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HEPATOPROTECTIVE ACTIVITY OF *BALIOSPERMUM AXILLARE* ROOT ON EXPERIMENTAL LIVER INJURY IN RATS

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ABSTRACT:

The present experimental study was to evaluate the methanol extract of root of *B. axillare* for hepatoprotective activity against carbon tetrachloride-induced hepatotoxicity in experimental rats. *B. axillare* root extract exhibited significant ($P \leq 0.001$) hepatoprotective activity by reducing carbon tetrachloride-induced change in biochemical parameters that was evident by antioxidants and enzymatic examinations. The plant root extract may interfere with free radical formation, oxidative stress and lipid peroxidation, which may conclude as hepatoprotective agent. The results were comparable with the standard drug silymarin. From this extract, the active constituents-stigmasterol, ceryl alcohol, octacosanol-1, β–sitosterol, betulin, betulinic acid and lupeol were isolated by IR, ^{1}H NMR, ^{13}C NMR and MS studies. Chronic toxicological studies revealed that the LD_{50} value is more than the dose of 3g/kg body weight.

KEY WORDS: *Antioxidants; Baliospermum axillare; carbon tetrachloride, Markar enzymes.*

INTRODUCTION:

Baliospermum axillare Blume (Euphorbiaceae) is a stout leafy undershrub,

native to Dehra Dun (India) and grows in shady places along the Sub-Himalayan forest tracts, where it often forms a considerable portion of the under growth. It is well known for "Jaundice" in the indigenous system of medicine and commonly sold as 'Dantimul' by drug-dealers. The roots of this plant are purgative, often used in combination with aromatics, in constipation with flatulence and in anasarca and Jaundice. Dose is about a 'Tola' every morning in flatulence and retained secretions, anasarca and Jaundice. Roots and leaves have similar properties and are used in the indigenous medicine in dropsy and general anasarca (Husain et al., 1980; Chopra et al., 1999). The hepatoprotective activity through antioxidant status of root extract of this plant are not experimentally established. Free radical generation and lipid peroxidation of hepatocellular membrane are often implicated as positive factors for the onset of carbon tetrachloride (CCl_4) induced hapatocellular damage (Recknagel, 1983; Slater, 1984). Antioxidants play a crucial role in hepatoprotective ability and hence, search for crude drugs of plant origin with this property has become a central focus on hepatoprotection today. Therefore, the present study was carried out to determine the hepatoprotective activity of *Baliospermum axillare* root extract on experimental liver injury in rats.

MATERIALS AND METHODS:

Animals

, male, wistar strain, albino rats weighing 150-170 were used. The animals were housed in standard laboratory conditions and maintained on rat diet (Lipton India Ltd.) and tap water ad libitum under a natural light dark cycle.

Plant material

The roots of *B. axillare* have been taken from an Ayurvedic shop (drug dealers), Chhoti Chopad Market, Jaipur, Rajasthan (India) and authenticated by Dr. N.J. Sarana, Associate Professor, Department of Botany, University of Rajasthan, Jaipur, India.

Extraction and isolation of active compounds

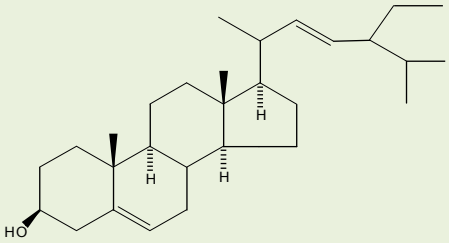
Shade dried roots of *B. axillare* have been powdered, packed in a Soxhlet apparatus and extracted exhaustively with methanol for 48 hours at 58°C to 60°C temperature. The extract was concentrated and dried using a rotary flash evaporator to give solid residue. The solid mass was then powdered, washed with petroleum ether to remove the fatty portions. Half of the extract was used for oral experimentation and rest of the part was then fractionated into chloroform-soluble, acetone-soluble and methanol soluble parts.

The methanolic extract so obtained (30 gms) was subjected to column chromatography. For this purpose, a column filled with Si-gel (600 gm). The purity of fractions was checked by qualitative thin-layer chromatography and HPLC using different solvent systems. After ascertaining the purity of

compounds, it was subjected for detailed spectral analysis (IR, ^{1}H NMR, ^{13}C NMR and MS) to establish the structure.

