e- ISSN 0975 – 9328
Print ISSN 2229 – 7472

International Journal of Phytopharmacology

Journal homepage: www.onlineijp.com

IJP

IN-VITRO STUDIES TO EXPLORE THE PHARMACOGNOSTIC, PHYSICOCHEMICAL, PHYTOCHEMICAL, TOXICITY AND HEPATOPROTECTIVITY OF *SMILAX OVALIFOLIA* ROOT

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ABSTRACT

The primary aim of the present study was to explore the pharmacognostic, physicochemical, phytochemical, toxicity and hepatoprotectivity of *Smilax ovalifolia* root. Standard procedures were implemented to assess the pharmacognostical, physicochemical and phytochemical parameters of *Smilax ovalifolia* root. Toxicity of *Smilax ovalifolia* root was assessed using MTT assay on BRL3A cell line. Hepatoprotectivity of *Smilax ovalifolia* root was assessed on paracetamol treated BRL3A cell line using MTT assay. Results of phytochemical screening have shown the presence of various phytochemicals including (a) Steroids and gums in petroleum ether extract, (b) Carbohydrates, steroids and flavonoids in chloroform extract, (c) Carbohydrates, tannins and phenolic compounds in methanol extract. Results of high performance liquid chromatographic analysis have shown the presence of several phytoconstituents including gallic acid and rutin. Results of toxicity study have shown that the root of *Smilax ovalifolia* was non-toxic to BRL3A cell line at the dose <1000 µg/mL. Results of hepatoprotectivity have shown that the root of *Smilax ovalifolia* has offered protection towards BRL3A cell line against paracetamol at a dose of 500 and 1000 µg/ml. The present study concludes that the root of *Smilax ovalifolia* is safe and offered substantial protection to the rat normal liver cell line against paracetamol toxicity. However, data gathered from the current study necessitate further validation using molecular level and *in-vivo* animal studies.

Key words: *Smilax ovalifolia*, Flavonoids, Hepatoprotective Activity, Hepatoprotectivity, Paracetamol Toxicity.

INTRODUCTION

Liver is a vital organ, which demonstrates a substantial role in the metabolism of toxins. However, few drugs of synthetic and natural origin may cause damage to the liver leading to cirrhosis and cancer. Despite the medical advancement, there is a scarcity of drugs that stimulate utility, protection, regeneration of hepatic cells. Hence, there is an immediate requirement for a safe hepatopotective agent (Gupta and chadha, 1995). Traditional system of medicine uses plants for the treatment of various ailments from time immemorial, particularly among tribal communities (Pareek, 1996). In India about 2000 drugs used are of plant origin (Dikshit, 1999). According to World Health Organization (WHO), there were about 20,000 medicinal plants globally and 15-20% were from India (Singh, 2000). About 80% of the population in the developing countries depends directly on plants for their healthcare (Mukhopadhyay, 1998).

Smilax ovalifolia is a small tree belongs to Smilacaceae family distributed in the forest area of the central and eastern parts of China, India, Myanmar, Nepal, Thailand and Vietnam (Alanis, 2005). *Smilax ovalifolia* is being used traditionally for the treatment of arthritis (Rama Shankar and Ramesh Babu Devalla, 2012). However, the present study was aimed to explore the pharmacognostical, physicochemical, phytochemical, toxicity and hepatoprotectivity of *Smilax ovalifolia* root.

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MATERIALS AND METHODS

Materials

Analytical grade reagents and chemicals were used in the study. The roots of *Smilax ovalifolia* were collected from southern part of the Eastern Ghats, Seshachalam forest, Tirupathi, Andhra Pradesh, India and authenticated by Dr. K. Madhava Chetty (Field Botanist, Assistant Professor, Department of Botany, Sri Venkateswara University). Fresh roots were washed in the running water and shade dried. Dried roots were crushed into course powder and packed in a black plastic bag and stored in an air-tight container for further studies (Wallis, 2011).

