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Effects of Flavonoids From *Andrographis Serpyllifolia* Leaves Extract on Carbon Tetrachloride-Induced Hepatotoxicity in Wistar Rats

KEYWORDS: Rutin, free radical, flavonoids, lipid peroxidation, carbon tetrachloride
Thank you

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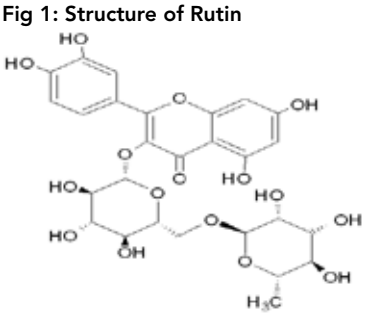
ABSTRACT *The current study investigated the effect of standardized extract of Andrographis serpyllifolia leaves on carbon tetrachloride-induced hepatotoxicity in Wistar rats. Hepatoprotective activity was analyzed by biochemical parameters like serum enzymes, Aspartate transaminase (AST), Alanine transaminase (ALT), Serum Alkaline Phosphatase (ALP) and total serum bilirubin (T.Bil) and antioxidant parameters like Lipid peroxidation (LPO), Superoxide dismutase (SOD), Catalase (CAT), and Reduced glutathione (GSH) against CCl4 induced hepatotoxicity. Treatment with standardized extract at dose level of 125, 250 and 500 mg/kg showed 19.84-47.74%, 27.84-55.86%, and 52.20-74.65% protection respectively with the depletion of AST, ALT ALP and T.Bil in serum, significantly reduced the lipid peroxidation (from 0.62±0.07 to 0.40±0.09) and SOD (from 204.2±13.1 to 101.2±0.8) and increased in levels of catalase (from 30.2±1.2 to 35.4±1.3) and GSH (from 40.5±2.8 to 59.6±3.7). Based on AST, ALT, ALP, Total bilirubin levels and antioxidant estimation, the ethanol extract of Andrographis serpyllifolia showed antihepatotoxic effect as compared to silymarin.*

Introduction

Liver injuries induced by CCl_4 are mediated through the formation of reactive intermediates such as trichloromethyl free radical ($CCl_3^{\bullet}$) and its derivative trichloromethyl peroxy free radical ($CCl3OO^{\bullet}$), generated by cytochrome P_{450} of liver microsomes. These free radicals are thought to react with membrane lipids leading to their peroxidation. As a result fats from the adipose tissue get translocated and accumulated in the liver which finally results in cell necrosis and consequent cell death (Recknagel et al, 1989). Membrane disintegration of hepatocytes with subsequent release of aspartate transaminase (AST), alanine transaminase (ALT), total serum bilirubin (T.Bil) and alkaline phosphatase (ALP) are some of the consequences of CCl_4-induced lipid peroxidation (Singh et al, 2008). The free radical scavenging and inhibition of oxidative stress of flavonoids is due to presence of a phenolic group. Rutin, also called quercetin-3-O-rutinoside, is a bioflavonoid comprised of quercetin and the disaccharide rutinose (α-L-rhamnopyranosyl-(1→6))-β-D-glucopyranose). Rutin possesses antioxidant (Yang et al, 2008; Boyle et al, 2000), antitumor (Deschner et al, 1991), anti-inflammatory (Aleksandrov et al, 1986) and antimutagenic potential (Bear and Teel, 2000), besides myocardial protection (Pozin et al, 1996) and immunomodulating activities (Chen et al, 2000).

Fig 1: Structure of Rutin



IUPAC Name: 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyloxy]-4H-chromen-4-one

Andrographis serpyllifolia (Family:Acanthaceae) is reported against digestive problems, snakebites, fever, cancer, inflammation, wounds (Sekhar et al, 2011), hypolipidemia (Chelluboina and Manga, 2010), jaundice (Manjunatha et al, 2004) and typhoid (Gupta et al, 2014). Ethanolic extract of *A. serpyllifolia* leaves has been found safe for long term administration (Gupta et al, 2014). Therefore, the present study was designed to evaluate the effects of flavonoids from ethanolic extract of *Andrographis serpyllifolia* (ASE) on carbon tetrachloride-induced hepatotoxicity in Wistar rats.

