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# *IN VITRO* ANTIOXIDANT AND HEPATOPROTECTIVE POTENTIAL OF *NEPENTHES KHASIANA* HOOK. F AGAINST ETHANOL-INDUCED LIVER INJURY IN RATS

**Tiewlasubon Uriah*, Mrityunjaya B Patil and Sivaiah Kumar**
Department of Pharmacognosy, KLE University's College of Pharmacy, Nehru Nagar, Belgaum - 59010, Karnataka, India.

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## ABSTRACT

**Purpose**: To evaluate the *in vitro* antioxidant and hepatoprotective activity of *Nepenthes khasiana* leaf extracts against ethanol-induced hepatotoxicity.

**Approach**: *In vitro* antioxidant activity was measured spectrophotometrically for the methanolic and aqueous leaf extracts of *N. khasiana* using different assays i.e DPPH, superoxide anion and hydroxyl radical scavenging activity. For hepatoprotective effect, the rats of either sex were administered with 30% w/v ethanol at a dose of 5 g/kg b.w. orally for three weeks. Methanolic extract of *N. khasiana* leaf was administered to the experimental rats at 100 mg, 200 mg and 400 mg/kg, p.o. for three weeks. The hepatic markers such as alanine aminotransferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), total bilirubin and total proteins were evaluated and supported by histopathological studies of the liver.

**Findings**: The methanolic leaf extract of *N. khasiana* exhibited strong antioxidant capacity with $IC_{50}$ values of 23.33±0.441, 62.75±0.713 and 38.38±0.425 µg/mL respectively and this extract was selected for hepatoprotective studies. The results showed that administration of methanolic leaf extract of *N.khasiana* reversed the liver damage due to alcohol as evident from histopathological studies of the liver. The HPTLC fingerprinting analysis of the methanolic leaf extract of *N. khasiana* at different wavelengths revealed presence of many compounds which may provide basic information regarding the isolation, purification, characterization and identification of marker chemical compounds of the plant species.

**Originality**: The present study is the first time evaluation of the hepatoprotective effect of the methanolic leaf extract of *N. khasiana* on ethanol-induced liver injury in rat models. The isolation and characterisation of the active principle require extensive research in the future.

**Conclusion:** From our study, the methanolic leaf extract of *N. khasiana* shows potent hepatoprotective effects, hence it can be used as a natural protecting agent against liver damage.

**Key words:** *N. khasiana; DPPH; ethanol- induced liver injury; hepatic markers.*

## INTRODUCTION

Reactive Oxygen Species (ROS) are naturally generated by metabolism of the normal cells in the body and are well recognised for playing a dual role in human physiological and pathophysiological processes [1,2]. ROS have beneficial effects at low or moderate concentrations and without the production of these species our body could not fight invading organisms or damaged cells, which plays a key role in disease-resistance, cell-mediated immunity and microbicide activity[3]. On the other hand, if these molecules react with hydrogen atoms from other molecules it leads to the formation of highly reactive free radicals. Thereby, a condition called "oxidative stress" arises when there is interference in the balance between ROS production and ROS removal. Oxidative stress is linked to the pathogenesis of several chronic human diseases including ethanol-induced hepatic injury [4]. ROS include superoxide anion radical, singlet oxygen, hydrogen peroxide, and the highly reactive hydroxyl radical[5]. To prevent the deleterious damages due to these highly reactive compounds antioxidant enzymes such as superoxide dismutase, catalase, peroxidases and glutathione peroxidase trap and scavenge these free radicals converting them into non-toxic compounds [6]. Nowadays, antioxidant therapy has gained immense importance worldwide. Use of synthetic antioxidants to prevent free radical damage has been reported to involve toxic side effects thus necessitating the search for natural antioxidants and free radical scavengers[7]. In recent years, various natural products and their effects on health, especially the suppression of active oxygen species by natural antioxidants from tea, spices and herbs, have been intensively studied. Therefore, inhibition of free radical induced oxidative damage by supplementation of antioxidants has become an attractive therapeutic strategy for reducing the risk of these diseases.

Alcohol abuse and alcoholism are worldwide health and socioeconomic problems among adults and the younger generation [8]. According to the report by WHO, alcohol related diseases in most developed countries and in the developing countries of Central and South America, Eastern Europe, and East Asia are one of the leading causes of death and disability[9]. Many mechanisms

*Correspondence : email : lasubon0210@gmail.com

contribute to a myriad of direct and indirect ill effects of alcohol and they interact in complex ways. Increased oxidative stress due to excessive production of free radicals plays a central role in the pathogenesis of liver disease and ultimately the pathway towards alcoholic liver disease[10]. Both acute and chronic ethanol exposure to humans and also to rats enhances the production of ROS causing damage to the liver. The development for a suitable treatment of alcoholism however still remains a challenging aim among several researchers. Modern medicine has little to offer protection to liver injury or help to regenerate hepatic cells. Because of severe undesirable side effects of synthetic products, evaluation on scientific basis for the traditional herbal medicines which are claimed to possess hepatoprotective activity may be a great benefit as an alternative therapy in alcohol induced liver diseases. Herbal drugs possessing natural antioxidants polyphenolics[11] such as anthraquinones, flavonoids, aromatic acids, and tannins have been found to have reactive oxygen species (ROS) scavenging and lipid peroxidation preventing effects and are considered to be safe, effective and inexpensive.

