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Isolation of Quercetin From The Peels of Apple and Evaluation of *In Vivo* Hepatoprotective Activity Against CCl₄ Induced Hepatic Necrosis In Rat Models

Monica Chopra¹, AsishBhaumik^{*2}, B. Prriyanka³, MD. Tajuddin⁴ and Ch. Ram Naresh⁵

¹Department of Pharmacy, MeeraBai Institute of Technology, Maharani Bagh, New Delhi-110065.

²Department of Pharmaceutical Chemistry, Anurag Pharmacy College, Ananthagiri, Kodad, Suryapet-508206, Telangana State, India.

³Department of Pharmaceutical Analysis, UCPS, Kakatiya University, Warangal-506009, Telangana State, India

⁴Department of Pharmacology, Anurag Pharmacy College, Ananthagiri, Kodad, Suryapet-508206, Telangana State, India.

ABSTRACT

The liver is the only human internal organ capable of natural regeneration of lost tissue; as little as 25% of a liver can regenerate into a whole liver. This is, however, not true regeneration but rather compensatory growth in mammals. The lobes that are removed do not regrow and the growth of the liver is a restoration of function, not original form. This contrasts with true regeneration where both original function and form are restored. The objective of the present work was the preliminary phytochemical screening and isolation of flavanoid quercetin and evaluation of *in vivo* hepatocytes regenerator potentiality of methanolic extract of peels of apple (ME-PLS-A) (commonly called Maluspumilaor erroneously called Malus domestica). The *in vivo* experimental data displayed that the elevated levels of SGOT, SGPT, ALP and Sr. bilirubin were mainly due to CCl₄ intoxication, reduced significantly (*P<0.05) in rats, after treatment with ME-PLS-A. Treatment with ME-PLS-A at a dose of 150 mg/kg b. w. decreased the SGOT: 6.61%, SGPT: 22.87%, ALP: 10.56%, and Serum bilirubin levels by 42.46%, (significantly) respectively, while at higher dose of 300 mg/kg b. wt. was more effective, causing a reduction of SGOT: 23.75%, SGPT: 41.49%, SALP: 22.56% and Sr. bilirubin: 55.83%, Silymarin is used as standard drug showed a significant reduction of level of SGOT: 54.79%, SGPT: 47.61%, SALP: 60.39% and Sr. bilirubin: 78.08% respectively receiving CCl₄ alone.

*Corresponding author

AsishBhaumik

Department of Pharmaceutical Chemistry,

Anurag Pharmacy College,

Ananthagiri, Kodad,

Suryapet-508206, Telangana State, India.

Email: bhaumik.asish@gmail.com

INTRODUCTION

Phytochemistry is the study of phytochemicals, which are chemicals derived from plants. Specifically, phytochemistry describes the large number of secondary metabolic compounds found in plants. Many of these are known to provide protection against insect attacks and plant diseases. They also exhibit a number of protective functions for human consumers. Phytochemistry can be considered sub-fields of Botany or Chemistry. Activities can be led in botanical gardens or in the wild with the aid of Ethnobotany. The applications of the discipline can be for Pharmacognosy, or the discovery of new drugs, or as an aid for plant. Phytochemicals exist as long as plants exist but we only know about hundred years about their existence. Medicinal plants are traditionally used all over the world. It is likely that the knowledge of traditional medicine developed through trial and error over many centuries. The Chinese have the oldest medicine system¹.

Today, most new pharmaceuticals are not discovered in plants but are new synthetic creations. Recently there is a renewed interest in the discovery of phytochemicals. This renewed interest is our awareness has already developed many chemicals, which still have to be discovered. New modern laboratory techniques have made it easier to discover and identify new phytochemical. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. They are non-essential nutrients, meaning that they are not required by the human body for sustaining life. It is well-known that plant produce these chemicals to protect themselves but recent research demonstrate that they can also protect humans against diseases. There are more than thousand known phytochemicals. Some of the well-known phytochemicals are lycopene in tomatoes, isoflavones in soy and flavanoids in fruits². The development of modern pharmacognosy took place a simultaneous advancement in the area of Organic chemistry, Biochemistry, Medicinal chemistry, Biosynthesis and modern methods and techniques of analysis like TLC, Paper chromatography, HPLC, UV-Visible, IR, NMR and Mass spectroscopy etc. Thus a wide variety of active principles were isolated from different parts of various plants and established to possess a wide range of pharmacological and antimicrobial activities.