Materials and Methods

Plant Material

Plant leaves were collected from different localities of Tirupati (Chittoor district, Andhra Pradesh, India) and authenticated by the botanist Dr K. Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati-517 502, Andhra Pradesh, India. A voucher specimen (Herbarium Accession No.136) was deposited in the herbarium for future reference.

Preparation of Extract

Shade-dried leaves of *Andrographis serpyllifolia* were powdered and extracted using 70% ethanol in a soxhlet extractor. The extracts were filtered and then concentrated under reduced pressure in a rotavapour (Buchi R-200 USA) at 40^0C and then freeze-dried in lyophilizer (Labconco, USA) to obtain solid residue (ASE, yield 20.0% w/w).

Phytochemical Screening and HPTLC Analysis

The ethanolic extract of *A. serpyllifolia* leaves (ASE) were analyzed for presence of alkaloids, steroids and terpenoids, saponins, flavonoids, tannins and phenolic compounds as described by Trease and Evans, 1989 and Harborne, 1993.

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HPTLC analysis was processed on pre-activated (1000C) Aluchrosep silica gel 60F254 HPTLC plates (S.D.fine-chem Ltd, Mumbai, India) along with quercetin and rutin plates were eluted in solvent system toluene : ethyl acetate : formic acid (5:4:1) for phenols. After development, the plates were dried and densitometrically scanned at wavelength 366 nm (WinCats software, CAMAG, Switzerland).

Animals

Sprague-Dawley rats (100-150g) of either sex were purchased from the animal house of the National Laboratory Animal Centre, Lucknow, India. They were kept under controlled conditions of temperature 27 ± 2^{0}C and relative humidity 44-56%, light/dark cycles of 12 hours respectively for one week before and during the experiments. Animals were provided with standard rodent pellet diet (Amrut, India) and the food was withdrawn 18-24 h before the experiment though water was allowed ad libitum. All experiments were performed in the morning accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmerman, 1983). The protocols were approved by Institutional Committee for Ethical use of Animals and Review Board (106/IAEC/RB/7-11).

CCl_4 induced hepatotoxicity

The animals were divided into seven groups, each consisting of 6 animals. Group I (control) was received a single daily dose of carboxymethyl cellulose (1ml of 1%, w/v, p.o. body weight). Group II received CCl_4 (1ml/kg body weight, i.p., 1:1 v/v mixture of CCl_4 and olive oil) alone while group III-V, VI and VII were administered orally (125-500mg/kg), rutin (100mg/kg) and silymarin (50mg/kg) respectively along with carbon tetrachloride as in group II. The ASE, rutin and silymarin were given daily while carbon tetrachloride was given for every 72h for 14 days. Animals were sacrificed 48h after the last dose of the drug. The liver samples were dissected and blood was collected (Rao et al, 2006).

Assessment of hepatoprotective activity

The collected blood were allowed to clot and serum was separated at 2500 rpm for 15 min and analyzed for biochemical parameters like serum enzymes, Aspartate transaminase (AST), Alanine transaminase (ALT) (Reitman and Frankel, 1957), Serum Alkaline Phosphatase (ALP) (King and Armstrong, 1934) and total serum bilirubin (T.Bil) (Jendrassik and Grof, 1938). The percentage protection was calculated as 100 × (values of CCl_4 - values of sample) / (values of CCl_4 - values of control).

Assessment of antioxidant parameters

Lipid peroxidation (LPO) was estimated by standard method of Okhawa et al, 1979 and expressed as nmol of malonialdehyde(MDA) formed/min/mg protein. Superoxide dismutase (SOD) activity was estimated by the inhibition of nicotinamide adenine dinuleotide (reduced)-phenazine methosulphate-nitrobluetetrazolium reaction system as adapted by Kakkar et a, 1984 and the results were expressed as units (U) of SOD activity/mg protein. Catalase (CAT) was estimated by method of Aebi, 1974 and results were expressed as µmol of H_2O_2 consumed/min/mg protein. Reduced glutathione (GSH) was determined according to the method of Ellmann, 1959 and expressed as nmol/g protein.

