml), superoxide anion radical scavenging activity (IC_{50}= 33.52 µg/ml) and LPO (IC_{50}= 621.75 µg/ml). HPLC of *V. faba* fruit extract showed presence of polyphenols *i.e.* gallic acid (70.81 mg/100g) and catechin (49.63 mg/100g) and showed a significant reduction in the formation of nicked calf thymus DNA against either Fenton's reagent or UV radiation. Supplementation of *V. faba* fruit extract conferred significant protection against cytotoxicity induced by acetaminophen in primary cultured rat hepatocytes in comparison to standard hepatoprotectant silymarin.

Conclusion: *V. faba* fruit extract possesses significant antioxidant, DNA damage protective and hepatoprotective activities and may be used for management of drug induced liver injury.

Keywords: Acetaminophen, Cytotoxicity, Free radical scavenging activity, *Vicia faba,* Rat hepatocytes

INTRODUCTION

The management of liver diseases has become a critical concern in medical science. Drugs available in the present system of medicine are associated with toxic effects and cause injury to the organ due to oxidative stress when taken in overdoses. Due to unique metabolism and close relationship with the gastrointestinal tract, liver is susceptible to injury from drugs and other substances. Acetaminophen (APAP) or paracetamol (PAR) hepatotoxicity, because of overdose, is the most frequent cause of acute liver failure [1].

A toxic metabolite N-acetyl p-benzoquinone (NAPQI) generated as a result of APAP metabolism which is inactivated by glutathione (GSH) and form APAP–GSH adducts. Hence, a toxic dose of APAP causes 90% depletion of hepatic GSH level. Thus, metabolite actively participate in binding of cysteine groups on proteins at decreased levels of GSH that form APAP protein adducts which covalently bind to liver DNA and eventually disrupt cellular homeostasis. Oxidative stress is a condition in which balance between reactive oxygen species and antioxidant defence mechanism has been disturbed. It has been suggested that the reactive oxygen species (ROS) are produced as a consequence of decreased levels of GSH after administration of APAP in hepatocytes and induce hepatotoxicity [2].

Human body has several mechanisms to counteract oxidative stress by antioxidants which are either naturally produced or externally supplied through foods or supplements. High intake of fruits, vegetables and whole grains due to presence of phytochemicals has been linked to lowered risks of many chronic diseases including liver diseases, cancer, cardiovascular disease, chronic inflammation and many degenerative diseases [3]. In the past, several herbal compounds have also been screened to test their ability to reduce or nullify acetaminophen induced hepatotoxicity The oldest medicinal herb '*Swertia chirata*' have been mentioned in Indian Ayurvedic medicine which is used for the treatment of liver disorders and was found to be a good hepatoprotector against carbon tetrachloride (CCl_4) and APAP induced toxicity in primary cultured rat hepatocytes [4]. It has been reported that vegetable like *Amorphophallus paeonifolius* possesses phytochemicals as flavonoids and steroids showed hepatoprotective activity against APAP induced liver damage in rats [5]. These results indicate that plants possess their own antioxidant activity which suppresses the damage induced by free radicals.

Phytochemicals such as flavonols, isoflovones, saponins, alkaloids etc. not be considered as nutritive compounds, they are known to possess protective activity against disease. These chemicals are produced by plants for their own protection but their pharmacological effects are of great interest and being explored extensively. Quercetin, a common flavonol which is taken as a positive control in the study, exhibits a broad range of pharmacological properties includes anti-proliferative effects on cancer cells due to its great antioxidant potential [6].

Vicia faba also known as horse bean, broad bean, fava bean, field bean, bell bean belonging to sub-family papilionaceae, family leguminosae are native to North Africa and Southwest Asia. *V. faba* pods, seed, leaves, stems and blossoms are rich in levo-dihydroxy phenylalanine (L-dopa), a substance used medically in the treatment of Parkinson's disease [7]. *V. faba* leaves contain saponins, steroids, tannins and alkaloids [8]. *V. faba* fruit extract also contains five compounds, catechin gallate, quercetin arabinoside, epicatechin glucoside, methyl epicatechin gallate and kaempferol glucoside sulphate [9]. The hypoglycaemic and anti-inflammatory properties of vicine and its aglucone divicine isolated from *V. faba* have also been reported [10]. *V. faba* are rich in tyramine and thus should be avoided by those taking monoamine oxidase inhibitors.

Raw *V. faba* also contain the alkaloids vicine and convicine which can induce hemolytic anemia in patient with the hereditary condition glucose-6-phosphate dehydrogenase deficiency. This potentially fatal condition is called favism after the fava bean [11]. It is reported that extract of dry heated *V. faba* seeds showed higher

inhibition of hemolysis compared to standards butylated hydroxyl anisole and α-tocopherol [12].

However, the hepatoprotective effect of *V. faba* fruit against cytotoxicity induced by acetaminophen in primary cultured rat hepatocytes has not been demonstrated. Hence, the present study focused on evaluating the antioxidant activity in fruit, seed and leaf of *V. faba* plant extracts. On the basis of good TPC and antioxidant activity, *V. faba* fruit extract was used for the study of HPLC analysis and DNA protection activity. Furthermore, the hepatoprotective activity of *V. faba* fruit extract was studied against acetaminophen induced cytotoxicity in rat liver.

