

In vivo and *In vitro* Antioxidant and Hepatoprotective effects of Classical ayurvedic formulation Punarnavashtak kwath against Ethanol induced hepatotoxicity

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ABSTRACT

Punarnavashtak kwath (PN) a classical Ayurvedic formulation reported in "Bhaishyajaratnavali" consisting of eight medicinal plants was evaluated for its *in vitro* and *in vivo* antioxidant activity and hepatoprotective effect. *In vitro* antioxidant activity of PN kwath was investigated by DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging activity, superoxide anion and nitric oxide radical scavenging activity and reducing power assay. *In vitro* antioxidant activity was found to be dose dependent. Hepatoprotective and *in vivo* antioxidant effect was evaluated by ethanol (3.7 g/kg, p.o for 45 days in all group except control) induced hepatic damage in rats. Pretreatment with PN kwath 100 mg/kg p.o for 45 days significantly prevented physical (increased liver wt), biochemical (serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), bilirubin and total protein level), histological (damage to hepatocytes) and functional changes (thiopentone induced sleeping time) induced by ethanol in liver. Further PN kwath showed antioxidant activity by increasing activity of GSH, SOD and CAT and by decreasing the level of thiobarbituric acid reactive substance (TBARS). The results were compared to that of reference standard silymarin (50 mg/kg p.o for 45 days). The findings suggest that PN kwath protects the liver cell from ethanol induced liver damages due to its antioxidative effect on hepatocytes.

Key words: Ethanol, Hepatoprotective activity, *In vivo* antioxidant activity; *In vitro* antioxidant activity, Punarnavashtak kwath

INTRODUCTION

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals.^[1] Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism.^[2] The most common reactive oxygen species (ROS) include superoxide (O_2^-) anion, hydrogen peroxide (H_2O_2), peroxy (ROO^-) radicals and reactive hydroxyl (OH) radicals. The nitrogen derived free radicals are nitric oxide (NO^-) and peroxy nitrite anion ($ONOO^-$). ROS have been implicated in over a hundreds of disease states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome.^[3] In

treatment of these diseases, antioxidant therapy has gained an immense importance. Worldover the medicinal properties of plants have been investigated in the recent scientific developments, due to their potent antioxidant activities, no side effects and economic viability.^[4] Flavonoids and phenolic compounds widely distributed in plants have been reported to exert multiple biological effects like antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic activity etc.^[5] They were also speculated to be a potential iron chelators.^[6-7]

Liver diseases remain a serious health problem. It is well known that free radicals cause cell damage through mechanisms of covalent binding and lipid peroxidation with subsequent tissue injury. Antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals.^[8] Numerous medicinal plants and their formulations are used for liver disorders in ethnomedical practices as well as in traditional systems of medicine in India.^[9]

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Alcohol dependency is a major health and socioeconomic problem throughout the world.^[10-11] It has been observed that most of the consumed alcohol is eventually broken down by the liver and the products generated and accumulated during alcohol metabolism (e.g. acetaldehyde) are more toxic than alcohol itself. In addition, a group of metabolic products called free radicals can damage liver cells and promote inflammation, impairing vital functions such as energy production. The body's natural defenses against free radicals (e.g. antioxidants) are inhibited by alcohol consumption, leading to increased liver damage.^[12] Despite great progress made in the field in the past two decades, development of suitable medications for the treatment of alcohol dependency or alcohol-induced health injury remains a challenging goal for management/treatment of alcohol induced hepatotoxicity.

Traditional medicines are effective in certain disorders and are based on experience in the use of plant products in amelioration of common diseases. In the present investigation Punarnavashtak kwath, a classical Ayurvedic polyherbal (Table 1) formulation mentioned in Ayurveda,^[13] consisting of *Boerhaavia diffusa* Linn, *Picrorhiza Kurroa* Royle ex Benth, *Tinospora cordifolia* (Willd.) Miers, *Zingiber officinalis* Rosc, *Berberis aristata* DC, *Terminalia chebula* Retz, *Azadirachta indica* A. Juss and *Tricosanthes dioica* Roxb. plants has been evaluated for its hepatoprotective action against ethanol induced hepatotoxicity. Traditionally this formulation is used in treatment of hepatic disorders and asthma. Many of the individual ingredients of the formulation are reported earlier for their protective activity against different models of experimental hepatotoxicity. An aqueous extract of thinner roots of *Boerhaavia diffusa* at a dose of 2 ml /kg exhibited marked protection of various enzymes such as SGOT, SGPT and bilirubin in serum against hepatic injury in rats.^[14] The active constituents of *Picrorhiza Kurroa* were effective in preventing liver toxicity and the subsequent biochemical changes caused by numerous toxic agents.^[15] *Picrorhiza* extract, when given at a dose of 3-12 mg/kg orally for 45 days, was also shown to be effective in reversing ethanol-induced liver damage in rats.^[16] The hepatoprotective action of *Tinospora cordifolia* was reported in one of the experiment in which goats treated with *Tinospora cordifolia*