Nepenthaceae is represented by a single genus Nepenthes which is popularly known as tropical pitcher plant or monkey cups or Indian pitcher plant [12]. About 70 species are found worldwide with Indo-Malaysia considered as the center of evolution. The one and only pitcher or insectivorous plant of India, namely *Nepenthes khasiana* Hook. F. named after the Khasi Hills is largely endemic to Meghalaya, North East India. Though their distribution is restricted to exceptionally small area, yet it is found in abundance in the natural habitats of the East Khasi hills *(Smit & Nohron* Villages*),* West Khasi Hills *(Mawsynram & Mawkyrwat* Villages*),* Jaintia Hills (Jarain village), and in the Garo hills (Nokrek Biosphere Reserve of Meghalaya)[13,14] and several other places . The vernacular name for this plant is *"Tiew-Rakot"* (Demon-flower) in Khasi, *"Kset Phare"(*a device for trapping insects) in Jaintia and *"Memang-Koksi "* (basket of devil) in Garo[15]. The liquid present inside the unopened pitcher of the plant is prescribed to diabetic patients, to cure night blindness, conjunctivitis, ear trou bles and gynaecological problems[16]. Many Khasi people consume the liquid as a digestive tonic and is taken in the morning. The crushed powder of the pitcher is given to patients suffering from cholera. Leprosy is being treated by applying a fine paste of the pitcher with its contents by the Garo traditional medical practitioners [17]. Detailed information on the traditional uses of this species is very scanty, is not documented anywhere and is passed on orally from generation to generation by the tribal people of this region. Considering these folkloric claims in view, the present study attempted to evaluate the possible hepatoprotective potential of *N. khasiana* leaf extracts in an animal model, against alcohol-induced liver injury in rats.

## MATERIALS AND METHODS

### Chemicals

Folin-Ciocalteu reagent, gallic acid, quercetin, aluminium chloride, trichloroacetic acid, 2-deoxy-2-ribose, trisHCl, ascorbic acid, thiobarbituric acid (TBA), ferric chloride, ammonium molybdate, nitrobluetetrazolium, methanol were procured from Hi-Media, Merck and Rankem. 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma Aldirch. Silymarin (10gms) was received as a gift sample from Zydus Cadila, Ahmedabad. Diagnostic Kits of ERBA Diagnostic Mannheim GmbH, Germany was used for biochemical parameters. All chemicals and solvents used for the experimental work were of analytical grade.

### Plant material

The leaves of *N. khasiana* were collected from the natural habitat of Nohron and Jarian , East Khasi Hills district of Meghalaya in the month of April to September. The botanical identification of the plant material was done by Dr. B. K. Sinha Scientist-E & H.O, at the Herbarium of the Botanical Survey of India, Eastern Regional Centre, Shillong, Meghalaya where a voucher of the specimen (accession number 081153 dated: 9th January 2013) was deposited.

### Preparation of extracts

The leaves were washed, dried under shade, pulverized and sieved. Two hundred g of the dried plant material was extracted by cold maceration separately with methanol and water under room temperature for seven days with occasional shaking and stirring. The solvent was removed by filtration through a muslin cloth followed by Whatmann filter paper, the filtrate obtained was centrifuged at 3500 x g for 10 min and the supernatant were pooled out. The supernatant was concentrated under reduced pressure using IKA Rotary Evaporator at 40° C. The concentrated mass obtained was kept in hot air oven at 40° C until it was completely dried, finally the product was ground to fine powder using glass mortar and pestle and stored for further studies.

### Animals

Adult Wistar albino rats weighing 150-200 g of either sex were used in the experimental work. The animals were housed in six groups, comprising of six animals each and maintained under standard conditions of temperature, humidity and 12/12 h light/dark cycles, fed with standard rat diet and clean drinking water *ad libitum* throughout the experimental schedule. The experimental design was approved by the Institutional Animal Ethics Committee (IAEC) of KLE College of Pharmacy, Belgaum, Karnataka (Reg. No. 221/CPCSEA; 16th June 2000), protocol approval resolution number KLECOP/IAEC/res.17-31/08/2013.

### Phytochemical screening

Preliminary phytochemical screening of the prepared methanolic and aqueous leaf extracts was performed as per the standard method[18, 19] to identify the presence of

### HPTLC fingerprinting analysis

The HPTLC fingerprinting analysis of the methanolic leaf extract of *N. khasiana* was developed whereby a sample volume of 5 µl were spotted in the form of bands of width 8 mm with a 100 µl syringe with the help of Linomat 5 applicator. The mobile phase for developing the chromatogram was composed of toluene:

chloroform: ethanol mixture in the ratio 4:4:1 (v/v/v). The study was carried out using a CAMAG-HPTLC instrumentation equipped with a CAMAG TLC scanner 3, CAMAG TLC visualizer and WINCATS software for data interpretation. The Rf values were recorded and the developed plate was screened and photo-documented at three different wavelength (λ max) of 240 nm (Fig.1), 366 nm (Fig.2) and 540 nm (Fig.3) respectively.