MATERIALS AND METHODS

Samples were collected from east regions of Uttar Pradesh and washed with tap water twice, air dried, powdered and finally stored in polythene bags at 4°C till further processing. The identification of plant was confirmed by Dr. T. Hussain, Department of Taxonomy, CSIR-National Botanical Research Institute (NBRI), Lucknow, India. The voucher specimens were also deposited in the institute library.

Chemicals and reagents

Gallic acid, quercetin and bovine serum albumin (BSA), nitro blue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), trichloroacetic acid (TCA), ferric chloride and sodium dodecyl sulphate (SDS) were procured from Sigma-Aldrich, St. Louis, USA. (3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was procured from Calbiochem (USA). Ascorbic acid, β-carotene, Folin Ciocalteau's phenol reagents were the product of E. Merk, Mumbai, India. Rest of reagents and chemicals used were of analytical grade. Solutions were prepared in deionized ultrapure water (Direct Q5, Millipore, Bangalore, India).

Extraction

Twenty gram of dried plant powder of fruit, seed and leaf were extracted with 70% ethanol until decolouration. The filtrates were pooled and concentrated to 10 ml on a rotary evaporator (BUCHI Rotavapor R-114, Switzerland) followed by lyophilisation in a freeze dryer (Heto Fd 3 drywinner). The powdered form of plant extract was stored at −4 °C and used for the determination of antioxidant activity.

Animals

Albino Wistar male rats (*Rattus norvegicus*) weighing 150±20 g were taken from Indian Institute of Toxicology Research (IITR) animal house and used for the experiments. Animals were kept under standard conditions of humidity (60–70%), temperature (25±2 °C) and a controlled 12 h light/dark cycle. Rats were fed pellet diet and water ad libitum. All the protocols were approved by the institutional animal ethics committee of IITR (ITRC/IAEC/01/2010).

Total phenolic content

Total phenolic content (TPC) of *Vicia faba* fruit extract (VFFE), *Vicia faba* seed extract (VFSF) and *Vicia faba* leaf extract (VFLE) were measured by using the method of Ragazzi & Veronese [13] and reported in terms of mg of gallic acid equivalent (GAE)/g of dry weight (DW). To 0.1 ml plant extract, 0.5 ml of Folin's reagent (1N) and 1.0 ml of sodium carbonate was added subsequently. The test mixture was mixed properly and kept at room temperature for 30 min and made up to 10 ml with distilled water. The absorbance of this solution was measured at 720 nm wavelengths. The TPC was reported as mg of gallic acid equivalent (GAE)/g of dry weight (DW) of plant extract.

Reducing power

Reducing power of the vegetable extracts was determined using a slightly modified method of the ferric reducing antioxidant power assay [14]. Each extract (1.0 ml) was mixed with 2.5 ml of phosphate buffer (0.1 mol/l pH 6.6) and 2.5 ml of 1% (w/v) potassium ferricyanide and incubated at 50 °C for 20 min. At the end of the incubation period, 2.5 ml of 10% (w/v) trichloroacetic acid (TCA) was added to terminate the reaction. The upper layer (2.5 ml) was diluted with equal volume of deionised water. Finally, 0.5 ml of 0.1% (w/v) $FeCl_3$ was added and after 10 min the absorbance was measured at 700 nm against a blank. Reducing power was expressed as ascorbic acid equivalents (1 ASE=1 mmol/l ascorbic acid). The ASE value is inversely proportional to reducing power.

Free radical scavenging activity

Free radical scavenging activity of the extracts was measured by using the DPPH stable radical according to Yen and Duh [15]. Each extract (0.1 ml) was added to freshly prepared DPPH solution (6×10^{-5} mol/l in 2.9 ml of HPLC grade methanol) and mixed vigorously. The reduction of the DPPH radical was measured by continuous monitoring of the decrease in absorbance at 515 nm until a stable value was obtained. The inhibition was determined according to the equation:

% inhibition = [(absorbance of blank−absorbance of sample)/ absorbance of blank] × 100.

The IC_{50} which represents the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, a parameter widely used to measure the antioxidant activity, was calculated from a calibration curve by linear regression.

Superoxide anion radical scavenging assay

This assay was based on the capacity of the extract to inhibit the reduction of NBT by using the method of Nishikimi *et al.* [16]. Three milliliters of reaction mixture containing different aliquots of plant extracts (50, 100, 150 and 200 µl) with 0.1 mol/l phosphate buffer (pH 7.8), 60 µmol/l PMS, 468 µmol/l NADH and 150 µmol/l NBT was incubated for 5 min at room temperature. The absorbance was taken after 6 min at 560 nm by using a UV–visible spectrophotometer. The percentage inhibition of superoxide generation was measured by comparing the absorbance of the control and that of the reaction mixture containing the test sample.

Lipid peroxidation assay

A modified thiobarbituric acid-reactive species (TBARS) assay method of Ohkawa *et al.* [17] was applied to measure the lipid peroxide formed, using egg homogenate as the lipid-rich medium. Egg homogenate (10% in 0.2 mol/l phosphate-buffered saline, 0.5 ml), plant extract (0.1 ml) and deionised water (0.85 ml) were mixed in a test tube. Finally, $FeSO_4$ (0.07 mol/l, 0.05 ml) was added to the reaction mixture and incubated at 37 °C for 30 min to induce lipid peroxidation. Thereafter, acetic acid (20%, 1.5 ml), TBA (0.8% prepared in 1.1% sodium dodecyl sulfate, 1.5 ml) and TCA (20%, 0.05 ml) were added, vortexed and then heated in a boiling water bath for 60 min. After cooling, butanol (5 ml) was added to each tube and centrifuged for 10 min at 1000 g. The absorbance of the organic upper layer was measured at 532 nm by using a UV–visible spectrophotometer (Jasco V-550, USA).

