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Safety Evaluation of *Rhazya stricta* Ethanolic Crude Alkaloid Extract on Some Vital Organs in Mice: Histological Study

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Abstract: Herbal and complementary medicine is one of the most promising therapies for cancer patients. *Rhazya stricta (R. Stricta)* played a major role in the treatment of many diseases in the Middle East and South Asia countries. Recently, it was used due to its anticancer effect on breast, brain and lung cancer. The main objective of the present study was to assess the safety of 2 doses of crude ethanolic alkaloid extract of *R. stricta* grown in western region of Saudi Arabia using *in vivo* histopathological evaluation of some vital organs, namely heart, liver and pancreas of adult male mice. Crude alkaloid extract was prepared using 70% ethanol. Albino mice were treated with *R. stricta* leaf extracts at doses of 20 mg/kg and 40 mg/kg and their effects on body weight, organ index and histological changes in heart, liver, kidneys and pancreas were investigated. Mild changes were observed in most organs at lower doses. In the treated group that received high dose, vascular congestion was observed as the most evident feature. In addition, mild changes in the form of karyomegaly and apoptosis were seen in liver of these mice. The present study showed that, at low dose *R. stricta* alkaloids extract has no adverse effects on the vital organs of mice (Heart, liver, kidney and pancreas). Additional *in vitro* and *in vivo* studies are required to establish the mechanism of action of individual "Active" fractions of *R. stricta* before advising to be used in clinical field.

Key words: *Rhazya stricta* · Liver · Heart · Pancreas · Mice · Histological Study

INTRODUCTION

Rhazya stricta (R. stricta) is a herb used in folkloric medicine and has been traditionally used for the treatment of many diseases in Middle East and South Asian countries [1]. R stricta is used for the treatment of inflammatory conditions, stomach problems and liver diseases [2, 3]. The leaves of *R. stricta* were effective to reduce blood glucose, blood cholesterol, urea and glycosylated haemoglobin [4, 5].

Recent studies have shown the anti-cancer activity of *R. stricta* extracts or its alkaloids. It is proved to have anti-carcinogenic, antioxidant and free radical scavenging properties [6]. Several studies (In Saudi Arabia, Pakistan and others) proved anticancer activity of *R. stricta* against a variety of cancers, including the malignancy of breast, brain and lung [7, 8].

In addition, hepatotoxicity and nephrotoxicity liability of *R. stricta* were studied earlier in a mouse model [8-10]. An exploratory study was conducted to investigate the

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effect of two different doses (20gm/kg and 40gm/kg body weight) of crude alkaloid extracts of *R. stricta* on mice liver, kidney, heart and pancreas. *In vivo* safety was evaluated using histopathological analysis of tissues from the control and experimental animals.

MATERIALS AND METHODS

Extraction of Alkaloids from *R. stricta* Leaves: The leaves from *Rhazya S.* were collected and dried as previously described by Baeshen *et al.* [11]. Briefly, freshly obtained leaves were allowed to dry at room temperature in darkness for 3 weeks. Once dried, the leaves were soaked in 70% ethanol for 3 days followed by filtration using simple filter paper. After the filtration process, ethanol was evaporated from the filtered mixture. This process was further repeated for 3 times to ensure maximum yield. The extract was diluted using 10% of HCl making final volume of 100ml, followed by the addition of chloroform. By the use of a separation funnel, the mixture was separated into two layers, alkaloids and non-alkaloids. Ammonium hydroxide (NH4OH) was added to the alkaloids and pH was tested using Litmus paper. The alkaloids extract was re-suspended in chloroform, followed by another phase of separation. The purified alkaloid extract was stored at 4°C.