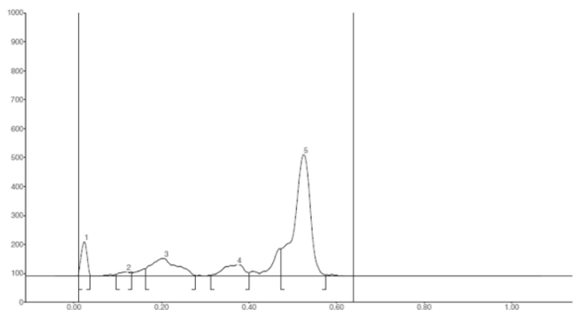


***Fig. 1****: HPTLC Chromatogram of the methanolic leaf extract of N. khasiana - Peak densitogram displayed (scanned at 240 nm)*

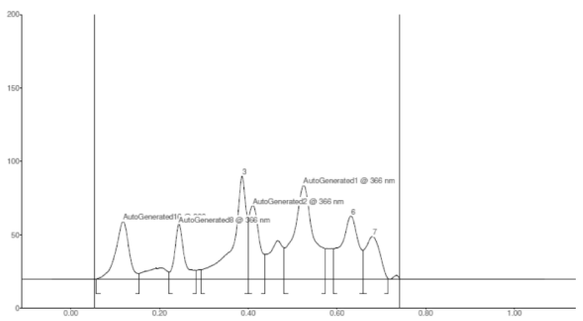


***Fig. 2****: HPTLC Chromatogram of the methanolic leaf extract of N. khasiana - Peak densitogram displayed (scanned at 366nm)*

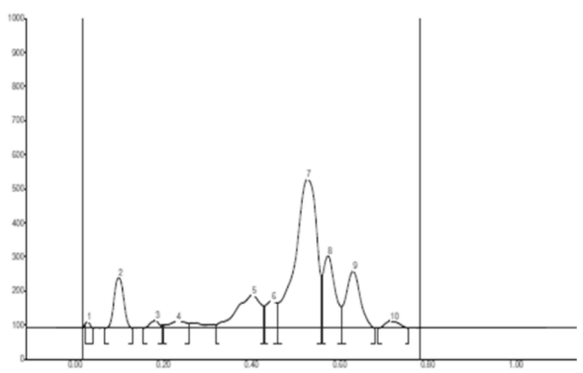


***Fig. 3****: HPTLC Chromatogram of the methanolic leaf extract of N. khasiana - Peak densitogram displayed (scanned at 540 nm)*

**Determination of total phenolic, total flavonoid and total antioxidant content**

Total phenolic content of the methanolic and aqueous leaf extracts of *N.khasiana* was determined by Folin–Ciocalteau assay [20]. The results were expressed as mg of gallic acid equivalents per g of dry extract. Determination of total flavonoid content was performed according to the colorimetric method described by Zhischen *et al*[21] and the flavonoid content was expressed as mg of quercetin equivalent per g of dry extract. The total antioxidant capacity was determined by phosphomolybdate method described by Prieto *et al*[22] with slight modification. The antioxidant capacity was calculated in terms of mg of gallic acid equivalent per g of dry extract. Standard curve of gallic acid is presented in Fig. 4A and that of quercetin in Fig. 4B.

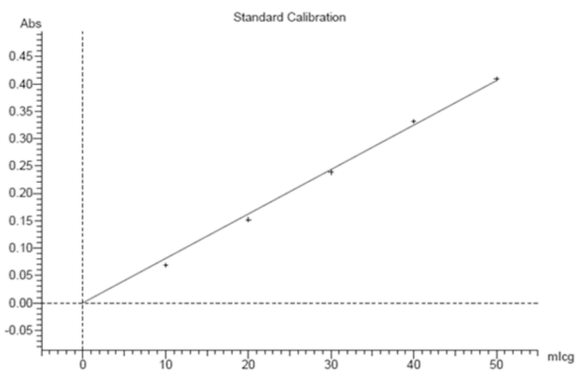



***Fig. 4 (A)****: Standard calibration curve of gallic acid*

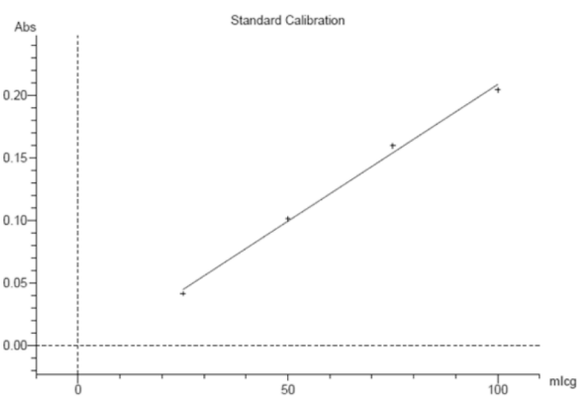



***Fig. 4 (B)****: Standard calibration curve of quercetin*

**FREE RADICAL SCAVENGING ASSAYS**

**DPPH radical scavenging activity**[23]

DPPH was prepared in methanol and the reaction mixture consist of 2ml of 0.1 mM DPPH solution added to the methanolic leaf extract of varying concentrations. Similarly, this was carried out with the aqueous leaf extract. The mixture was vortexed and allowed to stand for 30 min at room temperature. The absorbance was measured at 517nm against a blank. The percentage of antioxidant activity was calculated as follows:

Scavenging activity (% inhibition) = $[(A_c-A_s)/A_c]*100$ Eq…(1)

**Where,** $A_c$ = absorbance of control and $A_s$ = absorbance of sample

**Superoxide radical scavenging activity**[24]

For measuring superoxide anion scavenging activity the reaction mixture consist of 0.5 ml of NBT (0.3 mM), 0.5 ml NADH solution (0.936 mM), 1.0 ml extract and 0.5 ml

Tris-HCl buffer (16 mM, pH 8). The reaction was initiated by the addition of 0.5 ml PMS solution, incubated at 25°C for 5 min and the absorbance was measured at 560 nm. Quercetin was used as a standard. The percentage of inhibition was calculated by using the equation (1).

**Hydoxyl radical scavenging activity**[25]

The hydroxyl radical scavenging activity was assayed by the Deoxyribose method. The reaction mixture contained 3 ml of phosphate buffer (0.1M, pH 7.4), 1 ml of 2-deoxyribose (10mM), 0.5 ml of EDTA (20 mM), 0.5 ml of ferric chloride (20 mM), 3.8 ml of distilled water, various concentrations of the methanolic and aqueous leaf extracts, 1 ml of Hydrogen peroxide (10 mM) and 0.1ml of ascorbic acid (1mM). The above mixture was incubated at 37ºC for 1hr. Thereafter 5 ml of trichloro acetic acid (TCA) (2.8%; w/v) and 5 ml of 1% aqueous thiobarbituric acid (TBA) was added to the reaction mixture. It was heated for 15 min on a boiling water bath until pink color had developed. After cooling the absorbance was taken at 532 nm against a blank. The scavenging activity on hydroxyl radical was calculated by using the equation (1).

**Experimental design for hepatoprotective activity**

Rats were allocated randomly to six experimental groups of six animals each. The treatment groups were pretreated with the methanolic leaf extract of *N. khasiana* (100, 200 and 400 mg/kg/day per oral) for one week and the experiment was continued for additional three weeks, during which all the other groups except the normal group received ethanol (5 g/kg b.w, 30% w/v) once daily per oral and 2 h apart. The normal group received normal saline (1ml/kg b.w) in addition to their normal diet.

**Assessment of hepatoprotective activity**

Blood was collected from each group by retro orbital plexus in previously labelled blood collecting tubes and allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 3000 rpm for 15 min. The separated serum was used for the estimation of biochemical parameters.

**Biochemical estimations**

Biochemical parameters such as aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin and total protein were determined by using commercially available kits (ERBA Diagnostic Mannheim GmbH, Germany.) following the manufacturer's protocol supplied with the kits.

**Histopathology studies**

The liver tissues were removed after sacrificing by cervical dislocation, fixed in 10% buffered formalin and later dehydrated with alcohol. Liver tissues were cleaned and embedded in paraffin, cut into 3–5 µm sections, stained with the haematoxylin-eosin dye and finally, observed under a photomicroscope for morphological changes such as cell necrosis, ballooning degeneration, fatty changes or inflammation of lymphocytes.

**Statistical analysis**

The data for various biochemical parameters were analyzed by one way ANOVA followed by Tukey's multiple comparison tests using statistical soft wares. P values <0.01 were considered as statistically significant.

## RESULTS

**Extraction yield of plant material**

Extraction yield (w/w) of the methanolic and aqueous leaf extracts of *N. khasiana* (NKM & NKA) was found to be 12.10% w/w and 25.2% w/w. .

**Phytochemical investigation**

Preliminary phytochemical investigation reveals the presence of flavonoids, tannins as phenolic compounds, carbohydrates, and steroids presented in Table 1. High Performance Thin Layer Chromatography (HPTLC) analysis of the methanolic leaf extract of *N. khasiana* scanned at 254, 366 and 540 nm revealed the presence of numerous peaks with different Rf values as shown in Table 2. This indicates that many compounds may be present, which will provide basic information regarding the isolation, purification, characterization and identification of marker chemical compounds of the plant species.

***Table 1:*** *Preliminary phytochemical analysis of the methanolic and aqueous leaf extracts of Nepenthes khasiana*

| Phytoconstituents | Test performed | NKM | NKA |
|---|---|---|---|
| Carbohydrates | Molisch's test, Fehling's test, Barfoed's test, Benedict's test | + ve | + ve |
| Flavonoids | Shinoda test, Lead acetate test | + ve | + ve |
| Steroids | LibermannBurchard test, Libermann's test | + ve | + ve |
| Alkaloids | Dragendorff's test ,Mayer test,Hager's test, Wagner's test | - ve | - ve |
| Tannins and phenolics | 5% Ferric Chloride, dilute iodine solution, dilute $KMnO_4$ | + ve | + ve |

+ve: indicates the presence of phytoconstituents
-ve: indicates the absence of phytoconstituents

