

**ANTIHEPATOTOXIC ACTIVITY OF 5-HYDROXY 3,4', 6,7 - TETRAMETHOXY
FLAVONE FROM *ACHILLEA MILLEFOLIUM***

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Summary

The known flavone , 5- Hydroxy 3,4', 6, 7- tetramethoxy flavone from *Achillea millefolium* L. (*Compositae*) is found to possess significant antihepatotoxic activity against CCl₄ and Paracetamol induced hepatotoxicities (in-vivo) and Thioacetamide and Galactosamine induced hepatotoxicities (in-vitro) in rats . The activity was found comparable with Silymarin (50 mg / kg b. w. i. p.) . The activity of the compound was reported for the first time.

Key Words Antihepatotoxic activity , *Achillea millefolium* , *Compositae* , 5-Hydroxy 3,4',6,7-tetramethoxy flavone , Carbontetrachloride (CCl₄) , Paracetamol (Pcl) , Galactosamine (Galn) and Thioacetamide induced hepatotoxicity , in-vivo , in-vitro.

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The total aqueous extract of aerial parts of *Achillea millefolium* is used in the six of the polyherbal formulations listed for liver ailments¹, still lack any report on systematic screening for antihepatotoxic activity. Preliminary evaluation on antihepatotoxic activity of the drug was encouraging². The active fractions of the total aqueous extract afforded two compounds, Compound I, characterized as 5-hydroxy 3,4',6,7-tetramethoxy flavone while compound II could not be characterized completely.

Different flavanoids like Salvagenine, Artemetrine, 2,5-dihydroxy 3,4',6,7-tetramethoxy flavone, Casticin, 5-Hydroxy 3,4',6,7-tetramethoxy flavone are isolated from the plant. The plant is reported to contain other constituents like amide (N2-methyl propyl (EE) 2,4 decanamide) with mosquito larvicidal activity. Sesquiterpene lactones like Hydroxyachillin, Azulenes and proazulenes like achillin, a mixture of amino acids and sugars with antiinflammatory activity and other compounds like fatty acids viz. Myristic acid, Palmitic acid, Oleic acid, Cetyl alcohol, Tricontine³.

The present communication describes isolation, identification and evaluation of antihepatotoxic activity of compound I from the total aqueous extract of *Achillea millefolium* L. (Compositae).

Materials and Methods

Collection of plant material The dried aerial parts of *Achillea millefolium* were procured from Bombay Market of Crude Drugs. The plant was identified by comparing with the specimen available in the museum of Botany Department of M. S. University of Baroda and Central Drug Research Institute, Lucknow, India, the voucher specimen is deposited in the Pharmacy Department.

Chemicals: The chemicals utilized for the experiments were of AR grade available commercially in India. Silymarin was obtained as a gift sample from Ranbaxy Laboratories, New Delhi, India.

Preparation of extract and isolation of compound: The total aqueous extract was prepared by decoction method (yield 62.78 % w/w). The dried total aqueous extract (100 gm) was separately taken in Distilled water so as to adsorb it on required amount of Silica gel for Column Chromatography and was dried under vacuum. The Silica gel thus adsorbed with the extract was then extracted successively with chloroform (fraction I, yield 6.74 % w/w), acetone (fraction II, yield 22.68 % w/w) and methanol (fraction III, yield 12.67 % w/w). 28.63 % w/w of the remaining water soluble fraction was obtained. Fraction I, 10 gm was suspended in 45 ml of diethyl ether and filtered. The diethyl ether soluble fraction was chromatogrammed on Silica gel G as adsorbent and eluted with Toluene : Dioxane (8 : 2) as mobile phase. The TLC analysis revealed presence of the two spots, spot A, with Rf 0.86, designated as compound I, was detected as black spot after spraying with methanolic ferric chloride. The spot B with Rf 0.76, designated as compound II, was detected as blue fluorescent spot under 365 nm, also appeared as violet colour after spraying with vanillin – sulphuric acid reagent. The compounds were isolated by preparative TLC technique and were subjected to physicochemical and spectral studies. The compound II could not be isolated and identified due to low yield.

The dried fractions, Silymarin and the isolated compound were suspended in 4% w/v acacia mucilage. The suspension of the fractions, Silymarin and the isolated compound were administered to rats intra peritoneally at the doses of 50, 50 and 20 mg/kg body weight respectively.

