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Isolation and Characterization of Secondary Metabolite from *Amorphophallus paeoniifolius* for Hepatoprotective activity

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ABSTRACT

The present study was designed to isolate the flavonoid from the ethylacetate fraction of corm of *Amorphophallus paeoniifolius* by coloum chromatography using gradient elution method. The isolated flavonoid was characterized by spectral studies and screened for hepatoprotective activity on ccl_4 induced model. The flavonoid (Quercetin) was subjected to various biochemical parameters such as SGOT, SGPT, SALP, bilirubin, total protein and histopathology of rat liver were studied. The results were found to be significant by reducing the elevated enzyme levels, increasing the protein level and attenuating the damaged hepatocytes toward the normal texture. The results were further supported by histopathology of isolated rat liver. Therefore the present study justifies that the isolated flavonoid exihibits significant hepatoprotective activity.

Key words: *Amorphophallus paeoniifolius;* Quercetin; CCl_4; Hepatoprotective.

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INTRODUCTION

Hepatitis is one of the most prevalent diseases in the world [1] and drug related hepatotoxicity is the leading cause of acute liver failure among patients referred for liver transplantation because of an intentional or unintentional overdose of drugs in the United States [2]. Despite the fact that hepatic problems are responsible for a significant number of liver transplantations and deaths recorded worldwide [3], available pharmacotherapeutic options for liver diseases are very limited and there is a great demand for the development of new effective drugs.

It has been reported that flavonoid constituents of the plant possess antioxidant properties [4] and was found to be useful in the treatment of liver damage [5]. The earlier investigators have screened for the hepatoprotective property of the flavonoids compound, rutin, isolated from *Artemisia scoparia* , which is also claimed to have free radical scavenging activities against CCl_4 induced hepatotoxicity [6]. Hepatoprotective activity-guided fractionation of the methonolic extract of *Equisetum arvense* L. (Equisetaceae) resulted in the isolation of flavonoids and exhibited hepatoprotective activity [7].

In our previous study, the phytochemical investigations of ethylacetate extract of corm of *Amorphophallus paeoniifolious* revealed the presence of flavonoid by prelimnary test and t.l.c studies, and exhibited antioxidant and hepatoprotective activity[8]. Hence the current protocol is designed for isolatioin and characterization of flavonoids from ethylacetate fraction of *Amorphophallus paeoniifolious* and screened for hepatoprotective activity.

MATERIAL AND METHODS

Preparation of Crude Extract

About 4 kg of corm of *Amorphophallus paeoniifolius* was extracted with 90% ethanol. The obtained crude residue was dried and further fractionated with ethylacetate. The obtained residue was evaporated; dried and total yield was 9.2 gms. The fractionated residue from ethylacetate was used for separation of flavonoid by column chromatography.

Isolation of Flavonoid by Column Chromatography [9].

About 9 gms of ethylacetate fraction was chromatographed over silica gel and elution was carried out from Non-polar to polar solvents by gradient elution method.

1. Adsorbent : Silica gel (60-120 mesh)
2. Activation : 110^{o}C for 1 hour.
3. Length of the column : 45cm
4. Diameter : Outer 4.5 cm. Inner 2.5 cm
5. Length of the adsorbent : 30cm
6. Rate of elution : 10-15 drops/min.

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7. Volume of Elute collected : 10ml each.
8. Type of Elution : Gradient elution.

Preparation of Sample for Column

9 gm of ethyacetate fraction was dissolved in 20ml of methanol and mixed with 2gm of silica gel (60-120 mesh) and dried in vacuum oven at 45 ^{o}C. The material was then transferred to the column.

Gradient elution

Gradient elution was carried out by using Ethyl acetate, Ethylacetate: methanol, methanol:water in different proportion. The elution rate was adjusted to 10-15ml/minute. Different fractions like 1-10, 11-20, 21-50, 51-75, 76-100, 101-150, and 151-200 were eluted. TLC studies were carried out using chloroform: methanol (90:10) using anisaldehyde sulphuric acid as spraying agent for all the fractions. The fractions 1-150 did not exhibit any spots. But in between the 151-200 fraction exhibited single spot with Rf value 0.7 which showed yellow spot under u.v. light at 366nm. Fractions with similar spots were pooled together and concentrated at reduced pressure and temperature. The concentrated component after evaporation revealed yellowish crystalline powder represented in table. 01.