***In vivo* Safety Studies:** Experiments were performed on thirty, 10-weeks old male mice weighted 38-49grams, bred in the animal facilities of King Fahd Medical Research Center (KFMRC), King Abdulaziz University (KAU), Jeddah, Saudi Arabia, under a 12 hours light/dark cycle at a temperature of 25°C and relative humidity ranging from 60 to 70% throughout the experiment. They were given standard pellet diet and water ad libitum and kept for two weeks to acclimatize to the environmental conditions. The protocol met the approval of the Institutional Animal Care and Use Committee at KAU. The use of experimental animals was conducted in strict compliance with the rules and regulations established by the Research Ethics Committee at KAU.

The animals were allocated into 3 groups; G1, G2 and G3. The G1 (N= 10) was assigned as a control group, in which five animals were given 2ml of oral saline (0.9%) NaCl w/v and another five were given a saline mixed with DMSO (Used as solvent for *R. stricta* extract). The G2 and G3 were assigned as experimental groups, in which G2 (N=10) mice were given orally low dose of *Rhazya S.* alkaloid extract (20mg/kg of body weight) and G3 (N= 10) mice were given orally a higher dose (40mg/kg of body weight) of Rhazya alkaloid extract. The alkaloid extract was daily administered for a week. The choice of alkaloids dose and duration of treatment was based on previous studies carried out by our colleagues [11]. At the end of the experiment, all animals were anaesthetized by intra-peritoneal injection of phenobarbitone and the blood was withdrawn from orbital plexus. The blood serum was separated and stored at -80°C, followed by analysis for liver and kidney functions. An incision was made down the centre of animal's chest wall and heart was perfused with 10% neutral buffered formalin. The animal's kidney, liver, heart and pancreas were removed and weighed followed by the process of fixation in the same fixative and processed for paraffin embedded sections. The sections were cut (5µ thick) using a microtome, stained with H&E, examined and photographed by the light microscope.

Statistical Analysis: The results were expressed as Mean Â± Standard Deviation (SD). Differences between groups were assessed by one-way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS) software package for Windows. Post hoc testing was performed for inter-group comparisons using the least significance difference (LSD).

RESULTS

***In vivo* Safety Studies in Adult Mice:** Safety profile analysis of crude alkaloid extracts of *R. stricta* was carried out in a mouse model. Effects of *R. stricta* alkaloids on the cellular changes in various organs such as liver, kidney, heart and pancreas were analysed.

Effect of Crude Alkaloid Extracts of *R. stricta* on Body Weight and Organ Weight: There were significant changes in the body weight of G1 (P =0.038; P =0.038) and G2 (P =0.0239; P =0.039) on the 8th and 10th days of experiments, however, insignificant changes were observed in G3 (p=0.055). The comparison between all the three groups revealed insignificant differences in body weight (BW) throughout the course of the experiment, as shown in Table 1.

The effects of crude alkaloid extracts on different organ weights were also analysed. As shown in Table 2, no significant differences between liver, spleen, pancreas, right kidney, left kidney, heart and brain weights in various studied groups were observed, however significant increase in pancreas weight was observed at high dose as compared to low dose (P =0.014). As shown in Table 3, there was no significant difference in liver, spleen, pancreas, right kidney, left kidney, heart and brain

Table 1: Effect of crude alkaloid extracts of *R. stricta* on body weights of studied mice (Grams) in different groups.

Parameters	Control	Low dose	High dose
1st day body weight (g)	41.91±2.92	43.34±4.49	43.95±3.92
Significance		*P* =0.610	*P* =0.470; **P* =0.827
3rd day body weight (g)	41.24±1.91	42.61±4.72	44.46±3.43
Significance	*[1]P* =0.323	*P* =0.597; *[1]P* =0.115	*P* =0.230; **P* =0.478; *[1]P* =0.223
6th day body weight (g)	39.15±1.00	41.08±4.93	44.03±4.36
Significance	*[1]P* =0.143	*P* =0.494; *[1]P* =0.029	*P* =0.106; **P* =0.306; *[1]P* =0.869
8th day body weight (g)	38.91±1.19	39.17±5.31	42.58±4.17
Significance	*[1]P* =0.039	*P* =0.937; *[1]P* =0.049	*P* =0.284; **P* =0.282; *[1]P* =0.055
10th day body weight (g)	38.91±1.19	39.17±5.31	42.58±4.17
Significance	*[1]P* =0.039	*P* =0.937; *[1]P* =0.049	*P* =0.284; **P* =0.282; *[1]P* =0.055

Data are expressed as mean +/- SD. P: Significance versus control group; *P: significance versus low dose; P: Significance versus first day of the same group using one way ANOVA test.