Test animals: Non fasted Wistar rats of with initial body weight between 150 – 200 gm of either sex were used for the experiments. These were housed in polypropylene cages with temperature of about $24 \pm 2^{\circ}\text{C}$, R_H 40- 70 % and 12 hours light period (6:00 to 18:00 h). The animals were given pellet diet and deionized water *ad libitum*. The animals received human care and the experiments were carried out following the guidelines set by the Institutional Ethical Committee.

Protocols for induction of in – vivo carbontetra chloride hepatotoxicity and treatment⁴: The animals were divided into Control, Carbontetrachloride (CCl_4) and Test groups (CCl_4 + fractions(I-III), Silymarin and isolated compound.) each containing 6 animals in all the sets of experiments. 50% v/v CCl_4 (BDH) solution in olive oil (Olio Sasso, Italy) was used for administration.

Animals from the control group received single daily dose of 4% w/v aqueous acacia solution (1ml/Kg i.p.) on all four days and olive oil (1 ml/kg s.c.) on day 2 and 3.

Animals from CCl_4 group received single daily dose of 4 % w/v aqueous acacia solution (1ml/kg i.p.) for four days and CCl_4 solution 2 ml /kg s.c. o day 2 and 3, 30 min. later administration of aqueous acacia solution.

Animals from the test groups received single daily dose of the fractions I, II and III (50 mg/kg i.p.), Silymarin (50 mg/kg i.p) and isolated compound (20 mg/kg i.p) for four days. The animals were also administered toxicant CCl_4 (2ml/kg s.c) 30 min. later the administration of the test substances.

On the fourth day blood samples were withdrawn by puncturing orbital sinus and the blood samples were allowed to clot for 30-40 min. Serum was separated by centrifuging the clot at 37°C .

Protocols for induction of in – vivo paracetamol hepatotoxicity and treatment⁵ The animals were divided into Control, Paracetamol (Pcl) and Test groups (Pcl + fractions, Silymarin and isolated compound.) each containing 6 animals in all the sets of experiments. Pcl was suspended in 60% w/v aqueous sucrose solution.

Animals from the control group received single daily dose of 4% w/v aqueous acacia solution (1ml/Kg i.p.) on all three days and single dose of 60% w/v sucrose solution (1 ml/kg p.o.) on day 3.

Animals from Pcl group received single daily dose of 4 % w/v aqueous acacia solution (1ml/kg i.p.) for three days and single dose of Pcl suspension 3 gm /kg p.o. on day 3, 60 min. later administration of aqueous acacia solution.

Animals from the test groups received single daily dose of the fractions I, II and III (50 mg/kg i.p.), Silymarin (50 mg/kg i.p) and isolated compound (20 mg/kg i.p) for three days. The animals were also administered toxicant single dose of Pcl suspension (3 gml/kg p.o.) on day three , 60 min. later the administration of the test substances.

On the fifth day blood samples were withdrawn by puncturing orbital sinus and the blood samples were allowed to clot for 30-40 min. Serum was separated by centrifuging the clot at 37°C .

Assessment of Antihepatotoxic Activity : In case of in-vivo experiments the activity was assessed by estimating biochemical parameters . These included Serum Transaminases viz. Glutamyl Pyruvate Transaminase (GPT) and Glutamyl Oxalacetate Transaminases (GOT) using Reitman and Frankel Method⁶ , Alkaline Phosphatase using method reported by Bessay et al.⁷ and Total Bilirubin using Jendrassik's method⁸. The results are presented in Table 1.

Statistical Analysis⁹ : The results of Biochemical analysis are presented as Mean values \pm S.D. and % reduction was calculated by considering the difference between the control and toxicant as 100 % reduction . The statistical significance of the difference was analysed through one way analysis of variance (ANOVA) . The difference between the test group and control was analysed by least significant difference method at P= 0.05 confidence levels .

In –vitro Antihepato toxic Acivity Testing : Compound I (50 mg was dissolved in a mixture of 1ml of Dioxane and 2ml of water by vigorous shaking and the volume was made up to 50 ml with PBS (Phosphate Buffer Saline p H 7.4) (Concentration = 1000 ppm) : Solution A . The solution A was suitably diluted with PBS to get different concentrations viz. 100 and 10 μ g/ml .These concentrations were then tested for antihepatotoxic activity using in-vitro technique against Galactosamine and Thiocetamide induced hepatotoxicities.