Phytochemical test for isolated flavonoid

1. Shinoda test (Mg-Hcl reduction test): 1mg of isolated compound was dissolved in alcohol, to it few fragments of magnesium ribbon was added drop wise along the tube, which gave pink to red colour indicating flavanoids are present.
2. Zn-Hcl reduction test: To the test solution a mixture of zinc dust was added, few drops of concentrated Hcl was added dropwise along the side of the tube, which showed red colour indicating flvanoids present.
3. Ferric – Chloride test: To the test solution, freshly prepared ferric chloride solution was added, which showed bluish green to black colour.

Characterization of flavonoid Compound

Spectral data (fig.01 & fig.02)

Structures of isolated compound **AP-2** were established based on IR, ^{1}HNMR and mass spectral studies.

Yellow crystals, 200 mg. R_f 0.70, mp. found 314^{o}C, IR spectrum of AP-2 showed characteristic absorption band at 3406 cm^{-1} due to hydroxyl group. Another band at 1609 cm^{-1} attributed to stretching frequency of carbonyl group. The CH=CH stretching peak appeared at 2912 cm^{-1}

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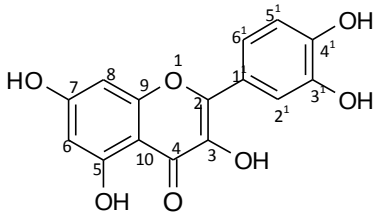
The ^{1}H NMR spectrum revealed the presence of a hydrogen bonded hydroxyl signal with C=O (C_5- OH) at δ 12.48. A singlet at δ 10.77 was assigned to C_3-OH proton. A singlet due to C_7-OH proton resonated at δ 9.58. A broad singlet at δ 9.36 which integrated for two protons was assigned to C_3' and $C_{4'}$ hydroxyl groups. Two singlets at δ 6.18 and δ 6.40 which integrated for a proton each were due to protons present on phenyl ring of flavanoid nucleus (H-6 and H-8). Two doublets at δ 6.88 and δ 7.67 were attributed to H_5' and H_6' protons. A peak due to C_2' proton appeared as singlet at δ 7.67.

The Mass spectrum of AP-2 showed molecular ion peak at m/z at 303.0 which corresponds to its molecular formula (C_{15} H_{10} O_7) and molecular weight.

All the above spectral data were consistent with the earlier reported data [8]. Based on these results the structure of the compound was confirmed as **Quercetin (AP-2)**



AP-2 (Quercetin)

EXPERIMENTAL PROTOCOL

Rats were divided into five groups comprising of 6 animals in each group. Group I served as normal control and received normal saline (5ml/kg p.o) for seven days. Group II was administered with CCl_4 in liquid paraffin (0.7 ml/kg, 1:1, v/v, i.p, on alternate days) [10, 11]. Group III was administered with silymarin (100mg/kg p.o) simultaneously with toxicant [12]. Group IV was treated with Quercetin ((25mg/kg p.o) suspension of quercetin were prepared by using Tween-80 and distilled water (2:8).

Assessment of Hepatoprotective activity

On the seventh day after administration of last dose of Quercetin, the rats were anesthetized by light ether anesthesia and blood was collected by making intra-cardiac puncture. It was allowed to coagulate for 30 min and serum was separated by cold centrifugation at 2500 rpm for 15 min. The centrifugate was used to estimate the serum SGPT, SGOT [13], SALP [14] and total protein [15] content were determined. Finally the rat liver were isolated and subjected for histopathological changes.

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Statistical analysis

The data were expressed as Mean ± S.E.M (n=6). The data were analyzed using one way ANOVA followed by multiple comparison tests. $p<0.01$ were considered statistically significant.

RESULTS

Rats treated with CCl_4 (0.7ml/kg body weight) suffered from hepatotaoxicity. The serum levels of SGPT, SGOT and bilirubin level were significantly elevated and protein level was significantly decreased as shown in Table 02. Pretreatment with Quercetine (25mg/kg body weight) for 7 days significantly decreased enzyme levels and bilirubin level. Meanwhile it showed increase in protein level in the blood, when compared to control and CCl_4 treated group ($p<0.01$) and ($P>0.001$). Results were also comparable with standard drug silymarin (100mg/kg p.o).