Pharmacognostical evaluation of *Smilax ovalifolia* root

Macroscopic parameters including colour, shape, size, taste and odour were evaluated (Khotton *et al*., 2009; Anonymous, 1966; Trease and Evans, 2002) for the *Smilax ovalifolia* root. For the microscopic evaluation, fresh root of *Smilax ovalifolia* was boiled for few minutes in water and the softened root was transversally sliced, which was stained using 0.1%w/v phloroglucinol and observed under microscope (Kokate, 2010). For the powdered microscopy, small quantity of the powdered *Smilax ovalifolia* root was soaked in water along with few drops of 0.1% w/v phloroglucinol for few minutes. Subsequently, soaked powder was stained using 1% safranine for few minutes on a glass slide and observed under the microscope. The characteristic features of cell components were observed and their photographs were taken using photomicrography (Dinesh Kumar *et al*., 2012; Krishnan, 1992; Savithramma *et al*., 2011).

Physicochemical evaluation of *Smilax ovalifolia* root

Physicochemical parameters such as moisture content (Kokate, 1986), total ash value (Ministry of Health and Family Welfare, 2001), acid insoluble ash (Siddiqua *et al*., 2010), water soluble ash (Kyari, 2008), alcohol soluble extractive value (Siddiqua *et al*., 2010) were performed for the *Smilax ovalifolia* root as the reported methods.

Preparation of extracts

Various extract *Smilax ovalifolia* root was carried out as per the reported methods (Sajjad Khan *et al*., 2009; Harborne, 1998). Briefly, about 500 Gms of course powdered root of *Smilax ovalifolia* was weighed and subjected to successive extraction process using various solvent including petroleum ether, chloroform, ethyl acetate and methanol at 50°C temperature for 3 days. Subsequently, the solvent were filtered through muslin cloth and the filtrate was concentrated using Buchi rotary evaporator until a soft mass (Table 1), which was preserved in desiccator for the further studies.

Phytochemical evaluation of *Smilax ovalifolia* root

Various extracts of *Smilax ovalifolia* root were subjected to preliminary phytochemical screening for the detection of various chemical constituents including carbohydrates (Mohammed Sarfaraj Hussain *et al*., 2011; Nishaa *et al*., 2013), glycosides (Nishaa *et al*., 2013), fixed oils and fats (Kokate *et al*., 1996), proteins and free amino acids (Khandalwal, 1998), saponins (Brain, 1957; Khandelwal, 2002), tannins and phenolic compounds, phytosterols (Radhika *et al*., 2010), alkaloids (Jain *et al*., 2011), flavonoids (Peach *et al*., 1955), gums and mucilage's (Peach *et al*., 1955).

High performance liquid chromatographic (HPLC) analysis of *Smilax ovalifolia* root

High performance liquid chromatographic analysis of *Smilax ovalifolia* root extract was performed using HPLC (Shimadzu SPD10A) equipped with a UV-Vis Abs. variable wave detector. The column used was Phenomenex 5 µm C18 (2) 100 Å, LC Column 250 x 4.6 mm. The mobile phase for the study was prepared as follows. HPLC grade water was adjusted to pH 3 using 10% orthophoshoric acid and this solution was sonicated for 15 min than filtered through 0.45 micron filter paper, from this 300 ml was transferred to reagent bottle and 700 ml of HPLC grade methanol was added and sonicated for 15 min. The mobile phase ratio was 70:30. The flow rate was set at 1 ml/min. The absolute calibration curve method was used for the calculation of the concentration (Madhira Geetha *et al*., 2013).

About 25 mg of *Smilax ovalifolia* root was taken in 10 ml standard flask and made up the volume to 10 ml with methanol. The final concentration of the extract was adjusted to 2500 µg/ml (Sample 1). Similarly, about 10 mg of Gallic acid was taken in 10 ml standard flask and made up the volume with methanol, from that 0.1 ml was taken and diluted to 10 ml. The final concentration was adjusted to 100 µg/ml (Sample 2). Likewise, about 10 mg of Rutin was taken in 10 ml standard flask and made up the volume with methanol. The final concentration was adjusted to 1000 µg/ml (Sample 3). The above mentioned samples (Sample 1,2&3) were injected and chromatogram was recorded.

