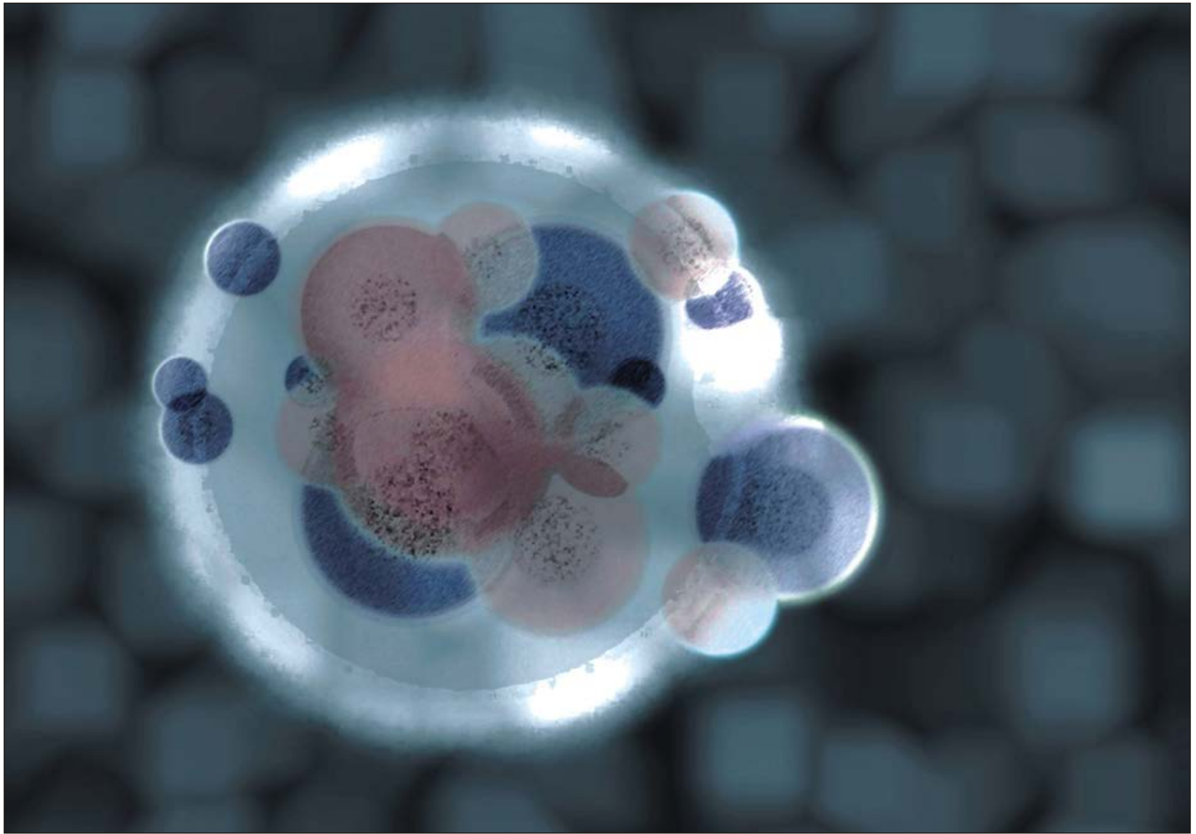

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Antioxidant and Hepatoprotective Potential of Isolated Fraction of *Revia hypocrateriformis*

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Abstract: Successive Ethanolic Extracts (SEE) of *Rivea hypocrateriformis* (RH) which recorded highest polyphenolic contents was subjected to fractionation by column chromatography. All fractions were tested for *in vitro* antioxidant activity. Out of these fractions, SEE2 possessed significant antioxidant activity. This fraction was further tested for *in vivo* antioxidant and hepatotoxicity in rat liver using carbon tetrachloride (CCl_4), showed significant activity. Further, this claim was confirmed by pretreatment of mice with SEE2 shortened the duration of pentobarbitone induced necrosis. The HPLC analysis was performed for same fraction evidenced the presence of gallic acid and lupeol along with other polyphenol when compared with markers.

