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ARTICLE

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CHEMICAL SCREENING AND EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF *OROXYLUM INDICUM* (KURTZ.) SEED

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Oroxylum indicum (Kurtz.), commonly known as Shyonaka, described in Ayurveda under the broad group of drug Dashamula, and is widely available throughout India. The plant is reputed for its anti-inflammatory effect. Several advanced chemical study with this plant has already been conducted and about 28 varieties of chemical compounds are obtained. Most important compounds are baircalein and Ellagic acid (EA). Both these two compounds exhibit important biological activities like anti-inflammatory on neuro-inflammation, anti-cancer and hepatoprotective effect. Potent hepatoprotective activity was observed in CCl4 induced hepato-toxicity and ethanol induced isolated goat liver. Probable mechanism of action of O. indicum may be due to free radical scavenging activities. The anti-inflammatory activity is due to its property of suppression the pro-inflammatory mediators like TNF-α, IL-6, NF-κB-p65 and iNOS[1].

Introduction

Chronic liver disease (CLD) and its associated complications cause significant mortality, morbidity, and economic burden. World Health Organization (WHO) published data of global burden of liver disease that show the burden of CLD is large and increasing, primarily owing to the increasing burden of nonalcoholic fatty liver disease and alcohol-related liver disease (ALD). Middle Eastern, Northern African, and Asian regions of the globe are mostly affected by hepatitis different viruses. Middle Eastern and North African regions also are affected by nonalcoholic fatty liver disease, and Eastern European, West African, and Central Asian regions are affected by ALD[2]. There is increasing overall importance of alcoholic liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD) as causes of liver disease over and above viral causative factors. The expanding size of the population and an increasing life expectancy in the country are important demographic determinants of this change. In keeping with this, encouraging health system response strategies have been adopted in India[3].

Despite significant advances in the understanding of the pathogenesis of alcoholrelated liver injury, there are no FDA-approved treatments for ALD. At present, abstinence remains the cornerstone for successful treatment of ALD. Aside from treatment of the underlying addiction, aggressive nutritional intervention and 'off-label' use of various pharmaco-therapies aimed at the underlying mechanisms of injury represent our approach to treating ALD[4]. In present situation it is increased that herbal medicines accompanied by increased laboratory investigations into the pharmacological properties of the bioactive ingredients and their ability to treat various

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diseases. Efforts are therefore needed to establish and validate evidence regarding safety and practice of Ayurvedic medicines. *Oroxylum indicum* (Linn) Ver. Kurtz. (Family Bignonaceae), is commonly known as the tree of Damocles, Indian caper, Indian trumpet flower and Indian trumpet tree (fig.1)

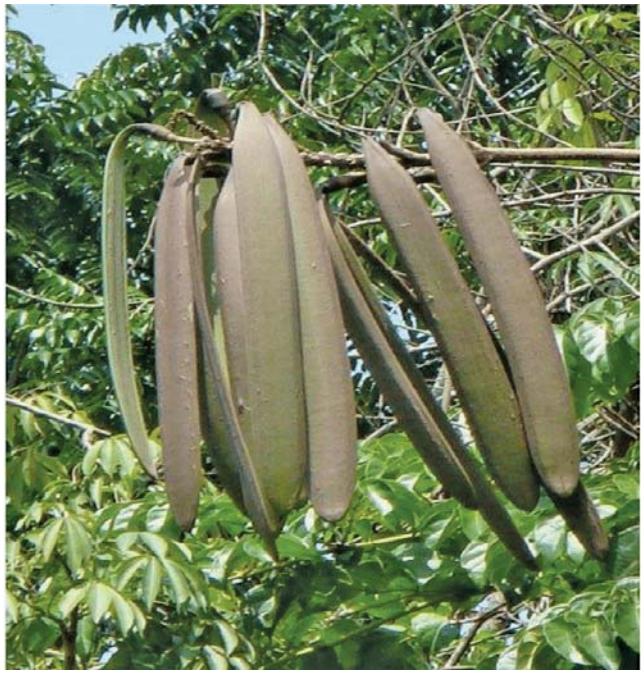


Fig 1. *Sonyaka* (*Oroxylum indicum)* Linn. Vent. Kurtz.

