Estimation of biomarkers berberine and gallic acid in polyherbal formulation punarnavashtak kwath and its clinical study for hepatoprotective potential

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Punarnavashtak (PN) kwath is a classical Ayurvedic formulation mentioned in Ayurvedic literature "Bhaishyajyaratnavali" for hepatic disorders and asthma. Standardization and clinical trial to support its efficacy are lacking. So, in the present study, standardization of PN kwath was done by using biomarkers, gallic acid and berberine, and its hepatoprotective activity was evaluated by clinical study to rationalise the traditional use of this formulation. PN kwath was standardized by HPTLC (High performance thin layer chromatography) using gallic acid and berberine as biomarkers and was subjected to clinical study. For clinical study patients attending outpatient clinics, with an evidence of liver disease were included in the study. During the study period, patients who fulfilled inclusion criteria were randomly assigned. The recommended dose was 20 ml kwath daily for 8 weeks. All the patients underwent clinical examination and laboratory investigations for liver functions tests before the commencement of therapy. Thereafter, clinical assessments were done after 8 weeks of treatment. The results showed significant changes in liver functions tests [serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), total bilirubin]. There was no report of adverse effects attributable to this formulation. Our results suggest that PN kwath showed significant hepatoprotective activity. Berberine and gallic acid were found to be 0.08 and 4.9%, respectively. Our results suggest that PN kwath showed significant hepatoprotective activity due to presence of various phytoconstituents and support its traditional uses in liver disorder.

Key words: Berberine, clinical study, gallic acid, hepatoprotective, punarnavashtak kwath

INTRODUCTION

Liver is an important organ actively involved in many metabolic functions and is the frequent target for a number of toxicants.[1] Hepatic damage is associated with distortion of these metabolic functions.[2] Liver disease is still a worldwide health problem. Unfortunately, conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effects.[3] In the absence of a reliable liver protective drug in modern medicine, there are a number of medicinal preparations in Ayurveda recommended for the treatment of liver disorders.[4] Ayurveda is one of the major health care systems developed since human civilisation in the Indian subcontinent, which is based upon the experiences with nature and natural resources. Scientific evidences to prove the rationale of using this formulation in health care are essential to develop and prevent cultural heritage.[5]

Many studies during the last two decades have shown that 20–30% of patients experience unwanted effects of herbal drugs and it seems that in ambulant patients this incidence is even higher. Though herbal medicines have been used since ancient times, there is need for safety evaluation of them. Proper clinical and phamacovigilance study of traditional medicines can ensure their safer use in the patient care.[6] There are a number of classical Ayurvedic formulations reported in Ayurveda but standardization and clinical trial to support their efficacy are lacking.

In light of these observations, we planned to evaluate clinically one Ayurvedic formulation "punarnavashtak (PN) kwath", mentioned in Ayurvedic literature "Bhaishyajyaratnavali", 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 for its hepatoprotective potential.[7] Traditionally, this formulation is used in the treatment of hepatic disorders and asthma. Individual ingredients of the formulation were reported earlier to exhibit protective activity against different models of experimental hepatotoxicity. An aqueous extract of thinner roots of *B. diffusa* exhibited

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in vivo hepatoprotective activity against hepatic injury in rats.[8] *P. kurroa* and its active constituents were effective in preventing liver toxicity caused by numerous toxic agents.[9,10] *Ti. cordifolia* showed significant *in vivo* hepatoprotective activity in CCl_4-induced hepatopathy in goats and *in vitro* inactivating property against Hepatitis B and E surface antigen.[11] The aqueous ethanol extract of *Z. officinalis* showed hepatoprotective effect against acetaminophen-induced acute toxicity due to its direct radical scavenging capacity.[12] *B. aristata* and berberine (an alkaloid from *B. aristata*) were found to be protective against both paracetamol- and CCl_4-induced liver damage.[13] *Te. chebula* extract was found to prevent the hepatotoxicity caused by the administration of rifampicin (RIF), isoniazid (INH) and pyrazinamide (PZA).[14] The aqueous extract of *A. indica* leaf was found to offer protection against paracetamol-induced liver necrosis in rats.[15] *Tr. dioica* was reported as a hepatoprotective agent in ferrous sulphate ($FeSO_4$) intoxicated rats.[16] Polyherbal formulations have synergistic potentiative agonistic/antagonistic pharmacological agents within themselves, which work together in a dynamic way to produce therapeutic efficacy with minimum side effects.

