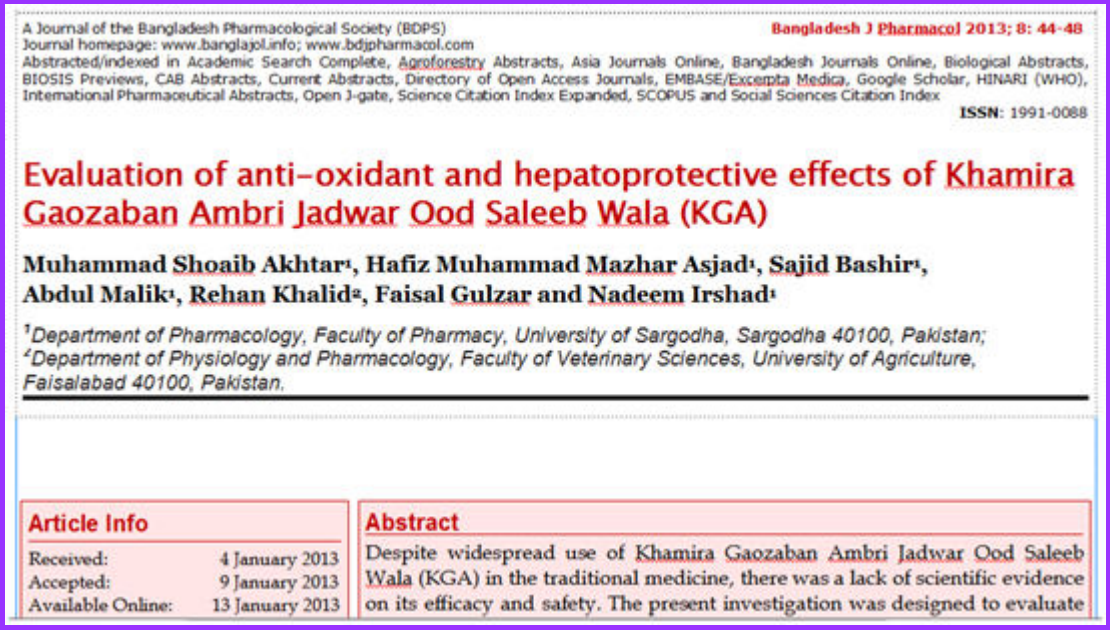

A Journal of the Bangladesh Pharmacological Society (BDPS)
Journal homepage: www.banglajol.info; www.bdjpharmacol.com
Abstracted/indexed in Academic Search Complete, Agroforestry Abstracts, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index
Bangladesh J Pharmacol 2013; 8: 44-48
ISSN: 1991-0088

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Article Info
Received: 4 January 2013
Accepted: 9 January 2013
Available Online: 13 January 2013

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BJP

Bangladesh Journal of Pharmacology

Research Article

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Article Info

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DOI: 10.3329/bjp.v8i1.13183

Cite this article:
Akhtar MS, Asjad HMM, Bashir S, Malik A, Khalid R, Gulzar F, Irshad N. Evaluation of anti-oxidant and hepatoprotective effects of Khamira Gaozaban Ambri Jadwar Ood Saleeb Wala (KGA). Bangladesh J Pharmacol. 2013; 8: 44-48.

Abstract

Despite widespread use of Khamira Gaozaban Ambri Jadwar Ood Saleeb Wala (KGA) in the traditional medicine, there was a lack of scientific evidence on its efficacy and safety. The present investigation was designed to evaluate its *ex vivo* anti-oxidant and *in vivo* hepatoprotective properties against carbon tetrachloride toxicity in albino rats. At first phytochemicals analysis of test preparation was conducted to estimate its total phenolic and flavonoid contents. Then their anti-oxidant activity was determined by various tests and compared it with standards ascorbic acid and rutin. Afterwards, hepatoprotective activity was studied against carbon tetrachloride-induced liver damage by determining SGOT, SGPT, ALP, total cholesterol, bilirubin and total proteins contents in the serum of rats before and after treatment. This suggests that the hepatoprotective activity of formulation is possibly attributed to its free radical scavenging properties.

Introduction

In South East Asia region, many herbs are used in different combinations in the preparations of patent herbal formulations (Sharma et al., 1991). However, only little proportions of hepatoprotective plants as well as their formulations used in traditional medicine have been evaluated scientifically for their safety and efficacy (Subramonium and Pushpangadan, 1999). Some herbal preparations are available as standardized extracts with known ingredients or even pure compounds which are pharmacologically evaluated (Schuppan et al., 1999).