Rat hepatocytes were isolated using the modified method of Seglen¹⁰ by circulating enzymatic perfusion. The hepatocytes were divided into three groups with three sets in each and the following protocol was followed.

Group I: Control: 0.1 ml of Hepatocyte suspension + 0.1 ml Vehicle (2 % v/v Dioxane in PBS) + 0.8 ml PBS .

Group II: Toxicant: 0.1 ml Hepatocyte suspension + 0.1 ml Toxicant Solution (20 μ g/ml Thioacetamide and 40 μ g/ml Galactosamine solution in Distilled water.) + 0.8 ml PBS.

Group III: Test: 0.1 ml Hepatocyte suspension + 0.1 ml Toxicant + 0.1 ml Test Substance + 0.7 ml PBS.

In case of in-vitro antihepatotoxic activity testing, assessment of the activity was carried out by determining % viability using Trypan Blue Exclusion test¹¹ and Oxygen uptake using Geilson's Oxygraph¹². The results are presented in Table 2.

Results

Characterization of Compound I

Pale yellow to greenish needle shaped tiny crystals , m.p. 145 – 146⁰C, λ_{\max} (NaOH) 226.5 η m and 286.5 η m, Soluble in Chloroform , Diethyl ether , Dioxane, Sparingly soluble in methanol , insoluble in water , Black coloration with Methanolic ferric chloride solution , Red coloration with Mg / HCl test . C = 63.6 % , H = 4.6 % , O = 31.8 % ; m/e = M⁺ = 328, C₁₉H₁₆O₇ ; FT-IR (Nicolet Instruments Inc. , Model 170, sx Magna 550) : (cm⁻¹) 3375, 1600, 1500-1400, 1300-1000, 1247, 1715, 950-970, Mass (V.G. Micromass,7070 H) m/e = 328, 343, 299, 242, 214, 201, 170; H¹ NMR (Make : Varian , USA, VXR 300S with 5 mm & 10mm probe for solution studies for both H¹ and C¹³ NMR) (ppm) in CDCl₃ :12.68(s), 6.4(d), 7.5(d), 7.76(d), 6.94(d), 3.86(s); C¹³ NMR : 28.87, 55.523, 56.102, 56.304, 60.803,127.158, 158.708, 182.662. The compound was identified as 5-Hydroxy 3,4', 6,7 –tetramethoxy flavone based on the reported data¹³.

Carbon tetrachloride and Paracetamol induced hepatotoxicity

Administration of CCl₄ and Pcl resulted in elevated levels of SGPT, SGOT, Alkp and T.Bil. by three to four folds indicating development of hepatotoxicity. Administration of the different fractions and the total aqueous extract resulted into significant reduction in biochemical parameters in case of groups treated with the total aqueous extract, fraction I and remaining water soluble fraction. The group treated with the fractions II and III could not reduce the elevated biochemical parameters indicating no protection could be offered by the fractions. Pretreatment with the isolated compound, compound I, 5-hydroxy 3, 4',6,7 tetramethoxy flavone, 20 mg/kg b.w.i.p. significantly reduced GPT levels (101.28 and 141.98 %) while GOT levels could be reduced to 99.8 and 110.12 % in, 39.2 & 17.97 % in Alkp and 55.28 & 43.64 % in T. Bil. as compared to CCl₄ and Pcl respectively. (Table 1 and 2)

Table 1 : Effect of Different fractions of the total aqueous extract of *Achillea millefolium* on CCl₄ and Pcl intoxicated rat liver.