Toxicity study of *Smilax ovalifolia* root

Toxicity of *Smilax ovalifolia* root was assessed using MTT assay on BRL3A cell line as per the reported methods (Francis *et al*., 1986; Vasanth *et al*., 2010; Wei-Lun Wong *et al*., 2012; Mohamed Saleem *et al*., 2010; Ao *et al*., 2009). Briefly, the monolayer cell culture (BRL3A cell line) was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using DMEM medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial

monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37° C for 24 hour in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted at 24 h interval. After 24 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line. Growth inhibition = 100 – [(Mean OD of individual test group) / (Mean OD of control group) x 100].

Hepatoprotectivity of *Smilax ovalifolia* root

Hepatoprotectivity of *Smilax ovalifolia* root was assessed on paracetamol treated BRL3A cell line using MTT assay (Alshawsh *et al*., 2011). Briefly, the monolayer cell culture (BRL3A cell line) was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium. 50 µl of DMEM with 4000 µg paracetamol/ 50 µl of different non-toxic concentrations of test drugs were added. The plates were then incubated at 37° C for 24 h in 5% CO_2 atmosphere. After 24 h, the cell supernatants were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage cell viability was determined, based on which the percentage protection offered by test and standard drugs was calculated over the ethanol control

RESULTS AND DISCUSSION

Pharmacognostical evaluation of *Smilax ovalifolia* root

The root of *Smilax ovalifolia* was displayed in figure 1. It was 15 - 30 cm long, 1-2 mm thick, red in colour externally with characteristic taste and aromatic odour. Transverse sections of *Smilax ovalifolia* root (Fig. 2) showed xylem vessels, medullary rays, cortex, cork, endodermis and phloem. Xylem shows a wide zone, consisting of lignified pitted vessels found in single as well as in groups of 2-3, scattered throughout xylem region. Medullary rays consist of bi-to triseriate, lignified and radially elongated parenchymatous cells, narrow in the xylem region and wider in the phloem region. Phloem consists of isodiametric, thin-walled, parenchymatous cells, a few containing rhomboidal crystals of calcium oxalate. Root of powdered *Smilax ovalifolia* (Fig. 3) appears dull yellow, showing fragments of cork cells about 4-5 rows of tangentially elongated, thick-walled cells. Cortex cell consists of thin-walled polygonal sclerenchymatous cells. Lignified stone cells and phloem, lignified fibres, non-lignified fibres and rhomboidal shaped calcium oxalate crystals.

Physicochemical evaluation of *Smilax ovalifolia* root

Physiochemical analysis of *Smilax ovalifolia* root includes moisture content, total ash value, acid insoluble ash, water soluble ash and extractive values were performed in triplicate and the results are summarized in Table 2.

Phytochemical evaluation of *Smilax ovalifolia* root

Phytochemical evaluation of *Smilax ovalifolia* root have showed the presence of various phytoconstituents including (a) Steroids and gums in petroleum ether extract, (b) Carbohydrates, steroids and flavonoids in chloroform extract, (c) Carbohydrates, tannins and phenolic compounds in methanol extract (Table 3).

HPLC analysis of *Smilax ovalifolia* root

HPLC analysis of *Smilax ovalifolia* root showed the presence of various phytochemical (Fig 4). Of all phytochemicals, Gallic acid and Rutin were found in significant quantities (Table 4), which were compared with respective standards (Fig. 5, 6).

Toxicity of *Smilax Ovalifolia* root

Toxicity of *Smilax ovalifolia* root was assessed using MTT assay on BRL3A cell line and the results are summarized in table 5. Various concentration of *Smilax ovalifolia* root showed toxicity towards BRL3A cell line. However, the CTC_{50} was found to be >1000 µg/ml. Hence, the powdered root of *Smilax ovalifolia* haven't shown any significant toxicity towards BRL3A cell line.

Hepatoprotectivity of *Smilax ovalifolia* root

Hepatoprotectivity of *Smilax ovalifolia* root was assessed on paracetamol treated BRL3A cell line using MTT assay and the results were summarized in table 6. *Smilax ovalifolia* root has offered protection for BRL3A cell line against paracetamol at the dose of 500 and 1000 µg/ml (Table 6). However, standard drug silymarin has offered significant protection for BRL3A cell line against paracetamol at the dose of 200µg/ml.

