Screening of Hepatoprotective Effects of Four Different Brands of Polyherbal Bitter Formulations against Tetrachloromethane (CCl4) Induced Hepatotoxicity In Wistar Rats

Kasim, L. S[1]., Ewuoso O. V [1]., Etim, E. I.[2] , Daodu, J.O.[1] Aderinola, A. A.[3]

[1]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, Olabisi- Onabanjo University, Sagamu, Ogun, State. Nigeria

[2]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, University of Uyo, Uyo, Akwa Ibom, State. Nigeria

[3]Department of Pharmacology, College of Health Sciences, Olabisi- Onabanjo University, Sagamu, Ogun, State. Nigeria

ABSTRACT

Polyherbal bitters are a diverse group of formulation products used to strenghten and improve the whole digestive system in the body as well as nervous system. The aim of this study is to evaluate the phytochemical constituents and the hepatoprotective activity against tetrachloromethane (CCl_4) induced liver injury in wistar rats for four different brands of polyherbal bitter formulations.The phytochemical tests were carried out with standard procedures while thirty (30) rats were used for hepaprotective study with weights ranging from 180 – 260g. The animals were divided into six groups, group 1 served as control and received 0.9% normal saline, group 2 – 6 received CCl_4 intraperitoneally on alternate days (five days) for a peroid of ten days , while group 2 – 5 were administered (2.6 ml/kg/day) of each of the four different brands of polyherbal bitter formulations (SAMPLES Y, S, L, B) respectively daily for nine days. The animals were anaesthesized after the last dose of carbon tetrachloride and blood samples were taken via cardiac puncture for hematological and biochemical analysis after which the animals were euthanized and four organs (liver, kidney, lungs and brain) were removed for histological analysis. The CCl_4 toxicity in group 6 significantly increased Alanine Aminotransferase (ALT) Aspartate Aminotransferase (AST) and Alkaline Phosphatase (ALP) (39.10 ± 2.4, 131.4 ± 0.73, 208.60 ± 4.6 respectively as compared to normal control group 1 of ALT, AST and ALP, 30.36 ± 1.24, 3.96 ± 1.27, 24.04 ± 3.81 respectively, (P<0.05). It was observed that there was a decrease in enzyme biomarkers (Aspartate and Alanine Transaminase) of liver injury in the herbal mixture treated groups. In the microscopic studies, CCl_4 induced toxicity showed haemorrhages, fatty changes and necrosis. All the four herbal formulations conclusively showed marked beneficial effects as revealed in biochemical and histological parameters. It can be concluded that the four polyherbal bitters protect the liver. The present findings demonstrated the efficacy of polyherbal liquid formulations in protecting the liver in CCl_4 induced hepatotoxicity in Wistar rats.

Keywords: Polyherbal bitter formulations, hepatoprotective, tetrachloromethane, hepatotoxicity.

INTRODUCTION

Herbal drugs have gained importance and popularity in recent years because of their safety, efficacy and cost effectiveness. The Indian Traditional Medicine like Ayurveda, Siddha and Unani are predominantly based on the use of plant materials. One of the important and well documented uses of plant products is their use as hepatoprotective agents. Hence, there is an ever increasing need for safe hepatoprotective agent (Eisenberg *et al*.,2001).

Polyherbal bitters are a diverse group of formulated products that share the common characteristic of a bitter taste. They are used to strengthen and improve the whole digestive system in the body as well as the nervous system. Bitters also act to increase the vital energy centers in the body hence they have such a broad effect on the entire physiology, tone, and function of the body.

Corresponding Author. E-mail: kasimls@yahoo.co.uk

In spite of tremendous strides in modern medicine, there are hardly any drugs that stimulate liver function, offer protection to the liver from damage or help regeneration of hepatic cell. The management of liver disorders by a simple and precise herbal drug is still an intriguing problem. Several Indian medicinal plants have been extensively used in the Indian traditional system of medicine for the management of liver disorder. Some of these plants have already been reported to possess strong antioxidant activity (Hawk *et al*., 2012).

