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HPTLC FINGER PRINT AND HEPATOPROTECTIVE ACTIVITY OF VASAKASAVA AGAINST PARACETAMOL INDUCED HEPATOTOXICITY

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ABSTRACT

The present study was undertaken to evaluate the hepatoprotective activity of prepared Vasakasava in paracetamol intoxicated rats. Vasakasava was prepared by fermenting the decoction of *Adhatoda vasica* leaves using a solution of jaggery, flowers of *Woodfordia fruticosa* (family: Lythraceae), and other powdered ingredients with subsequent stirring in a jar. After placing seven layers of mud smeared cloth, the jar was kept undisturbed. Further, HPTLC was performed to determine the amount of vasicine in Vasakasava. The antihepatotoxic effect of Vasakasava (0.45 and 0.90ml/250g p.o.) was tested against paracetamol induced liver toxicity (2g/kg p.o.). After 7 days of respective treatment, animals were sacrificed, MDA and reduced GSH were evaluated for oxidative stress, hepatic serum enzymes and histopathology was performed to investigate the degree of hepatic injury. HPTLC analysis revealed the presence of 0.032% vasicine in the formulation. Pre-administration of Vasakasava restored the levels of SGOT, SGPT, and ALP to 77.85, 78.97, and 68.27 IU/L, respectively (at 0.90ml/250g p.o.) and 81.1, 153.4 and 87.66 IU/L respectively (at 0.45ml/250g p.o.). Similarly, the level of total bilirubin and direct bilirubin was also normalized. The hepatoprotective effect was supported by histopathological examination of liver tissue. As a conclusion, Vasakasava possesses the potent hepatoprotective effect against paracetamol induced liver damage.

Keywords: Liver enzymes, glutathione, malondialdehyde, silymarin

INTRODUCTION

Ayurveda is a comprehensive natural and practiced health care discipline originated in India around 5000 BC.¹ In the past few decades, this Indian classical system of medicine has achieved preeminent importance and surpassed synthetic drugs in the realms of quality, safety, and efficacy.²⁻⁴ In fact, 80% of the people in the developing countries depend upon traditional herbal medicines for their primary health care purpose.⁵

Ayurvedic herbal medicines are majorly prepared by the active ingredient's transference through different manufacturing processes.^{4,6} Asavas, one of the polyherbal hydroalcoholic formulations, is prepared by fermentation of infusion of plant material (Hima) in a solution of sugar or jaggery for a specified period of time. It helps in facilitating the extraction of active principles present in the drug.⁴

Vasakasava (VA), an Ayurvedic hydroalcoholic formulation, that one may often stumble upon in various treatises of Ayurveda, is used to cure kasa, raktapitta, kasaya, sotha, swasa, etc.⁷ Besides the above mentioned maladies, it has also proved substantially useful to treat infertility in females according to Ayurveda Saar Sangraha. It is a self-fermented galenical that contains about 11 crude drugs, i.e. Vasaka, Dhataki, tweak, Ela, Patra, Kesara, Kankol, Sunthi, Marich, Pippali, and Ushir.

The main ingredient of VA is '*Adhatoda vasica*' which contains vasicine as the main chemical constituents. Though *Adhatoda vasica* has been pharmacologically reported to have significant hepatoprotective activity,⁸ none the less standardization and pharmacological evaluation to support the efficacy of VA is still

lacking. Therefore, in the present study, standardization of VA was done by analyzing various parameters including HPTLC. Furthermore, its hepatoprotective activity was evaluated against paracetamol (PCM) induced hepatic necrosis in rats.

MATERIALS AND METHODS

Procurement and authentication of herbal ingredients

Vasaka (*Adhatoda vasica*) and all the other herbal ingredients were collected from Jalandhar, Punjab and authenticated from Guru Nanak Dev University, Amritsar, Punjab (Ref. No. 1064). Guda was authenticated from KLE's Shri BMK Ayurveda Mahavidyala, Shahpur, Belgaum, Karnataka (Ref. No. CRF/CRI/32/2013).

Procurement of drugs and chemicals

PCM and silymarin (SM) was procured from Frankfinns Laboratories, Ludhiana and Ningbo Hi Tech Biochemicals Co. Ltd., China, respectively.

