Hepatoprotective activity of ethanolic extract of *Alysicarpus vaginalis* against nitrobenzene-induced hepatic damage in rats

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**Abstract**

*Alysicarpus vaginalis* (L.) DC commonly known as Alyce clover, belongs to the botanical family *Fabaceae.* It is widely distributed in India, Pakistan, Sri Lanka, Africa and Australia. In India it is traditionally used by the tribals for diuretics, leprosy, pulmonary troubles and back pain. The present study aimed to evaluate the hepatoprotective activity of ethanol extract of *A. vaginalis* aerial parts in nitrobenzene (NB) induced hepatic injury in Wistar rats. Liver injury was induced in rats by single oral administration of of NB (50 mg/kg bw). One days after NB induction, the rats were treated with ethanolic extract of *A. vaginalis* orally at the doses of 200 mg/kg orally significantly (P< 0.05) and dose-dependently reduced and normalized the serum marker enzymes, and increased the antioxidant enzyme status as compared to that of NB control group. Further more it was confirmed by histopathological studies. This study concludes that *A. vaginalis* demonstrated promising hepatoprotective agent in NB induced hepatic damaged rats.

1. Introduction

Liver plays an important role in the biotransformation of foreign compounds and is particularly vulnerable to toxic chemical assaults. There is a wide variety of hepatotoxins which damage liver. Nitrobenzene (NB) is considered a hazardous air pollutant and has proven to be an animal carcinogen. It is classified as a group B2 chemical according to the 1986 Cancer guide lines (Cattley et al., 1994) i.e. a likely human carcinogen. Metabolism of NB produce intermediates such as Nitrosobenzene (NOB) and phenylhydroxylamine (PH) that play an important role in the process of NB carcinogenesis (Howard et al., 1983). Following accidental NB poisoning in humans, the highest concentration was found in the liver, brain, blood and stomach (International Programme on Chemical Safety, 2003).

Several endogenous protective mechanisms have been evolved to limit the injury caused by hepatotoxins (Keppler et al., 1968). But endogenous protective mechanisms are unable to protect completely from the toxic effects, hence the additional protective mechanism of dietary antioxidants may be of a great importance, which would protect the liver from hepatotoxins. Many natural and artificial agents possessing antioxidative properties have been used to prevent and treat hepatopathies induced by oxidative stresses. Approximately 80% of the world population is almost entirely dependent on traditional medicines (Meenakshi et al., 2010; Balamurugan 2015). These are not only used for primary health care not just in rural areas in developing countries, but also in developed countries as well where modern medicines are predominantly used. Several plants are known to exhibit potent antioxidant and hepatoprotective po-
tential (Senthilkumar et al., 2008; Antonisamy et al., 2015).

The leaf juice were used for the improvement of eye sight and earache (Tirkey, 2006). The entire plant was used for the treatment of renal calculi (Ediriweera, 2007). Root of this plant is widely used in kidneys, diuretics; leprosy and pulmonary troubles (Burkil, 1985). In view of this consideration, it seems reasonable to hypothesize that the functional effects of A. vaginalis may be particularly important in preventing and diminishing NB -induced hepatotoxicity. As such, this study was focused on evaluating the protective potentials of aerial part of A. vaginalis in NB-induced hepatotoxicity.

2. Materials and methods

2.1. Plant material and extraction

The aerial part of A. vaginalis were collected in December 2008 from the foot hill of Kanniyyakumari, Tamil Nadu India. The materials were identified and authenticated by Dr. G.V.S. Moorthy, Botanical Survey of India Coimbatore. Voucher No: (BSI/SC/5/25/09-10/Tech.1604). The collected materials were thoroughly washed in water, chopped, air dried at 35–40°C for a week and pulverized in electric grinder. 1 g of powder was then extracted in 5 ml of ethanol. The ethanol extract was then made to powder with the help of rotary evaporator under reduced pressure.