PLANT PROFILE

Botanical information: The apple is a deciduous tree, generally standing 1.8 to 4.6 m (6 to 15 ft) tall in cultivation and up to 12 m (39 ft) in the wild⁴. When cultivated, the size, shape and branch density are determined by rootstock selection and trimming method. The leaves are alternately arranged dark green-colored simple ovals with serrated margins and slightly downy undersides. Blossoms are produced in spring simultaneously with the budding of the leaves, and are produced on spurs and some long shoots. The 3 to 4 cm (1.2 to 1.6 in) flowers are white with a pink tinge that

gradually fades, five petaled, with an inflorescence consisting of a cyme with 4–6 flowers. The central flower of the inflorescence is called the "king bloom"; it opens first, and can develop a larger fruit [6]. The fruit matures in late summer or autumn, and cultivars exist with a wide range of sizes. Commercial growers aim to produce an apple that is 7.0 to 8.3 cm (2.75 to 3.25 in) in diameter, due to market preference. Some consumers, especially those in Japan, prefer a larger apple, while apples below 5.7 cm (2.25 in) are generally used for making juice and have little fresh market value. The skin of ripe apples is generally red, yellow, green, pink, or russeted although many bi- or tri-colored cultivars may be found. The skin may also be wholly or partly russeted i.e. rough and brown. The skin is covered in a protective layer of epicuticular wax. The exocarp (flesh) is generally pale yellowish-white though pink or yellow exocarps also occur.



Fig-1: Flowering plant and plant bearing apples



Fig-2: Apples

Scientific classification

Kingdom:	Plantae
(unranked):	Angiosperms
(unranked):	Eudicots
(unranked):	Rosids
Order:	Rosales
Family:	Rosaceae
Genus:	<i>Malus</i>
Species:	<i>Malusdomestica</i>
Binomial name	
<i>Malusdomestica</i>	

Pharmacological activity of apples [8, 9]

1. Apples might help stave off Alzheimer’s disease: The health benefits of apples include the potential to ward off Alzheimer’s disease. Apples contain quercetin, a powerful antioxidant that protects brain cells from degeneration in rats and might do the same in humans. Dr. RamaniSoundararajan from Dalhousie Medical School and Dr. VasanthaRupasinghe at the Nova Scotia Agricultural College found that the flavonoids in Red Delicious apples had strong neuroprotective effects.

2. Apples can help prevent high blood pressure: There is overwhelming evidence that one-third of all cancer cases and half the incidences of cardiovascular disease and hypertension can be attributed to diet. Because apples are high in potassium, a mineral that helps control blood pressure, they can help reduce the risk of stroke.

3. Apples can protect your heart: University of California-Davis researchers found that apples and apple juice may help slow the oxidation process that is involved in the build up of plaque that leads to heart disease. Participants added only two apples or 12 ounces of apple juice to their diet daily and positive effects were evident after only six weeks.

4. Apples can help reduce cholesterol: A medium apple provides five grams of fibre-more than most cereals. They’re also packed with pectin, a soluble fibre that reduces cholesterol. Pectin prevents cholesterol from building up in the lining of blood vessel walls, thus reducing the risk of atherosclerosis and heart disease.

5. Apples offer protection against colon cancer:When the natural fibre in apples ferments in the colon, it produces chemicals that help fight the formation of cancer cells, according to new research from Germany.

6. Apples can help asthma sufferers: A study completed at Aberdeen University found there is strong evidence that a healthy diet rich in anti-oxidants and vitamins is good for asthma. Researchers found that when children sipped on apple juice their symptoms were relieved. Dr Peter Burney, who

led the project, believes that the ‘phytochemicals’ in apples, such as flavonoids and phenolic acids, help calm inflammation in the airways.

7. Apples provide bone protection: Researchers believe that a flavonoid called phloridzin-found only in apples-may protect post-menopausal women from osteoporosis and may also increase bone density. Boron, another ingredient in apples, also strengthens bones.

8. They May Have Prebiotic Effects and Promote Good Gut Bacteria: Apples contain pectin, a type of fiber that acts as a prebiotic. This means it feeds the good bacteria in your gut. Your small intestine doesn’t absorb fiber during digestion. Instead, it goes to the colon, where it can promote the growth of good bacteria. It also turns into other helpful compounds that circulate back throughout your body. New research suggests this may be the reason for some of the protective effects of apples against obesity, type 2 diabetes and heart disease.

9. Substances in Apples May Help Prevent Cancer: Several studies have shown a link between eating apples and a lower risk of cancer. More specifically, test-tube studies have explored the ways in which the plant compounds in them can combat cancer. One study in women reported that eating apples was linked to lower rates of death from cancer. They may lower cancer risk in several ways, including with antioxidant and anti-inflammatory effect .

10. Apples May Protect Against Stomach Injury From NSAIDs: The class of painkillers known as nonsteroidal anti-inflammatory drugs (NSAIDs) can injure the lining of your stomach. A study in test tubes and rats found that freeze-dried apple extract helped protect stomach cells from injury due to NSAIDs. Chlorogenic acid and catechin are two compounds that were particularly helpful.

11. The Antioxidants in Apples May Help Protect Your Brain in Old Age: Most research focuses on apple peel and flesh. However, apple juice may potentially have benefits for age-related mental decline. In animal studies, juice concentrate reduced harmful reactive oxygen species (ROS) in brain tissue and minimized mental decline. Apple juice may help preserve acetylcholine, a neurotransmitter that can decline with age. Low levels of acetylcholine are linked to Alzheimer’s disease. Researchers who fed elderly rats whole apples found that a marker of the rats’ memory was restored to the level of younger rats. That being said, whole apples contain all the same compounds as apple juice. It is always a healthier choice to eat your fruit whole.