Preparation of Vasakasava

The decoction of Vasaka was prepared, kept in the jar (already fumigated by dhupan karama) with subsequent addition of a solution of jaggery and dhataki pushpa i.e. flowers of *Woodfordia fruticosa* of the family Lythraceae (fermentation initiator). The prakshepa dravyas, viz. twak (stem bark of *Cinnamomum zeylanicum* of family Lauraceae), ela (seeds of *Elettaria cardamomum* of family Zingiberaceae), patra (leaves of *Cinnamomum tamala* of family Lauraceae), nagkesar (stamen

of *Mesua ferrea* of family Calophyllaceae), kankol (fruits of *Piper cubeba* of family Piperaceae), sunthi (rhizomes of *Zingiber officinalis* of family Zingiberaceae), marich (fruits of *Piper nigrum* of family Piperaceae), pippali (fruits of *Piper longum* of family Piperaceae) and ushira (roots of *Vetiveria zizanioides* of family Poaceae) were powdered and added in the formulation with subsequent stirring. The jar was kept undisturbed after placing seven layers of Kaparmitti (mud smeared cloth). Onset and cessation of sound showed the beginning and completion of the fermentation respectively.^{9,10}

Analytical study of Vasakasava

Organoleptic characters: The sample of VA was analyzed for different characters such as color, odor and taste.

Standardisation parameters: Various standardization parameters such as total solid content, sugar content (reducing and non-reducing both), specific gravity, pH, viscosity, refractive index, and alcohol content were determined by the method described in The Ayurvedic Pharmacopoeia of India (2008).¹¹

HPTLC profiling: HPTLC fingerprinting and quantification of vasicine as marker was done for the decoction of vasaka, the formulation before, during and after the completion of fermentation. CAMAG HPTLC system (CAMAG, Germany) with a Linomat five sample applicator was used for the analysis. TLC was performed on pre-coated silica gel HPTLC aluminum plates 60F254. The standard solution of vasicine was prepared by dissolving one mg of vasicine in ten ml of methanol. The test sample was prepared by dissolving 1000 mg of sample in ten ml of methanol and filtered. The mobile phase was prepared as ethyl acetate: methanol: ammonia in the ratio of 8:2:0.5. Standard and test solutions (ten µl) were applied to the plate by fine capillary. The plates were visualized at 254 nm. After derivatisation (CAMAG-Dip tank for one min), the plates were examined for the appearance of different bands at different R_f.^{12,13}

Evaluation of *in vivo* hepatoprotective activity of Vasakasava

Albino wistar rats (180–200 g) were selected for the study. They were maintained at a controlled temperature of 25–28°C with 12 h light/dark cycles and fed a standard diet and water *ad libitum*. Animal studies were conducted according to the regulations of the Institute Animal Ethics Committee (IAEC) and the protocol was approved by the CPCSEA (ATRC/09/2013).

Animals were randomly divided into six groups. Group I served as normal control. Group II (positive control) received dist. water, group III received SM, and group IV and V received VA at doses 0.45 ml/250 g and 0.90 ml/250 g, respectively once a day for seven days.¹⁴ On the fifth day, after the administration of the respective treatments, all the animals of the group II, III, IV, and V were administered with PCM 2 g/kg orally.¹⁵ On the seventh day, two hours after the respective treatment, the blood samples were collected by retro-orbital bleeding and centrifuged at 6000 rpm for ten min to obtain serum for the analysis of SGPT, SGOT, ALP and bilirubin. The animals were sacrificed and liver and kidney were collected for the estimation of GSH using the method described by Ellmann (1959)¹⁶ and MDA using TBARS assay.¹⁷

Histopathological studies: The liver and kidney specimens obtained from all the groups of animals were fixed in ten percent

formalin (24 h), and then stained with haematoxylin-eosin for microscopic evaluation of the histopathological architecture.¹⁸

Statistical analysis

Results were reported as mean ± SEM (Standard error of mean). The obtained data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. Statistical significance was determined at p value less than 0.05 and 0.01.

RESULTS

Organoleptic characters

The color of VA was dark brown, odor and taste were alcoholic and sweet, and appearance was clear without froth.

Standardization parameters

VA is a classical formulation, but its standard values have not been mentioned in Ayurvedic Pharmacopoeia of India. The various standardization parameters of VA have been presented in Table 1.

Table 1: Standardisation parameters of vasakasava

Parameters	Results
Total Solid Content	54.25%W/V
Specific Gravity	1.14 W/W
Refractive index	1.322
Viscosity	1.398 poise
pH	4.14
Alcohol content	7%
Reducing sugar	29.34%
Non reducing sugar	1.13%

HPTLC profiling

The decoction of vasaka was analyzed using HPTLC fingerprinting to confirm the presence of biomarker compound i.e. vasicine. The retention factor and concentration in terms of percentage for standard vasicine, decoction of vasaka, formulation before fermentation, during fermentation, and the final formulation (VA) has been presented in Table 2. HPTLC chromatogram of standard vasicine and of the various samples has been shown in Figure 1. HPTLC peak chromatogram explains the number of major and minor components present in the formulation. The presence of vasicine was confirmed by comparing the R_f values and the spectra of the standard bands.