have shown significant clinical and hemato-biochemical improvement in CCl₄ induced hepatopathy. Extract of *Tinospora cordifolia* has also exhibited *in vitro* inactivating property against Hepatitis B and E surface antigen in 48-72 h.^[17] The aqueous ethanol extract of *Zingiber officinalis* showed hepatoprotective effect against acetaminophen-induced acute toxicity, mediated either by preventing the decline of hepatic antioxidant status or due to its direct radical scavenging capacity.^[18] *Berberis aristata* and berberine (an alkaloid from *Berberis aristata*) were found to be protective against both paracetamol and CCl₄ induced liver damage and also showed MDME (microsomal drug metabolizing enzymes) inhibitory activities.^[19] *Terminalia chebula* extract was found to prevent the hepatotoxicity caused by the administration of rifampicin (RIF), isoniazid (INH) and pyrazinamide (PZA) (in combination) in a sub-chronic mode.^[20] The aqueous extract of *Azadirachta indica* leaf was found to offer protection against paracetamol induced liver necrosis in rats.^[21] *Tricosanthes dioica* was reported as a hepatoprotective agent in ferrous sulphate (FeSO₄) intoxicated rats.^[22] Poly herbal formulation have synergistic potentiative agonistic/ antagonistic agent within themselves that work together in a dynamic way to produce therapeutic efficacy with minimum side effects.

There is lack of scientific data regarding pharmacological evaluation of PN kwath, consequently it was considered worthwhile to screen PN kwath for its hepatoprotective activity. To understand the mechanisms of its pharmacological actions, *in vitro* and *in vivo* antioxidant activity of PN kwath was investigated.

MATERIALS AND METHODS

Collection of plants and Preparation of formulation

Punarnava, Galo, Tricosanthes, Neem were collected from medicinal garden of APMC College of Pharmaceutical Education and Research (January 2008) and other plants (*Picrorhiza*, *Berberis*, Harde and Ginger) were collected from market. All the plants were authenticated by the botanist, H.N.S.B Science College, Himatnagar and voucher specimen of all plants were kept in department of Pharmacognosy

Table 1: Composition of Punarnavashtak kwath.

No	Botanical name	Family	Part used
1	<i>Boerhaavia diffusa</i> Linn.	Nyctaginaceae	Root
2	<i>Azadirachta indica</i> A. Juss.	Meliaceae	Bark
3	<i>Tricosanthes dioica</i> Roxb.	Cucurbitaceae	Whole
4	<i>Terminalia chebula</i> Retz.	Combretaceae	Fruit
5	<i>Zingiber officinalis</i> Rosc.	Zingiberaceae	Rhizome
6	<i>Tinospora cordifolia</i> (Willd.) Miers.	Menispermaceae	Stem
7	<i>Berberis aristata</i> DC.	Berberidaceae	Stem
8	<i>Picrorhiza Kurroa</i> Royle ex Benth	Scrophulariaceae	Root

APMC College of Pharmaceutical education and research. (APMC 0801 to 0808). Kwath (decoction) was prepared by boiling powder of all drugs (Table 1) in equal quantity in proportion of 16 times of water reduced to one fourth and strained in cloth.^[13] Filtrate was evaporated and dried under reduced pressure. Yield of extract was 10% w/w.

Materials

Diagnostic kits were purchased from span diagnostic Ltd (Baroda, India) Reduced glutathione and thiobarbituric acid were purchased from Kemphasol (Mumbai, India). Tris-HCl was from Loba Chemie Pvt Ltd. (Mumbai, India). All other chemicals were obtained from SD fine chemicals. (Mumbai, India).

Preliminary phytochemical screening

The dried extract of kwath was subjected to the preliminary phytochemical analysis for the presence of different phytoconstituents.^[23]

Acute toxicity study

Swiss albino mice of either sex weighing between 25-30 g were divided into ten groups of six animals in each.^[24] The control group received normal saline (2 ml / kg, p.o). The other groups received 100, 200, 300, 600, 800, 1000, 2000, 3000, 5000 mg/kg of the test extract, respectively. Immediately after dosing, the animals were observed continuously for the first 4 h for any behavioral changes. They were then kept under observation up to 14 days after drug administration to find out the mortality if any. The observations were made twice daily, one at 7 a.m. and another at 7 p.m.