MATERIALS AND METHODS

Materials

All the chemicals used in the experiments were of analytical grade. Berberine and gallic acid (purity 99%) were purchased from Natural Remedies Pvt. Ltd. (Bangalore, India). All the solvents used in the experiments were of analytical grade.

Collection of Plants and Preparation of Formulation

Punarnava, Galo, Tricosanthes and Neem were collected from medicinal garden of APMC College of Pharmaceutical Education and Research (January 2008) and other plants (Picrrorhiza, Berberis, Harde and Ginger) were collected from market. All the plants were authenticated by the botanist Mr. M. M. Prajapati, H.N.S.B Science College, Himatnagar, and voucher specimens of all plants were kept in Department of Pharmacognosy, APMC College of Pharmaceutical education and research (APMC 0801–0808). PN Kwath (decoction) was prepared by boiling the powder of all drugs [Table 1] in equal quantities in a proportion of 16 times of water reduced to one fourth and strained in a cloth. The filtrate was evaporated and dried under reduced pressure. The yield of extract was 10% w/w.

Preliminary Phytochemical Screening

The dried extract of kwath was subjected to the preliminary phytochemical analysis for the presence of different phytoconstituents.[17]

Acute Toxicity Study

Swiss albino mice of either sex weighing between 25 and 30 g were divided into 10 groups of six animals in each. The control group received normal saline (2 ml/kg p.o.), while 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 hours for any behavioural changes. They were then kept under observation up to 14 days after drug administration to find out mortality, if any. The observations were made twice daily, once at 07:00 a.m. and again at 07:00 p.m.[18]

Methodology for Estimation of Berberine and Gallic acid in Punarnavashtak Kwath

Apparatus

Spotting device: Linomat V Automatic Sample Spotter (CAMAG, Muttenz, Switzerland)
Syringe: 100 µl (Hamilton, Bonaduz, Switzerland)
Thin layer chromatographic (TLC) chamber: Glass twin trough chamber (20 × 10 × 4c m) (CAMAG)
Densitometer: TLC Scanner 3 linked to Win Cats software (CAMAG)
HPTLC plates: 20 × 10 cm, 0.2 mm thickness precoated with silica gel 60 F_{254} (E. Merck, Mumbai, India)
Experimental conditions: Temperature 25±2°C; relative humidity 40%

Preparation of solutions

Standard solution of berberine

A stock solution of berberine was prepared by dissolving 2 mg of accurately weighed berberine in methanol and making up the volume to 25 ml with methanol. From this stock solution, standard solutions of 1.6-4.8 µg/ml were prepared by transferring aliquots (0.2–0.5 ml) of stock solution to 10-ml volumetric flasks and adjusting the volume with methanol.

Standard solution of gallic acid

A stock solution of gallic acid was prepared by dissolving 5 mg of accurately weighed gallic acid in methanol and making up the volume to 50 ml with methanol. From this stock solution, standard solutions of 2–12 µg/ml were prepared by transferring aliquots (0.2–1.2 ml) of stock solution to 10-ml volumetric flasks and adjusting the volume to 10 ml with methanol.

Table 1: Composition of PN kwath

Botanical name	Family	Part used
Boerhaavia diffusa Linn.	Nyctaginaceae	Root
Picrorhiza kurroa Royle ex Benth	Scrophulariaceae	Root
Berberis aristata DC.	Berberidaceae	Stem
Tinospora cordifolia (Willd.) Miers.	Menispermaceae	Stem
Terminalia chebula Retz	Combretaceae	Fruit
Azadirachta indica A. Juss.	Meliaceae	Bark
Zingiber officinalis Rosc.	Zingiberaceae	Rhizome
Tricosanthes dioica Roxb.	Cucurbitaceae	Leaf

Preparation of sample solution

One gram of PN kwath was exhaustively extracted with 25 ml of methanol (3 × 25 ml). The extract was dried completely and the stock solution (5 mg/ml) was prepared.

Calibration curve for berberine and gallic acid

Exactly 10 µl of each of the standard solutions of berberine and gallic acid was applied in triplicate on TLC plates. The plates were developed in a solvent system of toluene:ethyl acetate:methanol:formic acid (3:3:0.2:0.8) at 25±2°C and 40% relative humidity up to a distance of 8 cm. After development, the plates were dried in air and scanned densitometrically at 366 nm for berberine and at 280 nm for gallic acid. The peak areas were recorded. Calibration curves of berberine and gallic acid were prepared by plotting peak areas versus concentration.