High-performance liquid chromatography analysis

The dried extracts were dissolved in HPLC grade methanol (1.0 mg/ml), filtered through sterile 0.22 µm Millipore filter and subjected to qualitative and quantitative analysis by using a Shimadzu LC-10A (Kyoto, Japan) HPLC instrument. The instrument was equipped with a dual-pump LC-10AT binary system (Shimadzu) HPLC, a UV detector SPD-10A (Shimadzu), and a Phenomenex Luna RP, C18 column (4.6 × 250 mm). Data were integrated by using Shimadzu Class VP series software. Separation was achieved with acetonitrile/water containing 1% acetic acid in a linear gradient program, starting with 18% acetonitrile, changing to 32% in 15 min and finally to 50% in 40 min. Results were obtained by comparison of peak areas (γ max =254 nm) of the samples (mg/100g) with that of standards [18].

DNA damage assay

Induction of DNA scission by Fenton's reagent and UV rays was measured on calf thymus DNA according to the procedure described by Lee *et al.* [19] with some modifications.

DNA fragmentation induced by Fenton's reagent

Briefly, each extracts (1.0-5.0 µg/ml) and 500 ng calf thymus DNA in 1xTE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.2), were

incubated for 10 min at room temperature followed by the addition of Fenton's reagent (30 mM H_2O_2, 50 µM ascorbic acid and 80 µM $FeCl_3$). The reaction mixture was incubated for 60 min at 30 °C. The reaction mixture had final volume of 20.0 µl including phosphate-buffer saline (PBS) in 0.5 ml microcentrifuge tubes. After incubation, the samples were mixed with 3 µl of gel loading dye (0.15%) bromophenol blue and 80% (w/v) glycerol and immediately loaded into a 1.5 % agarose gel. The running buffer contained 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA in which gel was electrophoresed in a horizontal slab gel apparatus for 1.5 h (60 V/30 mA). After electrophoresis, the gels were photographed with Gel Doc system (Alpha Innotech). Catechin and silymarin (2.5 µg/ml) were used as positive controls.

DNA fragmentation induced by UV rays

The methodology is same as given in Fenton's reagent experiment, except the nicking of calf thymus DNA was introduced by adding 2 µl H_2O_2 (20.5 mM) in the presence of UV rays (302 nm).

Isolation of primary rat hepatocytes

Hepatocytes were isolated from overnight fasted rat after subjecting the liver to two-stage collagenase perfusion with HEPES buffer [20]. Cell viability was checked by trypan blue exclusion test. Cell preparations with more than 95% viability were used for subsequent experiments. Isolated hepatocytes were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum and 1% of 10,000 units Penicillin, 10 mg Streptomycin, 25 µg Amphotericin B, 1 mM sodium pyruvate, 2 mM glutamine under an atmosphere of 5% CO_2-95% air in an incubator (Thermo-forma) with controlled humidity at 37 °C. The cells were seeded at a density of 1.0×10^4 cells/well (counted on hemocytometer) in 0.1% collagen pre-coated 96 well plates and used for drug exposure experiments after being cultured for 24 h.

Assessment of cell viability and *in vitro* treatment schedule

Hepatocytes were treated with various concentrations of acetaminophen to determine the inhibitory concentration (IC_{50}). Acetaminophen was dissolved in 1% DMSO, filtered through 0.22 µm filter and used for subsequent treatment. The cells were pre-incubated with varying concentration of extract 2.5-7.5 µg/10^4 cells (45 min) prior to treatment with acetaminophen (60 min). Hepatocytes were also treated (control vehicle) with 0.1% dimethyl sulphoxide (DMSO) but no changes were observed (data not shown). For each set of experiment silymarin (5 µg/10^4 cells) was used as a recovery control (a known hepatoprotectant) whereas acetaminophen (675 µM) was taken to induce toxicity. Fifty percent cell viability was observed at the dose of 675 µM acetaminophen per 1.0×10^4 cells.

The viability of cultured cells was determined by MTT reduction assay. This assay is dependent on the reduction of MTT by mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured by spectrophotometer [21]. MTT was added into each treated and untreated well at the final concentration of 5 mg/ml for 4 h followed by addition of 200 µl DMSO to dissolve the MTT formazan crystals. The optical density of each well was measured at 530 nm using a Spectramax PLUS 384 microplate reader (Softmax pro version 5.1; Molecular Devices, USA). The data was expressed as a percentage of viability as compared to control i.e. untreated cells.

Superoxide dismutase activity (SOD)

SOD activity is based on the spectrophotometric assessment of the inhibition of nitro blue tetrazolium-NADH and phenazine methosulphate (PMS) mediated formazan formation. Briefly, to 10 µl of cell lysate, 90 µl of 30 nM sodium tetra pyrophosphate buffer (pH 8.3), 30 µl of 0.3 mM nitro blue tetrazolium, 10 µl of 0.96 mM PMS and 40 µl of deionised water were added. The reaction was initiated by addition of 20 µl 0.72 mM NADH. After incubation at 30 °C for 90 s, the reaction was stopped by the addition of 50 µl glacial acetic acid. Absorbance was measured at 560 nm. Fifty percent inhibition of formazan formation under assay condition in 1 min is taken as one unit of enzyme activity/minute [22].