2.2. Experimental animals

Wistar albino rats of both sex, weighing between 160 and 180 g were used for the study. The animals were housed in standard polypropylene spacious rat cages with stainless steel top goal, under hygienic conditions. Animals were provided standard pellet food manufactured by Hindustan Lever Ltd., India and Aqua gaurd ST 2000 filtered well water ad libitum. The animals were quarantined for 1 week, prior to the experiments to acclimatize to laboratory conditions. The study protocol was approved by the IAEC (Institutional Animal Ethics Committee, Govt. of India).

2.3. Toxicity studies

The acute toxicity study was performed for ethanolic extract of aerial parts of A. vaginalis were performed using wistar strain of albino rats. The animals were kept fasting for overnight providing only water, after which the extracts were administered orally in increasing dose (100-2000 mg/kg bw) and found safe up to 2000 mg/kg bw. One-tenth of the maximum dose of the extract tested for acute toxicity was selected for evaluation of hepatoprotective activity, i.e., 200 mg/kg (Handa and Anupama 1990).

2.4. Treatment of animals

The rats were divided in to four groups, each group consisting of six animals. Group I served as the control; in group II, rats received NB (50 mg/kg bw) orally as a single dose in group III, rats were subjected to induction and treatment, NB was injected orally and treatment with ethanolic extract (200 mg/kg bw) orally was started a day after the injection for a period of 30 days. In group IV, rats were treated with positive control silymarin (25 mg/kg bw) orally for 30 days. In group V, rats were treated with the ethanolic extract alone orally for 30 days.

2.5. Preparation of the samples for biochemical studies

The animals were anesthetized using chloroform and sacrificed. Blood was collected directly from the heart of each animal and the clot was centrifuged for 5 min minutes at 1500 rpm to separate serum and for biochemical analysis. Liver tissues were removed immediately and washed in ice-cold saline, and 10% homogenate was prepared using 0.1 M Tris–HCl, pH 7.4, a part of liver sections were dissected out for histopathological examinations.

2.6. Biochemical estimations

The liver marker enzymes like alanine transaminase (ALT) and aspartate transaminase (AST) (King 1965), alkaline phosphatase (ALP) (King and Armstrong 1980) total bilirubin (TB) (Gupta et al., 2007). The liver supernatant was used for the estimation of superoxide dismutase (SOD) (Misra and Fridovich 1972). The unit of enzyme activity is defined as the enzyme required to give 50% inhibition of epinephrine to adrenochrome. Catalase (CAT) (Sinha 1972). In this method, dichromate in acetic acid was reduced to chromic acetate. This was measured colorimetrically at 610 nm, glutathione peroxidase (GPx) (Rotruck et al., 1973) here the reaction between glutathione remaining after the action of GPx and 5,5'-dithiobis-(2-nitrobenzoic acid) to form a complex that absorbs at 412 nm. Lipid peroxidation (LPO) (Hogberg et al., 1974). Malondialdehyde (MDA), formed as an end product of the peroxidation of lipids served as an index of oxidative stress.

2.7. Histopathological examination

After weighed, part of the hepatic tissue was immediately collected from the same lobe of the liver and fixed in a 10% (v/v) neutral formalin solution for 24 h. Subsequently, hepatic tissue was dehydrated in a series of ethanol solutions from 75% to 100%. After dehydration, the tissue was embedded in paraffin, cut into 5 μm sections, stained with the haematoxylin-eosin dye and observed under a photomicroscope.

2.8. Statistical analysis

The values were expressed as mean ± SD. The statistical analysis was carried out by oneway analysis of variance using SPSS (version 10) statistical analysis program. Statistical significance was considered at p<0.05.