Figure 1. *Smilax ovalifolia* root

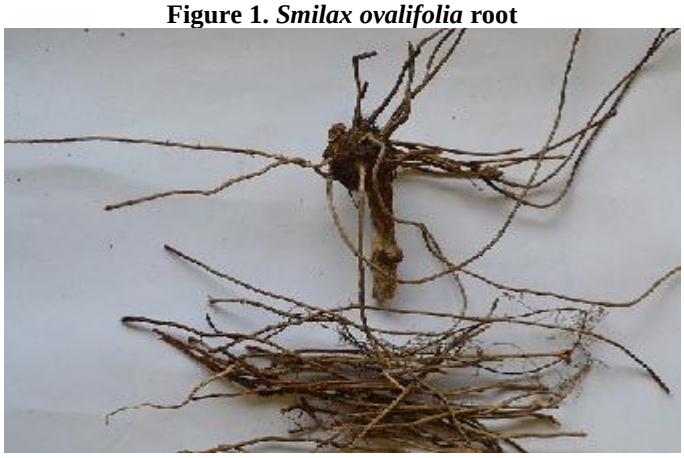

Figure 2. Transverse Section of *Smilax ovalifolia* root

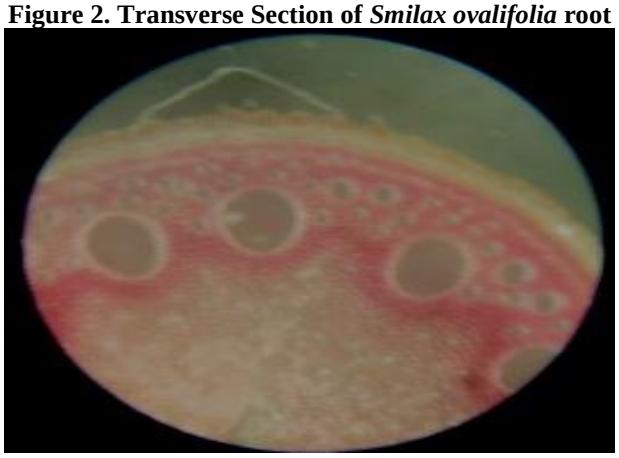

Figure 3. Powder characteristics of *Smilax ovalifolia* root