***Table 2****: Data pertaining to HPTLC fingerprint of the methanolic leaf extracts of Nepenthes khasiana*

| Sr.No | Wavelength (nm) | No. of peaks | Rf value | Percent area (%) |
|---|---|---|---|---|
| 1 | 254 | 5 | 0.04,0.13,0.28,0.40,0.58 | 6.99,1.74,15.99,8.36,66.93 |
| 2 | 366 | 7 | 0.15,0.28,0.40,0.44,0.57,0.66, 0.72 | 10.87,7.57,20.17,10.89,26.34, 15.63,8.53 |
| 3 | 540 | 10 | 0.04,0.13,0.20,0.26,0.43,0.46, 0.56,0.61,0.68,0.76 | 0.41,7.15,1.05,1.81,11.96,4.38, 47.06,12.72,11.89,1.56 |

**Total Phenolic, Total Flavonoid and Total Antioxidant Content**

Our result reveals that the total phenolic content of NKM and NKA was 178.8±2.13 & 176.5±2.10 mg GAE/g of the dry extract, the total flavonoid content was 312.7±3.97 and 115.7±1.03 mg QAE/g of dry extract and the antioxidant content was 304.45±3.42 & 279.95±2.78 mg GAE/g of the dry extract respectively as shown in Table 3.

***Table 3:*** *Total phenolic, Total flavonoid, and Total antioxidant content of leaf extracts of Nepenthes khasiana*

| Sr.No. | Name of extracts | Total phenolic (mg GAE /g) of dry extract | Total flavonoid (mg QE /g) of dry extract | Antioxidant capacity (mg GAE /g) of dry extract |
|---|---|---|---|---|
| 1 | *Nepenthes khasiana* (Methanolic) | 178.8± 2.13 | 312.7±3.97 | 304.45.±3.42 |
| 2 | *Nepenthes khasiana* (Aqueous) | 176.5 ±2.10 | 115.7±1.03 | 279.95±2.78 |

The average values of three calculations are presented as mean ± SEM (n=3)

**Free radical scavenging activity**

The DPPH, superoxide anion and hydroxyl radical scavenging activities of NKM, NKA and standard drug at different concentrations are given in Figure 5 (A-E). In the DPPH scavenging assay the $IC_{50}$ (the concentration required to scavenge 50% of radical) values of Quercetin, NKM and NKA were 3.962 µg/mL, 23.33 µg/mL and 80.34 µg/mL. In the superoxide anion scavenging assay the $IC_{50}$ values of Quercetin, NKM and NKA were 12.7 µg/mL, 62.75 µg/mL and 123.41 µg/mL. In the hydroxyl radical scavenging assay the IC 50 values of Quercetin, NKM and NKA were 9.16 µg/mL, 38.38 µg/mL and 111.6 µg/mL, respectively.

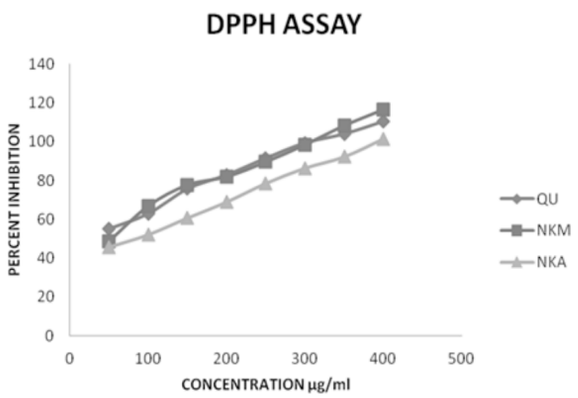



***Fig. 5 (A)**: Antioxidant activity of the extracts of N. khasiana determined by DPPH radical scavenging activity*

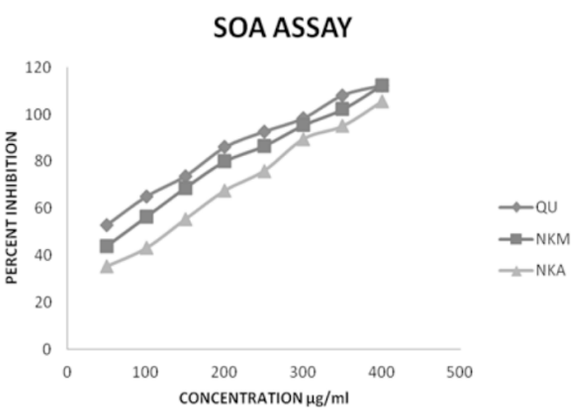



***Fig.5(B)**: Antioxidant activity of the extracts of N. khasiana determined by Superoxide anion radical scavenging activity*

**Hepatoprotective effect of the methanolic leaf extract of N. khasiana on ethanol-induced liver damage in rats**

The activities of various biochemical enzymes in the normal, ethanol control and treated groups are presented in figure 6 (A-E). Ethanol intoxication in rats produced serious liver injury as indicated by the significant elevation of serum ALT, AST, ALP in the ethanol administered group as compared to the normal group. However, upon co-administration of the methanolic leaf extract of *N. khasiana* at different doses,