The CCl_4 induced cirrhosis of the liver is an adequate model of alcoholic cirrhosis in humans and the histological and biochemical changes that develop in the CCl_4 treated animals were found to mimic human cirrhosis observed in several etiological types (Perez-Tomayo, 1983). Acute administration of carbon tetrachloride to rodents especially rats and mice induce centrilobular necrosis and steatosis and the chronic administration leads to fibrosis and cirrhosis of the liver (McLean *et al.,* 1969). CCl_4 induced liver damage by the free radical mechanism consists of two stages. The initial phase of injury to the liver is caused by the free radical of CCl_4 namely trichloromethyl radical (CCl_3 -). The second phase of injury is by the cascade of events in the metabolism of the trichloromethyl radical (CCl_3) which leads to lipid peroxidation (Timbrell, 1991). Since the mode of hepatic injury by CCl_4 is biphasic, it can be used for predicting the mode of action of the hepatoprotective agent. Many studies have been carried out using CCl_4 induced liver injury in rodents for the screening of herbal remedies.

Moreover commercial formulations like Picroliv (Dwivedi *et al.,* 1990) and Liv 52 (Kataria and Singh, 1997) and extracts from plants like *Ginkgo biloba* (Ashok et *al.,* 2001) and *Acanthus ilicifolius* (Babu *et al.,* 2001) have been tested and found effective against CCl_4 induced liver injury, *in vivo*. Herbs and herbal preparations are generally viewed as safe by the general public. But along with the beneficial components they may contain many bioactive compounds which could impose potentially deleterious effects. The prime objective of this study was to evaluate the hepatoprotective activity of four herbal mixtures in the western part of Nigeria against CCl_4 induced hepatotoxicity in wistar rat.

MATERIALS AND METHODS

Experimental animals

Thirty (30) Wistar rats used for this study were procured from an animal farm in University of Ibadan, Nigeria where they were reared commercially. They were relocated to the animal facility of the Faculty of Pharmacy, Olabisi Onabanjo University, Sagamu, Ogun State, Nigeria. The animals were allowed food broilers finish (Animal care) and water *ad libitum*. Animals were maintained in standard laboratory conditions at room temperature to acclimatize with the environment. During the period of acclimatization they gained weight of averagely 50-60g bringing them to 200-260g.

The study was conducted between October and November 2015, after approval by the animal ethics committee of the University.

Polyherbal bitter formulations.

Four different brands of polyherbal bitter formulations (SAMPLES Y, S, L, and B) duly registered with National Agency for Food and Drug Administration and Control (NAFDAC) and within shelf life from different pharmaceutical companies were randomly selected and purchased from a pharmacy outlet in Ogun state, Nigeria. The dose 2.6 ml/kg/day of each of the sample was calculated based on the average (human) adult dose juxtaposed with the animal's average body weight in each group. The samples were administered orally.

Preliminary phytochemical screening tests

The four polyherbal bitter formulations were subjected to various qualitative tests for the detection of presence of various phytochemical constituents (Harborne 1973).

Hepatoprotective effects

This was carried out according to the method of Perez–Tomayo, (1983). Carbon tetrachloride (CCl_4) was used to induce hepatic injury. It was diluted in liquid paraffin at a ratio of 1: 2 and administered intra-peritoneally at a dose of 2ml/kg body weight and it was administered once in every 48 hours for 10 days. Oral administration of 2.6 ml/kg/day of each of the polyherbal bitters (SAMPLES Y, S, L, B) to groups 2, 3, 4 and 5 respectively was carried out once daily for 9 days. The dose regimen of CCl_4 followed in the present study is sufficient to induce hepatotoxicity as evidenced by increase in plasma level of the liver function enzymes. The animals were anaesthesized 24 hours after the last administration of CCl_4, via cardiac puncture and blood samples were collected in EDTA vials and heparinized vials for haematological and biochemical studies respectively. After the blood collection, the animals were euthanized and four different organs (liver, kidney, lungs and brain) were removed for histological studies using Haematoxylin and Eosin staining technique (Drury and Wallington, 1976; Ellis *et al*., 1978).