The following compounds were isolated by eluting the column with petroleum ether : benzene, benzene, benzene : ethyl acetate at different concentrations and melting points (M.P.) yielded : stigmasterol (I), ceryl alcohol (II), octacosanol-I (III), β-sitosterol (IV), betulin (V), betulinic acid (VI), and Lupeol (VII).

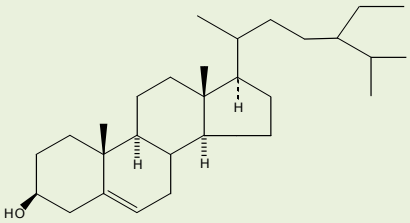
I. Stigmasterol($C_{29}H_{48}O$)
(M.P. 166°C-167°C)



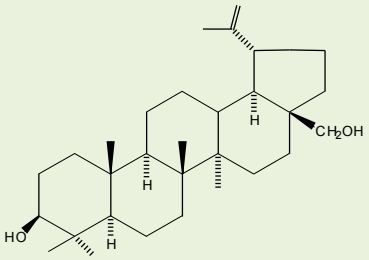
II. Ceryl alcohol($C_{26}H_{54}O$) $CH_3–(CH_2)_{24}–CH_2–OH$
(M.P. 80°C-81°C)

III. Octacosanol-I($C_{28}H_{58}O$) $CH_3–(CH_2)_{26}–CH_2–OH$
(M.P. 85°C-86°C)

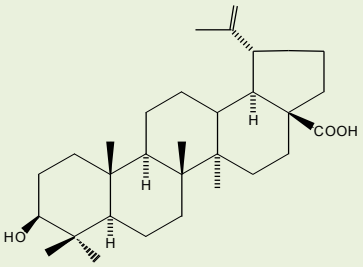
IV. □-sitosterol($C_{29}H_{50}O$)
(M.P. 136°C-137°C)



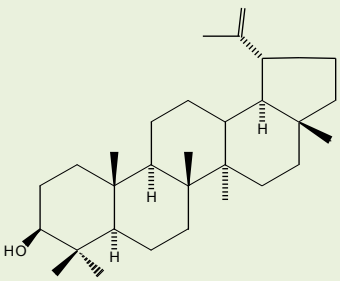
V. Betulin($C_{30}H_{50}O_2$)
(M.P. 254°C-256°C)



VI. Betulinic acid($C_{30}H_{48}O_3$)
(M.P. 316°C-318°C)



VII. Lupeol($C_{30}H_{50}O$)
(M.P. 215°C-216°C)



Chemicals

All chemicals and reagents have been used of analytical grade and obtained from Sigma Chemicals Company (St. Louis, MO, USA). The kits of SGOT (batch no. 61105), SGPT (batch no. 60805), GGT (batch no. 34004) were purchased from Accurex Biomedical Pvt. Ltd., Mumbai, India and LDH (lot no. 6854), ALP (lot no. 7093), total bilirubin (lot no. 6801) and total protein (lot no. 6808) were purchased from Span Diagnostics Ltd., Surat, India. The standard drug silymarin was purchased from Metro Scientific Chemicals, Jaipur, India.

Chronic toxicity of *B. axillare* root extract

The root extract was administered to the test groups in graded doses ranging upto 3g/kg body wt. and the rats were observed for signs of toxicity and mortality for 15 days afterward. The extract was found to be a practically non-toxic when given orally to rats and its LD_{50} value was found to be higher than 3g/kg body wt. The minimum dose levels viz. 50 and 100 mg/kg body wt. were used for the experimentation (Porchezhian and Ansari, 2005).

Experimental design

After acclimatization, the animals were divided in to following groups of 6 rats each :

Group I : Untreated rats were kept on normal diet and served as control.

Group II : Rats were intoxicated with carbon tetrachloride (1 ml/kg b wt/once a week, IP with olive oil, 1:1) for 15 days.

Group III : Rats received 50 mg/kg b wt/day, PO with olive oil of *B. axillare* root extract and CCl_4 as group II, for 15 days.

Group IV : Rats received 100 mg/kg b wt/day, PO with olive oil of *B. axillare* root extract and CCl_4 as group II, for 15 days.