Preparation of stock solution for quantification of polyphenol and Flavonoids

Stock solution of PN kwath 1mg/ml in distilled water was prepared and used for estimation of polyphenol and flavonoids

Quantification of Polyphenol in PN Kwath

Total phenol content in plant extracts was generally determined according to the Folin-ciocalteu method. This colorimetric method is based on the reduction of a Phosphotungstate phosphomolybdate complex by phenolics to blue color products in alkaline conditions.

Each of the 100 µl of samples taken in to 25 ml volumetric flask, to which 10 ml of water and 1.5 ml of Folin Ciocalteu reagent were added. The mixture was then kept for 5 min. and to it 4 ml of 20% w/v sodium carbonate solution was added, the volume was made up to 25 ml with double distilled water. The mixture was kept for 30 minute until blue color developed. The samples were then observed at 765 nm in UV- visible spectrometer Shimadzu, UV-1601,

Japan. The % of total phenolic was calculated from calibration curve of Gallic acid plotted by using similar procedure.^[25-27]

Quantification of total flavonoids

Aluminum chloride colorimetric method was used for flavonoids determination.^[28] The method is based on the quantification of yellow color produced by the interaction of flavonoids with AlCl₃ reagent. 1 ml of sample from stock solution was mixed with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with UV Visible spectrophotometer. The % of total flavonoid was calculated by plotting calibration curve of standard flavonoid (Rutin).

In vitro antioxidant activity

Preparation of stock solution for in vitro antioxidant activity

Methanolic extract of PN kwath was taken and stock solution 1mg/ml was prepared. From the stock solution different concentration of solution was prepared.

DPPH free radical scavenging activity

4.3 mg of DPPH (1, 1-Diphenyl -2-picrylhydrazyl) was dissolved in 3.3 ml methanol; it was protected from light by covering the test tubes with aluminum foil. 150 µl DPPH solution was added to 3 ml methanol and absorbance was taken immediately at 516 nm for control reading. Different concentrations of kwath were taken and the volume was made uniformly to 150 µl using methanol. Each of the samples was then further diluted with methanol up to 3 ml and to each, 150 µl DPPH was added. Absorbance was taken after 15 min. at 516 nm using methanol as blank on UV-visible spectrometer Shimadzu, UV-1601, Japan. IC₅₀ value for kwath was calculated.^[29]

Super Oxide free radical scavenging activity

100 µl Riboflavin solution [20 µg], 200 µl EDTA solution [12 mM], 200 µl methanol and 100 µl NBT (Nitro-blue tetrazolium) solution [0.1 mg] were mixed in test tube and reaction mixture was diluted up to 3 ml with phosphate buffer [50 mM]. The absorbance of solution was measured at 590 nm using phosphate buffer as blank after illumination for 5min. This is taken as control. Different concentrations of kwath were diluted up to 100 µl with methanol, to each of this, 100 µl Riboflavin, 200 µl EDTA, 200 µl methanol and 100 µl NBT were mixed in test tubes and further diluted up to 3 ml with phosphate buffer. Absorbance was measured after illumination for 5 min. at 590 nm on UV visible spectrometer Shimadzu, UV-1601, Japan. IC₅₀ value for kwath was calculated.^[30-31]

Nitric Oxide scavenging activity

Different concentrations of the kwath were taken in separate tubes and the volume was uniformly made up to 150 μ l with methanol. To each tube 2.0 ml, of sodium nitroprusside (10 mM) in phosphate buffer saline was added. The solutions were incubated at room temperature for 150 minutes. Similar procedure was repeated with methanol as blank which served as control. After the incubation, 5 ml of Griess reagent was added to each tube including control. The absorbance of chromophore formed was measured at 546 nm on UV visible spectrometer Shimadzu, UV-1601, Japan. Curcumin was used as positive control.^[32-34]

The DPPH free radical scavenging activity, super oxide free radical scavenging activity and nitric oxide scavenging activity were calculated using the following formula:

$$\% \text{Reduction} = \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \times 100$$

Reducing power assay

The reducing power of phenolic samples was determined by the method of Jayaprakasha et al.^[35] Different concentrations of kwath dissolved in methanol (1 ml) were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. After incubation 20 min at 50°C, 2.5 ml of 10% trichloroacetic acid was added to the mixtures. After centrifugation, 2.5 ml of the upper layer was diluted with distilled water and 0.5 ml of 0.1% ferric chloride was added. The absorbance was measured at 700 nm. Increase in absorbance of the reaction indicated the reducing power of the polyphenol test samples.