Key words: Antioxidant, hepatoprotective, gallic acid, lupeol, HPLC

INTRODUCTION

Presense of antioxidants in foods or body delays oxidation of oxidizable substrate. Antioxidants may help the body to protect itself against various types of oxidative damage caused by reactive oxygen species, which are linked to a variety of diorders including cancer, diabetes, shock, arthritis, and acceleration of the ageing process (Shahidi, 1997). Several groups of polyphenols (anthocyamins, tannins, flavanones, isoflavones, resveratrol and ellagic acid) are currently used as antioxidants in the industry as nutraceuticals or functional foods (Espin *et al*., 2007). RH is climbing shrub known for a large number of biological activities such as antidiabetic, antiimplantation, in treatment of burning and piles, antidepressant, anticancer and analgesic properties (Dhawan *et al*., 1980; Shivalingappa *et al*., 1999). Chemically it known to contains amino acid and sugar (Dhore *et al*., 2001). In literature survey, no evidence was reported for biological and bio-chemical investigation of RH. In the present study an attempt has been made for evaluation of antioxidant and hepatoprotective activities along with phytochemical investigations.

MATERIALS AND METHODS

Plant material: Aerial parts of *R. hypocrateriformis* were collected in the month of August- September from Amravati District, Maharashtra and authenticated by Prof. Dr. Bhowagaokar, VIHS, Amravati, Maharashtra, India. A voucher specimen (AMT-36) has been preserved for future reference.

Extraction and fractionation: The dried powder material (10 kg) was extracted successively extracted with petroleum ether (SPE), chloroform (SCE), ethanol (SEE) and water (SAE) as per the increasing order of their polarity. All solvents were evaporated to dryness under pressure using rotary flash evaporator to obtain crude extracts. The Successive Ethanol Extract (SEE) was selected for further fractionation based on polyphenolic content. SEE was subjected to column chromatography over silica gel (60-120 mesh) using varying proportion of chloroform: methanol (90:10, 75:25, 50:50, 0:100 v/v) as eluent. All fractions were collected and concentrated to dryness on a rotary flash evaporator.

These fractions were screened for *in vitro* antioxidant activity.

Animals: Wistar albino rats (150-250 g) and Swiss albino mice (25-35 g) each of either sex, obtained from the Institute's animal house were used. Rats were housed under standard laboratory conditions and were fed commercial rat feed (Lipton India Ltd., Mumbai, India) and boiled water, *ad libitum*. All animal experiments were carried out according to institutional animal ethical committee (Approval letter no. GCPA/ IAEC/ 2011/ 1245).

Total phenolic content (TP): The TP content was determined by Folin-Ciocalteau colorimetric method of Singleton *et al.* (1999). The TP content was calculated from calibration curve of gallic acid and expressed as gallic acid equivalents.

In vitro antioxidant capacity

DPPH and ABTS radical scavenging assay: ABTS and DPPHÿ$^{+}$ quenching ability was measured according to Kalaskar and Surana (2011). The antiradical activity was expressed as IC_{50} (μg mL^{-1}).

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Nitro blue tetrazolium (NBT) reduction assay: Effect of scavenging superoxide radical was determined by the nitroblue tetrazolium reduction method of Fu *et al.* (2010). The absorbance of samples was measured at 560 nm against blank.

Lipid peroxidation inhibition activity by ferric thiocyanate method (FTC): The FTC method was adapted from Fu *et al.* (2010). All measurements were made in triplicate and averaged. The inhibition rate was calculated using the equation.

$$\frac{Ac-As}{Ac} \times 100$$

where, Ac is absorbance of control; As is absorbance of sample.

CCl_4 induced hepatotoxicity: CCl_4 induced hepatotoxicity in rats carried out according to Kalaskar and Surana (2011). All studies were carried out in double dose, 50 mg and 100 mg kg^{-1} body wt. Samples of organ, livers and kidneys from all the animals were dissected at the end of experiment, washed and used for histological studies and biochemical characterization.

Pentobarbitone-induced sleeping time studies: Pentobarbitone induced sleep studies carried out according to Kalaskar and Surana (2011). All groups of animals were given pentobarbitone (PBT, 40 mg kg^{-1}, IP), 2 h after CCl_4/vehicle treatment. The time between loss of righting reflex and its recovery was recorded.

Histopathological studies and Biochemical determinations: After blood draining, liver samples were excised from the control and treated groups of animals and washed with normal saline separately. All samples were fixed in 10% buffered formalin for 48 h. Each sample was stained with haematoxylin-eosin (H and E) for photomicroscopic observations of the liver histological architecture and homogenates of liver and kidney were used for determination of SOD, CAT, MDA, and GSH.

HPLC analysis of SSE2: HPLC analysis confirmed the presence of gallic acid and lupeol in the SEE2 fraction using gallic acid and lupeol (Sigma-aldrich Chemie, Steinheim, Germany) as standard markers. The separation of the components was performed on Phenomenix C18 column (250×4.6 mm I.D., 5 μm particle size). The separation of gallic acid was achieved using methanol + water (70:30 v/v) as mobile phase, flow rate was adjusted to 0.7 mL min^{-1} and dectection was performed at 280 nm. While, for lupeol acetonitrile: water (90:10 v/v) was used as mobile phase, flow rate 1.0 mL min^{-1} and detected at 230 nm.