Lipid peroxidation determination (LPO)

In this assay, the evaluation of end product malondialdehyde (MDA) formed due to membrane lipid peroxidation was measured. Briefly, 10 µl of 1 mM butylated hydroxyl toluene (BHT), 75 µl of 1.3% thiobarbituric acid (TBA) was added. The lipids were isolated by precipitated them with 50 µl of 50% trichloroacetic acid. The mixture was then incubated at 60 °C for 40 min and then kept in ice for 15 min. The reaction was stopped by addition of 10 µl of 20% sodium dodecyl sulphate. This assay measures the amount of pink coloured MDA-TBA adduct at 530 nm and to account for the interference of phytochemicals, It is also read at 600 nm. The concentration of thiobarbituric acid reactive substances (TBARS) was expressed as nmoles of MDA formation in pre-treated hepatocytes using a freshly diluted 1,1,3,3-tetraethoxypropane for the standard calibration curve [23].

Nitric oxide determination (NO)

Nitric oxide formation was measured in all the treated and untreated hepatocytes by assaying nitrite, one of the stable end products of NO oxidation [24]. In brief, 100 µl of cell supernatant was incubated with 100 µl Griess reagent consisting of 1 % sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride and 2.5% H_3PO_4 at 37 °C, for 30 min and the absorbance was recorded at 540 nm. Nitric oxide formation was expressed as % of NO in the presence of samples in pre-treated hepatocytes.

Statistical analysis

All analytical data were subjected to an analysis of variance (ANOVA). Each value is mean±SD of three replications (n=3). Results were considered significant when *P<0.05, **P<0.01, *** P<0.001. Statistical analysis was done by using Prism software (Graph Pad Prism software version 3.0, USA).

RESULTS

Total phenolic content

Total phenolic content was measured in *V. faba* fruit extract, *V. faba* seed extract, *V. faba* leaf extracts and values were measured 104.90±4.14 mg of GAE/g of DW, 52.44±3.10 mg of GAE/g of DW and 51.10±1.44 mg of GAE/g of DW, respectively. These results showed that total phenolic content was higher in *V. faba* fruit extract followed by leaf extract and seed extract (table 1).

Reducing power

Reducing power is determined to measure the reductive ability of antioxidant which is evaluated by transformation of Fe (III) to Fe (II) in the presence of the plant extracts. Among different *V. faba* plant part extracts, fruit extract showed maximum reducing power (0.83 ASE/ml), whereas seed and leaf extract has the reducing power to the tune of 1.54 and 2.56 ASE/ml (table 1).

Free radical scavenging activity

V. faba fruit extract, seed extract and leaf extract were subjected for the evaluation of antioxidant activity using DPPH method. The results of free radical (DPPH) scavenging activity of plant extracts were expressed in the terms of inhibitory concentration (IC_{50}) which ranged from 31.27 to 170.59 µg/ml (table 1). *V. faba* fruit extract scavenges the radicals by 43.07, 64.66, 73.21 and 86.79 %, respectively when 25, 50, 75 and 100 µg/ml plant extract were added to the reaction mixture. However, *V. faba* seed extract required to achieve a 50 % reduction in DPPH radicals (IC_{50}) were 93.04 µg/ml followed by *V. faba* leaf extract (170.59 µg/ml). Hence, the IC_{50} value appeared highest in *V. faba* fruit extract.

Superoxide anion radical scavenging activity

Superoxide anion radical scavenging activity was further explored by assessing the superoxide radical quenching ability of extracts depicted in table 1. The results showed that *V. faba* fruit extract inhibited NBT reduction significantly. *V. faba* fruit extract showed 32.14, 42.42, 46.67 and 53.74% inhibition when 10, 20, 30 and 40 µg/ml *V. faba* fruit extract was added to reaction mixture. Whereas

V. faba seed showed 16.96, 22.53, 26.74, 30.11% inhibition and *V. faba* leaf extract showed 13.81, 15.76, 18.70, 23.24% inhibition at the same concentration. Data showed that *V. faba* fruit extract had better SARSA than the other parts.

Table 1: Estimation of total phenolic content and antioxidant activity (AOA) in *Vicia faba* fruit, seed and leaf extracts

S. No.	TPC[d] (mg of GAE/g of DW)	RP[e] (ASE/ml)	FRSA[f] IC_{50} (µg/ml)	SARSA[g] IC_{50} (µg/ml)	LPO[h] IC_{50} (µg/ml)
VFFE[a]	104.90±4.14	0.83±0.09	31.27±1.02[i]	33.52±3.52[i]	621.75±16.54[i]
VFSE[b]	51.10±1.44	1.54±0.33[i]	93.04±3.20[i]	54.88±1.75[i]	727.56±9.35[i]
VFLE[c]	52.44±3.10	2.56±0.25[i]	170.59±4.96[i]	79.61±1.23[i]	995.63±5.03[i]
Quercetin	-	0.14±0.18	5.34±0.44	11.58±0.51	106.79±1.09

[a] *V. faba* fruit extract (VFFE); [b] *V. faba* seed extract (VFSE); [c] *V. faba* leef extract (VFLE); [d] Total Phenolic content; [e] Reducing Power (RP); [f]Free radical scavenging activity (FRSA); [g]Superoxide anion radical scavenging activity (SARSA); [h]Lipid peroxidation (LPO); Values are mean±SD (n=3); [i]Results were considered signi ficant (P<0.001) when compared with standard quercetin.