Hematological and biochemical estimations:

Red blood corpuscles (RBC), white blood corpuscles (WBC), hemoglobin (Hb) and packed cell volume (PCV) were read on an Erma particle counter (Model PC 607 Erma Inc., Japan). The differential count cells in the blood smears were also read on an Erma particle counter (Model PC 607 Erma Inc., Japan) in addition to above, the biochemical parameters in the plasma and liver homogenate were estimated using Erba test reagent kits (Transasia biochemical Ltd., Bombay, India). The biochemical parameters estimated in blood include alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, blood urea nitrogen (BUN) and creatinine (CRE). in the liver homogenate alkaline phosphate (ALP),and lactate dehydrogenase (LDH) were estimated (Lai *et al*, 2007)

Statistical analysis

Statistical analysis of the results was performed using student't' test with 95% confidence level. All values were reported as mean ± SEM and a value of $P<0.05$ was considered statistically significant.

RESULTS

Phytochemical tests.

The phytochemicals present in the samples of polyherbal bitter formulations examined include combined anthraquinone, free anthraquinone, saponin, cardiac glycoside, tannin, saponin and carbohydrate as shown in table 3.1

Table 3.1: Results of Phytochemical Tests

Phytochemicals tested	Sample Y	Sample L	Sample S	Sample B
Combined Anthraquinone	---	----	++	----
Free Anthraquinone	---	---	++	---
Tannin	---	---	++	---
Alkaloids	----	----	----	---
Carbohydrates	+	_	_	_
Saponin	+	++	+	+
Cardiac glycosides	+	+	++	+

Keys: + Present in moderate quantity ++ Present in abundant quantity - Absent

Table 3.2: Effects of polyherbal bitters on biochemical parameters in plasma of rats subjected to CCl_4 toxicity

GROUP	AST (IU/L)	ALT (IU/L)	BUN (mg/dl)	CREA (mg/dl)	ALP (IU/L)	LDH (IU/L)	BILIRUBIN (mg/dl)	PROTEIN (mg/dl)
G 1 (control)	3.96 ± 1.27	30.36 ± 1.24	54.02 ±19.62	0.50 ± 0.18	24.04 ± 3.81	139.21 ± 9.43	0.191 ±0.024	11.98 ± 1.98
G 2 (CCl_4 +sample Y)	6.68 ±1.94*[a]	19.40 ± 5.34*	181.34 ± 20.33*	0.54 ± 0.28	173.22 ±24.32[a]	156 ± 10.05*	0.34 ± 0.311[a]	9.68 ± 1.09
G 3 (CCl_4 + sample S)	5.94 ± 2.11*	41.14 ± 19.44	131 ± 5.01*[a]	149.3± 14.65*[a]	0.18 ± .08*[a]	115 ± 2.10*[a]	0.214 ± 0.05*	12.22 ± 1.34*
G 4 (CCL_4 + sample L)	6.32 ± 1.93*	28.60 ± 4.16	136 ± 4.16[a]	0.23 ± 0.17*[a]	86.40 ±3.89[a]*	106 ± 6.76 *[a]	0.38 ± 0.14[a]	10.86 ± 0.34*
G 5 (CCl_4 + sample B)	6.90 ± 1.7*	28.06 ± 4.06*	76.85 ± 24.53*	160.4± 22.02[a]	0.90 ± 0.16*[a]	118.07 ± 24.05*	0.38 ± 0.128	8.54 ± 0.74
G 6 (CCl_4 alone)	131.40 ± 0.93*	39.10 ±2.40*	164.71 ± 5.28*	0.75 ± 0.07	208.60 ±4.60*	263.20 ± 5.45*	0.33±0.01*	8.32 ± 0.25

Result expressed as Mean ±SEM, SEM- Standard error of mean; *$p<0.05$ when compared with CCl_4 alone. [a]$p<0.05$ when compared with control (normal saline); AST= aspertate aminotrassferase; ALT = alanine aminotransferase; BUN = blood urea nitrogen; CREA = creatinine; ALP = alkaline phosphatase; LDH = lactate dehydrogenase; IU/L =international units per liter; mg/dl = milligram per decilitre