Table 2: Effect of crude alkaloid extracts of *R. stricta* on organ weights (Grams) of studied mice in different groups

Groups	Liver weight	Spleen weight	Pancreas weight	Right kidney weight	Left kidney weight	Heart weight	Brain weight
Control	2.08±0.07	0.13±0.02	0.24±0.01	0.36±0.03	0.39±0.02	0.22±0.02	0.49±0.07
Significance							
Low dose	2.06±0.27	0.18±0.06	0.17±0.03	0.34±0.07	0.35±0.07	0.20±0.04	0.53±0.06
Significance	*P* =0.918	*P* =0.308	*P* =0.293	*P* =0.482	*P* =0.277	*P* =0.536	*P* =0.522
High dose	2.20±0.22	0.18±0.06	0.34±0.11	0.36±0.04	0.33±0.03	0.22±0.05	0.52±0.09
Significance	*P* =0.489;	*P* =0.308;	*P* =0.170;	*P* =0.983;	*P* =0.114;	*P* =0.957;	*P* =0.632;
	**P* =0.396	**P* =1.000	**P* =0.014	**P* =0.462	**P* =0.531	**P* =0.541	**P* =0.858

Data are expressed as mean +/- SD. P: Significance versus control group; *P: significance versus low dose using one way ANOVA test.

Table 3: Effect of crude alkaloid extracts of *R. stricta* on organ indices (Grams) in different groups

Groups	Liver weight index	Spleen weight index	Pancreas weight index	Right kidney weight index	Left kidney weight index	Heart weight index	Brain weight index
Control	5.35±0.33	0.34±0.03	0.61±0.02	0.93±0.06	1.01±0.03	0.56±0.04	1.27±0.21
Significance							
Low dose	5.28±0.38	0.46±0.18	0.43±0.07	0.85±0.13	0.89±0.12	0.51±0.08	1.37±0.21
Significance	*P* =0.784	*P* =0.277	*P* =0.209	*P* =0.286	*P* =0.087	*P* =0.525	*P* =0.541
High dose	5.17±0.32	0.41±0.12	0.79±0.22	0.85±0.06	0.77±0.05	0.51±0.13	1.23±0.21
Significance	*P* =0.516;	*P* =0.519;	*P* =0.208;	*P* =0.286;	*P* =0.004;	*P* =0.547;	*P* =0.803;
	**P*= 0.681	**P* =0.610	**P* =0.012	**P* =0.998	**P* =0.064	**P* =0.970	**P* =0.361

Data are expressed as mean +/- SD. P: Significance versus control group; *P: significance versus low dose using one-way ANOVA test

weights in different treatment groups. However, significant increase in pancreas weight was observed at high dose as compared to low dose (P =0.012) and significant decrease in left kidney weight index was detected at high dose versus control (P =0.004).

Effect on Organ Histology

Effect on Mice Liver: Liver of male mice stained with H&E showed normal lobular structure, hepatocytes radiate from the central veins (CV) to the periphery (Fig. 1A) where portal elements were found (Bile duct, portal vein, hepatic artery) (Fig. 1B). Hepatocytes have slightly basophilic cytoplasm, rounded central, vesicular nuclei. Occasionally bi-nucleated cells were seen. Blood sinusoids lined by endothelial cells were observed among hepatocytes.