MATERIALS AND METHOD

Chemicals and drugs

Silymarin was used as standard drug and other chemicals used for the extraction and phytochemical screening was provided by Institutional store.

Experimental animals

White male albino Wister rats weighing about 200-250 g was used. They were obtained from the animal house of C.L. BaidMetha College of Pharmacy, Chennai. They were kept under observation for about 7 days before the onset of the experiment to exclude any intercurrent infection, had free access to normal diet and water. The animals were housed in plastic well aerated cages at normal atmospheric temperature (25 ± 5 °C) and normal 12- hour light/dark cycle under hygienic conditions. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of CPCSEA: IAEC/XXIX/01/2017.

Extraction method [10]

Weigh 5 g of peels of applespaste (can be mashed to prepare a paste) transfer into 250 ml of round bottomed flask. Add a mixture of 50 ml of methanol and 60 ml of dichloromethane. Heat the mixture under reflux for 5 min on water bath with frequent shaking. Filter the mixture under suction and transfer the filtrate to a separating funnel. Wash this mixture containing bioactive compounds with three portions of 150 ml each with sodiumchloride solution. Dry the organic layer over anhydrous magnesium sulphate. Filter and evaporate most of the solvent in vacuum without heating and obtained Methanolic Extract of Peels of Apple (ME-PLS-A).

Phytochemical screening [11-13]

Preliminary Phytochemical screening of ME-PLS-A had shown the presence of various bioactive compounds such as carbohydrates, aminoacids and peptides, phytosterols, carotenoids, and polyphenols etc.

Isolation and characterization of bioflavanoidquercetin in ME-PLS-A

Thin layer chromatography (TLC) procedure: The TLC developing was set as twin through chamber were examined in various solvent systems, such as chloroform and methanol in the ratio 7:3, 1:1. The fractions were run on silica gel 60 F254 pre-coated aluminium plate, of 0.2 mm thickness. The optimal solvent for the identification of compound was determined by varying the ratios of solvents for developing the solvent system. Visualization was carried out by dipping the plate in vanillin-sulphuric acid reagent and heating till the colour of the spot appears. Retardation factor (R_f) was calculated using the formula: $R_f = \text{Distance moved by the solute} / \text{Distance moved by the solvent}$.

Infrared (IR) and nuclear magnetic resonance (NMR) analysis: IR spectra were recorded on Bruker Alpha TKBR and ATR spectrophotometer. ^1H and ^{13}C -NMR spectra were run on a Bruker AV NMR instrument equipped with 5 mm ^1H and ^{13}C operating at 500 MHz, respectively with tetramethylsilane (TMS) as an internal standard.

Protocol for the study of acute oral toxicity of ME-PLS-A.

In the present study acute oral toxicity of the ME-PLS-A performed by acute toxic class method according to OECD guideline-423 [14]. In this method the toxicity of extract tested using a step wise procedure, each step using three rat of single sex (female/male).

Evaluation of *in vivo* hepatocytes regenerator potentiality [15]

A total of 25 rats were taken and divided into 5 groups:

Treatment Groups

- (A) Group I: Normal Control Group (only the vehicle (1 ml/kg/day of 1% CMC; p . o.)
- (B) Group II: Negative Control CCl₄ 1 ml / kg (1:1 of CCl₄ in olive oil) I . p.
- (C) Group III: Low Dose Group [CCl₄ 1 ml / kg (1:1 of CCl₄ in olive oil) I . p + ME-PLS-A (150 mg/ kg b. w., p . o.)]. Treatment was given daily for seven days orally.
- (D) Group IV: High Dose Group [CCl₄ 1 ml / kg (1:1 of CCl₄ in olive oil) I . p + ME-PLS-A (300 mg/ kg b. w., p . o.)]
- (E) Group V: Positive Control/Standard Group [CCl₄ 1 mL/kg (1:1 of CCl₄ in olive oil) I . p.+ Standard Silymarin 50 mg/kg orally (p . o.) for 7 days] .

Biochemical analysis: On the 8th day, blood was collected by retro orbital puncture, under mild ether anesthesia after 8 hr fasting. Blood samples were centrifuged at 3000 rpm for 20 mins. Serum was separated and stored at -20⁰C until biochemical estimations. All the biochemical parameters were determined by spectrophotometrically. The Serum samples were analyzed for SGPT, SGOT, ALP, serum bilirubin.

Histopathological analysis: Histopathological study was carried out by Fine needle aspiration biopsy of rat liver (FANB) [16] and the smear was stained by **H and E-stained** technique. The whole assessment was carried out at V.H.S Hospital in Chennai.

RESULTS AND DISCUSSION

Thin layer chromatography

In the TLC mobile phase solvent ratio of chloroform: methanol (1:1) showed R_f value of 0.46 equal to that of standard quercetin.