The plant is used traditionally for a wide diversity of ailments such as cancer, diarrhea, fever, ulcer, jaundice and arthritis[5].It is used as key ingredients of mostly used ayurvedic preparation *Dasamula*[6]. According to *Charaka Samhita*, this plant is described in *Sothahara Mahakashaya* (Anti-inflammatory group of drugs).

पाटलाग्निमन्थश्योनकविल्वकाश्मर्यकन्टकारिकावृहतीशालपर्णीपृश्निपर्णीगोक्षुरका

इति दशेमानि श्वयथुहराणी भवन्ति ।। च.सं.सु. ४/१६

The plant is also known with other names as *Soshana, Nata, Mandukaparni, Arula* etc. According to Ayurvedic classic text books the bark of the *Shyonaka* is indicated for *Atisara* or diarrhoea and root is suggested for the treatment of *Shotha* or Oedema[7,8]. According to *Acharya Bhavamishra* documentation, the unripe fruit of this plant has *Vata Pitta-hara* property and when the fruit is ripen then the character of this plant is *Vata-vardhaka.* Principal chemical constituent of this plant is Ellagic acid but there are some other chemical constituents like Baicaleine, Tetuine, Oroxindin, aloe-emodin, Chrysin β-sitosterol, etc[7] (fig. 2).

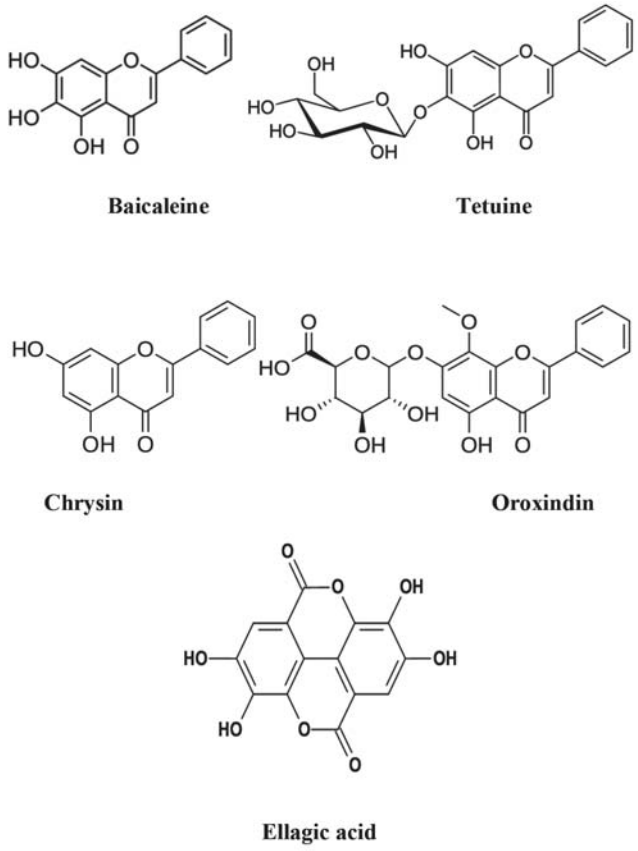


Fig.2. Different Chemical Constituents of *Oroxylum indicum*

Materials and Methods

Selection and identification of plant. The plant *Oroxylum indicum* is collected from North East region of India, particularly from Gangtok, Sikkim. Secondly, the authentication of the plant sample (BIPS/PSP/SKB-001/2021) was done from Botanical Survey of India, Howrah (vide authentification number BSI/PLANT CHEM/0002/2022 dated, 07.01.2022) and voucher specimen is preserved accordingly.