Group V : Rats received 25 mg/kg b wt/day, PO with olive oil of Silymarin and CCl_4 as group II, for 15 days.

Biochemical analysis

After the 24 hours of last dose delivery, all rats of each treated group were anesthesized with ether and blood was collected by cardiac puncture. Serum was separated by centrifugation at 2500 rpm at 37°C for 20 min and analysed for SGOT, SGPT, ALP, GGT, LDH, total bilirubin and total protein, using diagnostic kits. After the collection of blood, liver was immediately excised, washed with cold saline, blotted, minced and homogenized for SOD (Marklund and Marklund, 1974), CAT (Aebi, 1984), GSH (Moron et al., 1979), GPx (Paglia and Velentine, 1967), LPO (Ohkawa et al., 1979) determination. Afterthat, a liver microsomal fraction was prepared (Schneider and Hogeboom, 1950) and the cytochrome *P*-450 enzyme content in this fraction was measured from a reduced carbon monoxide difference spectrum (Omura and Sato, 1964), respectively.

Ethical aspects

The study was approved by the ethical committee of the University Department of Zooolgy, Jaipur, India. Indian National Science Academy, New Delhi, (INSA, 2000) guidelines were followed for maintenance and use of the experimental animals.

Statistical analysis

All the results were expressed as mean ± SEM and analysed using the students *t*-test. A probability value of $P \leq 0.05$ was considered as significant.

RESULTS:

The results of the biochemical parameters revealed that the administration of CCl_4 to rats caused significant ($P \leq 0.001$) liver injury as evidenced by markar enzymes and antioxidant defense system through liver and serum contents (Table I and II).

Table I depicts that the activities of hepatic-antioxidants such as SOD, CAT, GSH, GPx and enzyme-cytochrome *P*-450 were decline significantly ($P \leq 0.001$) alongwith significant ($P \leq 0.001$) elevation of lipid peroxidation, upon CCl_4 administration alone to rats (group II) when compared with group I (vehicle control). These altered hepatic levels were dose-dependently, brought about towards normalization by *B. axillare* root extract against CCl_4-induced alterations in the hepatic antioxidants and enzyme levels. The degree of protection was observed statistically similar in both silymarin (Group V) and higher dose (100 mg/kg) of *B. axillare* root extract (group IV).

Carbon tetrachloride caused a significant ($P \leq 0.001$) elevation in serum- enzymatic activities of SGOT, SGPT, ALP, LDH, GGT and total bilirubin alongwith concurrent decline in total protein levels in comparison to vehicle control (group I). In contrast, treatment with *B. axillare* root extract showed dose-dependent restoring effect on CCl_4-induced alterations in serum levels of treated rats in group III and IV (Table II). The elimination of hepatic injury by silymarin (group V) and higher dose (100 mg/kg) of *B. axillare* root extract (group IV) was significantly ($P \leq 0.001$) Similar (Table II).

DISCUSSION:

Hepatocytes injury caused by toxic species, which usually are metabolites. In case of CCl_4 intake by experimental animals, the toxic species are altered the liver metabolism through oxidative damage, which are similar to that of acute viral hepatitis (Bishayee *et al*., 1995). It is now generally accepted that the oxidative damage in liver by CCl_4 is the result of reductive dehalogenation, which is catalyzed by *P*-450 and forms the highly reactive trichloromethyl free radical ($CCl_3^{\cdot}$). This then readily interact with molecular oxygen to form the trichloromethyl peroxy radical ($CCl_3COO^{\cdot}$) (Brent and Rumack, 1993). Both radicals are capable of covalently binding to proteins or lipids or of abstracting a hydrogen atom from an unsaturated lipid, which initiate lipid peroxidation and liver damage and by

play a significant role in the pathogenesis of diseases (Williams and Burk, 1990). Therefore, the suppression of *P*-450 can result in a reduction in the level of reactive metabolites.

The lipid peroxidative degradation of biomembranes is one of the principle cause of oxidative damage of hepatocytes induced by CCl_4 (Kaplowitz et al.,1986). Because lipid peroxidation is viewed as a complicated biochemical reaction involving free radicals, oxygen, metal ions and a host of other factors in the biological system. Lipid peroxidation is the focus of intense activity in relation to its possible involvement in health and disease (Floyd, 1990).