Fourier transforms infrared (FT-IR) spectrum analysis

The FT-IR spectrum of isolated compound was shown in fig. 2 and their corresponding characteristic peak positions were listed in **table 1**. The broad absorption peak at around 3290 cm⁻¹ was assigned to the OH stretching vibration of phenol. C=O aryl ketonic stretching vibrations are observed at 1668 cm⁻¹. The absorption peaks positioned at 1612 cm⁻¹, 1516 cm⁻¹ and 1429 cm⁻¹ are assigned to the C---C, C=O and C=C aromatic stretching vibrations respectively. OH bending

vibrations of phenols were observed at 1359 cm⁻¹. The absorption peak at 1315 cm⁻¹ and the peaks at the lower frequencies between 950 cm⁻¹ and 600 cm⁻¹ were assigned to the C-H bending vibrations of aromatic hydrocarbons. C-O stretching vibrations of aryl ether and phenols were observed at 1240 cm⁻¹ and 1210 cm⁻¹ respectively. C-CO-C stretching and bending vibrations of ketones were observed at 1163 cm⁻¹, which confirms that the isolated compound is flavonoid quercetin. This result is in good agreement with the previous literature for molecular structure of quercetin²⁰.

Table-1: Peak positions and probable inter-atomic bonds of isolated quercetin

Peak position	Inter-atomic bond
3290.58	O-H stretching vibration of phenol
1668.24	C=O Aryl ketonic stretch
1612.16	C--C Aromatic ring stretch
1516.26	C=O aromatic stretch
1429.54	C=C aromatic stretch
1359.37	O-H bending of phenols
1315.58	C-H bond in Aromatic hydrocarbon
1240.55	C-O stretch of Aryl ether
1210.97	C-O stretch of phenol
1163.60	C-CO-C stretch and bending in ketone
932.70, 815.46, 705.65, 596.88	C-H bending of aromatic hydrocarbons

NMR spectrum of isolated compound

NMR studies were carried out to confirm the positions of proton and carbon binding sites. The isolated compound displayed a better resolved ¹H-NMR spectrum. The ¹H-NMR spectrum of the isolated compound showed aromatic hydrogen groups from 6.18-7.66 ppm and phenolic-OH groups from 9.36-12.48 ppm respectively. The ¹³C-NMR spectrum showed carbonyl group at 176.2 ppm and aromatic carbon group from 93.8-164.3 ppm. The corresponding ¹H NMR and ¹³C NMR peak positions for isolated compound were shown in table 2.