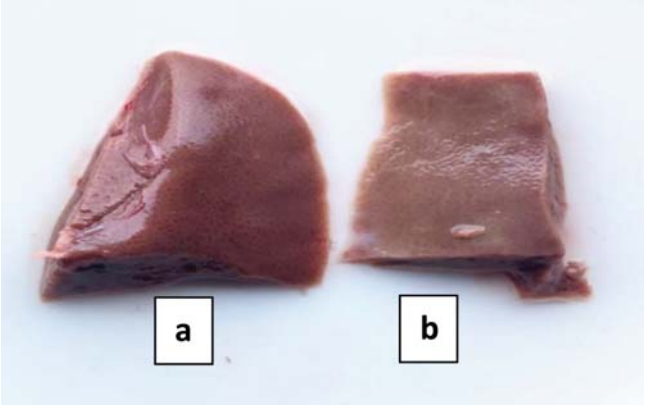


Fig 3. The unperfused liver (a) turns into perfused liver (b) with 0.9% NaCl solution

Extraction of plant. The coarsely powdered dried seed of *O indicum* weighing 3 g was soaked in 200 ml of solution prepared with 100 ml of 50% ethanol and 100 ml of DDW. The solution mixture was rotated in automated rotator for 30 minutes, cooled down and then kept for overnight in 4^0C temperature freezer. The mixture was filtered through Whatsman'0' number filter paper in a conical flask. The filtrate is collected and the precipitate residue was discarded. The filtrate was thereafter placed in glass petridishes and placed over water bath at 100^0C for about 3 hours till the liquid part completely evaporated. The material was collected and further placed in vacuumed drier so that the material became sticky. The material was collected by scratching with a sterile spatula. The extract was preserved in aseptic sealed container at 4^0C temperature. The yield of the extract was 0.3708 g (~370 mg).

Pharmacognostic study. Different Pharmacognostic studies like moisture content, total ash and pH value of the *o. indicum* seed was evaluated. Moisture content (%), of the hydro-alcoholic extract of *O. indicum* was screened and an amount of 7.94 g test sample was used for this experimental purpose. The initial weight of test sample was taken after placing the sample in petridish and then places in hot air oven for 20 minutes and the reducing weight was calculated. This process was repeated for consecutive five times. Total ash content (%) was measured by converting the mineral constituents to the respective oxides at 600°C. An amount of 3.594 g test sample was used for experimental purpose. The initial weight of test sample was taken with china dish and then placed in the muffle furnace for 2-3 hours. After the experiment was completed and cooled down, it was observed that the test sample was totally carbone free and looking white in colour. To measure the pH of *O. indicum* an amount of 20 mg / ml liquid extracted test sample (*O. indicum*) was taken and measured with pH instrument.

Chemical screening. Chemical screening was done by three methods as TLC, HPTLC and HPLC. *Rf Value (Retention factor / Perferdation factor) of TLC* was calculated by following formula[9]

$$\text{Rf Value} = \frac{\text{Distance travelled the solute}}{\text{Distance travelled the solvent}} = \frac{A}{B}$$