The efficacy of any hepatoprotective drug is dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic physiology that has been disturbed by CCl_4 and other hepatotoxicants (Senthilkumar et al., 2005).

In our present investigation, the measurement of lipid peroxidation in the liver is a convenient method to monitor oxidative cell damage. Inhibition of elevated LPO has been observed in *B. axillare* extract and silymarin treated groups due to its antioxidant and free radical scavenging activities through reestablishment of biomembranes of hepatic parenchymal cells.

The inhibitory effect of CCl_4 on cytochrome *P*-450 level was also compensated by *B. axillare* extract and silymarin through maintenance of its normal level. The role of *B. axillare* extract in the protection of CCl_4-mediated loss in cytochrome *P*-450 content may be considered as an indication of improved protein synthesis in the hepatic cells (Mandal et al., 1993).

The generation of reactive oxygen species (ROS) leading to oxidative damage due to O_2^- is contained by dismutation with SOD, which converts the reactive O_2 to H_2O_2, if not scavenged by catalase caused lipid peroxidation by an increase in the generation of hydroxyl radicals. Hence, a decrease in SOD and catalase levels in hepatocytes of CCl_4 treated rats may lead to increased accumulation of reactive products resulting oxidative damage of liver (Shirwaikar et al., 2004). GSH plays a critical role in important cellular functions, which includes the maintenance of thiol status of proteins, the destruction of H_2O_2, lipid peroxides, free radicals, translocation of amino acids across cell membranes, the detoxification of foreign compounds, and the biotransformation of drugs (James and Hrabison, 1982). The decreased level of GSH in CCl_4-treated rat liver may be due to its use by the excessive amount of free radicals. This depletion not only compromises cellular defenses against attack by reactive molecules but also has profound effects on normal hepatocellular function (Gupta et al., 2005). Glutathione peroxidase is a isoenzyme, act as a free radical scavenger and traps peroxyl radicals before they can initiate lipid peroxidation (Ray and Husain, 2000). Therefore, the depleted level of GPx, throught to be utilized in the liver. Oral administration of *B. axillare* root extract to rats showed significant elevation in depleted SOD, CAT, GSH and GPx levels due to an antioxidant activity of *B. axillare* root extract through scavenged the free radicals. Further, a significant protection against CCl_4-induced hepatic antioxidant aberrations was achieved with the silymarin treatment.

In the assessment of liver damage by carbon tetrachloride, the determination of enzyme levels such as SGOT, SGPT, ALP, GGT and LDH were increased remarkably in plasma by the release of these enzymes from hepatic parenchymal cells, which were indicating a considerable hepatocellular injury (Bishayee et al., 1995). Oral treatment with *B. axillare* root extract and silymarin attenuated these increased enzyme activities produced by CCl_4 and a subsequent recovery towards normalization of these enzymes strongly suggests the possibility of *B. axillare* root extract being able to improve the condition of hepatocytes so as to cause accelerated regeneration of parenchymal cells, thus protecting against membrane fragility decreasing the leakage of markar enzymes into the circulation stabilization of serum-total bilirubin and total protein levels through the administration of extract is further a clear indication of the improvement of functional status of the hepatic cells (Bishayee et al., 1995).

In conclusion, biochemical alterations observed in hepatic injury seems to be mainly due to an oxy-radical-mediated mechanism, involving lipid peroxidation, under conditions of reduced antioxidant levels that scavenge superoxide, hydrogen peroxide and lipid peroxides. The results yet available are overwhelming, which suggests that *B. axillare* root extract may be helpful in quenching free radicals and induction of an in vivo antioxidant defense system by the isolated active compounds like stigmasterol, ceryl alcohol, octacosanol-I, □-sitosterol, betulin, betulinic acid and lupeol which are present in it. Therefore, the current findings suggest the hepatoprotective capability of *B. axillare* roots through its antioxidant properties and induction of in vivo antioxidant defense system.

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Table I : Showing hepatoprotective activity by *B. axillare* root extract and silymarin on experimental liver injury through antioxidants defense and enzymatic levels.