Histopathological observations (fig.03)

Histopathology of normal rat liver revealed prominent central vein, normal arrangement of hepatic cells (A). Microscopic examination of CCl_4 treated liver section shows necrosis and fatty degeneration (B). Liver section treated from silymarin protected the structural integrity of hepatocyte cell membrane and recovery of hepatocyte cells (C). Quercetine treated groups showed maximum recovery of hepatocytes, attenuated the fatty degeneration and necrosis and finally exhibited a significant protection against CCl_4 induced liver toxicity. (D).

DISCUSSION AND CONCLUSION

CCl_4 may cause liver damage due to accumulation of fat (fatty liver), inflammation and centrilobular necrosis [16].

The liver damage is caused due to variety of reasons like drugs, toxic chemicals alcohol and viruses. To induce hepatotoxicity a convenient agent should be chosen amongst various toxicants; especially known to cause hepatic damage. The chemical agent of recurring incidences to cause hepatotoxicity is CCl4. [17].

In the present study CCl_4 treated group exhibited significant rise in the enzyme levels of SGOT and SGPT and in bilirubin level and decrease in protein level. The liver damage may be due to necrosis or fatty accumulation in the liver. The enzymes are sensitive to hepatic dysfunction, after liver damage protein levels are decreased due to deficiency. [18, 19]. However Quercetine treated groups showed significant protection against CCl_4 induced liver toxicity by decreasing the enzyme levels and in bilirubin level and increase in the protein level to normal value in the blood.

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The overall results indicate that isolated compound Quercetine from *A. paeoniifolius* play an important role in restoring the disturbed liver function in CCl_4 induced hepatic damage. Results were further supported by histopathology of rat liver.

Table.01 Gradient elution of Flavonoid by Column chromatography

Fraction	Solvents and it's ratio	Color of the elute	TLC solvents ratio	No. of spots & Rf. Values	Yield
01-10	Ethyl acetate	No colour	Chloroform: methanol (90:10)	No spot	--
11-20	Ethylacetate:Methanol (75:25)	No colour	-- do --	No spot	--
21-50	Ethyl acetate: Methanol (50:50)	No colour	-- do --	No spot	--
51-75	Ethylacetate:Methanol (25:75)	No colour	-- do --	No spot	--
76-100	Methanol:Water (75:25)	Yellowish brown	-- do --	No spot	--
101-150	Methanol:Water (50:50)	Light Yellow	-- do --	No spot	--
151-200	Methanol:Water (25:75)	Fluoroscent yellow	-- do --	Single spot (0.7)	200mg

Table 02. Effect of isolated compound of *Amorphophallus paeoniifolius* on CCl_4 induced hepatotoxicity

S.NO	Groups	SGPT (µ/ml)	SGOT (µ/ml)	Serum bilirubin (mg/ml)	Total Protein (mg/ml)
I	Control	127.65 ± 1.25	28.68± 4.48	1.06 ± 0.18	3.485 ± 0.28
II	CCl_4 (0.7ml/kg)i.p	248.3 ± 3.12 *	56.62±3.64 *	3.28 ± 0.13 *	2.590 ± 0.21 *
III	Silymarin (100mg/kg)+CCl_4	140.52±2.08 a	30.56± 0.89 a	1.18 ± 0.08 b	3.110 ± 0.23 a
IV	Quercetine(25mg/kg)+CCl_4	143.10± 1.48 b	34.26± 1.56 a	1.16 ± 0.21 b	3.112±0.28 b

$p<0.01$ against normal control; [a]$p<0.01$ against normal control; [b]$p < 0.001$ against hepatotoxic control.