Lipid peroxidation

Antioxidant activities of extract were further supported by LPO (table 1). *V. faba* fruit extract showed 20.70, 45.89, 57.14 and 70.32 % inhibition when 250, 500, 750 and 1000 µg/ml *V. faba* fruit extract were added to reaction mixture whereas *V. faba* seed extract and leaf extract showed 23.11, 34.63, 56.33, 63.84% and 20.65, 28.50, 38.84, 51.86%, respectively at same concentrations. Hence, in this study the IC_{50} appeared highest in *V. faba* fruit extract. This showed that the lipid peroxidation activity of fruit extract had more reduction in MDA formation in comparison to seed and leaf extract.

High-performance liquid chromatography analysis

Determinations of phenolic compounds such as gallic acid, catechin, hydroxybenzoic acid (HBA) and caffeic acid (CA) were analysed in *V. faba* fruit extract by using HPLC. The analysed values of gallic acid and catechin content in *V. faba* fruit were 70.81±8.77 and 49.63±3.58 mg/100g, respectively. While other phytochemical, hydroxybenzoic acid (HBA) and caffeic acid (CA) were not present in *V. faba* fruit extract.

DNA damage protection assay

The protective properties of *V. faba* fruit extract, standards silymarin and catechin against hydroxyl radical induced DNA damage was studied using *in vitro* method employing calf thymus DNA. The extract showed concentration dependent protection and neutralized the oxidative stress produced by Fenton's reagent and UV rays. The results were obtained through gel electrophoresis, showed that the extract of *V. faba* fruit signi ficantly protected against DNA strand breakage caused by Fenton's reagent (fig. 1) and UV rays (fig. 2).

In the absence of plant extracts, exposure of hydroxyl radical generated either by Fenton's reagent or UV rays to DNA caused a complete fragmentation of DNA in lane 4 of fig. 1 and lane 3 of fig. 2. Supplementation of extract to the calf thymus DNA at various concentrations (1.0-5.0 µg/ml) during the exposure of Fenton's reagent and UV rays showed the protection of DNA damage significantly at all the concentrations which are comparable to protection shown by catechin and silymarin.

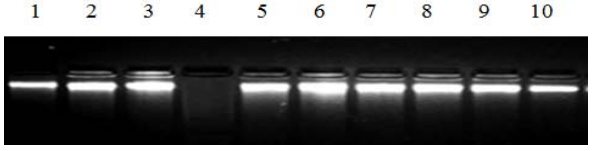

Fig. 1: Inhibitory effects of *V. faba* fruit extract (VFFE) on DNA nicking caused by Fenton's reagent. Lane 1: Calf thymus DNA (500 ng)+PBS; Lane 2: Calf thymus DNA+Fenton's reagent (60 min)+Catechin (2.5 µg/ml); Lane 3: Calf thymus DNA+Fenton's reagent (60 min)+silymarin (2.5 µg/ml); Lane 4: Calf thymus DNA+Fenton's reagent (60 min); Lane 5-10: Calf thymus DNA+Fenton's reagent (60 min)+VFFE (1.0, 1.5, 2.0, 2.5, 3.0 and 5.0 µg/ml)

Quantitative analysis of viable cells

Cell viability was assayed using MTT and measurement of the solubilised formazan dye at a wavelength of 530 nm. Isolated primary rat hepatocytes were treated with varying concentration of extracts 1.0-7.5 µg/10^4 cells to study its effect on cell viability. All the tested concentrations of extract were found to be non-toxic since no cytotoxicity was observed on treatment of hepatocytes with any of the tested concentrations of extract with respect to control (fig. 3).

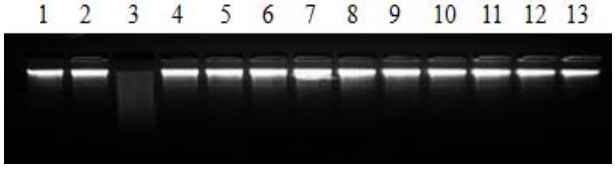

Fig. 2: Inhibitory effects of *V. faba* fruit extract (VFFE) on nicked DNA caused by UV induced hydroxyl radicals. Lane 1: Calf thymus DNA (500 ng)+PBS; Lane 2: Calf thymus DNA+H_2O_2 (20.5 mM); Lane 3: Calf thymus DNA+H_2O_2 (20.5 mM)+UV (8 min); Lane 4-7: Calf thymus DNA+H_2O_2 (20.5 mM)+UV (8 min)+VFFE (1, 1.5, 2.5 and 5.0 µg/ml); Lane 8-11: Calf thymus DNA+H_2O_2 (20.5 mM)+UV (8 min)+silymarin (1, 1.5, 2.5 and 5.0 µg/ml); Lane 12-13: Calf thymus DNA+H_2O_2 (20.5 mM)+UV (8 min)+Catechin (2.5 and 5.0 µg/ml)