In vivo hepatoprotective activity

PCM administration induced a significant increase in the serum levels of SGOT, SGPT, ALP and bilirubin in the positive control rats as compared to the normal rats, indicating the liver damage. Treatment with SM and VA has significantly brought down the elevated levels of SGPT, SGOT, ALP and bilirubin [Table 3].

In the present study, the GSH levels were found to get depleted in PCM treated group in comparison to the normal group. Moreover, a significant rise in hepatic MDA was observed in the PCM treated group as compared to the normal group. Pretreatment with SM as well as VA significantly restored the levels of GSH and MDA.

Table 2: HPTLC profile of different samples

Track	Sample ID	Volume applied	Peaks	R _f	Conc. (%)
1	Vasicine standard	10 μ l	1	0.56	-----
2	Decoction of vasaka	10 μ l	9	0.55	0.443
3	Formulation before fermentation	10 μ l	6	0.55	0.049
4	Formulation during fermentation	10 μ l	5	0.55	0.045
5	Formulation (VA)	10 μ l	5	0.54	0.032

Table 3: Effect of vasakasava on biochemical parameters, hepatic glutathione levels and MDA level in paracetamol induced liver injury

Parameter	Normal	Positive control	Standard (SM)	VA (0.45ml/250g)	VA (0.90ml/250g)
SGOT (IU/L)	33.06 $\pm$ 2.815***	94.94 $\pm$ 3.851	47.44 $\pm$ 3.991***	81.10 $\pm$ 1.891*	77.85 $\pm$ 1.355**
SGPT (IU/L)	40.45 $\pm$ 3.027***	172.11 $\pm$ 4.869	58.63 $\pm$ 4.292***	153.40 $\pm$ 2.985**	78.97 $\pm$ 3.118***
ALP (IU/L)	47.17 $\pm$ 1.611***	101.89 $\pm$ 3.819	60.30 $\pm$ 2.547***	87.66 $\pm$ 2.588**	68.27 $\pm$ 1.862***
Total Bilirubin (mg/dl)	0.62 $\pm$ 0.058***	2.70 $\pm$ 0.143	1.28 $\pm$ 0.039***	2.38 $\pm$ 0.074*	2.23 $\pm$ 0.069**
Direct Bilirubin (mg/dl)	0.29 $\pm$ 0.034***	0.87 $\pm$ 0.036	0.47 $\pm$ 0.024***	0.77 $\pm$ 0.008*	0.686 $\pm$ 0.013***
GSH (n mol/g of tissue)	100.39 $\pm$ 2.682**	58.57 $\pm$ 4.411	7.09 $\pm$ 2.270***	0.42 $\pm$ 2.358*	83.21 $\pm$ 2.614***
MDA (n mol/g of tissue)	0.86 $\pm$ 0.178***	4.63 $\pm$ 0.137	2.39 $\pm$ 0.110***	3.89 $\pm$ 0.159**	2.98 $\pm$ 0.126***

Results are expressed as mean $\pm$ SEM (n=5). Data were analyzed by one-way ANOVA followed by Dunnett's test; * P <0.05; ** P <0.01; *** P <0.001 versus positive control; VA= Vasakasava; SM= Silymarin

Figure 1: HPTLC chromatogram of standard vasicine and different samples

Figure 2: Effect of silymarin and Vasakasava on Paracetamol-induced hepatotoxicity in rats

Figure 3: Paracetamol induced hepatotoxicity and its prevention by Vasakasava. Paracetamol form a reactive metabolite NAPQI by cytochrome P450. NAPQI covalently binds to proteins, decreases glutathione store, and increases oxidative stress and lipid peroxidation. Paracetamol insult also leads to direct injury of hepatic cells by migration and activation of neutrophils, increased production of cytokines. NAPQI: N-acetyl-p-benzoquinone imine, PAF: platelet-activating factor.

Histopathological examination of liver: The hepatoprotective effect of VA was confirmed by histopathological examination of the liver tissue of different groups [Figure 2]. The histological profile of the rats intoxicated with PCM showed disarrangement and degeneration of normal hepatic cells with intense necrosis. In the liver sections of the rats treated with low dose of VA, there was a moderate hepatoprotective activity. However, rats treated with SM and high dose of VA showed very less disarrangement and degeneration of hepatocytes, indicating marked hepatoprotective activity.