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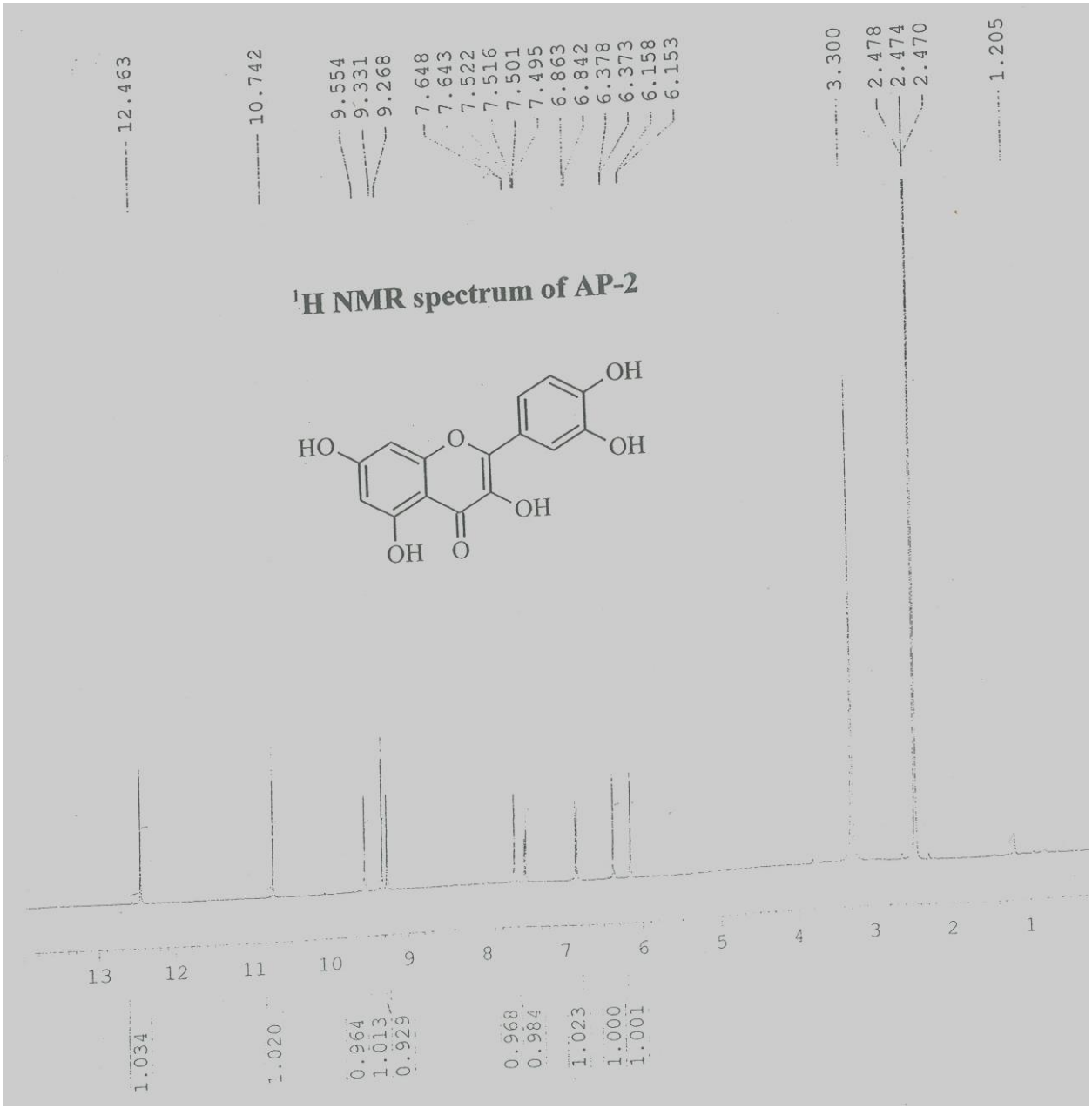