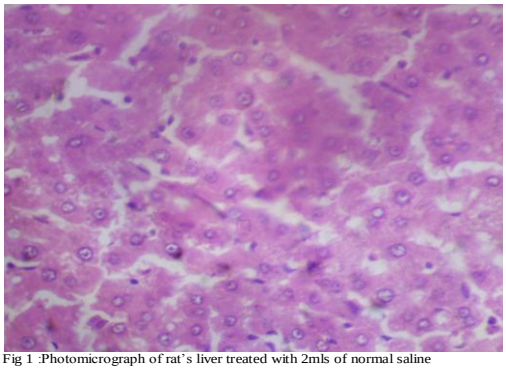

Fig 1 :Photomicrograph of rat's liver treated with 2mls of normal saline showing normal epithelium (H&E staining 40X)

GROUP 6 (CCl_4 ALONE)

Biochemical tests

The results of polyherbal bitters on some biochemical parameters are shown in table 3.2

Histological tests The results of histological studies using Haematoxylin and Eosin staining technique (Drury and Wallington, 1976) are presented in fig. 1 to 7.

DISCUSSION

The phytochemical components present in the samples of polyherbal bitter examined as shown in table 3.1 include combined anthraquinone, free anthraquinone, saponin, cardiac glycoside, tannin, saponin and carbohydrate.

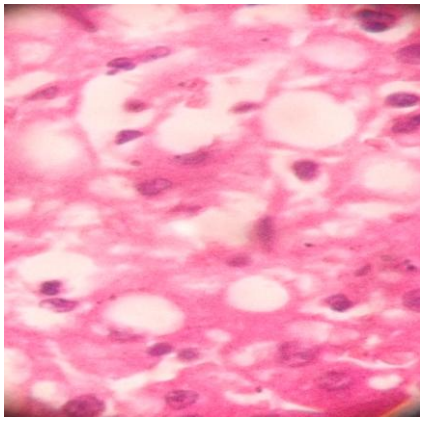

Fig 2: Photomicrograph of rat's liver treated with CCl_4 showing mild liver fatty change (initial stage of hepatic damage) Mild cellular change

Sample Y contains carbohydrate and cardiac glycosides in moderate quantity. Carbohydrates are energy builders which could help overcome fatigue. (Singh *et al*., 1991).

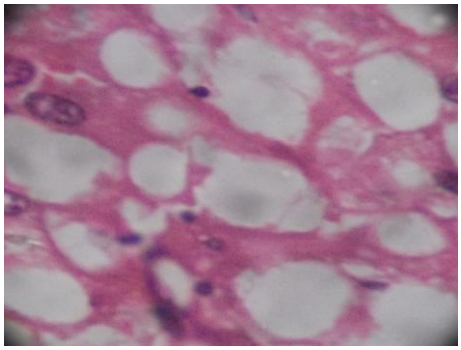

Fig 3: Photomicrograph of rat's liver treated with sample Y showing Mild liver fatty change (initial stage of hepatic damage) Mild cellular change

Mild portal vein vascular congestion (H&E staining 40X):

Sample S contains combined anthraquinone and free anthraquinone in abundant quantity, saponin, and cardiac glycosides in moderate quantity. Free and combined anthraquinone are known to increase bowel movement and the presence is responsible for the use of bitters in treating constipation. This is due to the fact that sample S contains senna, aloe and rhubarb.

Cardiac glycosides are drugs used in the treatment of congestive heart failure and cardiac arrhythmia and these glycosides are found as secondary metabolites in several plants.