DISCUSSION

VA, an Ayurvedichydroalcoholic formulation, is a self-fermented galenical that contains about 11 crude drugs, i.e. Vasaka, Dhataki, Twak, Ela, Patra, Kesara, Kankol, Sunthi, Marich, Pippali and Ushir. It is used to cure kasa, raktapitta, kasaya, sotha, swasa etc.⁷ The main ingredient of VA is '*Adhatoda vasica*' which contains vasicine as the main chemical constituent.

In the standardization of Ayurvedic formulation, HPTLC profiling of an Ayurvedic formulation has often been employed for the determination of its bioactive components.¹⁹ In this regard, VA was analyzed by comparing the absorption spectra at different formulation steps i.e. from a decoction of the plant upto final formulation.

Then, the ability of VA to offer protection against hepatotoxicity induced by 'over the counter drug', PCM was explored. Liver plays a central role in transforming and clearing the chemicals, but it is susceptible to the toxicity from certain medicinal agents, like PCM, when taken in overdose or sometimes even within therapeutic range.²⁰ Liver injuries induced by various hepatotoxins have been recognized as a major toxicological problem for years.^{1,21}

At therapeutic dose, PCM is metabolized by glucuronyl and sulfotransferases. However, at toxic doses, a higher percentage of PCM is oxidized by cytochrome-450 enzymes to highly reactive N-acetyl-p-benzoquinone imine (NAPQI). This intermediate can alkylate and oxidize intracellular GSH and protein thiol group, resulting in GSH depletion and increased lipid peroxidation as shown in the Figure 3.^{1,8,15,22,23} These events lead to the disruption of calcium homeostasis, mitochondrial dysfunction, and oxidative stress and may eventually culminate in cellular damage and death.^{18,24}

The present study revealed a considerable hepatocellular injury in the rats exposed to PCM, which was confirmed by many parameters like elevation in the serum activities of SGOT, SGPT, ALP and bilirubin levels, and a decrease in the GSH and an increase in the MDA levels in liver tissue. It is well established that elevation in the SGOT, SGPT, and the ALP is indicative of cellular leakage and loss of functional integrity of the hepatic cell membranes implying hepatocellular damage.²⁵ Serum bilirubin on the other hand is related to the functional status of the hepatic cell.²⁵ It is produced when heme is catabolized. Hepatic cells render bilirubin water-soluble and therefore easily excretable by conjugating it with glucuronic acid prior to secretion into bile by active transport. Hyperbilirubinemia may result from the increased production or decreased excretion of bilirubin due to liver damage or any obstruction in the excretory ducts of the liver.²⁶ Administration of standard SM and VA at different dose levels attenuated the elevated serum enzyme activities and bilirubin in the PCM treated rats and caused a subsequent recovery towards normalization.

The standard SM is basically a polyphenolic flavonoid isolated from the fruits and seeds of the milk thistle plant *Silybum marianum* (family Asteraceae).^{21,27} Its antihepatotoxic activity is due to a synchronous effect of principal flavonolignans.²² The protection provided by SM appears to rest on the properties like free radical scavenging and anti-lipid peroxidation, ability to increase the cellular content of GSH, ability to regulate membrane permeability, capacity to regulate nuclear expression by means of a steroid-like effect, and inhibition of the transformation of stellate hepatocytes into myofibroblasts, which are responsible for the deposition of collagen fibres leading to cirrhosis.²⁸

GSH, an endogenous non enzymatic antioxidant, is a critical determinant of tissue susceptibility to oxidative damage.^{25,29} Decreased GSH level in the PCM treated groups is due to binding of NAPQI to GSH to yield non-toxic 3-GS-yl-PCM. This is accompanied by the covalent binding of remaining quinone with cellular macromolecules leading to cell death.³⁰ NAPQI not only reduces GSH, but also increases the level of reactive oxygen species resulting in oxidative stress.³⁰ These reactive species initiates lipid peroxidation by binding to membrane phospholipids. Hence, the level of lipid peroxide (MDA) is a measure of membrane damage and alterations in the structure and function of cellular membranes.²⁹ It was confirmed from the present study that the standard SM as well as VA significantly reduced the lipid peroxidation and also restored hepatic GSH content towards normal in PCM intoxicated rats.

Furthermore, the hepatoprotective effect of the VA was accomplished by the histology of liver tissue. The PCM intoxicated rats showed disarrangement and degeneration of normal hepatic cells with intense centri-lobular necrosis, while rats pre-treated with SM and VA showed less disarrangement and degeneration of hepatocytes, indicating marked regeneration activity.

CONCLUSION

Vasakasava possesses the potent hepatoprotective effect against paracetamol induced liver damage. This effect might be attributed to its phytoconstituents and its free radical scavenging activity. The future scope of the research remains in exploiting the signaling pathways of the active components of VA thus, enabling effective disease targeting.

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