Hepatoprotective and in vivo antioxidant activity

Healthy male albino Wistar rats 180-250 g were used. They were collected from animal house, Zydus Cadila Pharmaceuticals, Ahmedabad. The animals were grouped and housed in poly acrylic cages, with not more than two animal per cage and maintained under well-controlled conditions of temperature (27 \pm 2 °C), humidity (55 \pm 5 %) and 12/12 h light-dark cycle were used for the study. Conventional laboratory diet and tap water were provided *ad libitum*. The protocol of the experiment was approved by the Institutional Animal Ethical Committee as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (Proposal no. 08/02, APMC, Himatnagar)

The animals were divided into four groups. Each group consisted of six rats. Normal rats were kept in group I. The rats of group II were given ethyl alcohol (3.7 g/kg) for 45 days.^[36] Group III animals received simultaneous

feeding of both ethyl alcohol (3.7 g/kg) and PN kwath (100 mg/kg p o) for 45 days. Group IV animals received simultaneous feeding of both ethyl alcohol (3.7 g/kg) and Silymarin (50 mg/kg p o) for 45 days. After last dose of PN kwath, thiopentone sodium was injected (40 mg/kg i.p) in all group to record sleeping time.^[37]

Experimental analysis was carried out 24 h after the last dose of ethyl alcohol. Liver was isolated and used for antioxidant activity and histopathological analysis

Biochemical studies

Blood was taken from rats by puncture of the retro-orbital plexus, after 24 h of last dose of ethyl alcohol and allowed to coagulate at 37°C for 2 h, serum was separated by centrifugation at 3000 rpm for 10 min and analyzed for various biochemical parameter namely SGPT, SGOT,^[38] SALP,^[39] serum bilirubin,^[40] and protein content.^[41]

In vivo antioxidant activity

For estimating antioxidant activity, animals were sacrificed and their liver was excised, rinsed in ice-cold normal saline, followed by 0.15 M Tris-HCl (pH 7.4) blotted dry and weighed. A 10% w/v of homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation.^[42] A part of homogenate after precipitating proteins with trichloro acetic acid (TCA) was used for estimation of glutathione.^[43] The remaining homogenate was centrifuged at 15000 rpm for 15 min at 4°C. The supernatant thus obtained was used for the estimation of super oxide dismutase^[44] and Catalase.^[45]

Histopathological studies

Paraffin sections (7 μ m thick) of buffered formalin-fixed liver samples were stained with hematoxylin-eosin to study the histological structure of control and treated rats liver.^[46]

Statistical analysis

Results are expressed as mean \pm S.E.M. The statistical difference was analyzed by one way analysis of variance followed by Tukey-kramer multiple comparison test, significance was calculated as the P value, and P values of less than 0.05 were regarded as statistically significant.

RESULTS

Preliminary phytochemical studies revealed the presence of alkaloid, tannin, saponin, flavonoid and bitter principle in PN kwath.

In acute toxicity study, it was observed that there was no mortality at any of the tested doses (Up to 5000 mg/kg) till the end of 14 days of observation.

Total phenolic and Flavonoid Content

Total phenolic and flavonoids compound were found to be present $7.24 \pm 0.037\%$ and $2.95 \pm 0.13\%$ respectively in PN kwath

In vitro antioxidant activity

PN kwath possessed significant antioxidant activity in DPPH assay with IC_{50} value $14.97 \mu\text{g/ml}$ and was compared with the reference drug ascorbic acid (Table 2). PN kwath also possessed significant nitric oxide scavenging activity with IC_{50} value $97.10 \mu\text{g/ml}$ and superoxide free radical scavenging activity with IC_{50} value $6.40 \mu\text{g/ml}$ was compared with reference drug (Table 3 & 4). The reducing power of PN kwath was increased with increasing dosage (Figure 1) showed significant antioxidant activity.

Hepatoprotective activity

Treatment of rats with ethanol produced an increase in the weight of wet-liver. Rats pretreated with silymarin and PN kwath showed significant decrease in wet-liver weight compared to toxic group (Table 5). Hepatic damage induced by ethanol caused significant rise in marker enzymes SGPT, SGOT, ALP, bilirubin and decrease the level of protein. Oral administration of PN kwath was seen to lower significantly the levels of marker enzymes (SGPT, SGOT, ALP, and Bilirubin) and significantly increased the protein level in rats, compared to ethanol treated group (Table 6). A significant reduction in thiopentone induced sleeping time was observed with PN kwath treated animals compared to ethanol treated animals (Table 5). Effects of PN kwath were compared with standard reference drug silymarin.

In vivo antioxidant activity

The effect of PN kwath on lipid peroxidation (TBARS), glutathione, super oxide dismutase and catalase levels are

Table 2: Effect of PN kwath on DPPH free radical scavenging activity.