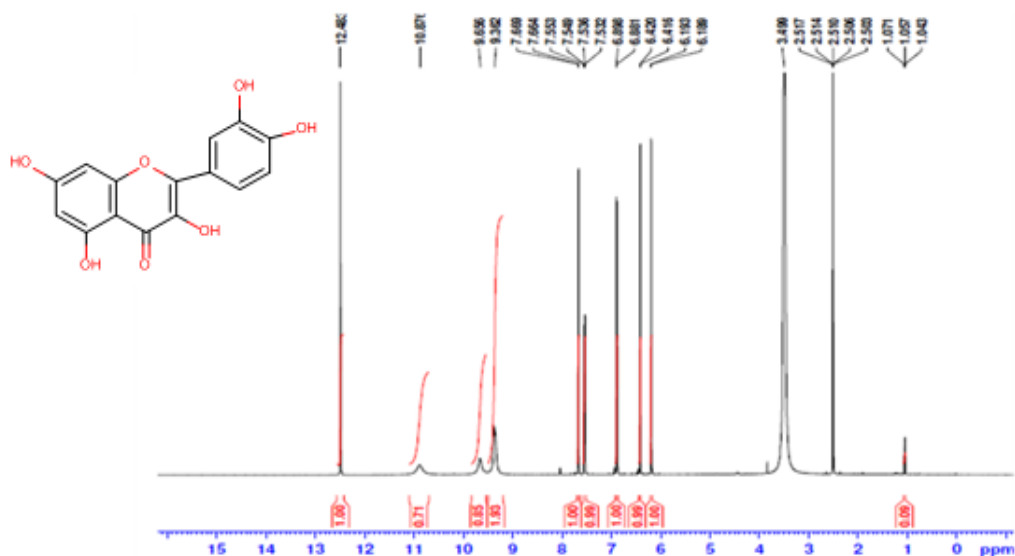


Fig -3-A: ¹H NMR Spectrum of the isolated compound-quercetin

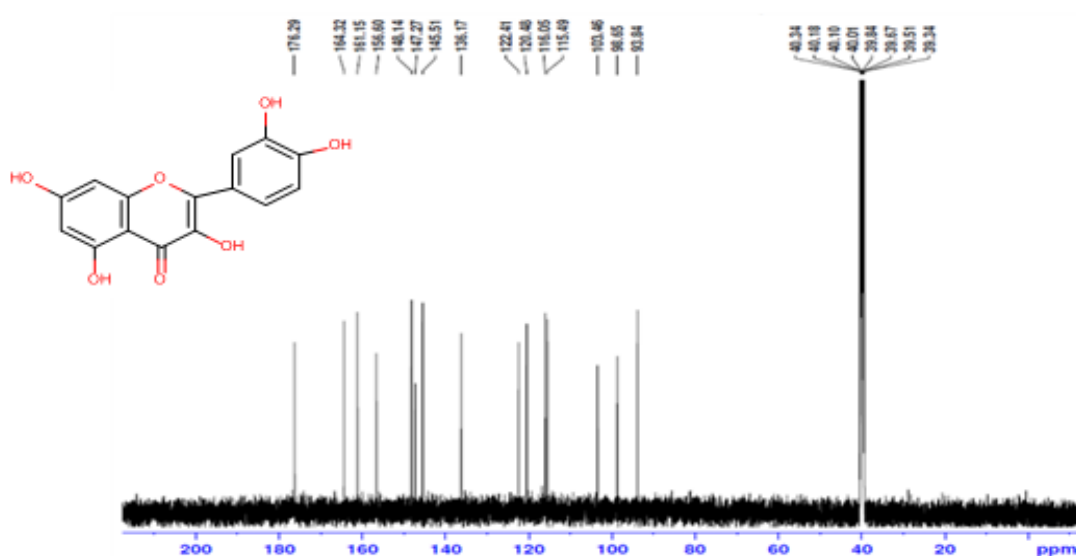


Fig-3-B: ¹³C NMR Spectrum of isolated compound-quercetin

Table-2: ¹H NMR and ¹³C NMR data for isolated compound quercetin

¹ H NMR spectrum of quercetin	¹³ C NMR spectrum of quercetin
6.19 (d, 1H, <i>J</i> = 7.2 Hz, <i>Ar-H</i>)	93.8, 98.6, 103.4, 115.4, 116.0 (<i>Ar-C</i>)
6.41 (d, 1H, <i>J</i> = 6.9 Hz, <i>Ar-H</i>)	120.4, 122.4, 136.1, (<i>Ar-C</i>)
6.88 (d, 1H, <i>J</i> = 5.1 Hz, <i>Ar-H</i>)	145.5, (<i>Ar-C</i>)
7.54 (q, 1H, <i>J</i> = 6.9 Hz, <i>Ar-H</i>)	147.2, (<i>Ar-C</i>)
7.66 (d, 1H, <i>J</i> = 7.4 Hz, <i>Ar-H</i>)	148.1, (<i>Ar-C</i>)
9.36 (s, 2H, <i>Ar-OH</i>)	156.6, (<i>Ar-C</i>)
9.65 (s, 1H, <i>Ar-OH</i>)	161.1, (<i>Ar-C</i>)
10.87 (s, 1H, <i>Ar-OH</i>)	164.3, (<i>Ar-C</i>)
12.48 (s, 1H, <i>Ar-OH</i>)	176.2, (<i>Ar-C=O</i>)

Liquid chromatography/tandem mass spectrometry (LC/MS/ MS identification)

The isolated plant compound was analyzed by LC-MS-MS. It has been successfully applied for a quick separation and identification of the isolated compounds from fenugreek. The chromatogram of the isolated compound. The fragment pattern m/z 302.95 was found in its first order mass spectrum, and it is speculated that they may correspond to the fragment patterns of quercetin. Comparison to the reference substance and a mass spectral library system confirmed that the isolated compound is found to be quercetin.