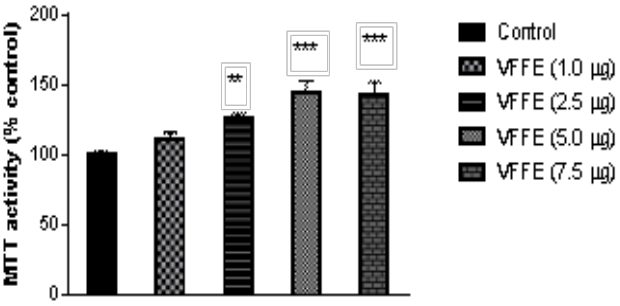

Fig. 3: Survival rate of primary rat hepatocytes at different concentrations (2.5 µg/10^4 cells, 5.0 µg/10^4 cells, 7.5 µg/10^4 cells) of *Vicia faba* fruit extract (VFFE). Values are mean±SD (n=3). Results were considered signi ficant (P<0.01, ***P<0.001) when compared with control**

Hence, in order to ascertain their cytoprotective effects against APAP toxicity, 2.5, 5.0 and 7.5 µg/10^4 cells concentrations of the extracts and silymarin were selected for further experiments. During pre-treatment schedule, 24 h cultivated hepatocytes were incubated with selected concentrations of extract for 45 min before subjecting them to oxidative stress of APAP for 60 min. A positive correlation between dose-response in terms of viability was seen. Cells pre-incubated with VFF extract (7.5 µg/10^4 cells) showed significant protection and cell survival was found to increase by 42.58 % (P<0.001) as compared to APAP stressed cells. Cells pre-treated with silymarin (7.5 µg/10^4 cells) also showed the protective effect and the viability was found to increase by 59.34 % (p<0.001) with respect to APAP treated cells (fig. 4).

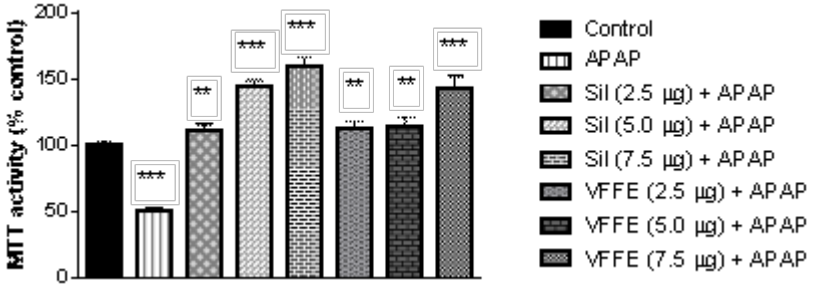

Fig. 4: Survival rate of acetaminophen stressed primary hepatocytes at selected concentrations (2.5 µg/10^4 cells, 5.0 µg/10^4 cells, 7.5 µg/10^4 cells) of silymarin and VFFE. Values are mean±SD (n=3). Results were considered signi ficant (P<0.01, ***P<0.001) when APAP compared with control and extracts and silymarin compared with APAP**

Table 2: Dose dependent antioxidant activity in cells treated with *V. faba* fruit extract, silymarin and acetaminophen

Pre-treatments	SOD[a]	LPO[b]	NO[c]
Control	11.31±0.53	0.38±0.017	99.33±1.14
APAP (675 µM)	4.80±0.45[d]	0.63±0.015[d]	125.74±4.42[d]
V. faba (2.5 µg/10^4 cells)	8.81±0.65[d]	0.43±0.018[d]	62.38±3.97[d]
V. faba (5.0 µg/10^4 cells)	9.06±0.81[d]	0.25±0.035[d]	58.02±1.73[d]
V. faba (7.5 µg/10^4 cells)	10.06±0.24[d]	0.14±0.027[d]	53.44±3.36[d]
Silymarin (5.0 µg/10^4 cells)	12.82±0.52[d]	0.24±0.014[d]	60.45±2.06[d]

[a]Superoxide dismutase (Units/min/10^4 cells); [b]Lipid peroxidation (nM MDA formation/10^4cells); [c] Nitric oxide (%); Values are mean±SD (n=3); [d]Results were considered signi ficant (P<0.001) when APAP compared with control and *V. faba* extracts and silymarin with APAP.

Superoxide dismutase activity

Cultured hepatocytes when treated with APAP (675 µM) showed superoxide dismutase activity of 4.80 units/min/10^4 cells which was 2.3 fold lower than the untreated cells (table 2). Cells that were treated with silymarin at 5.0 µg/10^4 cells showed 2.6 fold increases in superoxide dismutase activity (p<0.001) with respect to APAP treated hepatocytes. The cells pre-treated with VFFE at 5.0 µg/10^4 cells showed 1.9 fold increase SOD activity with respect to APAP treated hepatocytes which indicate that VFFE is comparable to silymarin and act as a strong antioxidant.

Lipid peroxidation determination

Oxidative stress induced in hepatocytes by free radical generation due to APAP caused 0.63 nM MDA formation/10^4cells (P<0.001). In hepatocytes pretreated with extract, the peroxidative decomposition of the lipids was reduced from 0.43 nM MDA formation/10^4 cells at a concentration of 2.5 µg/10^4 cells to 0.14 nM MDA formation/10^4 cells at a concentration of 7.5 µg/10^4 cells with respect to APAP treated cells (table 2). The results showed that VFFE had the capacity to reduce the MDA formation which is comparable to standard antioxidant silymarin.