Quantification of berberine and gallic acid

Exactly 10 µl of the sample solution was applied in triplicate on a precoated silica gel 60 F_{254} TLC plate (0.2 mm thickness) with the Linomat V Automatic Sample Spotter. The plate was developed in the solvent system of toluene:ethyl acetate:methanol:formic acid (3:3:0.2:0.8) and scanned at 366 nm for berberine and at 280 nm for gallic acid. The peak areas and absorption spectra were recorded. The amount of berberine and gallic acid in the sample was calculated using the respective calibration curves.

Validation of the method

ICH guidelines were (CPMP/ICH/381/95; CPMP/ICH/281/95) followed for the validation of the analytical procedure. The method was validated for precision, repeatability and accuracy. The repeatability of the method was checked by repeated scanning of the same spot of berberine (32 ng) and gallic acid (400 ng) seven times and was expressed as coefficient of variance (% CV). Variability of the method was studied by analysing aliquots of standard solution of berberine (16, 40, 64 ng/spot) and gallic acid (400, 800, 1200 ng/spot) on the same day (intraday precision) and on different days (interday precision) and the results were expressed as % CV. Accuracy of the method was tested by performing recovery studies at three levels (60, 100 and 120% addition). The percent recovery as well as average percent recovery was calculated. For the determination of limit of detection and limit of quantitation, different dilutions of the standard solutions of berberine and gallic acid were applied along with methanol as blank and determined on the basis of signal to noise ratio.

Methodology for Clinical Study of Punarnavashtak Kwath

Inclusion criteria

All the patients aged between 18 and 70 years having abnormal liver functions tests were included.

Exclusion criteria

Patients having evidence of extensive disease that required hospitalisation and pregnant women were excluded from the study.

Withdrawal criteria

The subject may voluntarily withdraw from the trial at any time without giving any reason. A subject can be removed from the trial for the following medical or administrative reasons:

1. Hypersensitivity to the study drug or any component of the medication and
2. Adverse drug reaction severe enough in the investigator's opinion to stop further administration of the drug.

Study procedure

Open labelled, nonrandomised, active controlled clinical study of PN kwath was conducted at Divyajyot Ayurvedic Research Foundation (Shri Dardi Narayan Seva Mandal Hospital, Paldi, Ahmedabad). The study protocol, case report forms (CRFs), regulatory clearance documents, formulation-related information and informed consent forms (in English and Hindi) were submitted to the institutional ethics committee and approved by the same (Protocol No: DARF 03). Trial was registered in clinical trial registry of India (CTRI/2009/091/000719, 22-09-2009). Patients attending outpatient clinics, with an evidence of liver disease were included in a study and informed about the study drug, its effects, duration of the study and overall plan of the study. The patients were included in the clinical study only after written informed consent was obtained from each of them. The history was noted by interviewing the patient. Thorough clinical examination and symptomatic evaluation was carried out and the details were noted down in the CRF. Patients were advised to take PN kwath 20 ml (50 mg/ml) daily for a period of 2 months. Physical measurements includes weight, height, Body Mass Index (BMI) and biochemical parameters including serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), total bilirubin and protein were taken before and after treatment with PN kwath.

Statistical Analysis

The statistical analysis was performed by paired *t*-test. The results were expressed as the mean±SEM to show variations in a group. Differences are considered significant at a *P* value <0.05.

RESULTS AND DISCUSSION

In the absence of a reliable liver protective drug in modern medicine, there are a number of medicinal preparations

in Ayurveda recommended for the treatment of liver disorders.[16] Scientific evidences to prove the rationale of using these formulations in health care are essential to develop cultural heritage.[17] Proper clinical and phamacovigilance study of traditional medicines can ensure their safer use in patient care.[18] In the present investigation, PN kwath, traditionally used in hepatic disorder, was evaluated for its hepatoprotective activity by subjecting it to clinical study.

Preliminary phytochemical screening showed the presence of alkaloids, tannin, flavonoid, saponin, and bitter principle in PN kwath [Tables 2 and 3].