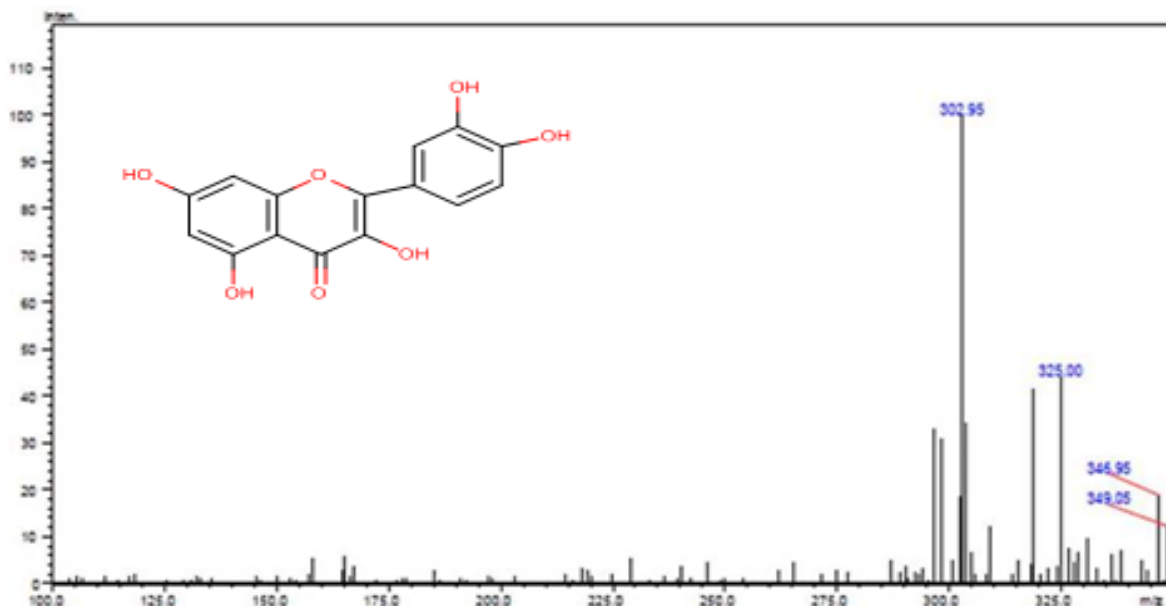


Fig-4: The total ion chromatogram of the isolated compound

Acute toxicity studies

- (i) Acute oral toxicity studies were performed according to the OECD guideline 423 method.
- (ii) This method has been designed to evaluate the substance at the fixed doses and provide information both for hazard assessment and substance to be ranked for hazard classification purposes.
- (iii) The **ME-PLS-A** was administered initially at a dose of 2000mg/kg b.w and 1% CMC (p.o) and observed 14 days mortality due to acute toxicity.
- (iv) Careful observation were made at least thrice a day for the effect on CNS, ANS, motor activity, salivation and other general signs of toxicity were also observed and recorded.
- (v) Since no sign of toxicity observed at 2000 mg/kg b.w. to the group of animals, the **LD₅₀** value of the **ME-PLS-A** expected to exceed 2000 mg/kg b. w. and represented as class 5 (2000 mg/kg < LD₅₀ < 2500 mg/kg).

(vi) From the toxicity studies the data revealed that all the extracts proved to be non toxic at tested dose levels and well tolerated by the experimental animals as there **LD₅₀** cut of values > 2000 mg/kg b. w.

Evaluation of *in vivo* hepatocytes regenerator potentiality

Statistical analysis

The data (Table) were expressed as mean ± SD. Statistical differences at *P < 0.05 between the groups were analyzed by one-way ANOVA followed by Dunnett's Multiple Comparison Test using Graph Pad Prism 5.04 Instate software package. The data's were compared with group II i.e. Negative Control group.

Biochemical analysis

The present study displayed that ME-PLS-A possessed a significant hepatoprotective activity. The declining of plasma enzyme level is a prognostic status of the hepatoprotective action of the drug. Protection of hepatic damage caused by carbon tetrachloride administration was observed by recording SGOT, SGPT, SALP and Serum bilirubin levels in different groups²⁷. The transport function of the hepatocytes is disturbed in hepatic injury, causing the leakage of enzymes due to altered membrane permeability²⁸.

The *in vivo* experimental data displayed that the elevated levels of SGOT, SGPT, ALP and Sr. bilirubin were mainly due to CCl₄ intoxication, reduced significantly (*P<0.05) in rats, after treatment with ME-PLS-A. Treatment with ME-PLS-A at a dose of 150 mg/kg b. w. decreased the SGOT: **6.61%**, SGPT: **22.87%**, ALP: **10.56%**, and Serum bilirubin levels by **42.46%**, (significantly) respectively, while at higher dose of 300 mg/kg b. wt. was more effective, causing a reduction of SGOT: **23.75%**, SGPT: **41.49%**, SALP: **22.56%** and Sr. bilirubin: **55.83%**, Silymarin is used as standard drug showed a significant reduction of level of SGOT: **54.79%**, SGPT: **47.61%**, SALP: **60.39%** and Sr. bilirubin: **78.08%** respectively receiving CCl₄ alone.

Histopathological analysis

The results of FANB of liver cells of rats of control, CCl₄ treated and treated with ME-PLS-A were represented in (Fig-8). FANB of liver cells of rats revealed that the liver treated with CCl₄ showed a high degree of damage characterized by piecemeal necrosis and portal tract necrosis, interface hepatitis due to expanded portal tract by infiltration of lymphocytes, plasma cells and macrophages, fulminant necrosis which is characterized by submassive and massive necrosis.

Table-3: for the assessment of serum biochemical parameters

Treatment Group	AST(SGOT) IU/L	ALT(SGPT) IU/L	ALP(SALP) IU/L	Sr. bilirubin mg/dL
Group I	52.25±0.356	48.11±0.1533	51.95±0.1531	0.7368±0.00673
Group II	202.5±0.3038***	209.4±0.1065***	390.9±1.499***	7.383±0.1014***
Group III (ME-PLS-A, 150 mg)	189.1±0.1533***	161.5±0.09425***	349.9±0.1698***	4.261±0.05351***

Group IV (ME-PLS-A, 300 mg)	154.4±0.07149***	122.5±0.07424***	302.7±0.08570***	3.261±0.05351***
Group V, 50 mg	91.55±0.1502***	109.7±0.1453***	154.8±0.1229***	1.631±0.2323***

Table-4: for the decrease percentage (%) of serum biochemical parameters

Treatment Group	AST(SGOT) %	ALT(SGPT) %	SALP %	Sr. bilirubin %
Group III (ME-PLS-A, 150 mg)	6.61	22.87	10.56	42.46
Group IV (ME-PLS-A, 300 mg)	23.75	41.49	22.56	55.83
Group V, 50 mg	54.9	47.61	60.39	78.08