Conc ($\mu\text{g/ml}$)	% Inhibition	IC_{50} $\mu\text{g/ml}$	R^2
4	17.50 ± 2.51		
6	23.14 ± 1.40		
8	27.40 ± 1.37	14.97	0.995
10	34.70 ± 2.09		
15	50.68 ± 2.09		
Std (ascorbic acid)		8.75	0.9919

The sign R^2 is correlation of Regression
Values are means \pm SD (n=3).

Table 3: Effect of PN kwath on Nitric oxide scavenging activity

Conc ($\mu\text{g/ml}$)	% Inhibition	IC_{50} $\mu\text{g/ml}$	R^2
20	10.74 ± 0.36		
40	21.40 ± 1.11		
60	33.29 ± 1.65	97.105	0.9947
80	40.22 ± 1.73		
100	51.42 ± 1.65		
Std (Curcumin)		17.72	0.9921

Values are means \pm SD (n=3).
The sign R^2 is correlation of Regression.

Table 4: Effect of PN kwath on Super oxide free radical scavenging activity.

Conc ($\mu\text{g/ml}$)	% Inhibition	IC_{50} $\mu\text{g/ml}$	R^2
4	36.61 ± 1.49		
6	49.70 ± 0.30		
8	57.08 ± 0.52	6.40	0.990
10	69.46 ± 1.16		
15	88.99 ± 0.12		
Std (Ascorbic acid)		4.77	0.990

Values are means \pm SD (n=3).
The sign R^2 is correlation of Regression.

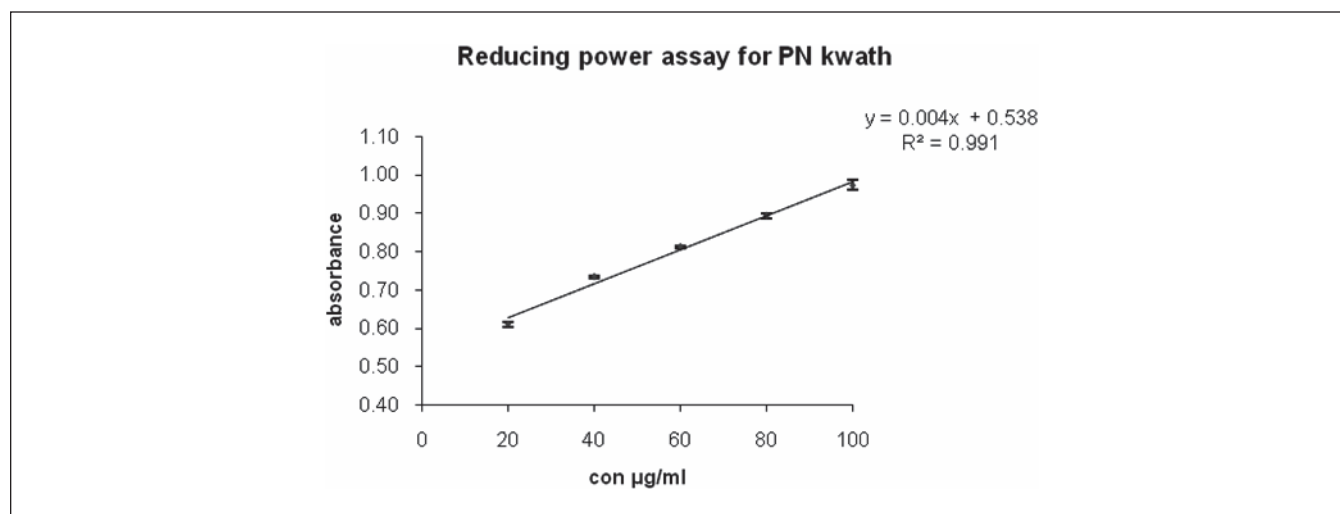


Figure 1: Reducing power of PN kwath at different concentrations. Each value represents means \pm SD (n=3). High absorbance at 700 nm indicates high reducing power.

shown in Table 7. Thiobarbituric acid reactive substance levels were significantly increased in the ethanol treated rats when compared with the normal rats. Treatment with PN kwath significantly prevented the increase in TBARS levels and brought them near to normal level. GSH and CAT activity were significantly increased in PN kwath treated groups. PN kwath also showed increase in activity of SOD compare to toxicant but it was not significant. The effects of PN kwath were comparable to that of standard reference drug silymarin.