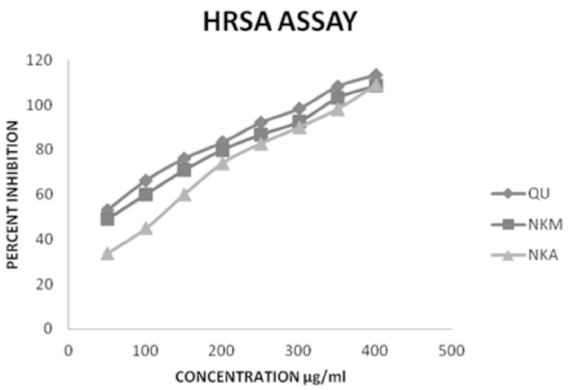



***Fig. 5(C)**: Antioxidant activity of the extracts of N. khasiana determined by Hydroxyl radical scavenging activity*

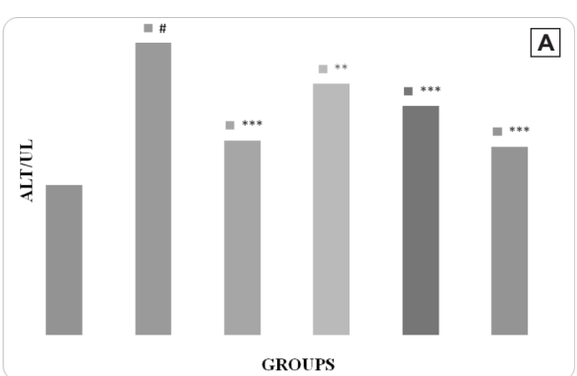
*QU; Quercetin, NKM; methanolic leaf extract of N. khasiana, NKA; aqueous leaf extract of N. khasiana.*

the results obtained showed a significant reversal in the levels of these enzymes as shown in Table 4. The activity of the methanolic leaf extract at the dose of 400 mg/kg was comparable to that of the reference drug Silymarin with a significant reduction in the ALT, AST, ALP and total bilirubin levels. A significant decrease in the total protein level was witnessed in the ethanol treated rats when compared to the normal group. Following treatment with the methanolic leaf extract, there was significant increase in the protein level at 400 mg/kg b.w. similar to the standard group treated with Silymarin.

***Table 4:** Hepatic markers assessment in the serum of normal and ethanol-administered rats*

| GROUP | ALT (IU/L) | AST (IU/L) | Alk .P (IU/L) | TB (mg/dl) | TP (mg/dl) |
|---|---|---|---|---|---|
| Normal | 37.67 ± 1.520 | 93.17± 1.922 | 99.67± 3.095 | 0.2667 ± 0.03333 | 6.300 ± 0.2989 |
| Alcohol induced (5 g/kg/day) | 73.50 ± 2.045 # | 187.3 ± 2.404 # | 209.8 ± 2.688 # | 1.451 ± 0.1626 # | 3.913 ± 0.2359 # |
| Silymarin (50mg/kg b.w) and 30% w/v ethanol | 48.87± 2.819 *** | 120.5 ± 3.314*** | 126.5 ± 5.334 *** | 0.3500 ± 0.02236*** | 5.633 ± 0.2261*** |
| *N.khasiana* extract (100 mg/kg/day) and 30% w/v ethanol | 63.10 ± 0.8491* | 173.3 ± 3.432* | 188.5± 5.284 ** | 1.004 ± 0.08762** | 4.913 ± 0.2467* |
| *N.khasiana* extract (200 mg/kg/day) and 30% w/v ethanol | 57.50 ± 1.945 *** | 159.0 ± 2.295*** | 141.0± 2.160 *** | 0.7108 ± 0.03339*** | 5.163± 0.1471** |
| *N.khasiana* extract (400 mg/kg/day) and 30% w/v ethanol | 47.33± 2.418 *** | 133.8 ± 2.761*** | 128.0± 0.3651*** | 0.4347 ± 0.04180*** | 5.600 ± 0.08563 *** |

N=6, Values are expressed as mean ±S.E.M with six animals in each group.* indicates statistical significance ***p<0.0001, **p<0.001, *p<0.01 vs ethanol intoxicated group, # p<0.0001 vs normal group