In the current study, a polyherbal hepatoprotective formulation Khamira Gaozaban Ambri Jadwar Ood Saleeb Wala (abbreviated here as KGA) was selected for pharmacological evaluation. The criteria for selection included: i) claims in traditional medicine, ii) reports of presence of flavonoids and flavones contained in the constituent plants, iii) semi-solid formulation easy for oral administration, iv) established hepatoprotective activity of one or more plants, and v) sufficiently long shelf life. This herbal preparation has been traditionally used since centuries for treatment of various hepatic diseases and is considered curative and even hepatoprotective, nervine, general and cardiac tonic.

Nair (2006) has studied the protective effect of Tefroli- a polyherbal mixture (tonic) on cadmium chloride-induced hepatotoxicity in rats and determined serum bilirubin and other enzymes such as transaminases and phosphatases of both serum and liver (Nair, 2006). The current study was designed to evaluate the anti-oxidant and hepatoprotective activities of a reputed formulation KGA. The selected formulation has been reported to contain extracts/powdered plant drugs including *Borago officinalis, Coriandrum sativum, Bombyx mori,*

Salvia haematodes, Centaurea behen, Santalum album, Mellisa parviflora, Lallemantia roylean, Ocimum gratissimum, Lavendula stoechas, Cheiranthus cheiri, Mathiola incana, Ambra grasea, Delphinium denudatum, Paeonia emodi and *Pandanus tectorius* in a syrupy base (Ahmad et al., 2010).

Material and Methods

The polyherbal formulation of KGA manufactured by Hamdard Laboratories, Karachi, a reputed Unani Pharmaceutical concern was purchased from local market of Sargodha, Pakistan. Folin-Ciocalteu reagent, diagnostic kit, ascorbic acid, rutin, silymarin, carbon tetrachloride and other chemicals were purchased from Merck, Germany. Wister albino rats of both sexes were used for hepatoprotective study.

Estimation of total phenolic contents

Total phenolic contents of the test formulations were determined by Folin- Ciocalteu reagent. To 0.5 mL samples of each formulation added 1 mL of 10:100 µg/mL ethanolic gallic acid solution which was added to 9 mL of distilled water in a 25 mL flask. A blank reagent was prepared by taking 10 mL distilled water and 1 mL Folin- Ciocalteu's phenol reagent with shaken vigorously. After 5 min, 10 mL 7.5% w/v sodium carbonate solution was added and volume was made with distilled water. The absorption was read after 90 min, at room temperature at 750 nm with a spectrophotometer, and calibration curve was drawn (Pal and Shukla, 2003). Total phenolic contents in KGA formulation was calculated from the graph.

Estimation of total flavonoids

It was carried out by aluminum trichloride colorimetric method (Marinova et al., 2005) and 0.5 mL KGA was extracted with 50 mL of 80% aqueous methanol on an ultrasonic bath for 20 min. An aliquot (2 mL) of the extract was centrifuged for 5 min at 14000 rpm. 1 mL of aliquot was mixed with 2 mL aluminum trichloride in methanol (2% w/v). Blank was prepared from 1 mL of standard solution and diluted to 25 mL with methanolic acetic acid (0.5% v/v). The absorbance of probe solution against standard solution was determined at 420 nm after 30 min. All the determinations were carried out in triplicate. Results were express as total (%) flavonoids contents (TFC) in formulations as quercetin equivalent was calculated by following formula:

$$TFC\ (\%) = \frac{\text{Absorbance x Dilution factor}}{E^{1\%}_{1\ cm} \text{ x Weight of sample (mL)}} \text{ x } 100$$

$E^{1\%}_{1cm}$ = Specific absorption of the quercetin $ALCl_3$ complex (500)

DPPH radical scavenging activity

The scavenging activity of KGA was measured *in vitro* by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay. About 0.1 mM solution of DPPH in 100% ethanol was prepared and 1 mL of this solution was added to 3 mL of each KGA dissolved in ethanol at different concentrations (10–100 µg/mL). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm with a spectrophotometer. The IC_{50} value of the drug was compared with that of ascorbic acid, which was used as the standard (Roy and Burdon, 1994). The ability to scavenge the DPPH radicals was calculated using the following formula:

$$\text{DPPH scavenged (\%)} = \frac{\text{Absorbance (control - test)}}{\text{Absorbance control}} \times 100$$

Nitric oxide scavenging activity

The reaction mixture (3 mL) containing sodium nitroprusside (10 mM, 2 mL), phosphate buffer saline (0.5 mL) and extract or standard solution (0.5 mL) was incubated at 25°C for 150 min. After incubation, 0.5 mL of the reaction mixture containing nitrite was pipetted and mixed with 1 mL of sulphanilic acid reagent (0.3% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 mL of naphthylethylene diamine dihydrochloride (1%) was added to it, mixed and allowed to stand for 30 min. A pink colored chromophore was formed in diffused light. The absorbance of these solutions was measured at 547 nm against the corresponding blank solutions. The IC_{50} value is the concentration of sample required to inhibit 50% of nitric oxide radical (Salma et al., 2004). All determinations were performed in triplicates.

