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Impact of differential dosage of *Azadirachta indica* A. Juss. leaf-extract on alcohol-induced hepatopathy of Albino Rat

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ABSTRACT

Acclimatized Albino Rats (Wister variety) of either sex were divided into 8 groups having 5 rats each; reared feeding on pulses, gram and bread. First 4 groups were non-alcoholic groups namely groups-A1, A2, A3 and A4. Group A1 was the Normal Control Group and Groups- A2, A3 and A4 were administered orally with water- extract of *Azadirachta indica* leaves in dosage of 200µg/kg, 400µg/kg and 600µg/kg of body weight for 4 weeks. In rest 4 groups namely B1, B2, B3 and B4, alcoholic hepatopathy was induced by oral administration of 1000µl of 30% ethanol daily for 4 weeks. Groups- B2, B3 and B4 were administered orally with water- extract of *A. indica* leaves in dosage of 200µg/kg, 400µg/kg and 600µg/kg of body weight, parallel to alcohol administration. Investigations of Aspartate Transferase (AST), Alanine Transferase (ALT), Alkaline Phosphatase (ALP) activities in blood serum; Lipid Peroxide (LPO), Glutathione-S-Transferase (GST) and Cytochrome p450 (CYP) activities in liver homogenate after the experimental period leads to a conclusion that *A. indica* leaf- extract is not an ideal hepatoprotector. In lower dosage (less than 200µg/kg) though it showed some hepatoprotective role, it was found to be highly toxic in higher dosage (over 200µg/kg) and have adverse impact on liver.

Keywords: Hepatopathy, alcoholism, toxicity, Albino Rats.

INTRODUCTION

Azadirachta indica A. Juss., also known as Neem, or Indian Lilac is a tree in the mahogany family Meliaceae. It is one of two species in the genus *Azadirachta*, and is native to India and the Indian sub-continent including Nepal, Pakistan, Bangladesh and Sri Lanka. Typically it grows in tropical and semi-tropical regions. Its fruits and seeds are the source of neem-oil¹. Neem is a fast-growing tree that can reach a height of 15–20 meters (49–66 ft), rarely to 35–40 metres (115–131 ft). It is evergreen, but in severe drought it may shed most or nearly all of its leaves. The branches are wide and spreading. The fairly dense crown is roundish and may reach a diameter of 15–20 meters (49–66 ft) in old, free-standing specimens. The opposite, pinnate leaves are 20–40 centimeters (7.9–15.7 in) long, with 20 to 31 medium to dark green leaflets about 3–8 centimeters (1.2–3.1 in) long. The terminal leaflet is often missing. The petioles are short. The (white and fragrant) flowers are arranged in more-or-less drooping axillary panicles which are up to 25 centimeters (9.8 in) long. The inflorescences, which branch up to the third degree, bear from 150 to 250 flowers. An individual flower is 5–6 millimeters (0.20–0.24 in) long and 8–11 millimeters (0.31–0.43 in) wide. Protandrous, bisexual flowers and male flowers exist on the same individual tree. The fruit is a smooth (glabrous) olive-like drupe which varies in shape from elongate oval to nearly roundish, and when ripe is 1.4–2.8 centimeters (0.55–1.10 in) by 1.0–1.5 centimeters (0.39–0.59 in). The fruit skin (exocarp) is thin and the bitter-sweet pulp (mesocarp) is yellowish-white and very fibrous.

The mesocarp is 0.3–0.5 centimeters (0.12–0.20 in) thick. The white, hard inner shell (endocarp) of the fruit encloses one, rarely two or three, elongated seeds (kernels) having a brown seed coat².

A. indica leaves are dried in India and placed in cupboards to prevent insects eating the clothes and also while storing rice in tins³. Neem is a key ingredient in non-pesticidal management (NPM), providing a natural alternative to synthetic pesticides. Neem seeds are ground into a powder that is soaked overnight in water and sprayed onto the crop. To be effective, it is necessary to apply repeatedly, at least every ten days. Neem does not directly kill insects on the crop. It acts as an antifeedant, repellent, and egg-laying deterrent, protecting the crop from damage. The insects starve and die within a few days. Neem also suppresses the hatching of pest insects from their eggs. Neem cake is often sold as a fertilizer⁴. Neem oil has been shown to avert termite attack as ecofriendly and economical agent⁵.