Low dose of Rhazya's alkaloids (20mg/kg; G2) did not markedly alter liver architecture. Mild changes were observed in the form of slight dilation and congestion of portal vessels (Fig. 1C). Nuclear changes were the most evident observation. There was variation of nuclear size where some hepatocytes showed large size nuclei known as karyomegaly. Kupffer cell nuclei which lined the blood sinusoids looked prominent (Fig. 1D). High dose of *Rhazya* alkaloid (40 mg/kg; G3) produced more vascular dilation and congestion of both central and portal veins compared to low dose. More cells showed karyomegaly while few scattered apoptotic cells (Shrunken cells with dark cytoplasm and nuclei) were seen (Fig.1E). Mild bile duct proliferation was observed. No signs of fatty infiltration or necrosis were seen (Fig. 1F).

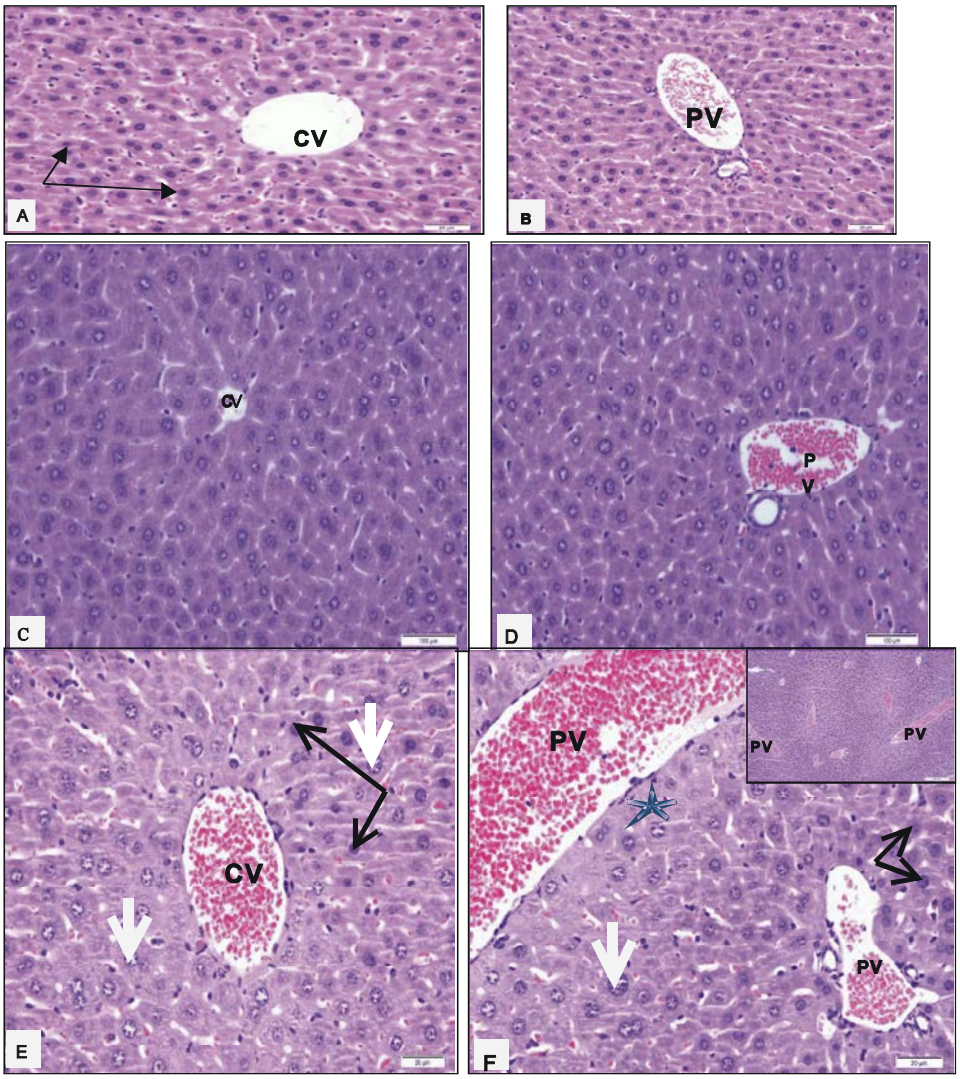

Fig. 1: Histological analysis of mice's livers in treatment groups-1, 2 and 3 using H&E staining. A. Histological image of G1 liver (Control mice) stained with H&E showing normal architecture of hepatocytes (Arrows) around central vein (CV). B. Histological image of G1 liver (Control mice) is showing normal architecture of hepatocytes around portal vein (PV). C. Mice's livers of G2 group treated with low dose of Rhazya stricta alkaloid extract are showing variation in nuclear size near central vein. Many cells have large size nuclei (Karyomegaly). Some cells are binucleated while few cells are shrunken and have dark nuclei as an early sign of apoptosis. D. G2 group liver shows mild dilation and congestion of portal vein with variation in nuclear size. Many cells showed large size nuclei (Karyomegaly) and bile duct. E. Histological image of G3 group treated with high dose of ARS is showing mice liver congestion of central vein, karyomegaly (White arrow) and apoptosis (Black arrow) of hepatocytes. F. High magnified part of mice liver (G3 group) near portal vein area with congested vessels, proliferated bile ducts (Star), karyomegaly (White arrow) and apoptosis (Black arrow)

Effect on Mice Heart: In untreated mice, histological `structures of the left ventricle showed cardiac fibres run in various directions. Both longitudinal and cross section could be seen. Coronary artery branches showed thin wall while capillaries between muscles are thin and invisible (Fig. 2A). Low dose of *Rhazya* alkaloids (20mg/kg) did not alter cardiac muscle appearance or cause any changes in coronary arteries walls (Fig 2B-D). However, high dose (40mg/kg) was found to cause congestion of blood capillaries located among cardiac muscle fibres. Few of those muscles showed degenerative patches. Coronaries showed no changes (Fig. 2E and F).