Histopathological study

Hepatocytes of the normal control group showed a normal lobular architecture of the liver. In the ethanol treated group the liver showed microvascular fatty changes and the hepatocytes were surrounded by large number of fat droplets. (Figure 2a, Figure 2b) Silymarin and PN kwath

pretreated groups showed minimal fatty changes and their lobular architecture was normal, indicating the hepatoprotective effect of these extracts (Figure 2c, 2d).

DISCUSSION

Although oxygen is essential for life, its transformation to reactive oxygen species (ROS) may provoke uncontrolled reactions. Such challenges may arise due to exposure to radiation, chemicals or by other means. Antioxidants may offer resistance against oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and some other mechanism.^[7]

Polyphenol and flavonoids are used for the prevention and cure of various diseases which is mainly associated with free

Table 5: Effect of Punarnavashtak kwath on different biochemical parameter in ethanol induced hepatotoxicity.

Group	SGOT(U/L)	SGPT(U/L)	ALKP (U/L)	Bilirubin(mg%)	Protein(mg/dl)
I (control)	40.33 ± 2.26	103.83 ± 7.31	130.17 ± 8.50	0.56 ± 0.03	5.65 ± 0.27
II (Toxicant)	151.17 ± 4.34 ^a	280.83 ± 9.51 ^a	288.50 ± 13.44 ^a	1.18 ± 0.09 ^a	3.88 ± 0.14 ^a
III (PN kwath)	94.67 ± 3.16 ^{***}	157.67 ± 10.58 ^{***}	186.83 ± 13.59 ^{***}	0.80 ± 0.04 ^{**}	5.23 ± 0.30 ^{**}
IV (Silymarin)	77.50 ± 6.67 ^{**}	128.67 ± 10.66 ^{***}	157.33 ± 10.3 ^{***}	0.66 ± 0.06 ^{***}	5.42 ± 0.26 ^{**}

Values are mean ± SEM of 6 animals in each group

^aP < 0.001 relative to control group.

^{***}P < 0.001 relative to Toxicant group.

^{**}P < 0.01 relative to Toxicant group.

Table 6: Effect of Punarnavashtak kwath on thiopentone induced sleeping time and weight of liver.

Group	Thiopentone sodium induced sleeping time		Liver wt gm/100gm bw
	Onset(s)	Duration(min)	
I (control)	202.50 ± 4.96	76.67 ± 4.94	3.95 ± 0.51
II (Toxicant)	53.33 ± 4.22 ^a	243.33 ± 4.77 ^a	6.42 ± 0.32 ^a
III (PN kwath)	163.50 ± 7.25 ^{***}	132.50 ± 8.83 ^{***}	5.25 ± 0.16 [*]
IV (Silymarin)	179.17 ± 9.44 ^{***}	120.63 ± 6.64 ^{***}	4.83 ± 0.14 [*]

Values are mean ± SEM of 6 animals in each group

^aP < 0.001 relative to control group

^{***}P < 0.001 relative to Toxicant group

^{*}P < 0.05 relative to Toxicant group

Table 7: Effect of Punarnavashtak on lipid peroxidation (MDA), superoxide dismutase (SOD), catalase and glutathione level in ethanol induced hepatotoxicity

Group	TBRAS	Gutathione	Catalase	SOD
	nmol/ mg protein	µg/mg protein	u/min/mgprotein	U/mgprotein
I (control)	1.92 ± 0.25	56.85 ± 4.26	36.36 ± 4.82	14.59 ± 1.76
II (Toxicant)	7.23 ± 0.70 ^a	31.87 ± 6.44 ^b	9.12 ± 1.65 ^a	4.02 ± 0.62 ^a
III (PN kwath)	3.14 ± 0.44 ^{***}	39.48 ± 3.61 [*]	23.75 ± 0.81 [*]	7.87 ± 0.65 [*]
IV (Silymarin)	2.49 ± 0.41 ^{***}	46.75 ± 7.80 [*]	25.02 ± 3.10 [*]	8.03 ± 0.63 [*]