Statistical Analysis

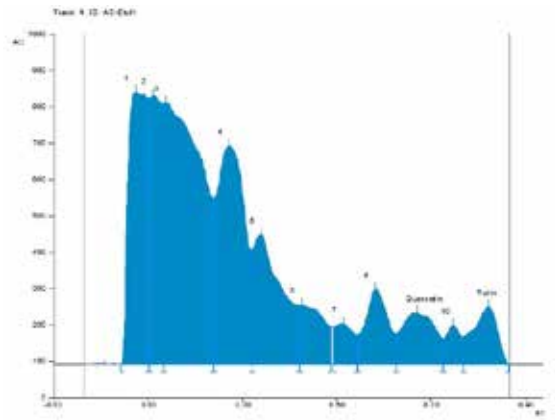
The data are expressed as mean±S.E.M. The difference among means has been analyzed by student's t-test, method of Woolson and Clarke, 1987. A value of P<0.05 was considered statistically significant.

Results

Phytochemical Screening and HPTLC

Phytochemical results showed the presence of alkaloids, carbohydrates, steroids, flavonoids, tannins and phenolic compounds. Quantitative HPTLC determination showed the presence of 0.08196% w/w of quercetin and 0.20461% w/w of rutin (a flavonoid) in ethanolic extract of *A. serpyllifolia* leaves (Fig 2).

Fig 2: HPTLC finger print profile of ethanolic extract of *A. serpyllifolia leaves*



Effect of ASE on AST, ALT, ALP and T.Bil levels

Result in table 1 showed that CCl_4 induced a significant increase in serum level of liver enzymes at the end of the experiment. Rutin, silymarin and test doses showed significant percentage protection with the depletion of AST, ALT ALP and T.Bil in serum as was raised by the induction of CCl_4. Therefore, ethanol extract of *A. serpyllifolia* (ASE) restored the altered level of enzymes significantly. ASE at dose level of 125, 250 and 500 mg/kg showed 19.84-47.74%, 27.84-55.86%, and 52.20-74.65% protection respectively.

Table 1: The effect of *A. serpyllifolia* on CCl_4-induced alterations in AST, ALT, ALP and T.Bil levels in rats

Treatment	AST (U/L)	ALT (U/L)	ALP (U/L)	T.Bil (mg/dl)
Control	96.66±1.67	56.64±0.40	212.52±1.39	0.171±0.10
CCl_4 (1ml/kg)	190.50±1.89^y	140.62±0.31^y	403.09±2.20^y	0.282±0.05^y
ASE (125mg/kg) + CCl_4	162.50±0.72^a	102.23±0.27^a	365.29±0.20^b	0.229±0.03
ASE (250mg/kg) + CCl_4	155.21±0.59^b	94.11±0.30^b	350.04±0.52^b	0.220±0.10^a
ASE (500mg/kg) + CCl_4	141.51±0.25^c	86.82±0.61^c	321.29±0.71^c	0.201±0.00^b
Rutin (100mg/kg) + CCl_4	101.71±1.56^c	60.35±0.21^c	234.02±3.12^c	0.182±0.13^c
Silymarin (50mg/kg) + CCl_4	97.52±1.51^c	57.53±0.72^c	230.12±0.32^c	0.190±0.04^c

Values are mean ± SEM for six rats, P: ^{y}<0.001 compared to respective control group, P: ^{a}<0.05, ^{b}<0.01 and ^{c}<0.001 compared to respective CCl_4 group

Estimation of LPO, SOD, CAT and GSH

Treatment with ASE at dose of 125-500 mg/kg significantly reduced the lipid peroxidation (from 0.62±0.07 to 0.40±0.09) and SOD (from 204.2±13.1 to 101.2±0.8) and increased in levels of catalase (from 30.2±1.2 to 35.4±1.3) and GSH (from 40.5±2.8 to 59.6±3.7). Rutin and Silymarin showed significant inhibition in lipid peroxidation (0.39±0.02 & 0.42±0.06) and SOD (103.1±9.5 & 105.6±8.8) and enhanced the activities of catalase (33.1±1.2 & 34.9±1.4) and GSH (59.4±3.5

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& 55.5±3.3) activity as compared to CCl_4 induced group II (Table 2).