3. Results

The ethanolic extract of A. vaginalis did not show any sign and symptoms of toxicity and mortality up to 2000 mg/kg. The activity of serum marker enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin (TB) analyzed in serum samples of...
different groups of rats are shown in Table 1. In group II there was a significant increase (p<0.05) in serum levels of ALT, AST, ALP and TB. But when the ethanolic extract of aerial part of *A. vaginalis* was given to group III, there was a significant decrease in the value, which tends to reach the normal values. In group IV, when treated with positive control silymarin the levels of liver marker enzymes get normalized. In group V, when the plant extract alone was given, the level approached the normal values.

The activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and lipid peroxidation analysed in liver homogenate of different groups of rats are shown in Table 2. Antioxidant enzymes such as SOD, CAT, GPx were analyzed in different groups of rat. In group II, there was a significant decrease (p<0.05) in the antioxidant enzyme and the lipid peroxidation levels were found to be increased. But when the ethanolic extract of *A. vaginalis* was given to group III, there was a significant increase in the antioxidant levels and lipid peroxidation levels were decreased. In group IV, when treated with positive control silymarin the antioxidant enzyme levels get increased and lipid peroxidation levels get reduced. In group IV, when the plant extract alone was given, the level approached the normal values.

The hepato protective effect of *A. vaginalis* was confirmed by histopathological examination of liver section of control and treated animals. The histopathological observation were explored in Figure 1. In group I (normal control) rats, liver showed normal histological architecture (a). In group II (NB control) showed marked inflammatory changes associated with fatty changes and confluent hepatic necrosis (b). Group III (NB+ *Alysicarpus vaginalis*) which is NB damaged and treated with the ethanolic extract showed almost normal architecture with lesser degree of inflammation (c). Group V (NB + Silymarin) when treated with positive control showed normal architecture with lesser degree of inflammation showing its potent hepatoprotective effects (d). Group V (*Alysicarpus vaginalis* only) showed normal liver (e).

4. Discussion

Liver has a great capacity to detoxicate toxic substances and synthesize useful metabolites. Serum levels of AST and ALT are the quite sensitivity indicators to evaluate the degree of hepatic damage. The
with ethanolic extract of *A. vaginalis* enzymes were significantly decreased in rats treated 1). On the other hand, the activities of these marker enzymes and bilirubin in NB-intoxicated (group nificant increase in the activities of serum liver mark-

As NB causes hepatotoxic effects, there was a sig-
nificant increase in the activities of liver marker en-
zymes and bilirubin in NB-intoxicated (group II) animals when compared to control rats (Table 1). On the other hand, the activities of these marker enzymes were significantly decreased in rats treated with ethanolic extract of *A. vaginalis* (group III) when compared to group II animals. Estimating the activities of serum marker enzymes, like aspartate transaminase, alanine transaminase, alkaline phosphatase can make assessment of liver function. When liver plasma membrane is damaged, a variety of en-
zymes normally located in the cytosol are released in the blood stream. Their estimation in the serum is a useful quantitative marker and type of hepatocel-
ular damage (Mitra et al., 1998). The serum marker enzymes (aspartate transaminase, alanine transami-

Free radicals are regularly produced in vivo as a re-
sult of carcinogen treatment causing oxidative stress that leads to damage of nucleic acids, proteins, and lipids, which play an important mechanistic role in the development of cancer (Waris and Ahsan 2006). Natural antioxidants are capable of inhibiting ROS production, and thereby, it reduces the intracellular oxidative stress (Feng et al., 2001). The antioxidant systems are major cell defenses, which protect mem-
branes and cytosolic components against damage induced by free radicals under diseased conditions (Janani et al, 2008).

A major defense mechanism involves the antiox-
didant enzymes, including SOD, CAT, and GPx, which convert active oxygen molecules into non-toxic compounds (Jain et al, 2008). In our study, the liver SOD, CAT, and GPx activities were significantly decreased where as lipid peroxidation levels were increased in NB –intoxicated animals, which were brought back to near normal in ethanolic extract of *A. vaginalis* treated animals with NB administration (Table 2). SOD is an endogenous enzymatic scavenger which can compete with the oxidative destruction of free radicals. Most of the SOD in tissues of cytoplasmic origin and contains Cu and Zn as essential prosthetic groups (Rathi et al, 2009). SOD catalyzes the break-
down of O$_2$ to O$_2$ and H$_2$O, and prevents the formation of OH$. Thus, SOD has been implicated as playing an essential defensive role against potential oxygen toxicity.