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

Herbal medicines are generally available as a mixture of more than one plant constituent. It is important to quantify the maximum possible number of markers in such herbal formulations by which the quality of the formulations may be assessed/assured.[19] Berberine is a hepatoprotective[17] marker compound that was reported in *B. aristata* and gallic acid was also reported as a hepatoprotective compound[17,20] Berberine and gallic acid were used as markers for standardization of PN kwath. TLC densitometric methods were developed using High Performance Thin Layer Chromatography (HPTLC) for the quantification of these two marker compounds from the polyherbal formulation, PN kwath. Solvent systems were optimised to achieve best resolution of the marker compounds from the other components of the sample extracts. Of the various solvent systems tried, the one containing toluene:ethyl acetate:methanol:formic acid (3:3:0.2:0.8) gave best resolution of berberine (R_f = 0.20) and gallic acid (R_f = 0.39) in the presence of other compounds in the sample extract and enabled the quantification of marker compounds [Figures 1 and 2]. The identity of the bands in the sample extracts was confirmed by comparing the R_f values and the absorption spectra by overlaying their UV absorption spectra with those of their respective standard using TLC Scanner 3. The purity of the bands due to berberine and gallic acid bands in the sample extract was confirmed by overlaying the absorption spectra recorded

Table 2: Preliminary phytochemical screening of PN kwath (chemical test)

Constituents	Test	Results
Alkaloid	Dragondorff test	+ve
	Hagers test	+ve
	Wagners test	+ve
Tannin	Gelatin test	+ve
	Lead acetate test	
Phenolic	Ferric chloride test	+ve
	Folinciocalteu test	
Saponin	Foam test	+ve
Carbohydrate	Molisch test	+ve
Flavonoid	Shinoda test	+ve

Table 3: TLC pattern of PN kwath (solvent system: toluene:ethylacetate:methanol:water 3:3:0.2:0.8)

Detection	R_f value	Inference
UV light at 254 nm	0.12, 0.29,0.39, 0.49,0.55	–
UV light at 366	0.13 (blue) 0.20 (yellow) 0.24 (blue) 0.38 (blue) 0.39 (blue) 0.54 (blue) 0.69 (blue) 0.73 (blue) 0.77 (blue)	–
Dragondorff reagent	0.20	Alkaloid
Anisaldehyde sulphuric acid	0.19 (brown) 0.49 (brown) 0.54 (violet) 0.65 (brown) 0.78 (blue) 0.90 (brown)	Bitter principle
Vanillin sulphuric acid	0.19 (blue) 0.29 (brown), 0.42 (brown), 0.53 (violet), 0.60 (violet), 0.64 (brown), 0.72 (violet)	Bitter principle and saponin
Ferric chloride reagent	0.39 (blue) 0.56 (blue)	Tannin

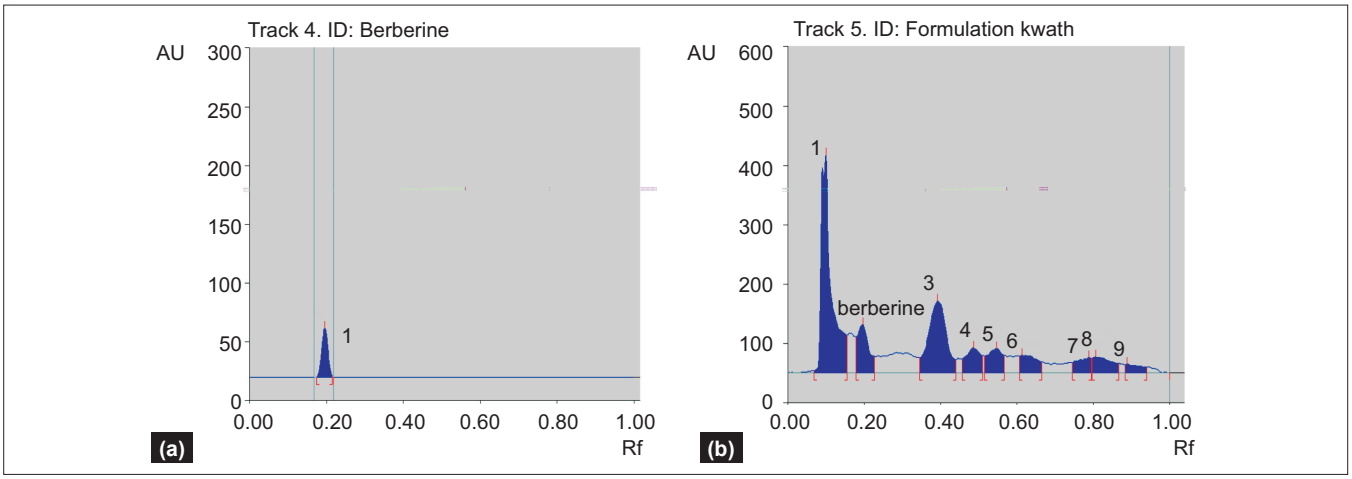

Figure 1: (a) Chromatogram for berberine; (b) chromatogram for berberine in kwath