Nitric oxide determination

Hepatocytes stressed cells treated with 675 µM of APAP was release 25.74% (p<0.001) more nitric oxide in comparison to untreated cells. The treatment of extracts showed dose dependent quenching of nitric oxide in primary rat hepatocytes (table 2). Nitric oxide generated by APAP was taken as 100% from which % inhibition was calculated for each extract. APAP was compared by control. The cells pre-treated with silymarin and VFF extract showed 39.55% and 41.98 % (p<0.001) quenching of nitric oxide at 5.0 µg/10^4 cells concentration in comparison to APAP treated cells.

DISCUSSION

In aerobic organisms, reactive oxygen species are continuously produced as a by-product of metabolisms and also produced on exposure to radiation, pesticides, ozone, tobacco smoke and other environment pollutions [25]. Several endogenous antioxidants such as superoxide dismutase act either independently, cooperatively or even synergistically against free radicals to increase the antioxidant defence system of the body. These antioxidants act as a guard against the harmful effects of reactive oxygen species either by scavenging them or converting them to non toxic compounds or chelating the ion required for their activation. Natural antioxidants such as phenolics and flavonoids are found in various plants which possess diverse biological properties such as anti-apoptotic, anti-ageing, anti-carcinogen, anti-inflammation, anti-atherosclerosis, cardiovascular protection and hepatoprotective activity [26].

Total phenolic content was estimated in different parts of *V. faba* plant extracts. In the present study, total phenolic content was detected maximum in fruit extract of *V. faba* in comparison to seed extract and leaf extract of *V. faba.* Phenolic compounds constitute a class of antioxidant agents acting as free radical terminators. The data demonstrate that polyphenols are widely distributed in vegetables. A high and significant correlation existed between antioxidant activity and total phenolic content of vegetables. Several studies indicate that total phenolic content is the major contributor for the antioxidant activity of vegetables [27].

In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+}/ferricyanide complex to $Fe^{2+}$by donating an electron which is an important mechanism of phenolic antioxidant action [28]. Okuda *et al.* [29] have reported that the medicinal plants having tannins showed significant reducing power which may be reduced liver injury by inhibiting the formation of lipid peroxides. Reducing power of plants is believed to react directly with peroxides as well as it also reacts with certain precursors and prevents from peroxide formation [30]. Our findings suggest that *V. faba* fruit extract showed significant reducing power among all the part of plant extracts. Therefore, this extract is capable of donating electrons and could react with free radicals or terminate chain reactions. In the present study, the range of reducing power is in agreement with the previous literature [31].

Free radical scavenging activity in the DPPH discoloration assay was used for evaluation of antioxidant capacity of *V. faba* plant extracts. Among all the other method, DPPH• method takes a short time to evaluate the antioxidant activities. It is one of stable organic nitrogen radical. In this assay, antioxidant donates its hydrogen ion to free radical. The presence of antioxidant is proportional to the disappearance of DPPH• in the reaction mixture. A freshly prepared DPPH solution exhibit a deep purple colour with an absorption maximum at 517 nm. The purple colour generated by DPPH•

radicals disappears in the presence of an antioxidant in the medium [32]. Our studies revealed that *V. faba* fruit extract has significant DPPH• radical scavenging activity in comparison to other plant parts. Results of antioxidant activity were reported in term of IC_{50} which is the concentration of an antioxidant necessary to inhibit the 50% of initial DPPH• radicals. The lower IC_{50} indicates higher antioxidant activity of plant extracts [33, 34].

The superoxide radical may act either as a reductant or as an oxidant. The superoxide anion radical scavenging activity of *V. faba* plant extracts were monitored by PMS-NADH-NBT reduction system, a non-enzymatic method. In this method, blue formazan is generated by reduction of yellow dye (NBT^{2+}) due to O_2• by PMS-NADH coupling reaction which is measured by spectrophotometer at 560 nm. The decrease in colour intensity showed that antioxidant present in the plant extracts scavenge the superoxide radical in the reaction mixture. Hydroxyl radical and singlet oxygen, a strong reactant, give a weak oxidant superoxide anion which contributes to oxidative stress. The addition of plant extract decreases the colour intensity of reaction mixture. In our study, *V. faba* fruit extract significantly reduces the superoxide anion and inhibit the formation of blue formazan complex which supports previous study by Singh *et al.* [35].

Oxidative stress in cells and tissues indicates the cellular injury in both plants and animals induced by lipid peroxidation. The lipid peroxyl radical (LOO·), which possesses intermediate reactive activity and a long lifetime, is known to cause various diseases *in vivo* [36]. Reactive hydrogens are present between methylene-CH_2-groups of polyunsaturated fatty acids which are primarily affected by lipid peroxyl radicals. Lipid peroxides are very reactive in nature and unstable. After decomposition, it forms a complex series of compounds including reactive carbonyl compounds. Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) is two major compounds of polyunsaturated fatty acid peroxides which are measured as an indicator of lipid peroxidation [37]. Antioxidant activity of extract is measured to inhibit lipid peroxidation (LPO) by TBA methods, a non-enzymatic method. The higher antioxidant activity of plant extracts showed their low IC_{50} value. The IC_{50} of *V. faba* fruit extract showed significant scavenging activity by TBA methods along with quercetin standard. Our study on lipid peroxidation inhibitory potential of vegetable plant extract is confirmed by the previous study on plant extract [38].