In Assam, *A. indica* leaves are traditionally used right from killing insects, fungus, bacteria and rodents and even as medicine in various human diseases. Common people have a belief that leaves of *A. indica* have hepatoprotective role and leaf-extract is administered orally for the treatment of jaundice. *A. indica* leaf-extract had been reported to be beneficial against paracetamol-induced hepatic damage.⁶ Phytochemical investigations revealed that *A. indica* bear some amount of antioxidant flavonoids⁷. But, several toxic compounds of limnoid group are also present in these including Azadirachtin⁸ which have active antifeedant phagodeterrent properties, so are used as insecticide. It may be a big question of modern mind that a single plant-leaves which can kill or minimize various groups of organisms due to its toxic ingredients how can it be of medicinal property for hepatostimulation and hepatoprotection?

Alcoholic hepatitis is a major social problem amongst lower middle-class people of the third world countries now a day. Though the synthetic antioxidants like “Metadoxine” (C₈H₁₁NO₃ .C₅H₇NO₃) to treat alcoholic hepatopathy are available, common people have a tendency to use plant based ethno-medicines.

In case of administration of ethno-medicinal components prepared from plant extracts, some problems of intoxications may occur as a single plant or part of it may comprise hundreds of ingredients. Though some may act as medicines, others may act as toxins and bring irreversible loss to some susceptible organs like liver. It is also not possible for the common men to isolate the beneficial ingredients (if present) from the harmful ones as it will require maximum sophistication and it will not be economically viable. Hence, toxicological investigation using laboratory animals is a necessary part of promotion of ethno-medicinal components for common men use in traditional ways.

In intoxication, (such as alcoholic intoxication) free radicals (H· & OH·) are formed which in turn bring lysis of the lipid bi-layer of the cell membrane by oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which methylene -CH₂- groups lie, that possess especially reactive hydrogen molecules. This phenomenon is known as “lipid peroxidation (LPO)”. As a result of this, the cells of soft tissues like Liver are destroyed and the contents of the cell are released to the body fluid (serum)⁹. The activities of the enzymes Aspartate Transferase (AST), Alanine Transferase (ALT) and Alkaline phosphatase (ALP) are mainly localized in the hepatocytes.

Hepatocyte destruction increases their level of activity in blood serum. Investigations of AST, ALT and Alkaline Phosphatase ALP activities in blood serum of the study animals are done along with study of Liver Lipid Peroxide (LLPO) to evaluate probable hepatotoxicity on administration of the *A. indica* leaf-extract.

GST (Glutathione S-Transferase) and CYP (Cytochrome p 450) are two main enzymes responsible for xenobiotic metabolism^{10,11}.

In case of entrance of any harmful foreign component to the body, the levels of their activities rise several times in various tissues including liver to detoxify the systems. GST and CYP activities in liver samples are investigated both in normal-control and the ethno-medicine administrated animals to find out the probable xenobiotic stress (which may lead to intoxication) on the administration of *A. indica* leaf-extract.

MATERIALS AND METHODS

To prepare the ethno-medicinal component fresh tender leaves of *A. indica* were homogenized adding deionized water and filtered through whatman's organic-grade filter paper. Filtrate was vacuum-dried at $50\pm 2^{\circ}$ C and obtained jelly-like substance was kept in air tight container in deep freeze. This was used within 3 days of preparation. Healthy Albino Rats (Wister variety) of either sex were reared fed on pulses, gram and bread. After acclimatization they were divided into 8 groups (having 5 rats each), namely groups-A1, A2, A3 and A4. Group A1 was the Normal Control Group and Groups- A2, A3 and A4 were administered orally with water- extract of *A. indica* leaves in dosage of 200 μ g/kg, 400 μ g/kg and 600 μ g/kg of body weight for 4 weeks. In rest 4 groups namely B1, B2, B3 and B4, alcoholic hepatopathy was induced by oral administration of 1000 μ l of 30% ethanol daily for 4 weeks¹². Groups- B2, B3 and B4 were administered orally with water- extract of *A. indica* leaves in dosage of 200 μ g/kg, 400 μ g/kg and 600 μ g/kg of body weight, parallel to alcohol administration. After the experimental period, blood samples are collected retro-orbitally from the inner canthus of the eye using micro-haemorit capillaries under light ether anesthesia and kept in separate labeled micro-centrifuge tubes.¹³ These are allowed to clot at room temperature for 20 minutes. The sera of respective blood samples are extracted by centrifugation and kept in separate labeled micro-centrifuge tubes in 0° to 4° C for biochemical assays. Then animals are sacrificed by high dose of ether anesthetization and livers are dissected out and kept in deep freeze in proper labeled vials. Measured amount of liver were homogenized with fixed amount of normal saline later on for the assays. A proper hygienic condition is provided to the study animals. No juvenile or pregnant individual is applied for the experimental purposes. The standard guidelines prescribed by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA 2003¹⁴) are followed during the study.