Statistical analysis: Results were expressed as Mean±SEM. Data were analyzed using one-way Analysis of Variance (ANOVA) followed by Dunnett's test. Value of $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Total phenolic contents: Phenolic compounds are considered to be the major contributors to the antioxidant capacity of plants. Some of diverse biological activities of plant may also be related to their antioxidant activity (Chung *et al.*, 1998). The phenolic content of RH was determined. The SEE had highest concentration of phenolics 55.16% amongst the fractions 32.12, 39.21, 25.33% for SPE, SCE and SAE, respectively. The SEE further subjected to column fractionation.

***In vitro* antioxidant activity:** Antioxidant activity of different fractions of SEE, SEE1, SEE2, SEE3 and SEE4 were evaluated by free radical scavenging by ABTS and DPPH, superoxide scavenging activity and lipid peroxidation. The concentration of each fraction required to inhibit each radical by 50% (IC_{50}) is shown in Table 1. The result revealed that the SEE2 fraction exhibits the most robust radical-scavenging activity amongst all fractions. Inhibition of DPPH and ABTS radical indicates its direct role in trapping free radicals by donating hydrogen atom or electron. The superoxide anion radical ($^{\bullet}O^{-}_{2}$) is the most common free radical generated *in vivo*. Suppression of $^{\bullet}O^{-}_{2}$ in presense of SEE was also observed. Also SEE inhibited lipid peroxidation may be due to termination of the radical chain reaction (Zhou *et al.*, 2010).

Hepatoprotective and *in vivo* antioxidant activities: Based on our *in vitro* antioxidant assays, SEE2 was chosen as the most potent fraction which was shortlisted for evaluation *in vivo* antioxidant and hepatoprotective potential.

Table 1: IC_{50} value of various fractions of SEE

	IC_{50} value (μg mL^{-1})			
Sample	DPPH	ABTS	Lipid peroxide	Superoxide
SEE1	51.19±1.01	48.07±0.25	76.55±0.24	69.15±0.01
SEE2	30.49±1.09	22.06±0.27	25.54±0.11	28.08±0.05
SEE3	35.04±0.05	33.43±0.86	42.34±0.54	48.96±0.22
SEE4	44.25±0.20	35.79±0.35	55.36±0.87	51.35±0.31
Ascorbic acid	20.61±0.26	19.81±0.24	-	-
Trolox	-	-	20.21±0.16	23.54±0.39

Values are the Mean±SEM, n = 3

Table 2: Effects SEE2 on rat serum parameters after CCl_4 administration

Groups	SGOT	SGPT	ALP	TB	DB	TP (g %)	PBT sleeping time (min)
	------------(IU L^{-1})--------------			-----------(mg dL^{-1})-----------			
Normal control	52.2±1.6	46.2±1.3	89.6±2.5	1.14±0.02	0.44±0.06	7.4±0.2	091.03±2.2
CCl_4 control	165.1±3.8^a	161.3±4.2^a	212.6±6.1^a	5.7±0.1^a	1.08±0.01^a	5±0.2^a	161.9±2.8^a
Silymarine	68.4±1.8^b	54.03±2.4^b	91.8 ±2.9^b	1.9±0.06^b	0.49±0.01^b	6.9±0.1^b	100.1±3.5^b
CCl_4 + SEE2(50 mg kg^{-1})	125.2±2.5^b	95.6±2^b	139±1.7^b	2.6±0.05^b	0.66±0.05^b	6.7±0.1^c	138.2±4.1^d
CCl_4 + SEE2 extract (100 mg kg^{-1})	94.3±2.1^b	67.07±1.6^b	98.6±1.4^b	1.4±0.04^b	0.58±0.01^b	6.7±0.1^b	115.8±1.5^c

Values are the Mean±SEM, n = 6. SGOT: Serum glutamate oxaloacetate transaminase, SGPT: Serum glutamate pyruvate transaminase, ALP: alkaline phosphatase, DB: direct bilirubin, TB: total bilirubin, TP: Total protein, PBT: Pentobarbitone (a) $p \le 0.01$ when compared with control. (b) $p \le 0.01$ when compared with toxicant. (c) $p \le 0.05$ when compared with toxicant. (d) $p \le 0.05$ when compared with toxicant.