Hepatoprotective activity

The hepatoprotective study was conducted in albino rats (150-250 g) against carbon tetrachloride induced toxicity and the study was got approved by the institutional ethics committee. The rats were kept by maintaining standard housing conditions and 12 hours light/dark cycle. They were maintained on standard diet in polypropylene cages and supplied with water *ad libitum*. Albino rats of either sex were divided into following group with six animals each: Group I: Normal, received normal rat fed and water; Group II: Control, CCl_4 hepatotoxic (0.7 mL/kg intraperitoneally); Group III: Standard drug treated, (Silymarin, 100 mg/kg, orally); Group IV: Polyherbal treated group (150 mg/kg disperse in 1% Tween 80 p.o); Group V: Polyherbal treated group (300 mg/kg disperse in 1% Tween 80 p.o).

The normal levels of serum liver enzymes were determined by withdrawing blood samples directly by puncturing the Retro-orbital plexus on the first day of study. Collected blood was centrifuged at 2,500 rpm for separation of serum. Then to the tested animals 0.7 mL per kg body weight, carbon tetrachloride (CCl_4) was

administered i.p for five days. On the sixth day enzymatic levels were recorded. After intoxication with CCl_4, tested formulation and standard silymarin were administered for five days. On the 11th day the serum levels were determined by using semi-autoanalyser against commercially available kits (Randox).

Statistical analysis

Data obtained from this work were analyzed statistically using Students' t-test and ANOVA (One- or Two -way) followed by a post test (Tukey-Kramer multiple comparison test). Differences between means will be considered significant at 5% level of significance ($p<0.05$).

Results

The polyherbal hepatoprotective formulation of Khamira Gaozaban Ambri Jadwar Ood Saleeb Wala (KGA) was found to contain 27.4 ± 0.2% of phenolics while the total flavonoid contents were 36.8 ± 0.2%.

The anti-oxidant activity was compared with standard ascorbic acid, the IC_{50} value of formulation was 60.8. The anti-oxidant activity of the polyherbal formulation showed significant action on free radicals (Table I).

Table I

Percentage inhibition of DPPH radical and IC_{50} values of KGA

Concentration (µg/mL)	%Inhibition*	
	Sample	Ascorbic acid
10	16.1 ± 1.2	30.2 ± 1.2
20	29.2 ± 0.1	48.6 ± 0.6
40	48.3 ± 0.1	62.7 ± 1.7
60	61.5 ± 1.1	74.8 ± 1.7
80	78.3 ± 0.8	81.0 ± 1.7
100	85.4 ± 0.1	90.6 ± 1.7
IC_{50} values	60.8	64.7

Data are mean ± SD; n = 3

Serum SGOT, SGPT and alkaline phosphatase levels were decreased in presence of polyherbal formulation (Table II). Polynerbal formulation increased the serum protein level and decreased the serum cholesterol and bilirubin levels in comparison to hepatotoxic rats (Table III).

Discussion

Hepatotoxins gets converted into radicals in liver by the action of enzymes and these attacks the unsaturated fatty acids of membranes in presence of oxygen to give lipid peroxides consequently. The functional integrity of hepatic mitochondria is altered leading to liver damage (Rubin, 1995).

During liver damage, cellular enzymes like SGPT, SGOT and ALP present in the liver cells leak into the serum, resulting in increased concentrations (Deb, 1998). CCl_4 is one of the most commonly used hepatotoxins in the experimental study of liver diseases. CCl_4 altered permeability of membrane resulting in leakage of hepatic marker enzymes (AST and ALT) from cells into the circulation. Hence, elevation of these enzymes in plasma acts as a reliable marker for assessing hepatotoxicity (Firdous et al., 2012). AST predominantly found in mitochondria of hepatocytes. ALT is more specific to liver, and thus is a better parameter for detecting liver injury. Serum ALP and bilirubin is also associated with liver cell damage. The ALT, AST and ALP activity and serum bilirubin level are largely used as most common biochemical markers to evaluate liver injury (Kozer et al., 2003; Girish et al., 2009). Administration of hepatotoxic agent caused a significant elevation of enzymes level such as AST, ALT, ALP and bilirubin level has been attributed to the damage structural integrity of liver indicating development of hepatotoxicity (Gutiérrez et al., 2009). The oral administration of Khamira at a doses (150 and 300 mg/kg) to rats caused a significant reduction in SGOT ($p<0.01$), SGPT ($p<0.01$) and ALP ($p<0.01$) levels almost near to the normal levels. Thus the decrease in