Serum-AST activity is measured by using AST (GOT) reagent kit (IFCC/Kinetic)¹⁵. Serum-ALT activity is measured by using ALT (GPT) reagent kit (IFCC/Kinetic).¹⁶ Serum-ALP activity is measured by ALP reagent kit (GSCC/Kinetic)¹⁷. Measurement of Liver Lipid Peroxidation is done by the photometric evaluation of molar extinction co-efficient of thiobarbituric acid¹⁸. GST activity in liver is measured by using GST Assay kit (Kinetic)¹⁹. CYP activity in liver is measured by using Cytochrome p450 assay kit (Kinetic)²⁰. Total protein estimation in liver samples is also done²¹ and GST and CYP activities are expressed as activity per mg of liver protein. Glucose, AST, ALT, ALP and Creatinine assay kits are procured from Ranbaxy-RFCL (India) LTD. GST and CYP assay kits are procured from Sigma-Aldrich Inc. (USA). Folin-Ciocalteu's phenol reagent (used in protein estimation) is procured from Sigma-Aldrich Inc. (USA). Thiobarbituric acid is procured from Research Fine Chem (India) LTD. The other reagents and chemicals are procured from Ranbaxy-Ranchem, (India) LTD. All the biochemical investigations and evaluations are done in a semi automated biochemistry analyzer ("Lab Life Chem-Master" manufactured by Ranbaxy- Diagonova LTD) with proper programming.

RESULTS

Results obtained during the period of investigation are statistically analyzed²² with the help of MS Excel and presented in the following table-

Experimental Rat Groups →	Non Alcoholic Rats				Alcohol Administered (Alcoholic) Rats			
	Group: A1 Normal Control Group	Group: A2 Rats Administered with <i>A.indica</i> leaf-extract (Dosage: 200µg/kg)	Group: A3 Rats Administered with <i>A.indica</i> leaf-extract (Dosage: 400µg/kg)	Group: A4 Rats Administered with <i>A.indica</i> leaf-extract (Dosage: 600µg/kg)	Group: B1 Rats Administered with Alcohol	Group: B2 Rats Administered with Alcohol and <i>A.indica</i> leaf-extract (Dosage: 200µg/kg)	Group: B3 Rats Administered with Alcohol and <i>A.indica</i> leaf-extract (Dosage: 400µg/kg)	Group: B4 Rats Administered with Alcohol and <i>A.indica</i> leaf-extract (Dosage: 600µg/kg)
Serum AST (IU/l)	275.774 ±0.951675	265.786 ±0.727438 -3.62% *	313.124 ±0.815467 +13.54% *	376.674 ±0.884492 +36.59% *	484.602 ±0.798144 +75.72% *	514.308 ±0.965497 +6.13% *	713.834 ±0.965244 +47.30% *	842.082 ±2.259304 +73.77% *
Serum ALT (IU/l)	74.294 ±0.132121	54.408 ±0.109973 -26.77% *	87.162 ±0.388798 +17.32% *	117.046 ±0.600222 +57.54% *	213.402 ±3.006179 +187.24% *	234.148 ±0.663803 +9.72% *	252.15 ±0.9705 +18.16% *	277.81 ±0.542559 +30.18% *
Serum ALP (IU/l)	814.478 ±0.599036	802.244 ±0.311939 -1.50% *	975.88 ±0.346973 +19.81% *	1234.378 ±7.786643 +51.55% *	2050.464 ±3.94118 +151.75% *	2270.038 ±5.355854 +10.71% *	2527.082 ±14.42398 +23.24% *	2754.004 ±14.79853 +34.31% *
Liver Lipid Peroxide (n mol/mg)	223.602 ±0.183995	221.232 ±0.21141 -1.06% *	273.994 ±0.778856 +22.54% *	296.964 ±0.97214 +32.81% *	474.368 ±0.862887 +112.15% *	495.968 ±0.429795 +4.55% *	547.31 ±1.405169 +15.38% *	713.16 ±0.777798 +50.34% *
Liver GST (µmol/min/mg of protein)	0.35344 ±0.000854	0.35332 ±0.000459 -0.034% ^{NS}	0.3691 ±0.001077 +4.43% *	0.38984 ±0.0026 +10.30% *	0.36796 ±0.00068 +4.11% *	0.3761 ±0.001453 +2.21% *	0.39698 ±0.000956 +7.89% *	0.43426 ±0.000989 +18.02% *
Liver CYP (µmol/min/mg of protein)	370.9236 ±0.153457	370.628 ±0.091837 -0.08% ^{NS}	381.406 ±1.090136 +2.83% *	396.906 ±0.934225 +7.01% *	395.0524 ±1.280091 +6.51% *	404.808 ±1.354021 +2.47% *	426.412 ±0.972715 +7.94% *	448.71 ±0.414753 +13.58% *
“N.S.” indicates Not significant and “*” indicates Significant at p<0.001 “+...%” and “-...%” indicate percent increase and percent decrease respectively.								