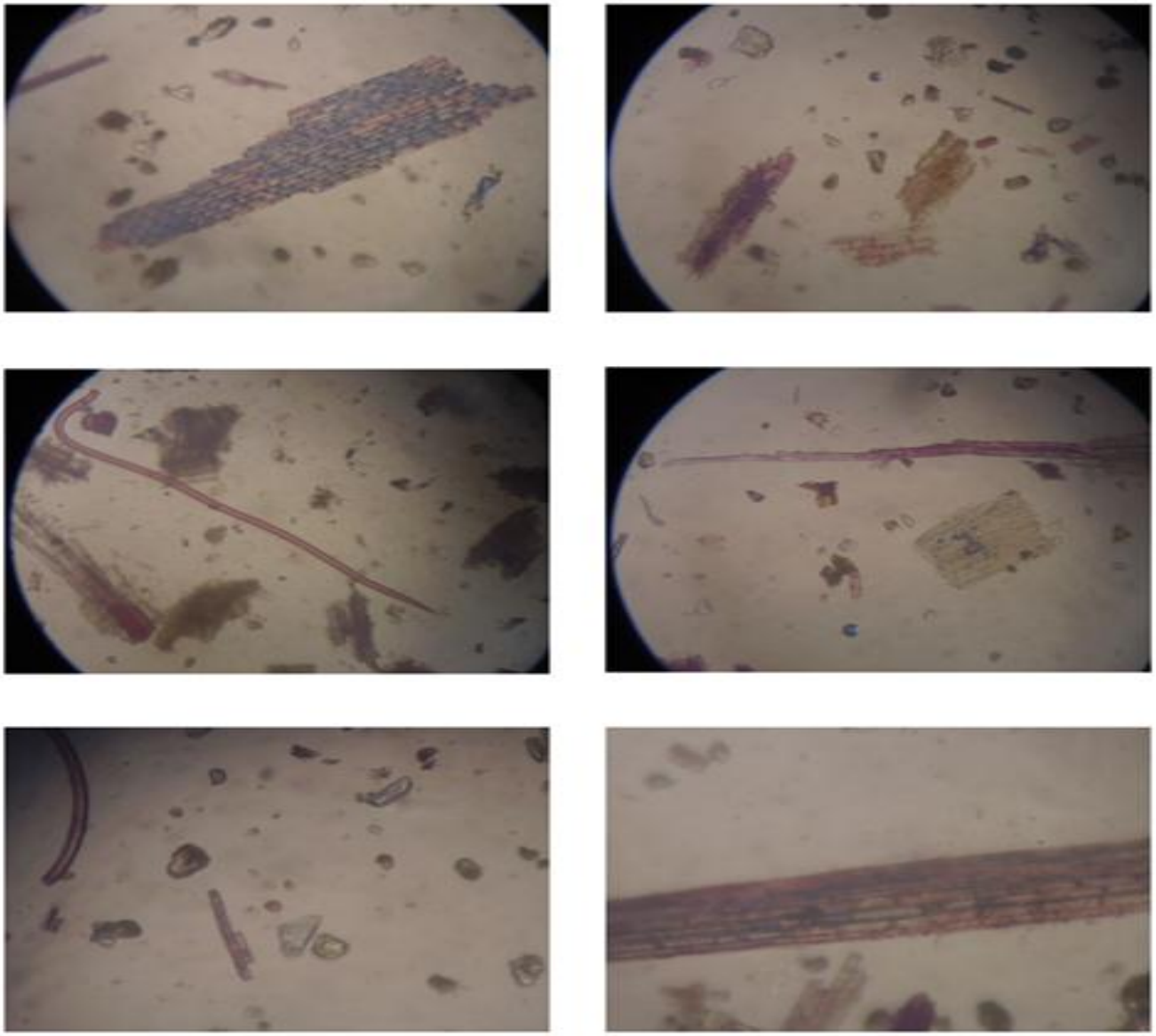

Figure 4. HPLC chromatogram of *Smilax ovalifolia* root

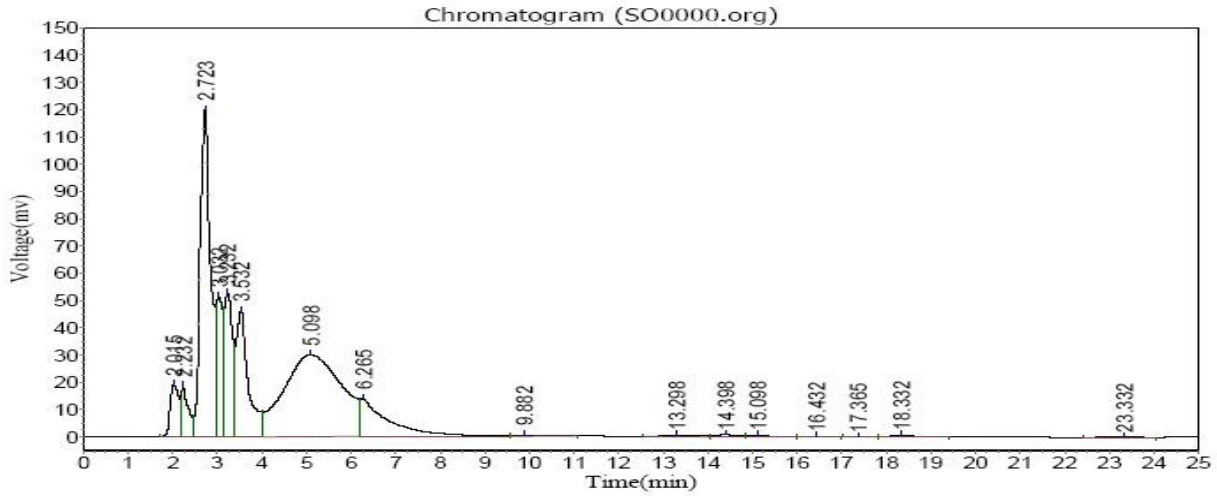

Figure 5. HPLC chromatogram of standard gallic acid

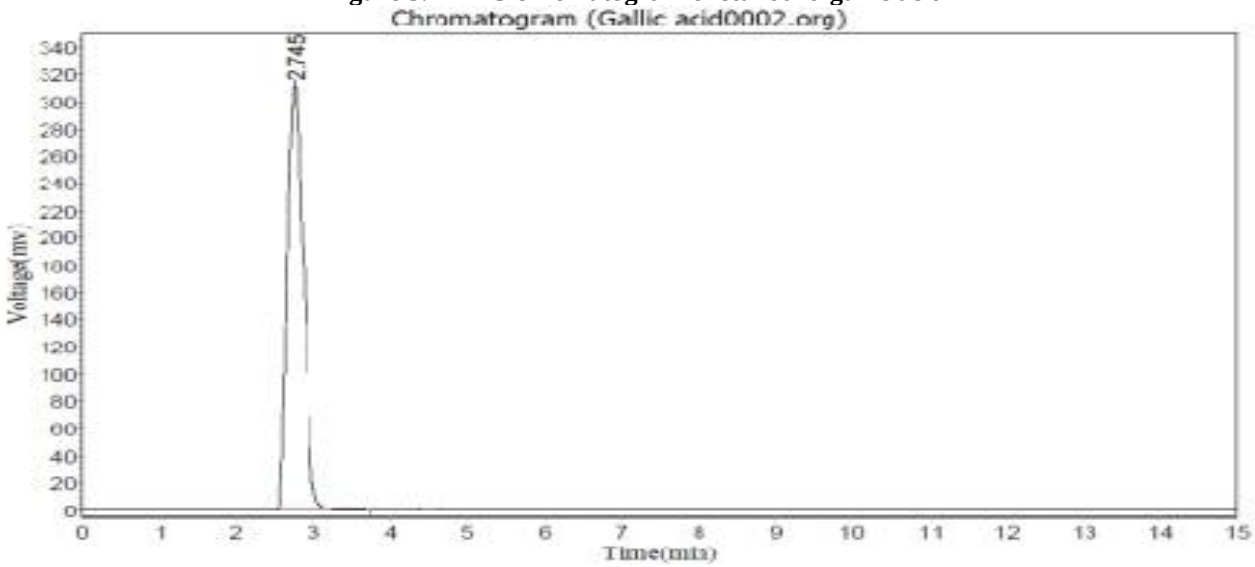