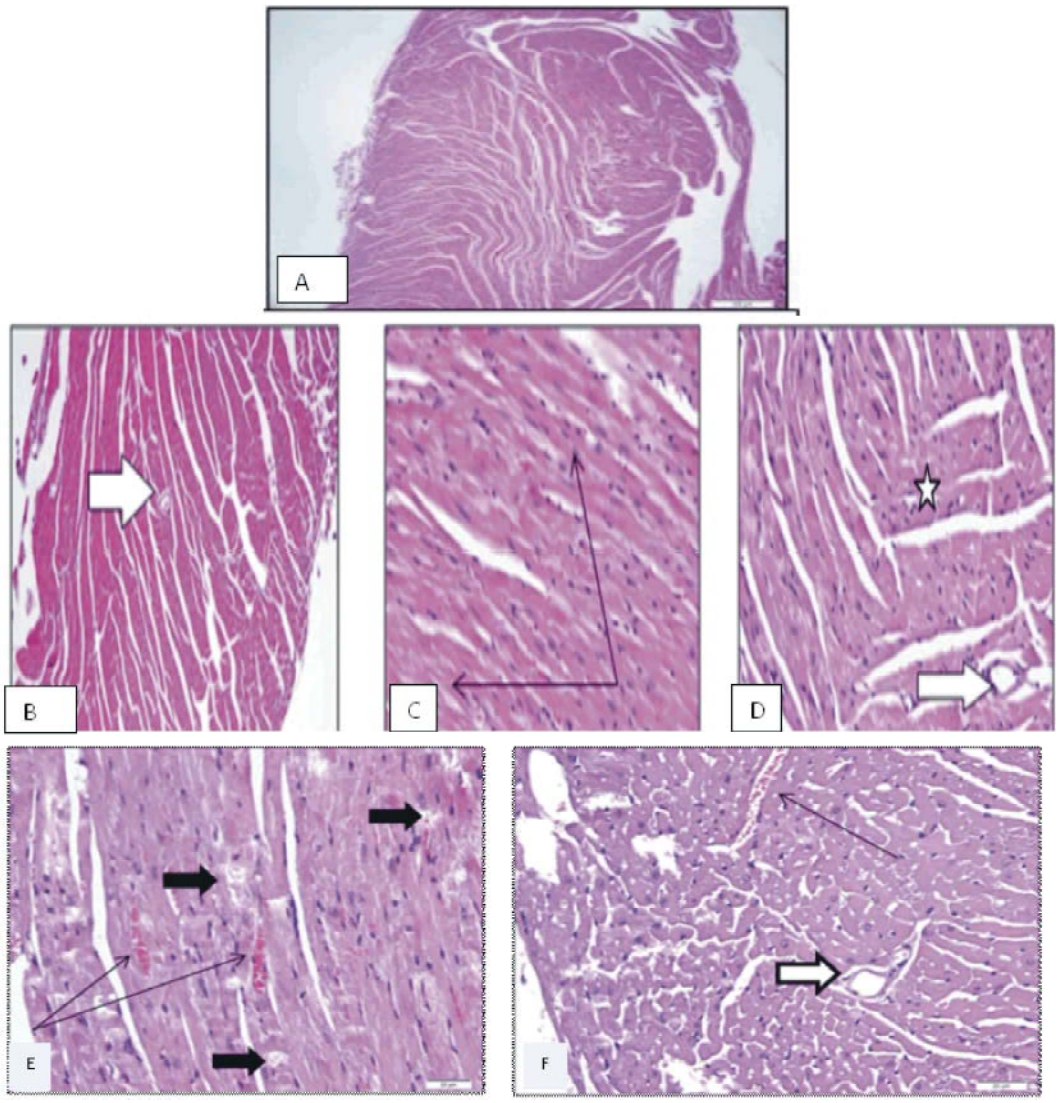

Fig. 2: Histological analysis of mice's hearts in group-1, 2 and 3 using H&E staining. A. Histological image of G1 heart (control mice) with normal histology. B. Histological images of G2 hearts showing part of left ventricle, C. longitudinal sections (black arrow) and D. transverse sections (star) in cardiac muscle fibres. The coronary branches are seen among cardiac fibres (white arrow). E. Showing longitudinal sections from high dose mice heart. The left ventricle showing congested capillaries (thin black arrows) and fibres with degenerative patches (thick black arrow). F. The coronary artery appears to be normal (White arrow)

Effect on Mice Pancreas: Control mice pancreas showed multiple lobes and lobules separated by loose scanty connective tissue. Inter lobar and interlobular vessels and ducts were seen along such vessels. The exocrine part of pancreas consists of acini, lined by cells that showed basophilic parts and apical acidophilic secretary granules. Endocrine part is represented by islets of Langerhans (II) (Fig. 3a). In case of G2 mice treated with low dose of *Rhazya* alkaloids (20mg/kg), vascular dilation and congestion of inter- lobar blood vessels were the only observed changes. Pancreatic ducts were also dilated and showed stasis of secretion (Fig. 3b). No apparent changes were observed in the acinar part or islets cells. Increasing the dose of *Rhazya* alkaloids (i.e. 40 mg/kg) resulted in marked dilation and congestion of inter- lobar blood vessels (Fig. 3c). Stasis of secretion within ducts was also observed. There was mild shrinkage in acini but no changes in the endocrine islet cells were observed.