DISCUSSION

On administration of *A. indica* leaf-extract with dosage 200mg/kg of body weight to the non-alcoholic rats (of Group A2), significant decreases of AST, ALT and ALP activities were observed in blood serum to with deviations of -3.62%, -26.77% and -1.50% respectively from normal control rats (of Group A1). Liver Lipid Peroxide also decreased with deviation of -1.06%. No significant augmentation of Liver GST and Liver CYP were marked with this dosage. These may be due to mild antioxidant property of the *A. indica* leaves, due to their flavonoid contents.

With dosage 400mg/kg of body weight, in non-alcoholic rats (of Group A3), there were enhancement of serum AST, ALT and ALP activities with +13.54%, +17.32% and +19.81% deviations respectively from normal control rats (of Group A1). Liver Lipid Peroxide, Liver GST and Liver CYP also shown similar trend with deviations of +22.54%, +4.43% and +2.83% respectively.

With dosage 600mg/kg of body weight, in non-alcoholic rats (of Group A3), there were enhancement of serum AST, ALT and ALP activities with +36.59%, +57.54% and +51.55% deviations respectively from normal control rats (of Group A1). Liver Lipid Peroxide, Liver GST and Liver CYP also shown similar trend with deviations of +32.81%, +10.30% and +7.01% respectively.

On oral administration of 30% ethanol to the rats of Group B1, significant increases of AST, ALT and ALP activities were observed in blood serum to with deviations of +75.72% , +187.24% and +151.75% respectively from normal control rats (of Group A1). Liver Lipid Peroxide also increased in these rats with deviation of +112.15%. Significant enhancement of Liver GST and Liver CYP were marked with +4.11% and +6.51% (from normal control rats) in this case. This is due to major hepatic on alcohol administration.

With administration of *A. indica* leaf-extract at dosage 200mg/kg of body weight, to alcoholic rats (of Group B2), there were enhancement of serum AST, ALT and ALP activities with +6.13% , +9.72% and +10.71% deviations respectively alcoholic rats of Group B1. Liver Lipid Peroxide, Liver GST and Liver CYP also shown similar trend with deviations of +4.55%, +2.21% and +2.47% respectively.

With administration of *A. indica* leaf-extract at dosage 400mg/kg of body weight, to alcoholic rats (of Group B3), there were enhancement of serum AST, ALT and ALP activities with +47.30%, +18.16% and +23.24% deviations respectively from alcoholic rats of Group B1. Liver Lipid Peroxide, Liver GST and Liver CYP also shown similar trend with deviations of +15.38%, +7.89% and +7.94% respectively.

With administration of *A. indica* leaf-extract at dosage 600mg/kg of body weight, to alcoholic rats (of Group B4), there were enhancement of serum AST, ALT and ALP activities with +73.77% +30.18% and +34.31% deviations respectively alcoholic rats of Group B1. Liver Lipid Peroxide, Liver GST and Liver CYP also shown similar trend with deviations of +50.34%, +18.02% and +13.58% respectively.

CONCLUSION

From this study this may be concluded that the toxic compounds present in *A. indica* leaves are more potent than the antioxidant flavonoids. Though in lower dosage administered to healthy individuals, these show mild hepatostimulant and hepatoprotective properties; in higher dosage it plays adverse role. In case of major hepatic disorders it may have very drastic role. It may be advised not to use *A. indica* leaves to treat major hepatic disorders as common men have no idea of proper dosage and they have no sophistications to separate the beneficial flavonoids from toxic components like the limnoids of these leaves.

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