Groups	SGPT (U/L) Mean \pm s.d. (% Reduction)	SGOT (U/L) Mean \pm s.d.. (%Reduction)	Alkp (U/L) Mean \pm s.d (%Reduction)	T.Bil(mg/dl) Mean \pm s.d (% Reduction)
Control	65.06 \pm 5.42	110.67 \pm 28.70	43.73 \pm 3.55	0.209 \pm 0.034
CCl ₄	302.47 \pm 17.54	464.84 \pm 43.58	188.72 \pm 12.80	2.035 \pm 0.438
Total Aq.Ext+CCl ₄	65.86 \pm 30.89* (100.89 %)	183.81 \pm 32.78* (79.34 %)	99.74 \pm 6.11* (61.36 %)	0.792 \pm 0.105* (67.70 %)
FI + CCl ₄	60.70 \pm 8.55* (101.83 %)	169.28 \pm 17.38* (83.45 %)	117.60 \pm 47.65** (49.05 %)	0.104 \pm 0.023* (105.65 %)
FII + CCl ₄	84.59 \pm 18.09* (92.62 %)	334.35 \pm 68.47* (36.84 %)	215.35 \pm 53.07 -	0.253 \pm 0.037* (97.5 %)
FIII + CCl ₄	192.03 \pm 58.35* (46.54 %)	420.58 \pm 37.62 (12.49 %)	125.30 \pm 36.34 (43.74 %)	0.320 \pm 0.089* (93.86 %)
Pcl	124.57 \pm 16.43	243.79 \pm 7.19	174.50 \pm 30.5	1.682 \pm 0.083
Total Aq. Ext + Pcl	55.35 \pm 9.86* (116.37 %)	125.05 \pm 11.66* (89.19 %)	59.77 \pm 11.65* (87.73 %)	0.576 \pm 0.074* (75.03 %)
FI + Pcl	59.98 \pm 12.96* (116.37 %)	140.82 \pm 40.38* (77.35 %)	77.32 \pm 16.65* (74.31 %)	0.576 \pm 0.074* (75.03 %)
FII + Pcl	112.65 \pm 16.64 (20.03 %)	213.11 \pm 35.58 (23.05 %)	154.16 \pm 21.30 (15.56 %)	1.232 \pm 0.299*** (30.52 %)
FIII + Pcl	109.97 \pm 6.83 (25.64 %)	239.66 \pm 10.28 (0.31 %)	148.65 \pm 14.20 (19.76 %)	1.180 \pm 0.18*** (34.26 %)
F IV + Pcl	64.68 \pm 14.66* (117.48 %)	98.62 \pm 21.77* (109.13 %)	60.45 \pm 17.77* (87.21 %)	0.948 \pm 0.212* (49.79 %)

No. of rats in each group = 6

P < 0.001; **P < 0.01; *** P < 0.05

Table 2 : Effect of the isolated compound I and Silymarin on biochemical parameters in rats intoxicated with CCl₄ and Pcl.

Groups	SGPT (U/L) Mean \pm s.d. (% Reduction)	SGOT (U/L) Mean \pm s.d.. (%Reduction)	Alkp (U/L) Mean \pm s.d (%Reduction)	T.Bil(mg/dl) Mean \pm s.d (% Reduction)
Control	65.06 \pm 5.42	110.67 \pm 28.70	43.73 \pm 3.55	0.209 \pm 0.034
CCl ₄	302.47 \pm 17.54	464.84 \pm 43.58	188.72 \pm 12.80	2.035 \pm 0.438
Comp. I +CCl ₄	61.97 \pm 3.25* (101.37 %)	113.17 \pm 14.17* (99.29 %)	131.74 \pm 17.8* (39.8 %)	1.025 \pm 0.135* (55.28 %)
Sil. + CCl ₄	78.34 \pm 6.38* (91.40 %)	169.47 \pm 17.87* (83.39 %)	120.67 \pm 8.47* (49.96 %)	0.794 \pm 0.038* (68.08 %)
Pcl	124.57 \pm 16.43	243.79 \pm 7.19	174.50 \pm 30.5	1.682 \pm 0.083
Comp. I + Pcl	40.13 \pm 9.15* (141.95 %)	97.19 \pm 13.04* (110.12 %)	151.0 \pm 18.46* (17.97 %)	0.965 \pm 0.171* (48.64 %)
Sil + Pcl	63.41 \pm 2.67* (102.85 %)	143.21 \pm 12.82* (83.39 %)	106.83 \pm 4.62* (51.74 %)	0.583 \pm 0.023* (74.42 %)

No. of animals in each group = 6; P < 0.001, Sil refers Silymarin

Thiacetamide and Galactosamine induced toxicity in rat hepatocytes

The isolated hepatocytes showed the % viability of 98.05 % and Oxygen uptake of 4.23 μ l / hr/mg of protein. Incubation of rat hepatocytes with Galn (40 μ g /ml) and Th (20 μ g/ml) resulted in 51 and 74.77 % viability in hepatocytes respectively. The Oxygen uptake was reduced by 52.28 and 76.27 % upon incubation with Galn and Th respectively. (Table 3)

Table 3 Effect of 5- Hydroxy 3,4',6,7 –tetramethoxy flavone on rat hepatocytes intoxicated with Th and Galn .