The ROS scavenging activity of SOD is effective only when it is followed by the actions of CAT and GPx, because the dismutase activity of SOD generates H$_2$O, which needs to be further scavenged by CAT and GPx (Ju et al., 2004). LPO, is through metal chela-
tion at the initiation level and also as a chain break-
er (Tripathi et al., 1996). Peroxy radicals are important agents that mediate lipid peroxidation there by damaging cell membrane. Scavenging of free radicals is one of the major antioxidant mechanisms to inhibit the chain reaction of lipid peroxidation. Reduced lipid peroxidation was revealed by significant de-
crease in MDA and hydroperoxidase level in extract groups. Simultaneously significant increase in SOD and CAT content of liver suggested antioxidant activ-
ity of *A. vaginalis* and silymarin. Administration with NB caused a significant increase in MDA concentra-
tion when compared with normal group (Rathi et al, 2010a). NB carcinogenicity is considered to correlate with its metabolic activation. It forms a number of phenolic compounds by oxidation and nitroxides by redu-
ction (Holder 1999). Reduction of nitro group plays a more potent role in NB carcinogenicity. Ni-
troreduction, which is driven by microsomal P-450s and NAD(P)H, can produce reactive nitro oxide inter-
mediates aromatic nitroso- and hydroxylamine com-
pounds, e.g. NOB and PH, associated with their reactive free radicals, e.g., the nitrosoation free radical and superoxide free radical (Mason and Holtzman 1975). In our laboratory, previously it was investigated that induction of nitrobenzene cause hepatic damage, which was treated with ethanolic extract of *Cayratia trifolia* (Guru Kumar et al., 2011).
In our previous study, the ethanolic extract of *A. vaginalis* revealed the presence of alkaloids, flavonoids, sterols, tannins, polyphenols, and triterpenoids (Rathi et al., 2010b). Alkaloids are plant-derived compounds with physiological activity, contain nitrogen in a heterocyclic ring with complex structure which possesses potent antioxidant activity (Chung and Shin 2007). The total phenolic content of *A. vaginalis* was found to be 2.7 mg gallic acid equivalent per gram dried plant, it also has strong free radical scavenging activity and showed significant antioxidant and peroxidant effect in vitro. It also exhibited anti proliferative activity against ovarian cancer cell lines (Rathi et al., 2010a).

The present results revealed that the extract of aerial part of *A. vaginalis* was able to protect the membrane integrity of hepatocyte against NB induced release of marker enzymes in the blood circulation. Preventing liver lesions from progressing to fibrosis and cirrhosis, and repairing parenchymal cell damage by stimulating liver regeneration are important mechanism for hepatoprotection. Perhaps the alkaloids, flavonoids, sterols, tannins, polyphenols, and triterpenoids present in *A. vaginalis* are responsible for the marked hepatoprotective effects, observed in the present study. Hence, it will be of great interest to isolate the active constituents of *A. vaginalis*. Further studies will be needed to identify the active compounds that confer the hepatoprotective protection of the *A. vaginalis* extract.

5. Conclusion

In conclusion, the result of this study seems to confirm that the ethanolic extract of *A. vaginalis* has a potent hepatoprotective action upon nitrobenzene – induced hepatic damage in rats and possess antilipid peroxidative and free radical scavenging activities. The present study thus justifies the traditional use of *A. vaginalis* in the treatment of liver disease and also point out that *A. vaginalis* warrants future detailed investigation as a promising hepatoprotective agent.

**Conflict of interest statement**

We declare that we have no conflict of interest.

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