Values are mean ± SEM of 6 animals in each group

^aP < 0.001 relative to control group

^bP < 0.01 relative to control group

^{***}P < 0.001 relative to Toxicant group

^{*}P < 0.05 relative to Toxicant group

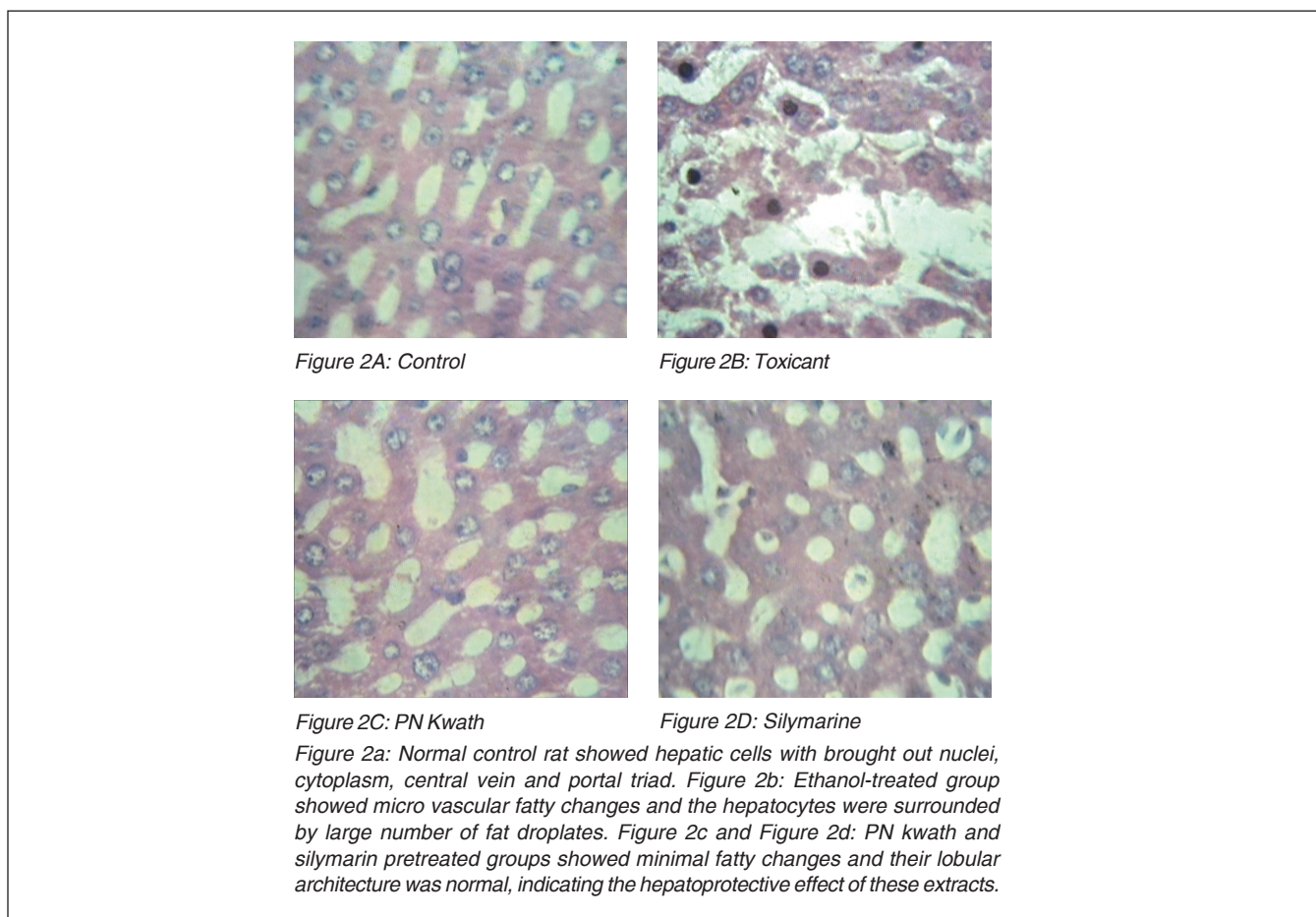


Figure 2: Photomicrograph of liver section taken from rats against ethanol induced hepatotoxicity (X45)

radicals. Significant amount of polyphenolic and flavonoid compound were present in PN kwath, which attributes to its rationality of possessing antioxidant activity.

DPPH radical is considered to be a model of lipophilic radical. A chain reaction in lipophilic radicals was initiated by lipid autooxidation. The radical scavenging activity of PN kwath was determined from the reduction in absorbance at 517 nm due to scavenging of stable DPPH free radical. The dose dependent inhibition of DPPH radical indicated that PN kwath causes reduction of DPPH radical in a stoichiometric manner.^[47]

Nitric oxide (NO) exhibits numerous physiological properties and it is also implicated in several pathological states.^[48] However, the specificity of this assay has been questioned since nitrite is one final product of the reaction of nitric oxide with oxygen, through intermediates such as NO₃, N₂O₄ and N₂O₃.^[49] Therefore the decrease in the nitrite production could also be due to interaction of the extract with other nitrogen oxides.^[50]

The *in vitro* superoxide radical scavenging activity is measured by riboflavin/ light/ NBT (Nitroblue tetrazoline) system

reduction. The method is based on generation of superoxide radicals by autooxidation of riboflavin in the presence of light. The superoxide radical reduces NBT to a blue colored formazone that can be measured at 560 nm. The capacity of the PN kwath to inhibit the colour to 50% is measured in terms of IC₅₀. Superoxide radical is known to be very harmful to the cellular components as a precursor of more ROS.^[31] The extract has been found to have significant superoxide radical scavenging activity, which ultimately adds to its antioxidant potential.