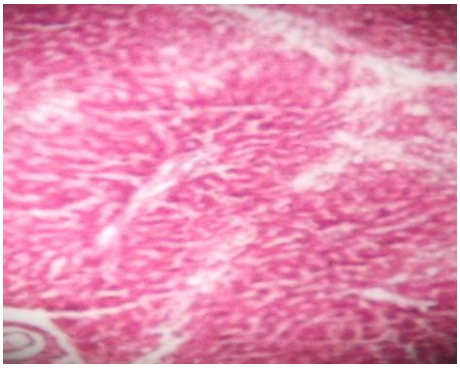

Fig 4: Photomicrograph of rat's liver treated with sample S showing mild liver fatty change (initial stage of hepatic damage) Mild cellular change

Mild portal vein vascular congestion (H&E staining 40X)

Samples L & B contain saponin, which has been reported to enhance natural resistance and recuperate powers of the body (Singh *et al.*, 1991)

Secondary metabolites are chemically and taxonomically diverse compounds with obscure function. They are widely used in the human therapy,

veterinary, agriculture, scientific research and countless other areas.

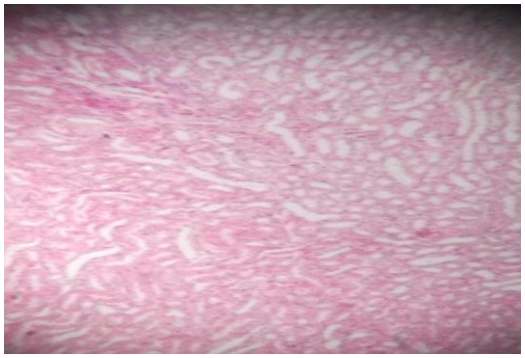

Fig 5: Photomicrograph of rat's liver treated with sample L showing mild liver fatty change (initial stage of hepatic damage) Mild cellular change

Mild portal vein vascular congestion (H&E staining 40X)

Hematological investigation revealed significant increase in white blood cell (WBC) count of the animals treated with samples Y (15.62±1.125), and L (17.7±1.3) when compared with that of the control (11.92±0.188). Sample S treated animals did not show any significant changes. There was also a significant increase in packed cell volume (PCV) of animals treated with samples B (46.60±0.812) and L (45.40±1.288), when compared with that of the control (42±0.707), $P<0.05$. There was no significant increase in red blood cells of all the treated animals.

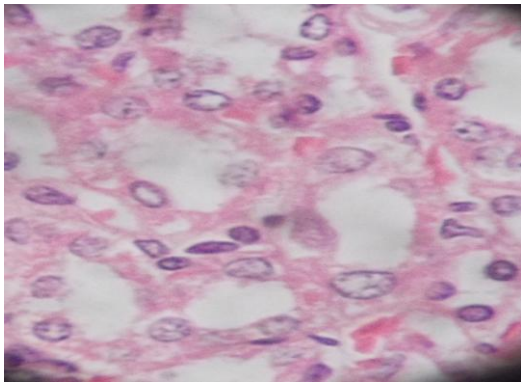

Fig 6: Photomicrograph of rat's liver treated with sample L showing mild liver fatty change (initial stage of hepatic damage) Mild cellular change

Mild portal vein vascular congestion (H&E staining 40X)

White blood cell or leukocytes are the cells of the immune system that work to defend the body against infections and other foreign matter. Most white blood cells are made in the bone marrow and are in the lymph tissue.

Packed cell volume is a measure of the proportion of blood volume that is occupied by red blood cells.

In the biochemical parameters as shown in table 3.2, Alanine transaminase or serum glutamic-pyruvic transaminase (SGPT) is a transaminase enzyme. It is most commonly associated with the liver, and it is most commonly measured clinically as a part of a diagnostic evaluation of hepatocellular injury to determine liver health when used in diagnosis (Giannini *et al*., 2005; Reichling and Kaplan, 1988; Lai *et al*., 2007).