Figure 6. HPLC chromatogram of standard rutin

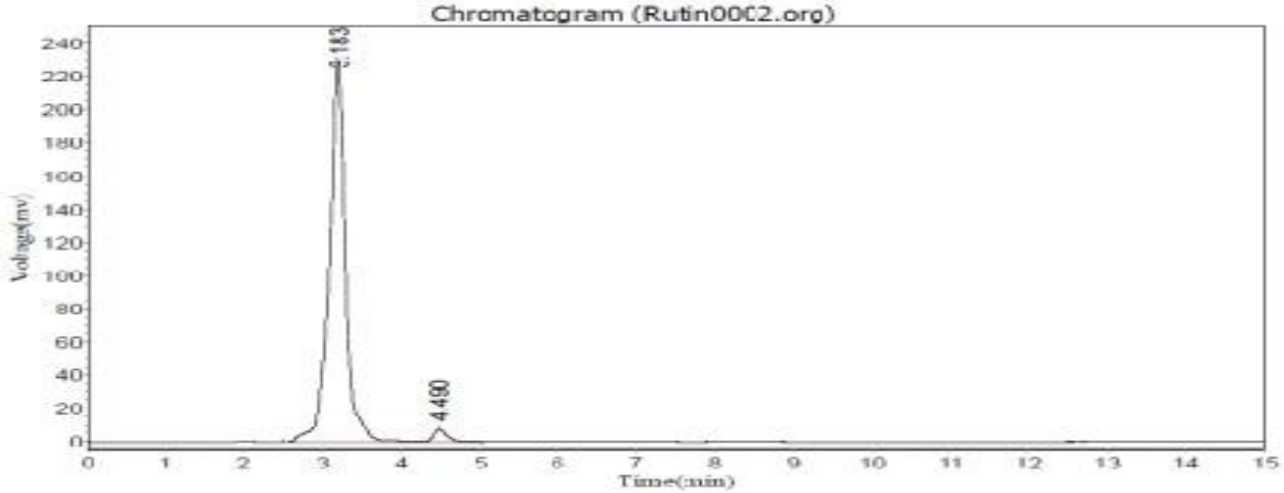

Table 1. Yield of extract using various solvents on *Smilax ovalifolia* root

Solvents	Yield
Petroleum ether	05.60 %
Chloroform	18.21 %
Ethyl acetate	13.26 %
Methanol	52.53 %