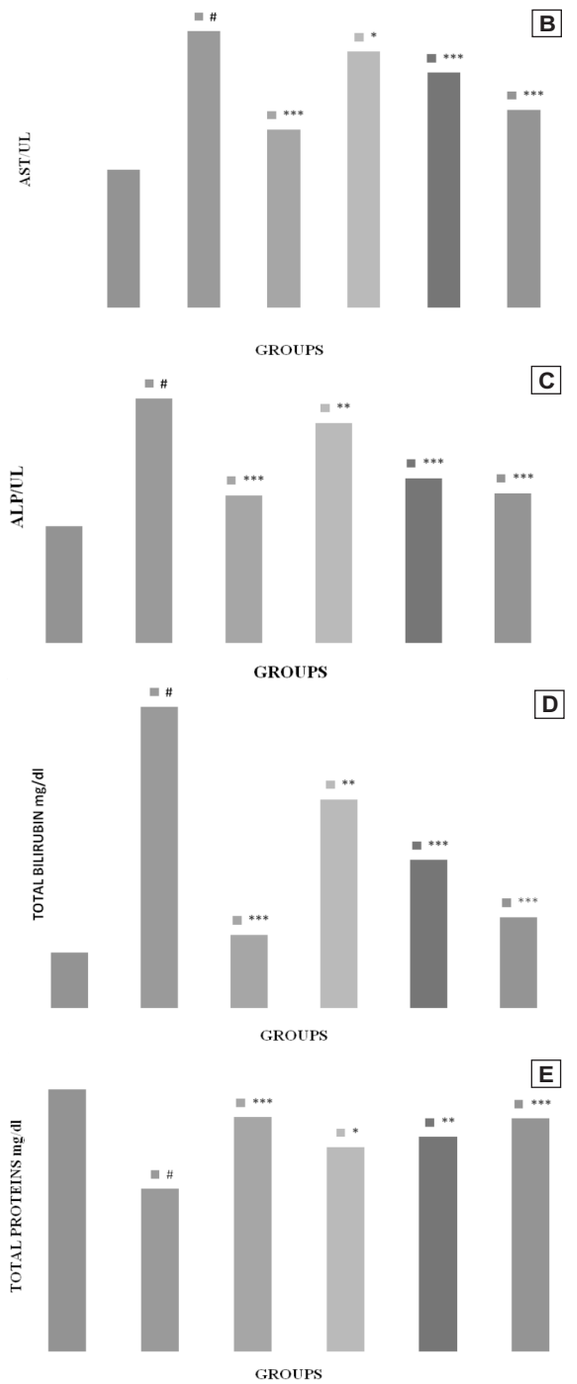



***Fig. 6****: Effect of the methanolic leaf extract of N. khasiana on serum biochemical parameters against ethanol (5 g/kg bw, 30% w/v) induced liver injury. (A) Representation of alanine aminotransferase. (B) Representation of aspartate aminotransferase. (C) Representation of alkaline phosphatase. (D) Representation of total bilirubin. (E) Representation of total proteins.*

*Values are expressed as mean ±S.E.M with six animals in each group. * indicates statistical significance ***p<0.0001, **p<0.001, *p<0.01 vs. ethanol intoxicated group, # p<0.0001 vs normal group.NKL: N. khasiana low dose, NKM: N. khasiana medium dose and NKH: N. khasiana high dose.*

**Effect on liver histopathological investigation**

The histopathological observation as shown in figure 7 supports evidence for biochemical results which demonstrated that the methanolic leaf extract of *N. khasiana* effectively protects the liver from alcohol induced hepatotoxicity. From the study, the liver of normal rats (Fig.7A) exhibited hepatic cells with well-preserved cytoplasm and nucleus. In contrast, the ethanol intoxicated rats (Fig.7B) revealed hydropic degeneration of hepatocytes with dense chronic inflammatory infiltrate in the portal triad region. The hepatocytes showed degenerative changes to the toxin. Treatment with Silymarin (Fig.7C) and higher dose, i.e 400 mg/kg b.w. of the methanolic leaf extract of *N. khasiana* (Fig.7F) reduced the ethanol induced morphological changes in the liver. Normal appearing hepatocytes arranged in cords radiating towards the periphery from the central vein was observed. Treatment with lower dose, i.e 200 mg/kg and 100mg/kg of the methanolic leaf extract of *N. khasiana* (Fig.7D & Fig.7E) shows mild to moderate inflammation and cellular infiltration in the region of the portal triad.

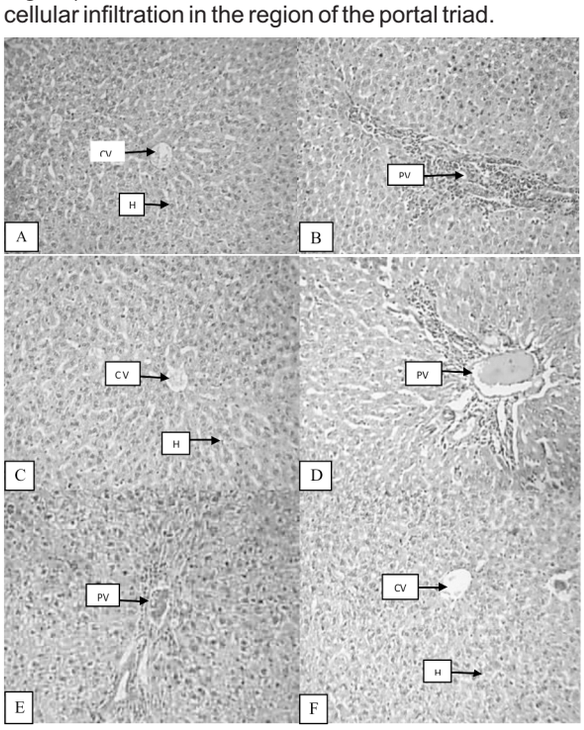



***Fig. 7****: Histopathalogical monograph of extract and standard (40× magnifications).*

*A. Normal Saline B. Ethanol (5 g/kg , 30% w/v) C. Silymarin (50mg/kg + ethanol 5 g/kg , 30% w/v) D. NK extract (100mg/kg + ethanol 5 g/kg , 30% w/v) E. NK extract (200mg/kg + ethanol 5 g/kg , 30% w/v) F. NK extract (400mg/kg + ethanol 5 g/kg , 30% w/v). (CV-Central vein, H-Hepatocytes, PV-Portal vein)*