HPTLC was performed with an amount of 20.0 µl each samples of S*honyka*, Gallic acid, Quercetine and Ellagic acid were injected one after another in HPTLC instrument of CAMAG Linomat 5 [Linomat5_210131" S/N 210131 (1.00.13)]. The size of 6.0 x 10.0 cm of HPTLC plate was prepared with HPTLC plates silica gel 60 F 254 for experimental purpose. HPTLC chromatograms were detected at two different wavelengths (254 and 366 nm) using AMD detector. The plate was spotted automatically and prepared chemical solvent system with ratio of Benzene : Chloroform : Methanol = 6:3:2 in TLC chamber. Plate was placed in TLC chamber and plate slides were running under TLC chamber. Plate was placed in TLC scanner instrument to identify chemical compounds and calculate Rf value automatically[10]. HPLC was performed with the stock solution of 1 mg/ml concentration the of standard phenolics acids (gallic acid, protocatechuic acid, gentisic acid, chlorogenic acid, *p*-hydroxy benzoic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, sinapic acid, salicylic acid and ellagic acid) and flavonoids (catechin, rutin, myricetin, quercetin, naringin, naringenin, apigenin and kaempferol) was set up in methanol. The working arrangements were set up by diluting the standard solution with the mobile phase solvent system. The standard & working solutions were filtered through 0.45 ìm PVDF-syringe filter before injecting in the HPLC instrument. Separate extraction of plant was done for this purpose in which 100g of each powdered plants were extracted twice with methanol and extraction was achieved with agitation for 18–24 h at ambient temperature. Concentrates acquired from the first and the subsequent extractions were pooled and concentrated using a rotary evaporator under reduced pressure to obtain viscous extracts which were further dried using a freeze drier. The dry extracts were stored at minus (-) 20°C until use. The dry extract obtained was weighed. HPLC analyses for the quantification of phenolic acids and flavonoids in the water extract was performed following method of Datta et al. 2019 using Dionex Ultimate 3000 liquid chromatograph including a diode array detector (DAD) with 5 cm flow cell and with Chromele on system manager as data processor. Separation was achieved by a reversed phase Acclaim C_{18} column (5 micron particle size, 250 x 4.6 mm). 20 µL of sample was introduced into the HPLC column. The method was validated according to the USP and ICH guidelines. The mobile phase contains methanol (Solvent A) and 0.5% aq. acetic acid solution (Solvent B) and the column was thermostatically controlled at 25^0C and the injection volume was kept at 20 ìl. A gradient elution was achieved by varying the ratio of solvent A to solvent B. Total analysis time per sample was 105 min. HPLC chromatograms were detected at three different

wavelengths (272, 280 and 310 nm) using a photo diode array UV detector. Each compound was recognized by its retention time and by spiking with standards under the same conditions[11].

Hepatoprotective Study on Goat Liver

Chemicals and reagents used. Chemicals and reagents like 0.9 % Normal saline, Phosphate-buffered saline (PBS), HBSS (Hank balance salt solution), Fetal Bovine Serum or FBS (SRL, India), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution and Di-methyl sulfoxide (DMSO) solution were used for this purpose.

Preparation of goat liver by perfusion technique. An amount of » 100 g of liver from healthy adult goat, was collected from local abattoir. It was used for animal experiment within 30 minutes of procurement. Liver was cut into small pieces of arround 4-5 g. It was perfused by injecting normal saline (0.9%) till the colour of the liver turned from red to white (fig 3) indicating complete ellimination of RBC from the liver.

Preparation of liver extract. The perfused liver pieces were kept in HBSS (pH 7.2) with intermittent oxygenation. HBSS solution at an amount of 5 ml was added in the perfused liver pieces.Thesepieces were then cut into more fine small pieces in a glass pestle. The solution of liver pieces thus produced, were filetred through muslin cloth. This filtrate was then centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded to remove cellular debris. This process was repeated twice to remove debris completely and get clear cell pellet. Cell pellet was then resuspended in 6 ml HBSS, supplemented with 1% FBS. HBSS was added to provide physiological enviroment and FBS was added to provide protein supplimentation to the isolated liver cells.The suspention was then placed over glass slide and observed under light microscope to detect cell population. The hepato- toxicity was induced with absolute alcohol.-The whole experiment was divided into 3 groups as follows-

i) Group –I : 50 µl HBSS + 450 µl liver cell suspension

ii) Group –II : 20 µl plant extract (5mg/ml) +50 µl alcohol + 450 µl liver cell suspension