Treatment design	SOD (μ mole/ mg protein)	CAT (μ mole H_2O_2 consumed/min/ mg protein)	GSH (n mole/g tissue)	GPx (n mole NADPH consumed/min/ mg protein	LPO (n mole MDA/ mg protein	Cytochrome-*P*-450 (n mole/ mg protein)
Control (vehicle treated) Group I	12.28 ± 0.44	65.35 ± 2.88	5.22 ± 0.27	15.39 ± 0.26	1.92 ± 0.08	5.21 ± 0.18
CCl_4 (1 ml/kg b wt, ip, once a week with olive oil, 1:1) Group II	5.82 ± 0.16[a]	36.15 ± 1.96 [a]	2.32 ± 0.13 [a]	8.42 ± 0.19 [a]	5.12 ± 0.18 [a]	2.08 ± 0.14 [a]
CCl_4 + *B. axillare* extract (50 mg/kg b wt/day, orally) Group III	8.26 ± 0.11 [a]	51.21 ± 1.32 [a]	3.98 ± 0.10 [a]	11.12 ± 0.30 [a]	4.21 ± 0.12[b]	3.01 ± 0.16[b]
CCl_4 + *B. axillare* extract (100 mg/kg b wt/day, orally) Group IV	11.20 ± 0.13[a]	63.20 ± 2.33 [a]	5.31 ± 0.18 [a]	14.85 ± 0.23 [a]	2.42 ± 0.14 [a]	5.10 ± 0.20 [a]
CCl_4 + Silymarin (25 mg/kg b wt/day, orally) Group V	11.68 ± 0.32 [a]	61.21 ± 2.12 [a]	6.30 ± 0.34 [a]	14.82 ± 0.27 [a]	2.21 ± 0.10 [a]	4.89 ± 0.15 [a]

Levels of significance : Data are mean ± SEM (n = 6)

a = P≤ 0.001 Group II compared with control (Group I)

a = P≤ 0.001; b = P ≤ 0.01 Group III compared with Group II

a = P≤ 0.001 Group IV and V compared with Group II

Table II : Showing hepatoprotective activity by *B. axillare* root extract and silymarin on experimental liver injury through serum parameters.

Treatment design	SGOT (IU/L)	SGPT ((IU/L))	ALP (KAU)	GGT ((IU/L))	LDH (IU/L)	Total bilirubin (mg/100 ml)	Total protein (gm/dL)
Control (vehicle treated) Group I	128.21 ± 2.10	108.32 ± 2.87	21.33 ± 1.30	9.52 ± 0.93	84.15 ± 2.57	0.85 ± 0.06	6.21 ± 0.22
CCl_4 (1 ml/kg b wt, ip, once a week with olive oil, 1:1) Group II	206.14 ± 2.87[a]	187.27 ± 3.09 [a]	35.10 ± 1.48 [a]	28.16 ± 1.41 [a]	142.22 ± 2.88 [a]	1.79 ± 0.10 [a]	3.14 ± 0.17 [a]
CCl_4 + *B. axillare* extract (50 mg/kg b wt/day, orally) Group III	176.10 ± 2.14 [a]	152.21 ± 1.97 [a]	29.14 ± 1.55[c]	20.21 ± 1.78[b]	110.17 ± 2.67 [a]	1.30 ± 0.13[c]	4.99 ± 0.16[a]
CCl_4 + *B. axillare* extract (100 mg/kg b wt/day, orally) Group IV	128.35 ± 3.15[a]	121.19 ± 2.37 [a]	24.15 ± 1.69 [a]	11.52 ± 1.72 [a]	92.22 ± 2.22 [a]	1.03 ± 0.07 [a]	6.28 ± 0.23 [a]
CCl_4 + Silymarin (25 mg/kg b wt/day, orally) Group V	122.20 ± 2.88 [a]	114.19 ± 1.42 [a]	23.85 ± 0.66 [a]	12.30 ± 1.42 [a]	80.10 ± 1.52 [a]	0.92 ± 0.08 [a]	6.88 ± 0.23 [a]

Levels of significance : Data are mean ± SEM (n = 6)

a = P≤ 0.001 Group II compared with control (Group I)

a = P≤ 0.001; b = P ≤ 0.01; c = P≤ 0.05 Group III compared with Group II

a = P≤ 0.001 Group IV and V compared with Group II