Table 3: Effects of SEE2 on liver and kidney SOD, CAT, MDA and GSH in CCl_4-intoxicated rats

Groups	SOD (U mg^{-1} protein)	CAT (U mg^{-1} protein)	MDA (nmol mg^{-1} protein)	GSH (mg g^{-1} protein)
Liver				
I	316.86±24.60	62.1±12.72	2.53±0.18	5.29±1.09
II	164.67±40.55^a	35.09±6.09^a	5.17±0.92^a	2.13±0.67^a
III	294.04±45.92^b	52.81±13.26^c	3.76±0.44^c	4.74±1.56^c
IV	253.94±45.78	41.34±9.61^c	2.95±0.51^c	4.29±0.58^d
V	279.95±36.67^c	51.04±13.33^b	3.06±0.73^c	4.38±1.08^d
Kidney				
I	287.17±15.29	72.18±10.66	1.33±0.42	5.17±0.19
II	139.81±32.26^a	35.65±8.070^a	3.81±0.63^a	3.01±0.73^a
III	260.54±56.34^b	61.11±15.87^c	2.61±0.69^c	4.44±0.68^c
IV	271.19±37.03^c	49.12±9.65^c	2.14±0.44^c	4.75±1.21^d
V	241.99±53.69^b	57.62±14.59^c	2.53±0.63^c	4.61±1.04^d

Values are mean±SD, Statistical significance is indicated by asterisks and double asterisks. SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde; GSH, glutathione. Group I: Control, Group II: CCl4, Group III: silymarin 100 (mg kg^{-1}), Group IV: SEE2 50 (mg kg^{-1}), Group V: SEE2 100 (mg kg^{-1}). (a) $p \le 0.01$ when compared with control. (b) $p \le 0.01$ when compared with toxicant. (c) $p \le 0.05$ when compared with toxicant. (d) $p \le 0.05$ when compared with toxicant

The CCl_4-induced hepatotoxicity model extensively used for the evaluation of hepatoprotective and *in vivo* antioxidant effects. CCl_4 is accumulated in hepatic parenchyma cells and metabolized to the $CCl^{\bullet}_3$ (Recknagel, 1983). $CCl_3^{\bullet}$ radical reacts very rapidly with oxygen to yield a highly reactive $CCl_3OO^{\bullet}$. These radicals react with proteins and lipids. They remove hydrogen atoms from unsaturated lipids thus initiating lipid peroxidation which causes loss of integrity of cell membranes and damage to hepatic tissue (Zhou *et al*., 2010). Concentration of enzymes viz., SGPT, SGOT, ALP, TP, TB and DB are considerably high in the cytoplasm. When liver cells are injured, these enzymes leak into the blood stream and causing significant rise in blood levels of these enzymes. The extent of liver damage is in proportion with the elevated serum levels of these enzymes. Alkaline phosphatase is the prototype of these enzymes that reflects the pathological alteration in biliary flow (Kalaskar and Surana, 2011). CCl_4 induced elevation of enzymatic activity in the serum is in line with high level of serum bilirubin content. The SEE2 induced suppression of the increased ALP activity with the concurrent depletion of raised bilirubin, suggest the ability of SEE2 to stabilize biliary dysfunction in rat liver. In this study pre-treatment of rats with SEE2 prior to CCl_4 administration caused a significant change in the values of SGOT, SGPT, ALP, TP, TB and SB) in a dose dependent manner. However, SEE2 (100 mg kg^{-1}) showed highest hepatoprotective activity (Table 2) almost comparable to the standard, Silymarin (100 mg kg^{-1}) treated group which was also supported by histological studies. The histological architecture of CCl_4 treated liver section showed marked massive fatty changes, necrosis, ballooning degeneration and the loss of cellular boundaries. However, necrosis was not observed in any groups treated with standard and SEE2, indicates that sufficient hepatotoxicity does not seem to be developed so as to cause necrosis (Fig. 1). Alternatively, reduction in the prolongation of pentobarbitone induced sleep in CCl_4 poisoned rats is further indicative of the hepatoprotective potential of the SEE2. It has been established that since the barbiturates are metabolized almost exclusively in the liver, the sleeping time after a given dose is a measure of hepatic metabolism.

Further levels of MDA, CAT, GSH and SOD reflect the extent of peroxidation damage. The decrease of key components of the antioxidant defence system, CAT, SOD and GSH may result in a lot of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide (Nanjan *et al*., 2007). The fact that SEE2 treatment reduced elevated MDA and increased levels of SOD, CAT, and GSH (Table 3), indicated its role in prevention of lipids peroxidation.