iii) Group – III: 50 µl alcohol + 450 µl liver cell suspension

All the 3 groups of mixtures were kept for 2 hours at 37^0C in bacteriogical incubator. After 2 hours, all these samples were centrifuged for 5 minutes at 1000 rpm (revolution per minute). Supernatant of different mixtures were collected and preserved in eppendorf at 4^0C for enzyme assay. The residual (pellet) was used for cell viability MTT assay and performed according to the description of Meerloo, *et al*. [12]. In the process of measurement of MTT an amount of 500 µl (0.5 %) solution of MTT was added in cell pellet. The colour of mixture was yellowish green. Then it was placed into bacteriogical incubator for 1 hour at 37^0C. After that, sample was collected from bacteriogical incubator and centrifuged for 5 minutes at 1000 rpm. Supernatant was discarded and cell pellet was used for next step. An amount of 500 µl of DMSO was added in the sample which turns into blue colour after breaking down of liver cells. The OD of the sample was measured against control in spectrophotometer at 570 nm. Aspartate transaminase (AST), Alanine transaminase (ALT) and Lactate Dehydogenase (LDH) were assayed according to conventional technique [13-17].

Results

Extraction of O. indicum. The 50% ethanol extracts of the plant *Oroxylum indicum* as done by conventional chemical methods yield 0.3708 g from 5 g of crude powder of seed.

Pharmacognostic study. It was observed from the study that the moisture content of the test sample (*O. indicum*) was 10.91% w/w, total ash was 5.57% w/w and pH was 5.40.

Chemical screening. The results of chemical screening of the 50% ethanolic extract of *Oroxylum indicum* were observed on the basis of the technique of TLC and HPLC.

Rf values of TLC. Rf values of *O. indicum* 50% ethanolic extract as observed with different solvents is depicted in table 1.

HPTLC. It was observed from the study that there were four end peaks of Rf value of *O. indicum* (0.30, 0.39, 0.51 and 0.57), four end peaks of Rf value of Gallic acid (0.32, 0.41, 0.57 and 0.84), seven end peaks of Quercetin (0.27, 0.43, 0.63, 0.73, 0.77, 0.86 and 0.99) and nine end peaks of Ellagic acid (0.26, 0.31, 0.35, 0.42, 0.62, 0.69.0.74, 0.88 and 0.95) respectively at 254 nm. These values at 366 nm were three end peaks of Rf values of *O. indicum* (0.23,

Table 1. Rf values of *O. indicum* in different solvents on TLC

Sl No	Chemical reagents	Ratio of chemical reagents	Solvent front (cm.)	Sample front (cm.)	Rf [Retention factor] value(cm.)
1.	Benzene : Chloroform	1:1	16	2	0.125
2.	Benzene : Chloroform : Methanol	6:3:1	15.5	1.6, 5.7, 9.6	0.1032, 0.3677, 0.6193
3.	Benzene : Chloroform : Methanol	6:3:2	16	3.5, 10.4, 11.6, 12.2	0.21875, 0.65, 0.725, 0.7625
4.	Chloroform : Methanol	9:1	15	4.2, 10.6, 11.9, 12.8	0.28, 0.7066, 0.7933, 0.8533

0.50 and 0.55), three end peaks of Rf value of Gallic acid (0.23, 0.56 and 0.84), six end peaks of Quercetin (0.07, 0.22, 0.59, 0.78, 0.85 and 1.03) and six end peaks of Ellagic acid (0.06, 0.20, 0.61, 0.87, 0.95 and 1.08) respectively (fig.4). This study indicates that the maximum chemical contents were Ellagic in the sample which resembles with the study of HPTLC.