at start, middle and end position of the band in the sample tracks. The methods were validated in terms of precision, repeatability and accuracy [Tables 4 and 5]. The relationship between the concentration of standard solutions and the peak response was linear within the concentration range of 8–64 ng/spot with a correlation coefficient of 0.997 for berberine and the concentration range of 200–1200 ng/spot with a correlation coefficient of 0.990 for gallic acid [Figures 3 and 4]. The average percent recovery at three different levels was found and the results are presented in [Table 6]. Berberine and gallic acid contents in PN kwath were found to be 0.08 and 4.9%, respectively. The method was found to be suitable for the quantification of these marker compounds in the herbal raw materials and polyherbal formulation.

Only a well-designed clinical study on a defined population can give meaningful results (positive or negative) about any therapeutic intervention and its safety and efficacy. A total of 14 patients with abnormal liver function parameters

Table 4: Method validation parameters for estimation of biomarker compounds by HPTLC

Parameters	Berberine	Gallic acid
Repeatability (% CV) (n = 7)	0.39	0.45
Limit of detection (ng/spot)	3	50
Limit of quantification (ng/spot)	8	150
Specificity	Specific	Specific
Linearity (correlation coefficient)	0.997	0.990
Range (ng/spot)	8–64	200–1200

Table 5: Intra- and inter-day precision study

Marker compound	Concentration (ng/spot)	Intraday precision*	Interday precision*
Berberine	16	1.38	1.49
	40	0.77	0.92
	64	0.90	0.97
Gallic acid	400	1.24	1.62
	800	1.18	1.15
	1000	0.67	0.92

*Relative standard deviation (% CV, n = 3)

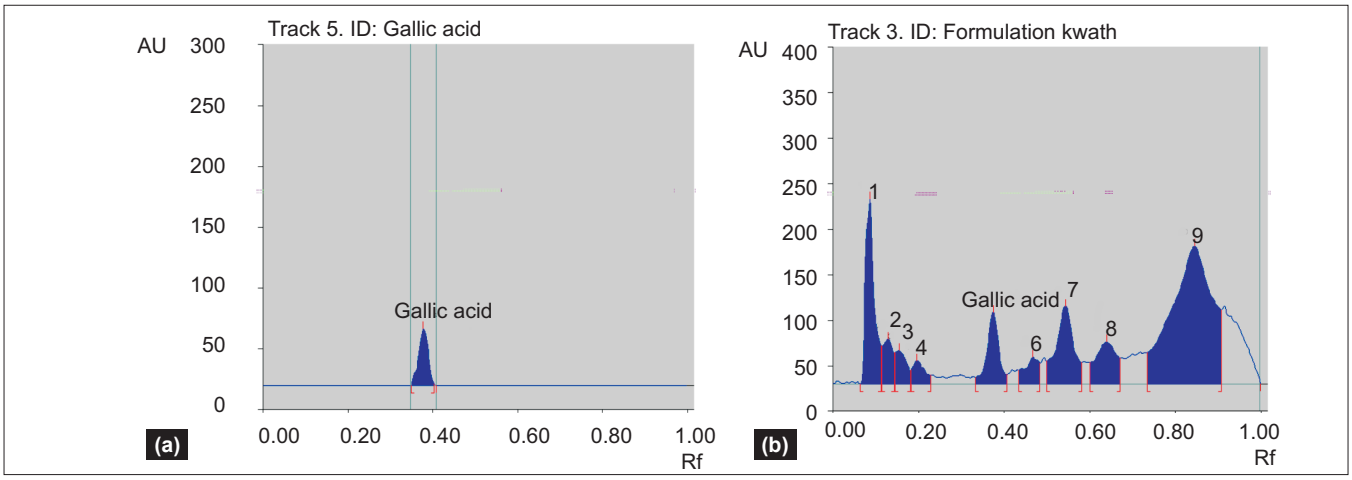

Figure 2: (a) Chromatogram of gallic acid; (b) Chromatogram of gallic acid in kwath