Fig.01

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Fig.02

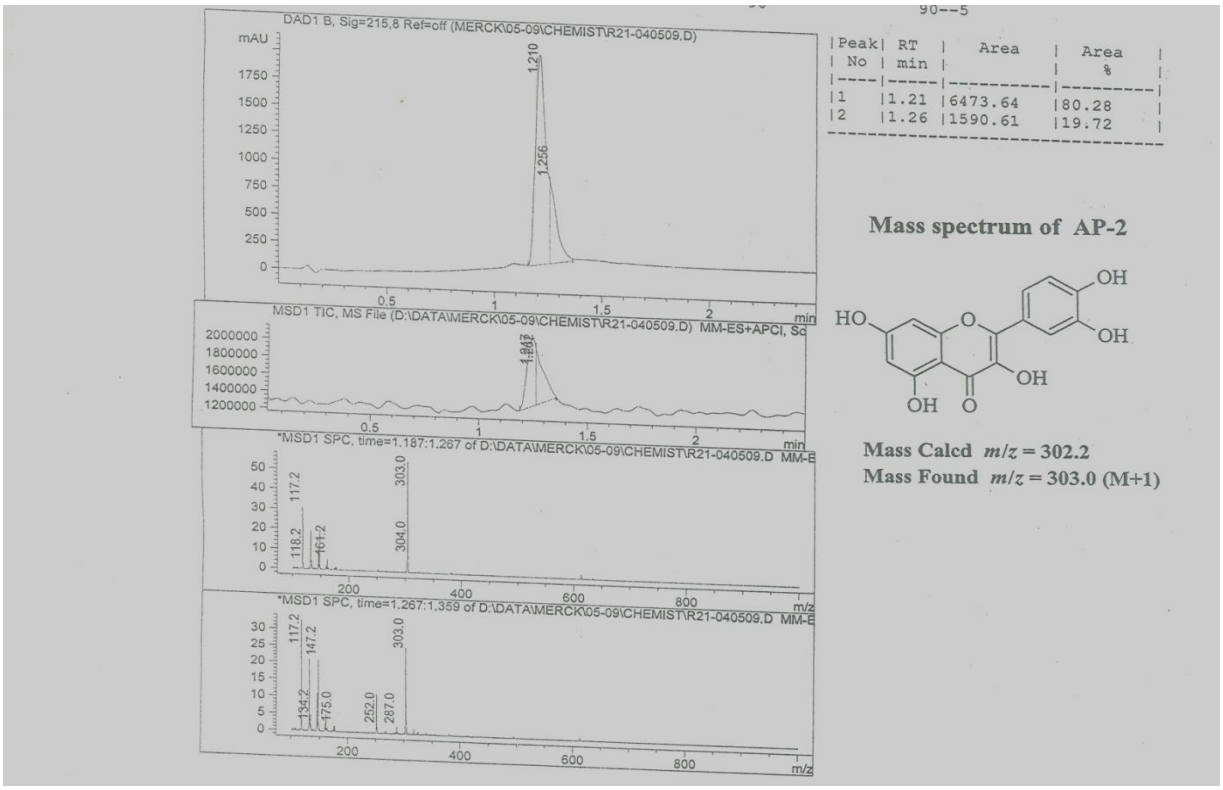
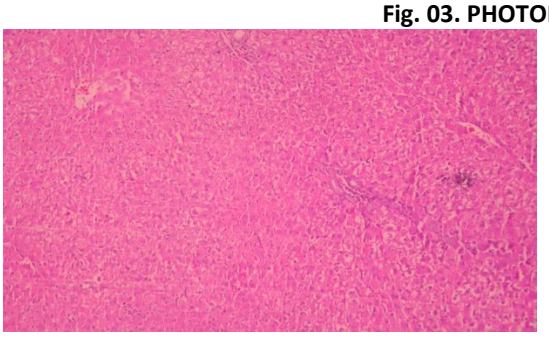
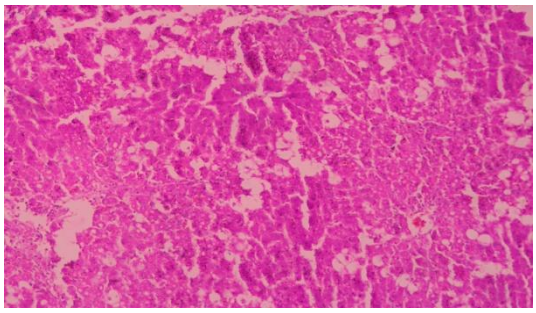


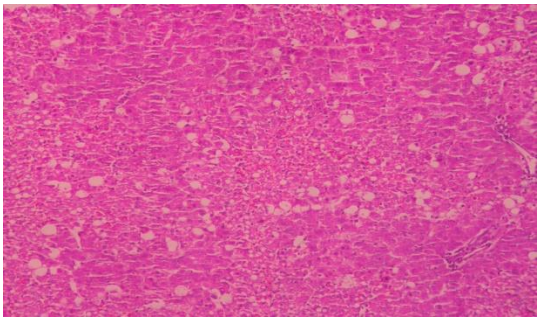
Fig. 03. PHOTOMICROGRAPHS OF RAT LIVER



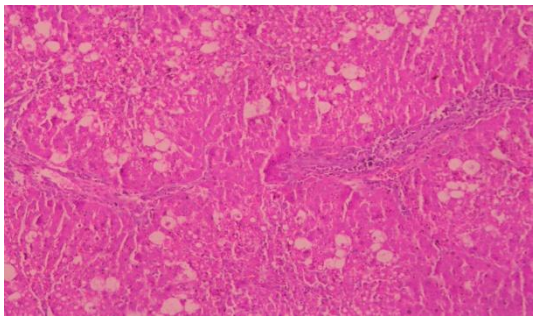
(A) NORMAL CONTROL



(B) CCl_4 TREATED



(C) SILIMARIN TREATED



(D) QUERCITIN TREATED

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