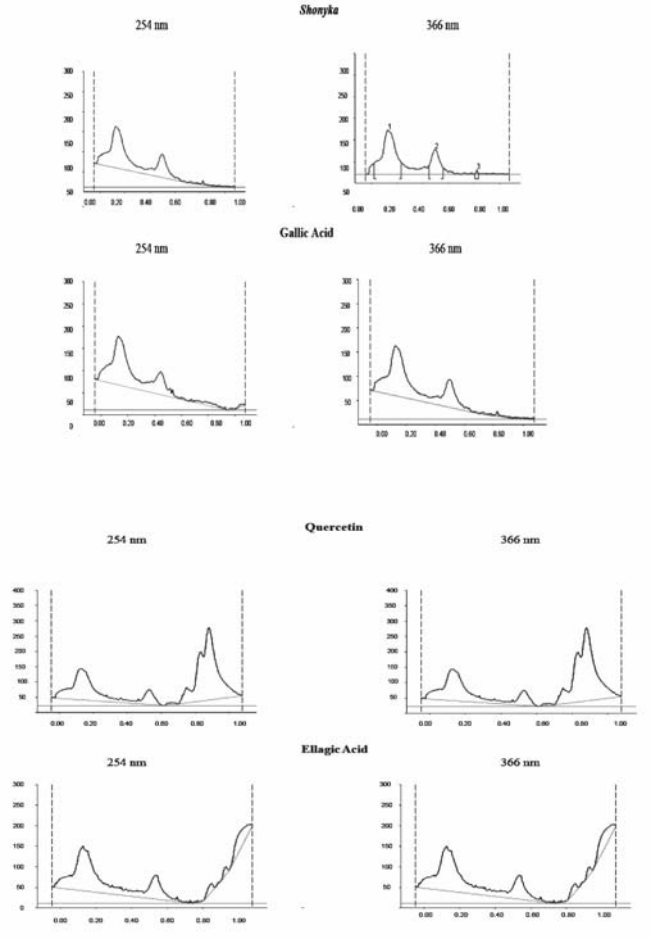


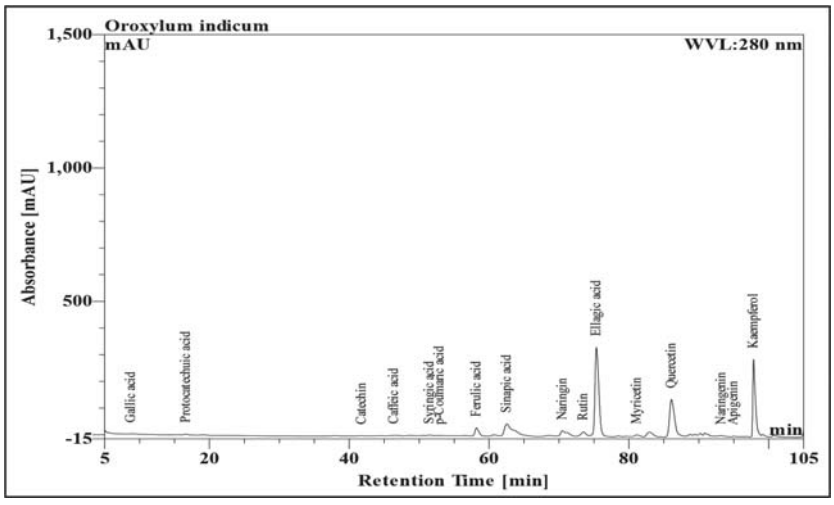
Fig.4. HPTLC Peaks of Shyonaka, Gallic acid, Quercetin & Ellagic Acid at 254 & 366 nm

HPLC. The quantification of phenolic acids and flavonoids in the extracts were carried out by the measurement of the integrated peak area and the contents were calculated using the calibration curve by plotting peak area against concentration of the respective standard sample. The data were reported with convergence limit in triplicate. It was observed in HPLC study that Ellagic acid was found highest in amount (38.87µg/mg). Quercetin (20.57µg/mg) and Kaempferol (16.17µg/mg) were found next in amount to Ellagic acid. There are many other varieties of phenolic acids and flavonoids chemical components of the methanolic extract of seed of *O. indicum* (fig.5).

Hepato-protective study. The results of the hepato-protective effect of the 50% methanolic extract of *Oroxylum indicum* were observed on the basis of the assay of MTT, AST, ALT and LDH. There were promising effects of reduction of these bio-markers with the treatment groups of mixture of liver extract suspension added with hydro-alcohol extract of *O. indicum* and alcohol (ethanol) with respect to alcohol or control groups (table 2).