Groups	Conc. (μ g/ml)	% Viability	Oxygen uptake (μ l/hr/mg of protein)
Control	-	98.05 \pm 1.25	4.13 \pm 0.11
Compound	1000	70.83 \pm 3.12	3.12 \pm 0.17
Th	20	24.73 \pm 2.54	0.98 \pm 0.032
Comp.I+ Th	1000	65.69 \pm 2.34**	1.42 \pm 0.074
Comp I+ Th	100	75.69 \pm 3.11*	2.42 \pm 0.028*
CompI + Th	10	90.66 \pm 5.50*	3.48 \pm 0.013*
Galn	40	50.01 \pm 5.03	1.98 \pm 0.053
CompI + Galn	1000	37.93 \pm 3.77	-
CompI+ Galn	100	35.46 \pm 3.13	-
CompI + Galn	10	87.76 \pm 2.93*	3.83 \pm 0.064*

The values of % Viability and Oxygen uptake are mean values of three readings. Comparison of the test group with respective toxicants*P < 0.001, **P < 0.01

Discussion

In order to isolate the active compound(s) from the total aqueous extract it was subjected to fractionation using lower polarity solvents and among the four fractions chloroform (Fraction I) and the remaining water soluble fraction (Fraction IV) were found active. The chloroform soluble fraction was subjected to TLC studies and by using the preparative TLC technique, the two compounds were isolated. Out of the two compounds, compound I, was identified as 5-hydroxy 3,4',6,7 tetramethoxy flavone while compound II could not be characterized completely.

Carbon tetrachloride leads to development of hepatotoxicity due to formation of toxic metabolites (free radicals of CCl₄) which leads to peroxidation of lipids of endoplasmic reticulum¹⁴. Results presented in Table 1 reveal that the pattern of % reduction of GPT, GOT Alkp and T.Bil. of group treated with compound I is similar to the group treated with Silymarin (50 mg/ kg b. w. i. p.). The structural similarity between the two compounds indicate that the compound may be protecting liver from the toxicants in the similar way as Silymarin. The mechanism of action of Silymarin is not clear but it can be proposed that it may be protecting liver against CCl₄ toxicity by inhibiting metabolism of CCl₄.

In case of Pcl treatment, hepatotoxicity is due to metabolism of Paracetamol through 2 electron oxidation process to N-acetyl-p-benzoquinone imine (NAPQI) by Cytochrome 2E1, 1A2, 3A4, and 2A6. The metabolite is detoxified by formation of Glutathione – Paracetamol conjugate. In case of toxic doses hepatic glutathione is decreased as much as 90 %, as a result the metabolite binds to Cysteine group of proteins and forms an adduct. The adduct is reported to be responsible for death of hepatocytes. The death is reported to be due to loss of mitochondrial or nuclear ion balance through increase in Ca⁺² followed by increase in mitochondrial Ca⁺² cycling leading to activation of proteases and endonucleases and consequently breaking of DNA strand. Thus inhibition of mitochondrial respiration has been investigated to be an important mechanism in Paracetamol induced hepatotoxicity¹⁵⁻¹⁹.

Galactosamine gets metabolized and induces hepatotoxicity by lowering the levels uridyl nucleosides (UTP . UDP) which results in inhibition of RNA synthesis and toxicity induced is similar to viral hepatitis in human being²⁰. Compound I being a flavanoidal molecule which is similar to Silymarin, found to offer protection to rat hepatocytes against Galn. The probable mechanism of action may be similar to Silymarin i.e. enhancement of ribosomal RNA synthesis as a result of stimulation of DNA dependent RNA polymerase A through increased incorporation of 3 (H) Uridene phosphate into liver RNA.

In case of treatment with Thioacetamide, hepatotoxicity is developed due to alteration of semi impermeable character of hepatocyte membrane, leading to imbalance in Ca⁺² ions and finally death of hepatocytes²¹. Here the toxicity is counteracted by the compound which may be due to stabilization of hepatocyte membrane. The activity was found comparable to that of activity of Silymarin.

The isolated compound I, 5-hydroxy 3,4',6,7 tetramethoxy flavone, was found to be non toxic to the hepatocytes since no significant differences in the values of % viability and Oxygen uptake was observed upon incubation with the compound alone at highest concentration of 1000 µg/ml and compared to the control group.

Conclusion

The findings thus establish potential medicinal value of the plant *Achillea millefolium* used in indigenous systems of medicines in India and also provide a lead for further detailed investigations on this plant in order to justify the use in polyherbal formulations prescribed in the treatment of liver disorders.

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