Table 2: The effect of *A. serpyllifolia* on LPO, SOD, CAT and GSH against CCl_4-induced hepatotoxicity

Treatment	LPO	CAT	SOD	GSH
Control	0.38±0.05	36.6±2.8	91.7±5.3	58.1±2.4
CCl_4 (1ml/kg)	0.68±0.05^x	25.4±1.6^x	246.7±6.2^y	38.1±3.6^y
ASE (125mg/kg) + CCl_4	0.62±0.07	30.2±1.2	204.2±13.1^a	40.5±2.8
ASE (250mg/kg) + CCl_4	0.50 ±0.02^b	31.1±1.2^a	165.1±10.2^b	55.9±3.1^b
ASE (500mg/kg) + CCl_4	0.40±0.09^c	35.4±1.3^b	101.2±0.8^c	59.6±3.7^c
Rutin (100mg/kg) + CCl_4	0.39±0.02^c	33.1±1.2^b	103.1±9.5^c	59.4±3.5^c
Silymarin (50mg/kg) + CCl_4	0.42±0.06^c	34.9±1.4^c	105.6±8.8^c	55.5±3.3^c

Values are mean ± SEM for six rats, P:^{x}<0.05 and ^{y}<0.001 compared to respective control group, P: ^{a}<0.05, ^{b}<0.01 and ^{c}<0.001 compared to respective CCl_4 group

Discussion

The present studies were performed to assess the hepatoprotective activity in Wistar rats, against Carbon tetrachloride as hepatotoxin. Liver has antioxidant defense system against free radical but excessive generation of free radicals leads to cell damage by lipid peroxidation resulting in liver injury (Kumar et al, 2008; Deshwal et al, 2011). It is well established that CCl_4 induces toxicity in liver cells maintaining semi-normal metabolic function. CCl_4 is bio-transformed by the cytochrome P_{450} system in the endoplasmic reticulum (ER) to produce metabolite, trichloromethyl free radical ($CCl_3^{\bullet}$) (Kshirsagar et al, 2009). Trichloromethyl free radical then combined with cellular lipids and proteins in the presence of oxygen to form a trichloromethyl peroxyl free radical ($CCl_3OO^{\bullet}$), which may attack on the membrane lipids of ER faster than trichloromethyl free radical. Thus, trichloromethyl peroxyl free radical leads to elicit lipid peroxidation and destruction of Ca^{2+} homeostasis and finally, results in cell death (Clawson et al, 1989; Reckengel et al, 1989). These changes of structures of ER and other membrane, loss of enzyme metabolic enzyme activation, reduction of protein synthesis and loss of glucose-6-phosphatase activation, lead to liver damage (Wolf, 1999; Azri et al, 1992). Among xenobiotics, CCl_4 are known to cause marked elevation in serum enzyme activities. In addition, elevated levels of hepatic serum enzymes are indicative of cellular leakage (Clawson et al, 1989). In present study, CCl_4 induced severe liver damage as evidenced by the significant elevation of serum levels of ALT, AST, ALP and T.Bil that indicates the severity of hepatocellular injury (Poli, 2000). Estimating the activities of serum marker enzymes, like AST, ALT, ALP can make the assessment of liver function when liver cell plasma membrane is damaged, a variety of enzyme normally located in the cytosol are released into the blood stream. Their estimation in the serum is a useful quantitative marker of the extent and type of hepato cellular damage (Mitra et al, 1998).

In accordance with phytochemical test results, it may be confirmed due to the presence of flavonoids in the ethanol extract of *A. serpyllifolia* could be considered as, responisible for the significant hepatoprotective activity. Treatment with extracts of *A. serpyllifolia* attenuated the increases in the activities of AST, ALT, ALP and T.Bil produced by CCl_4 indicating that extract of *A. serpyllifolia* protect liver injury induced by CCl_4 towards normalization. It has been hypothesized that one of the principal causes of CCl_4-induced liver injury is lipid peroxidation by free radical derivatives of CCl_4 ($CCl_3OO^{\bullet}$). The ethanol fraction showed better activity compared with other fractions.

Conclusion

On the whole, it can be concluded that the altered biochemical profiles due to CCl_4 exposure is reversed towards normalization by the extract of *Andrographis serpyllifolia*. Based on AST, ALT, ALP, Total bilirubin levels and antioxidant estimation the extract of *Andrographis serpyllifolia* showed antihepatotoxic effect as compared to silymarin.

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