## DISCUSSION

The capacity of the reactive oxygen species to react in the system has postulated many conditions in humans such as aging, arthritis, cancer, inflammation, and heart diseases.[26] Antioxidants thus, play an important role in neutralizing the excess of free radicals arising from the

consequences of their chemical reaction, thereby protecting the cells against their toxic effects and contribution to disease.[27] Secondary metabolites such as flavonoids and other dietary phenolic compounds are the subject of increasing scientific interest as they serve as sources of antioxidants, which provide beneficial effects to many cell systems in the body.[28] In our study, both the extracts of *N. khasiana* leaf exhibit effective scavenging potential and this might be attributed to the presence of flavonoids and phenolic compounds in the plant as evident from the preliminary phytochemical investigations. The total phenolic, total flavonoid and total antioxidant contents were higher in the methanolic leaf extract which indicates good antioxidant activity. The *in vitro* studies conducted on DPPH, hydroxyl, and superoxide reveals that the methanolic leaf extract when compared to the aqueous leaf extract has high potential to quench these radicals generated during alcohol metabolism. Indeed, these methods have proven the effectiveness of the extract comparable to that of quercetin. Many studies have reported that the antioxidant properties in different plant families are mainly due to the presence of high content of the bioactive compound, the flavonoids.[29-31]

Alcohol-induced liver damage results from associated nutritional deficiency and the direct toxic effects during alcohol breakdown, primarily in the liver. This leads to impaired digestion, reduced absorption and impaired utilization of nutrients [32] which may sensitize the cells to further injury. Excessive or long term use of alcohol contributes to alcoholic liver disease development by excess production of reactive oxygen species (superoxide, hydroxyl and hydroxyethyl radicals) which are generated during ethanol metabolism via CYP2E1 isozyme with cytochrome reductase. These may initiate membrane lipid peroxidation and cytokine release, which perpetuate liver injury.[33,34] The present study is the first attempt to test the hypothesis of the effect of the methanolic leaf extract of *N. khasiana* at varying doses that would possibly ameliorate alcoholic damage in experimental rats. Numerous studies have indicated that excessive ethanol intake induces the mass production of free radicals in the body, which are considered to be associated with alcoholic liver disease.[35] The extent of liver injury due to alcohol was being estimated by biochemical parameters and histopathological studies. Liver function marker enzymes like ALT, AST, ALP, TP and total bilirubin are important diagnostic tool for detecting hepatic dysfunction.[36] The findings in the present study are in agreement with the previous studies revealing that there is an elevation in the serum levels of ALT, AST, ALP and total bilirubin in the ethanol intoxicated rats when compared to the normal group.[37] This clearly indicates that there is a sign of hepatic injury which is due to leakage of cellular enzymes into the plasma. Ethanol administration for 21 days significantly increased all these serum enzymes, whereas animals treated with the methanolic leaf extract of *N. khasiana* had significantly reduced AST, ALT, and ALP levels in a dose dependant manner indicating that the extract provides protection against liver ultimately preserving the structural integrity of the liver from the toxic effects of ethanol. This may be due to the inhibitory effects on cytochrome P450, resulting in the hindrance of the formation of hepatotoxic free radicals.[38]

On the other hand, the decrease in the level of total protein observed in the ethanol intoxicated group suggested that there is destruction in the number of hepatic cells, which may result in decrease of hepatic capacity to synthesize proteins and accumulation of triglycerides leading to fatty liver.[39] Treatment with the methanolic leaf extract of *N. khasiana* leads to stabilization of serum protein level, which is another clear indication of the improvement of the functional status of liver cells. Histological observation supports the evidence of biochemical parameters which reveals major damage in the ethanol intoxicated rats, whereas treatment with various doses of the methanolic leaf extract of *N. khasiana* reduced the ethanol induced morphological changes in the liver.

In the present study, preliminary phytochemical analysis detected flavonoids, tannins and phenolic compounds, steroids and carbohydrates classes of phytochemical. Earlier reports revealed the presence of unknown flavonoids, phenolic compounds and leucoanthocyanins in some species of *Nepenthes*.[40] Due to the antioxidant potential of the flavonoids we hypothesized that the methanolic leaf extract of *N. khasiana* could possess hepatoprotective property which is evident from the high phenolic and flavonoid content of the plant. From the HPTLC fingerprinting analysis, it has been found that the methanolic leaf extract does not contain a single compound but has a mixture of compounds. Therefore, it may be established that the pharmacological activity shown by them are due to the cumulative effect of all the compounds together. Since there was no scientific evidence regarding the potential liver protective effect of this plant in an animal model, therefore the present study is focused to evaluate the hepatoprotective effect against alcohol-induced liver injury in rats to justify the use of this plant by the indigenous people of the region which was not documented elsewhere.

## CONCLUSION

The result of this study demonstrates that the methanolic leaf extract of *N. khasisana* (400 mg/kg b.w.) was found to be comparable with Silymarin and shows promising hepatoprotective effects. This effect could be due to the antioxidant property found in the extract. However, this would require further detailed investigation of the definitive mechanisms and isolation and characterisation of its active principles that might be useful as a natural protective agent not only against liver damage but also other free radical mediated injuries.

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