Table 2. Physicochemical analysis of *Smilax ovalifolia* root

Parameters	Values obtained on dry weight basis (% W/W)
Moisture Content	5.60 ± 0.09 %
Total Ash Value	5.85 ± 0.07 %
Acid Insoluble Ash	0.67 ± 0.25 %
Water Soluble Ash	0.83 ± 0.18 %
Alcohol Soluble Extractive Value	3.84 ± 0.09 %

Table 3. Phytochemical analysis of *Smilax ovalifolia* roots

S. No.	Tests	Pet Ether	Chloroform	Ethyl acetate	Methanol
1	**Carbohydrates & Glycosides**				
	Molisch's test	-	+	-	+
	Fehling's test	-	+	-	+
	Legal's test	-	-	-	-
	Borntrager's test	-	-	-	-
2	**Proteins & Free amino acids**				
	Million's test	-	-	-	-
	Biuret test	-	-	-	-
	Ninhydrine test	-	-	-	-
3	**Saponins**	-	-	-	+
4	**Tannins and Phenolic Compounds**				
	5% Ferric chloride solution	-	-	-	+
	10% sodium chloride	-	-	-	+
	10% lead acetate solution	-	-	-	+
5	**Phytosterols**				
	Salkowski test	+	+	-	-
	Liebermann Burchard test	+	-	-	-
6	**Alkaloids**				
	Mayer's reagent	-	-	-	-
	Dragendroff's reagent	-	-	-	-
	Hager's reagent	-	-	-	-
	Wagner's reagent	-	-	-	-
7	**Flavonoids**				
	Aq. NaOH	-	+	-	-
	Conc. H_2SO_4	-	+	-	-
	Shinoda's test	-	+	-	-
8	**Gums and Mucilage's**	+	-	-	-

Table 4. HPLC analysis of *Smilax ovalifolia* root

Parameters	Gallic Acid	Rutin
Purity	99	95
Sample area	1939698.5	742695.1
Standard area	4655800	3191292.5
Sample weight (mg)	25	25
Standard weight (mg)	10	10
Sample dilution (ml)	10	10
Standard dilution (ml)	100	10
Phytochemicals in 25 mg of extract	1.649 (6.5%)	8.843 (35.3%)

Table 5. *In-vitro* toxicity of *Smilax ovalifolia* root in BRL3A cell line by MTT assay

S. No.	Test Concentration (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	1000	23.14±1.8	>1000
2	500	21.02±0.3	
3	250	16.67±0.2	
4	125	13.34±0.7	
5	62.5	9.35±0.2	

Table 6. Hepatoprotective activity of *Smilax ovalifolia* root using paracetamol treated BRL3A cell line

S. No.	Test drugs	Test Concentration (µg/ml)	% Protection offered over Paracetamol control
1	*Smilax ovalifolia* root	1000	08.15±2.53
2	*Smilax ovalifolia* root	500	06.54±3.43
3	Silymarin	200	48.83±7.67

CONCLUSION

In this present study, we have investigated the pharmacognostic, physicochemical phytochemical, toxicity and hepatoprotectivity of *Smilax ovalifolia* root. The phytochemical screening of various solvent extracts have shown the presence of different phytoconstituents, which are responsible for various pharmacological activities. The root haven't shown any significant toxicity towards BRL3A cell line as the CTC_{50} was >1000 µg/ml. The root has offered protection for BRL3A cell line against paracetamol at the dose of 500 and 1000 µg/ml. The study concludes that the root of *Smilax ovalifolia* is safe and offered substantial protection to the rat normal liver cell line against paracetamol toxicity. However, data gathered from the current study necessitate further validation using molecular level and *in-vivo* animal studies.

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