The phytochemical analysis of SEE has shown high phenolic content. In the present study, the presence of

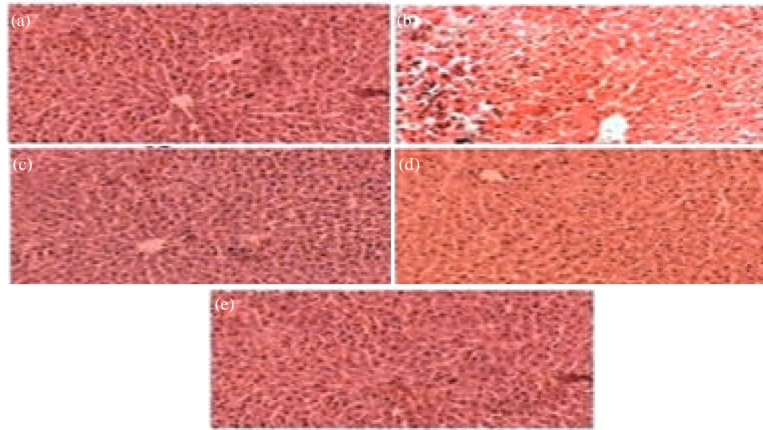

Fig. 1(a-e): Histopathological architecture in the liver of control and experimental rats treated with CCl_4 (H and E X100). a-Normal control, b-CCl_4 Control, c-Silymarin treated, d-SEE2 treated (50 mg kg^{-1}), e- SEE2 treated (100 mg kg^{-1})

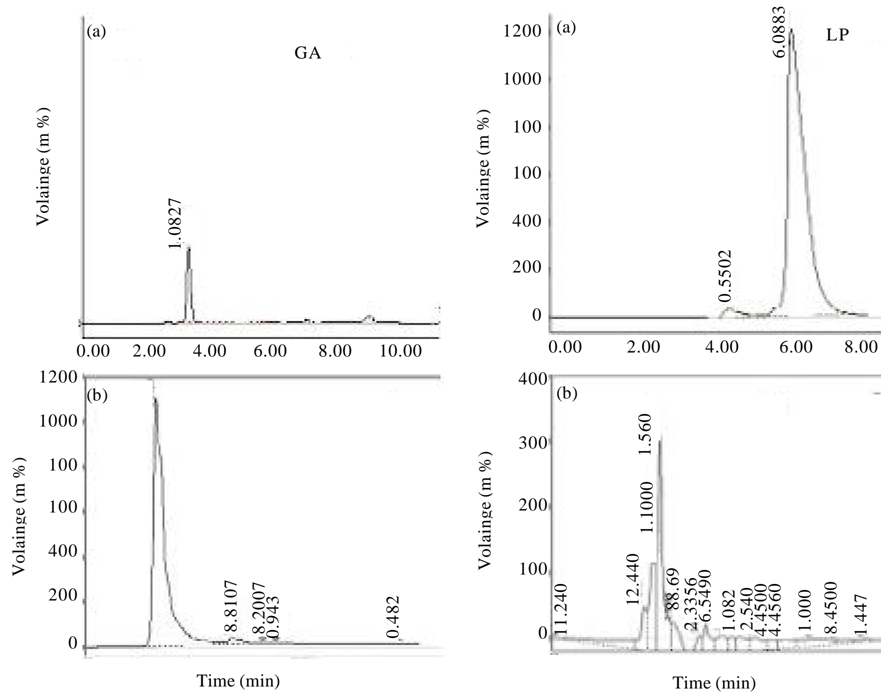

Fig. 2(a-b): HPLC chromatogram of (a) authentic standards (b) Compound identified in SEE2, GA-Gallic Acid, LP-Lupeol

gallic acid and lupeol in SEE2 was phytochemically confirmed (Fig. 2). Perhaps gallic acid, lupeol and other related phenolic compounds present in SEE2 may be responsible for its observed antioxidant and hepatoprotective activity. The antioxidant and free radical scavenging property of gallic acid (Kawashima *et al*., 1996) and lupeol (Preetha *et al*., 2006) was previously reported. These results suggest that prevention of superoxide radical generation (Yamashita *et al*., 2002) and binding of the gallate compounds to lipid membrane (Shahrzad *et al*., 2001) was the principal determining factor of antioxidant action.

CONCLUSION

Thus, it can be assumed that the possible mechanism of the hepatoprotective and anti-oxidant activities of SEE2 is due to the presence of polyphenolic constituents. It may be further concluded that the SEE2 is the most potent amongst all fractions, which may be due to presence of gallic acid, lupeol and other phenolics. In future further studies on identification of other phytoconstituents along with their biological evaluation should be necessarily carried out.

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