For the measurements of the reducing ability, the Fe₃⁺ to Fe₂⁺ transformation was investigated in the presence of PN kwath. The reducing power of PN kwath increased with increasing dosage.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, and prevention of continued hydrogen abstraction, reductive capacity and radical scavenging.^[51-53]

Liver can be injured by many chemicals and drugs. In the present study ethanol was selected as a hepatotoxicant to induce liver damage, since it is clinically relevant. Ethanol produces a constellation of dose-related deleterious effects in the liver.^[54] In chronic alcoholics, hepatomegaly occurs due to accumulation of lipids and proteins in hepatocytes,^[55] with an impaired protein secretion by hepatocytes.^[56] Water is retained in the cytoplasm of hepatocytes leading to enlargement of liver cells, resulting in increased total liver mass and volume^[57] as observed in the present study. This alcohol-induced increase in total wet-liver weight was prevented by pretreatment with PN kwath, thus indicating a hepatoprotective effect.

The hepatoprotective activity of the ethanolic extract was monitored by estimating serum transaminases, serum alkaline phosphatase and bilirubin, which are indicators of the functional state of the liver.^[58] The increase in the levels of serum bilirubin reflected the degree of jaundice, while increase in hepatic enzymes indicate cellular leakage and loss of functional integrity of cell membrane.^[59] It has been found that PN kwath effectively prevents ethanol-induced biochemical changes of liver toxicity.

Ethanol also alters the metabolic activity of hepatocytes, thereby inducing hepatic damage. Barbiturates are a class of xenobiotics that are extensively metabolized in the liver. Deranged liver function leads to delay in the clearance of barbiturates, resulting in a longer duration of hypnotic effect.^[60] In the present study, administration of thiopentone sodium to rats pretreated chronically with alcohol resulted in an increased duration of thiopentone sleep time. Pre-treatment with PN kwath decreased thiopentone-induced sleep time, an indirect evidence of their hepatoprotective effect.

Formation of ROS, oxidative stress and hepatocellular injury has been implicated in alcoholic liver disease. It has been documented that Kupffer cells are the major sources of ROS during chronic ethanol consumption, and these are primed and activated for enhanced formation of pro-inflammatory factors.^[61] Additionally, alcohol-induced liver injury has been associated with increased amount of TBARS.^[62] Indeed, PN kwath supplementation in our study was potentially effective in blunting TBARS, suggesting that PN kwath possibly has antioxidant property to reduce ethanol-induced membrane lipid peroxidation and thereby preserve membrane structure. It may thus be plausible that in our study, loss of membrane structure and integrity because of lipid peroxidation was accompanied with an elevated level of activities of SGOT, SGPT, ALP and bilirubin.

Our study further revealed that decrease in the activity of antioxidant enzymes SOD, CAT and GSH following ethanol exposure may be due to the damaging effects of free radicals,

or alternatively could be due to a direct effect of acetaldehyde, formed from oxidation of ethanol, on these enzymes. In our studies, it reveals that PN kwath could restore the activity of both these antioxidant enzymes and possibly could reduce generation of free radicals and hepatocellular damage.

Histological changes such as steatosis (fatty changes in hepatocytes) and perivenular fibrosis were observed in ethanol-treated (toxic) control group. Both the extracts prevented these histological changes, further indicating their hepatoprotective activity. All the histological changes observed were in correlation with the physical, biochemical, and functional parameters of the liver.

Ethanol, even after short-term consumption, induces CYP2E1 enzyme activity in doses that do not cause fatty changes. This enzyme accelerates alcohol metabolism with a resultant increase in acetaldehyde production.^[63] Acetaldehyde is thought to have a number of adverse effects like decreased transport and secretion of proteins owing to tubulin polymerization, enhanced vitamin metabolism and trace metals and drugs like paracetamol cause severe acute toxicity which is sometimes fatal.^[64-65]

Antioxidants exhibit hepatoprotective activity by blocking the conversion of ethanol to acetaldehyde.^[66] From the above studies it was found that PN kwath exhibited an *in vitro* antioxidant property.^[67,68] which may be responsible for the hepatoprotective activity of PN kwath. The presence of secondary metabolites like tannin, alkaloids, flavonoids, saponins and bitter principle in PN kwath may be responsible for the significant hepatoprotective activity. Detailed studies on the mechanism of action and phytochemical analysis are in progress at our laboratory.

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