V. faba fruit extract was quantified by high-performance liquid chromatography analysis and showed a good amount of gallic acid and catechin. Studies demonstrated that phenolics such as gallic acid, catechin possess many potential therapeutic properties including anti-cancer, antimicrobial and hepatoprotective activities [39]. Catechins are also known as flavanols. Catechin is a group that occupies an intermediary position in the tannin as a family of catechin tannins. The presence of catechin in green tea and fermented tea is associated with health protective, antimicrobial properties, cancer preventive properties and repair of damaged DNA [40]. In another work, it has been described that tannins, anthocyanins and phenolic acids were isolated from *V. faba* which are mainly responsible for the remarkable antioxidant and antimicrobial effect of this plant [41].

The protective effects of *V. faba* fruit extract on the hydroxyl radical mediated DNA damage by Fenton's reagent and UV rays were investigated. In the absence of *V. faba* fruit extract, exposure of hydroxyl radical generating system (Fe^{2+}concentration and UV+H_2O_2) caused complete fragmentation of DNA. Supplementation of *V. faba* fruit extract (1.0-5.0 µg/ml) to the calf thymus DNA during exposure of hydroxyl radical prevents DNA damage significantly. Hydroxyl radicals generated by irradiation or Fenton reaction are known to cause oxidative breaks in DNA strands to yield its fragmented forms [42, 43]. Our study was also supported by Sreelatha and Padma [44] which showed the inhibitory effect of plant extract on Fenton's reaction-mediated degradation of calf thymus DNA and found good hydroxyl radical scavenging potential.

Acetaminophen (Paracetamol) is a widely used as antipyretic, analgesic drug which produces acute hepatic damage on accidental overdosage. A highly toxic metabolite N–acetyl–p–benzoquinamine (NAPQI) is generated by a fraction of acetaminophen, which causes lipid peroxidation and other free radicals [45]. The protective effect of *V. faba* fruit extract and silymarin against cytotoxicity induced by acetaminophen in primary cultured rat hepatocytes was seen by measuring the activity of superoxide dismutase enzyme, lipid peroxidation and nitric oxide release.

Superoxide dismutase activity was measured by superoxide anion generated in PMS-NADH coupling reaction system by oxidation of NADH and assayed by the reduction of NBT. Antioxidant present in vegetable extract indicates $O_2^{\cdot-}$ quenching activity that decreases the absorbance at 560 nm of test sample [46]. In the present study, *V. faba* fruit extract showed dose dependent antioxidant activity in pre-treated primary hepatocytes and exhibited more effective protection at the all concentrations (2.5, 5.0 and 7.5 µg/10^4 cells) which was comparable with known hepatoprotectant silymarin (5 µg/10^4 cells). Administration of APAP decreased the hepatic SOD activity compared to unstressed cells whereas administration of extract significantly increased the hepatic SOD activity as compared to acetaminophen stressed cells of primary cultured rat hepatocytes. This indicates that free radical generation reduced in the presence of antioxidant which increases the hepatic antioxidant enzyme activities and diminishes oxidative stress to cells. Our study on superoxide anion inhibitory potential of vegetable plant extract is confirmed by the previous study of Kumari [47].

Lipid peroxidation is a major destructive process of liver injury due to acetaminophen administration. The activated free radical binds covalently to the macromolecules and induced peroxidation in polyunsaturated fatty acids. This leads to the formation of lipid peroxides which generate malondialdehyde (MDA). The defence mechanism of natural antioxidant at lower concentration indicates increased MDA concentration in primary cultured rat hepatocytes with hepatotoxins [48]. In the present study, treatment of *V. faba* fruit extract and silymarin in primary cultured rat hepatocytes against acetaminophen showed lower level of end product of lipid peroxidation i.e. MDA. Our study on lipid peroxidation was also supported by the study of Sharma *et al.* [45] which showed the inhibitory effect of probiotics against acetaminophen induced cytotoxicity in primary cultured rat hepatocytes.

Nitric oxide generates within the cell which is lipophilic and greatly diffusible solute. It takes part in the regulation of many physiological processes. It is involved in the mediation of neuronal signaling, hepatic metabolism, blood pressure, relaxation of smooth muscles and prevention of platelet aggregation. But the production of nitric oxide by the activated macrophages against pathogens may cause cellular injury to macrophages as well as to the neighbouring tissues. The excess nitric oxide can react with superoxide radicals leading to the formation of harmful peroxynitrite radicals that are responsible for the damage to macromolecules [46]. In this study, administration of *V. faba* fruit extract significantly reduced the production of nitric oxide by cells and protects them from the damaging effects of excess nitric oxide production. Our study was supported by a previous study of Tiwari and Kakkar [6] which showed the inhibitory effect of antioxidants camphene and geraniol against acetaminophen induced hepatotoxicity and found that excess nitric oxide production can be reduced in the presence of an antioxidant.

CONCLUSION

Hepatoprotective properties of plants have been ascertained due to the presence of its antioxidant. Our study indicates that hepatocytes treated with acetaminophen showed reduced cell viability, increased lipid peroxidation, nitric oxide release and decreased superoxide dismutase activity. However, supplementation of *Vicia faba* fruit extract conferred significant protection against acetaminophen induced injury to primary cultured rat hepatocytes. This is evident from decrease in lipid peroxidation, nitric oxide release and increase in superoxide dismutase activity. On the basis of above data, it may be concluded that *V. faba* fruit extract possess significant antioxidant, DNA damage protective and hepatoprotective activities. The outcome of the present study confirmed that *V. faba* fruit may be used as natural antioxidant in nutraceutical preparations and for management of drug induced liver injury after further necessary processing and toxicity studies.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest

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