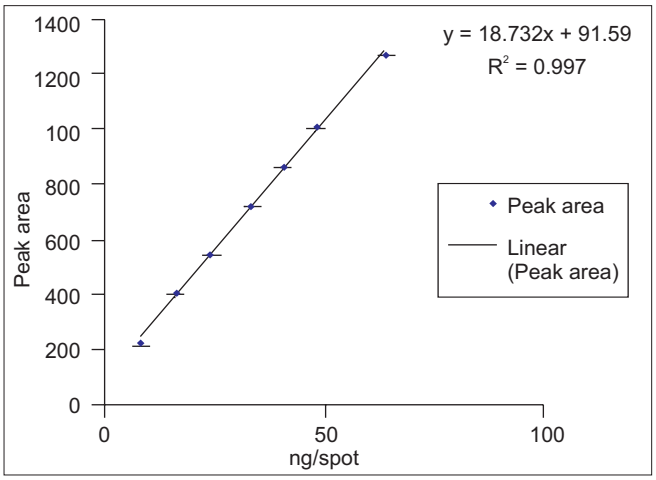

Figure 3: Calibration curve for berberine

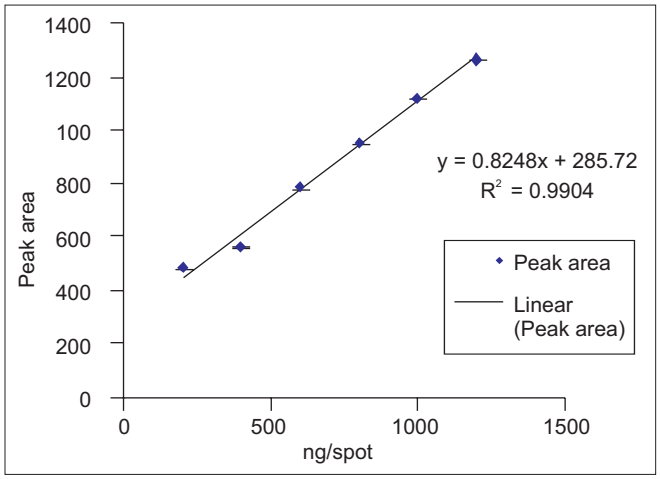

Figure 4: Calibration curve for gallic acid

Table 6: Recovery study of marker compound by HPTLC method

Marker compound	Amount present in the sample (ng)	Amount added (ng)	Amount found* (ng)	Recovery* (%)	Average recovery (%)
Berberine	20	12	31.54±0.50	98.56±1.55	
	20	20	39.77±0.58	99.41±1.45	99.17±1.11
	20	24	43.80±0.14	99.54±0.32	
Gallic acid	245	147	384.64±7.94	98.12±2.02	
	245	245	487.55±5.30	99.50±1.08	99.08±1.51
	245	294	537.00±7.72	99.63±1.43	

*Mean±standard deviation (SD, $n = 3$)

by institutional ethics committee of Shri Dardi Narayan Seva Mandal Hospital (Divyajyot Ayurvedic Research foundation, Paldi, Ahmedabad). Mean age of the patients was 56.78±4.25 years and the population consisted of equal ratio of males and females. After treatment for 2 months with PN kwath (20 ml, orally) there was significant ($P<0.05$) decrease in AST, ALT, ALP and bilirubin level in the patients having abnormal hepatic function [Table 7]. During treatment, there were no reports of adverse effects in terms of morbidity and mortality attributable to the drug. Our results suggest that PN kwath showed significant hepatoprotective activity which may be due to the synergistic effect of the various phytoconstituents like polyphenolic (gallic acid), flavonoids and alkaloid (berberine) present in the formulation and support traditional use of this formulation. For confirmation, further clinical study of this formulation in larger group of patients is recommended.

Table 7: Data for clinical study of PN kwath in the patient showing abnormal liver function test

Liver function test	Before treatment	After treatment
SGOT	98.71±12.29	62.00±8.77***
SGPT	128.43±24.58	74.71±13.53**
ALP	305.29±59.47	207.86±41.71***
Bilirubin	2.60±0.87	1.86±0.64*

Values are mean±SEM ($n = 14$)l; ***$P<0.001$significant compared to before treatment; **$P<0.01$ significant compared to before treatment; *$P<0.05$ significant compared to before treatment

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