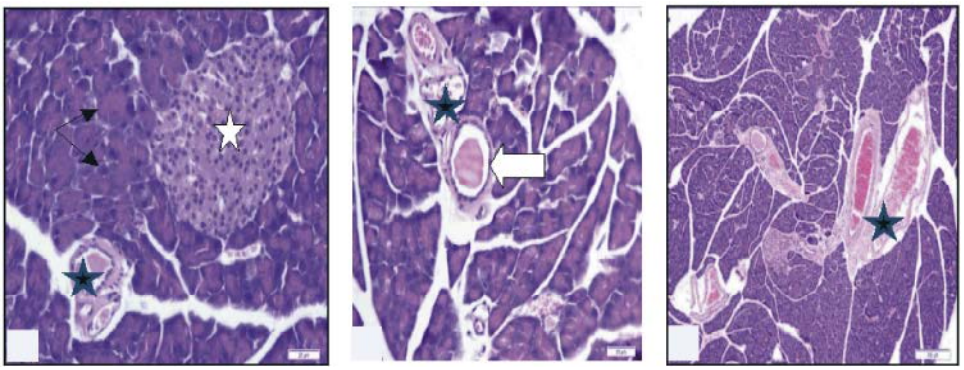

Fig. 3: Histological analysis of mice's pancreas in treatment groups-1, 2 and 3 using H&E staining. A. Histological image of G1 pancreas section (Control mice) showing islet (White star), exocrine acini & inter lobular vessels and ducts (black arrow). B. The low dose section (G2) is showing duct secretion (White arrow), congestion of inter lobar vessels (Black star) while acinar part showed slight or no changes. C. Sections from high dose treated mice pancreases (G3) showed marked congestion of interlobular vessels (Black star).

DISCUSSION

R. stricta is one of popular medicinal plant species, which has been in use as a treatment for variety of diseases in the southern regions of Asia. Along with its antioxidant activities, the phytochemical characteristics and pharmacological effects of *R. stricta* have been extensively studied [7, 12].

Our study points to the safety of these *R. stricta* alkaloids in a mouse model. Baeshen *et al*. [9] study on the breast cancer cell lines examined the role of apoptosis and cell cycle regulatory genes on MCF-7 and MDA-MB-231 cells. These authors concluded that crude alkaloid extracts of *R. stricta* may have a potential therapeutic effect in ER-negative breast cancer cells, although their prediction was based on the cell lines data without examining these total alkaloids extracts in primary tumors or in animal models.

The present study revealed that low dose *Rhazya stricta* alkaloids extract has no adverse effects on the vital organs of mice (heart, liver, kidney and pancreas) However, significant increase in pancreas weight and significant decrease in left kidney were observed at high dose as compared to low dose . Similar findings were reported by Marwat *et al.* [3] and Adam [13] who declared that ingestion *Rhazya stricta* in therapeutic doses is perfectly safe in human; however chronic administration of high doses in rats has shown variety of toxic effects including decrease in growth rate, dullness and hepatonephrotoxicity.

Liver is known to be very prone to the damage due to adverse effects of drugs used for treatment of various diseases in human. Moreover, liver is also involved in the detoxication of several toxic compounds. Pretreatment with *R. stricta* aqueous extract protected the livers of treated mice against paracetamol induced hepatotoxicity [9].

The hepatoprotective effect of *R. stricta* is comparable with silymarin (Ref.??). The extract of *R. stricta* leaves also showed anti-inflammatory [14] and significant antioxidant activities which may contribute to its hepatoprotective activity [3].This document my results that total crude alkaloid extracts of *R. stricta* did not reveal any adverse effects in major organs of mice and thus appears to be safe.

The findings of Ali *et al.* [15] showed clearly that crude extracts of *R. stricta* have marked effects on rat brain tribulin levels so it may be *R. stricta* that has antidepressant and sedative actions. On the other hand, Ali *et al.* [16] reported that pretreatment with *R. stricta* significantly protected mice against paracetamol induced biochemical changes and prolongation of pentobarbitone induced sleeping time. On the other hand, phytochemical studies on *R. stricta* showed the presence of alkaloids carboline and flavonoidal glycoside [3, 15, 17]. Phytochemical studies on *R. stricta* showed the presence of alkaloids (rhazimine, stemmadenine, vincadine and rhazimanine), carboline and flavonoidal glycoside.

CONCLUSIONS

The present study showed that, low dose *R. stricta* alkaloids extract has no adverse effects on the vital organs of mice (Heart, liver, kidney and pancreas). Additional *in vitro* and *in vivo* studies are required to establish the mechanism of action of individual "Active" fractions of *R. stricta* before advising to be used in clinical field.

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