Discussion and Conclusion

Human beings are exposed to various types of noxious agents in their daily life which results alteration of physiological activity leading to disease or decay. These noxious agents may be introduced from the source of environment through diets, water, air, land, etc. or from different artificial substances. Manifestations of diseases due to exposure to these agents or their by-products occur in human system due to pathological changes of various organs. Liver is an important organ of the body which performs one of the most valuable physiological activities like metabolism. Nutrition of different parts of body is provided by liver after a long process of biochemical changes, applicable for specific dietary substances. Maintenance of health of liver is important for maintaining of health in general of an individual. Liver may be damaged by various etiological factors like unhealthy diets, introduction to certain drugs and chemicals in system, alcoholic beverages, etc. manifesting different pathological conditions like hyperbilirubinaemia, hepatitis, cirrhosis, etc. It is also reported that about 107 medicinal plants and 58



[Absorbance m AU means - Mili Absorbance Unit, Retention time Indicates by Minutes, Wave Length- 280 nm]

Fig. 5. Phenolic Acids and Flavonoids in O. indicum by HPLC

Table 2. Results of Hepatoprotective Study of *O. indicum* in Goat Liver

Treatment groups	MIT (% Mean Viability)	AST		ALT		LDH	
		(IU/L)	(%) of HP	(IU/L)	(%) of HP	(IU/L)	(%) of HP
Alcohol + *O. indicum*	49.10	241.82	42.98	167.17	30.95	617.84	44.73
Alcohol	0.00	326.59	0.00	200.18	0.00	730.29	0.00
N / Control	100.00	129.36	100.00	93.51	100.00	478.91	100.00

Legend: IU/L = International unit per liter, HP = Hepato protection

compounds isolated from higher plants, can be utilized for the treatment of liver diseases[18].

The present research work was carried out in two phases as extraction and chemical screening of the seed of the *O. indicum* and *in vitro* hepato-protective activity on isolated goat liver. The Ellagic acid content is found maximum in the seed of *O. indicum* as envisaged through HPTLC ay 254 and 366 nm which resembles with the study of HPLC. The HPLC study of the 50% ethanolic extract of *O. indicum* reveals that it contains multiple phenolic and flavonoids group of compounds like gallic acid, catechin, p-coumaric acid, ferulic acid, sinapic acid, naringin, rutin, ellagic acid, quercetin, naringenin, apigenin and kaempferol. Among these compounds, Ellagic acid was found in maximum amount 38.87 µg/mg as studied through HPLC method. The hepato-protective effect of the 50% ethanolic extract of the seed of *O. indicum* was found far better than absolute ethanol treated group indicating promising role of *O. indicum* extract as hepato-protective agent. In accordance to Ayurvedic pharmacokinetic and pharmacodynamic mechanism, the plant possesses *Tikta, Kasaya* and *Madhura* rasa, *Laghu* and *Ruksha* guna, *Anushna* veerya and *Katu* vipaka. The *Tikta, Kasaya* and *Madhura* rasa are capable of reduction of Pitta dosa; *Laghu* and *Ruksha* guna can increase Vata dosa which is opposite to Pitta dosa; *Anushna* veerya is also responsible for reduction of Pitta dosa having Ushna quality and *Katu* vipaka that reduce Pitta as oppose to Vata dosa. The disease of liver or *Yakrita Vikara* according to Ayurveda is due to the influence of aggravation of Pitta dosa and hence the aim of treatment is to reduce it (*Pitta Shamak)*, which is satisfied by the plant *Shyonaka* or *Oroxylum indicum*. It may, therefore, be concluded that the plant extract can protect isolated fresh hepatic cells from alcohol induced hepato-toxicity and has no such cellular toxicity due to presence of phenoilic compounds. The study reveals a new avenue of Ayurveda to explore the hepatoprotective activity of *O. indicum* which was not directly mentioned in classical text. ❐

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