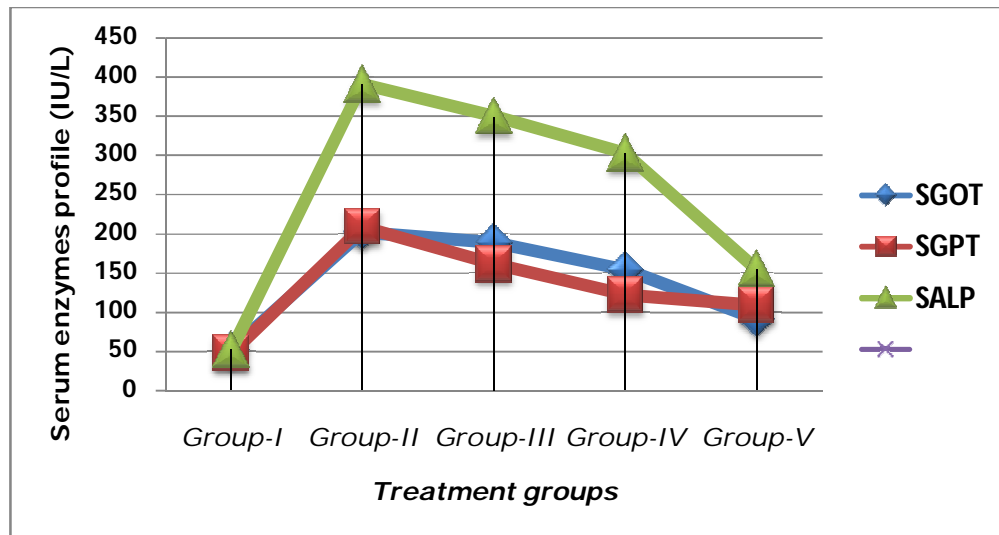


Fig-5: Comparison of serum enzyme levels in different treatment groups

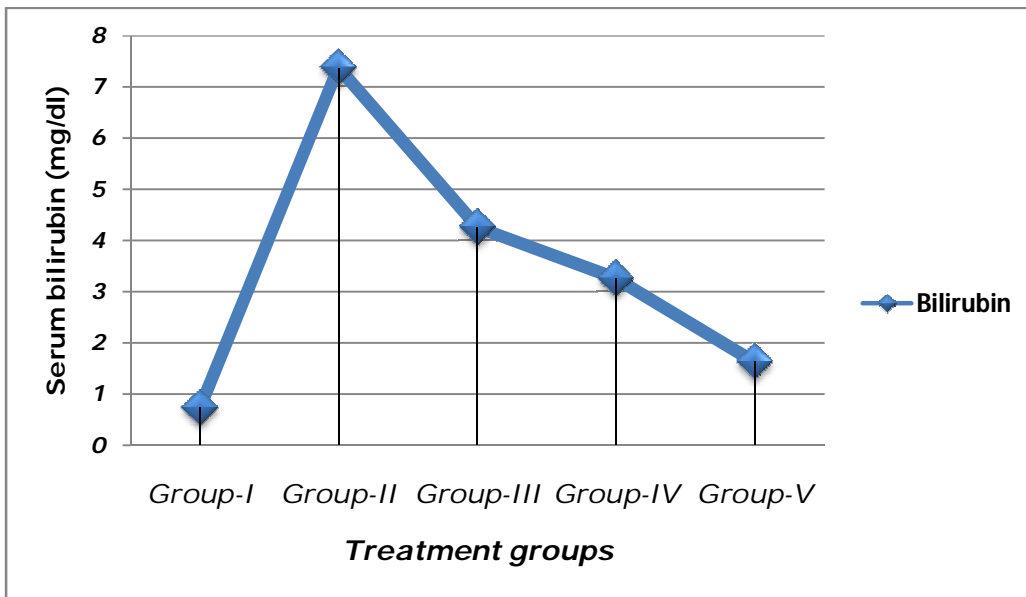


Fig-6: Comparison of serum bilirubin level in different treatment groups

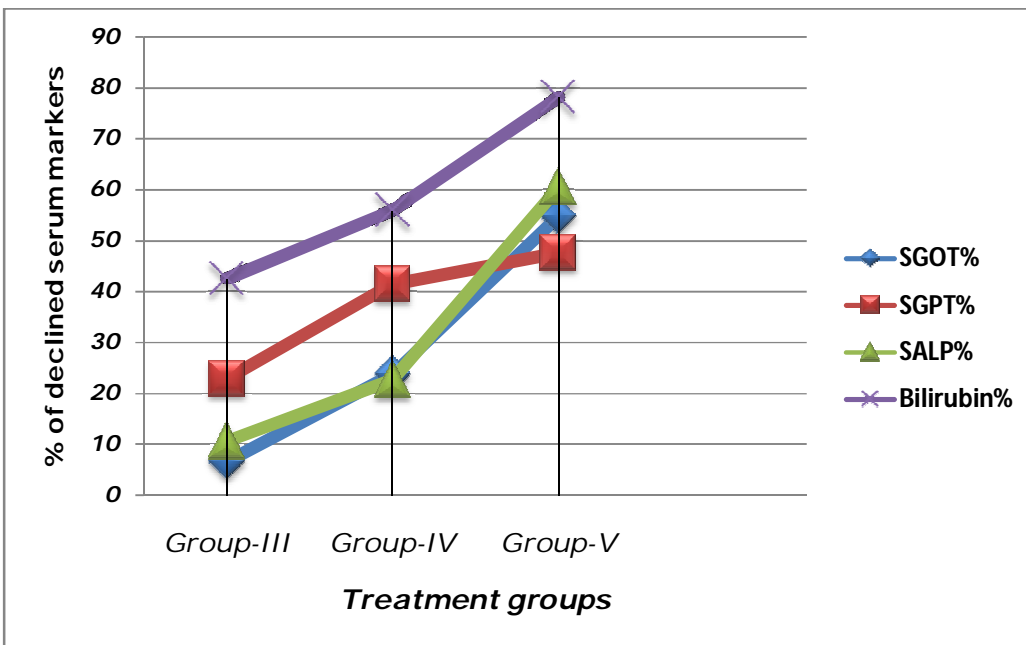


Fig-7: Comparison of % of declined serum biomarkers in different treatment groups

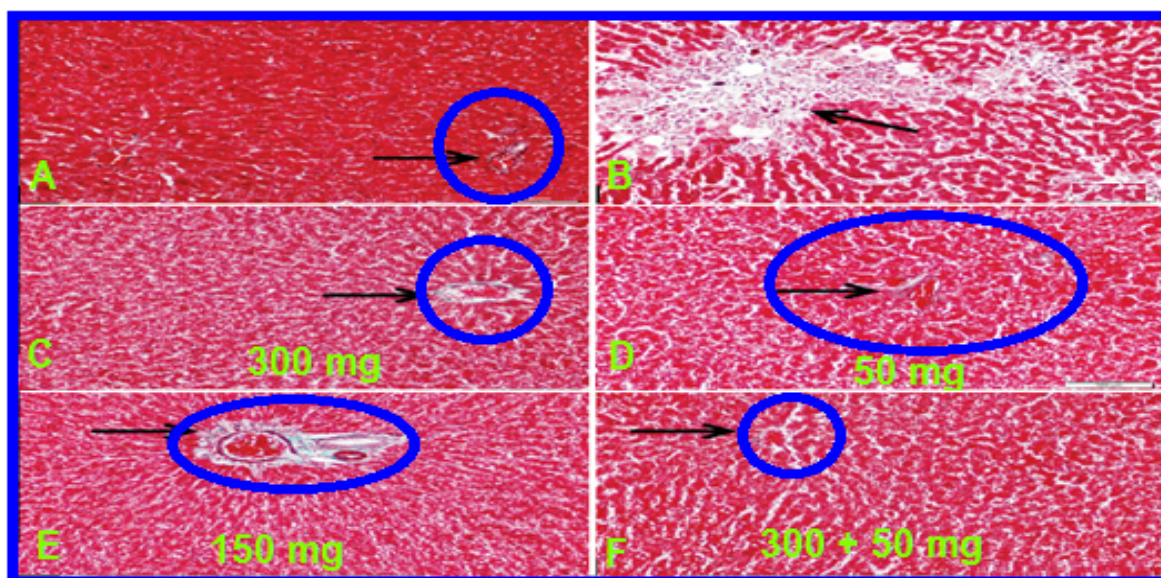


Fig-8:FNAB of the rat liver stained with H and E: (A) control rats, (B) carbon tetrachloride-intoxicated rats showing focal areas with massive degeneration, necrosis and inflammatory cellular infiltration, (C) carbon tetrachloride-intoxicated rats treated with ME-PLS-A alone revealing marked improvement of hepatocellular degeneration, (D) carbon tetrachloride intoxicated rats treated with silymarin showing moderate improvement of hepatocellular degeneration. (E) carbon tetrachloride-intoxicated rats treated with ME-PLS-A displayed few areas of little hepatic cells degeneration and focal areas of cellular infiltration improvement, but still there are scattered areas of degeneration and (F) carbon tetrachloride-intoxicated rats treated with silymarin and ME-PLS-A showing apparently normal liver tissue.

CONCLUSION

From the above experimental data, here we concluded that the ME-PLS-A contained various bioactive molecules which were confirmed by their qualitative confirmatory chemical tests and ME-PLS-A displayed the presence of bioactive flavanoid quercetin which was characterized by IR, ^1H and ^{13}C -NMR, HPLC-MS spectrometry and have the potential ability to restore as well as regenerate hepatocytes which were intoxicated with CCl_4 in rat model. The in vivo hepatocytes regenerator capacity of ME-PLS-A is mainly due to the presence of various bioactive molecules especially bioflavanoid quercetin.

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