Table II

Effect of KGA on serum SGOT, SGPT level and alkaline phosphatase level in hepatotoxic rats

Group	Serum SGOT (mg/dL)	Serum SGPT (mg/dL)	Serum alkaline phosphatase (U/L)
Normal	175.9 ± 1.7	78.3 ± 2.0	178.7 ± 1.0
Control	332.1 ± 1.8[a]	145.8 ± 0.8[a]	515.8 ± 3.0[a]
Hepatotoxic + Standard	220.2 ± 2.2[b]	102.8 ± 1.1[b]	213.9 ± 2.5[b]
Hepatotoxic + Polyherbal formulation (150 mg/kg)	250.8 ± 1.8[b]	106.9 ± 1.7[b]	252.7 ± 1.9[b]
Hepatotoxic+ Polyherbal formulation (300 mg/kg)	205.7 ± 1.7[b]	92.5 ± 0.7[b]	224.6 ± 1.6[b]

Values are given as mean ± SD (n = 6); [a]$p<0.01$ when compared with normal; [b]$p<0.01$ when compared with control

Table III

Effect of formulation on serum total protein, cholesterol and bilirubin levels in hepatotoxic rats

Group	Total protein (U/L)	Cholesterol (mg/dL)	Bilirubin (mg/dL)
Normal	8.2 ± 0.7	68.3 ± 1.0	0.8 ± 0.0
Control	6.1 ± 0.1[a]	121.8 ± 1.4[a]	2.8 ± 0.0[a]
Hepatotoxic + Standard	7.5 ± 0.0[b]	93.8 ± 1.8[b]	0.9 ± 0.0[b]
Hepatotoxic + Polyherbal formulation (150 mg/kg)	7.5 ± 0.0[b]	100.9 ± 2.1[b]	1.2 ± 0.0[b]
Hepatotoxic + Polyherbal formulation (300 mg/kg)	7.3 ± 1.7[b]	87.0 ± 1.8[b]	1.1 ± 0.0[b]

Values are given as mean ± SD; n = 6; [a]p<0.01 when compared with normal; [b]p<0.01 when compared with control

enzyme levels clearly pointed out the effectiveness of the formulation (Khamira) to normalize the functional state of the diseased liver. This is supported by the view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and regeneration of hepatocytes (Thabrew et al., 1987).

In recent years, plant extracts have been widely used as natural anti-oxidants because of the presence of polyphenolics compounds (Nuengchamnong et al., 2009). Percentage of phenolic compounds was determined in methanolic extracts of KGA by Folin-Ciocalteu reagent method and using Gallic acid as calibration standard. The results depicted in the Table I showed that Khamira Gaozaban Ambri Jadwar Ood Saleeb Wala has relatively sufficient phenolic contents i.e. 27.5 ± 0.2%. The presence of flavonoids in extracts has also been suggested to be important for their use in treating hepatic diseases and as anti-oxidant agents (Aguinaldo et al., 2005; Jiang et al., 2011). The present investigation showed that the polyherbal formulation could be a rich in flavonoids and polyphenols. The presence of these phenolic and flavonoid compounds, contribute diverse biological activities such as anti-carcinogenic, anti-inflammatory, and anti-atherosclerotic. These activities might be due to their antioxidant activity (Nuengchamnong et al., 2009). One way of determining anti-oxidant activity is by the use of the stable free radical DPPH (Molyneux, 2004; Brand Williams et al., 1995). The results of the activity of polyherbal formulation is not more comparable to L-ascorbic acid. This is understandable, since L-ascorbic acid is already in a pure form, while the crude plant extracts still need to be processed in order to isolate the compounds responsible for their anti-oxidant activity. However, this assay may be used to guide the fractionation and isolation of potential anti-oxidant compounds from these plant extracts (Moon and Shibamoto, 2009).

Conclusion

The test preparation of Khamira Gaozaban Ambri Jadwar Ood Saleeb Wala showed considerable hepatoprotective and anti-oxidant activity. By its free radical scavenging and metal chelating activity, Khamira Gaozaban Ambri Jadwar Ood Saleeb Wala besides hepatoprotective activity, might reduce the free radical generation and quench the radicals already formed and inhibit liver damage.

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