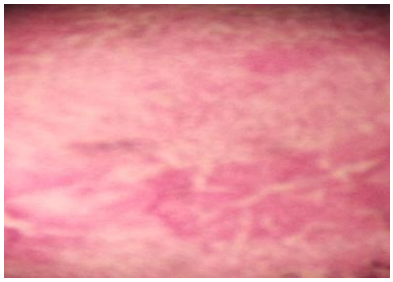

Fig 7. Photomicrograph of rat's liver treated with sample B showing mild liver fatty change (initial stage of hepatic damage) Mild cellular change

Mild portal vein vascular congestion (H&E staining 40X)

The ALT (39.10 ± 2.4) in group 6 (CCl_4 alone treated animals) is considerably higher than group 1 (untreated control) (30.36 ± 1.24) and the groups treated with polyherbal bitter i.e. group 2 (CCl_4 + sample Y) (19.40±5.344), 4 (CCl_4 + sample L) (28.6±4.165) and 5 (CCl_4 + sample B) (28.06±4.06), except group 3(CCl_4 + sample S) which is higher than group 1 (untreated control) and group 6 (CCl_4 alone treated animals). This may be due to the fact that rather than the drug preventing CCl_4 induced hepatic injury; it only caused more injury to the liver. Similarly the herbal mixture could have potentiated CCl_4 induced hepatic injury to a certain extent which lead to rise in ALT in group 3 and the same reason could be attributed to the lack of prevention of hepatic injury. It is known that agents that induce drug metabolizing enzyme tend to potentiate hepatic injury produced by compounds such as chloroform, carbon tetrachloride and halothane (Gopinath and Ford, 1975).

Aspartate transaminase or serum glutamic-oxaloacetic transaminase (SGOT) is a pyridoxal phosphate (PLP), Aspartate is found in liver, heart and skeletal muscle, kidneys, brain and white blood cell. It is one of the biomarkers of liver.

There is a significant increase in AST of the group treated with CCl_4 alone (group 6) (131.4±0.98) compared with group 2 (CCl_4 + sample Y) (6.68±1.94), 3 (CCl_4 + sample S) (5.94±2.11) 4 (CCl_4

+ sample L) (6.32±1.93), and 5 (CCl_4+sample B) (6.90±1.7) which has significant reduction after treatment with polyherbal bitter. In this study the hepatoprotective effect can be deciphered from the decrease in AST and ALT in the groups treated with polyherbal bitter i.e. groups 2, 3, 4 and 5 except group 3 in the case of ALT which has increased level when compared with CCl_4 alone treated group i.e. group 6

In an earlier study, the administration of isosafrole reduced the level of AST and ALT in mice treated with CCl_4 (similar to decrease in ALT and AST in this present study confirming the hepatoprotective effect of isasofrole (Zhao and O' Brien, 1996).

The decrease in ALT and AST indicate the level of protection offered by the herbal mixture.

Renal injury is known to increase plasma urea measured as blood urea nitrogen (BUN) (Miura *et al*., 1987). Increase in BUN was observed in group 2, 3, 5 and 6 compared to group 1. This means the samples did not have nephro-protective activity. However, creatinine, another biomarker of kidney damage which usually shoots up after extensive kidney damage (Faulkner and King, 1976), did not show any changes among the groups indicating nephro-protective effect of the herbal formulations.

Carbon tetrachloride is known to cause an increase in LDH levels (Korsrud and Grice, 1972). In table 3.2, an increase of LDH in liver homogenate was observed in CCl4 alone, when compared to untreated control, while LDH in the herbal mixture treated groups were similar to that of control indicating hepato-protective effect of these herbal formulations

Histological result revealed that the liver of all the animals treated with CCl_4 + polyherbal bitters showed mild liver fatty and cellular change compared to the CCl_4 alone treated animals which showed a mild protective activity of polyherbal bitter formulations on the liver. Other organs photomicrographs (kidney, lungs and brain) were normal in all the treated groups and control.

CONCLUSION

It can be concluded from this study that these samples of polyherbal bitter had hepatoprotective effect against CCl_4 induced hepatotoxicity (necrosis) in Wistar rats but the hepatoprotective activity was mild. This is evident from the histological analysis as all the liver of animals in group 2, 3, 4 and 5 still showed a mild fatty and cellular change after treatment. Based on the findings the herbal mixture can be considered as an herbal hepatoprotective agent after carrying out further pre-clinical efficacy and long term toxicity studies.

ACKNOWLEDGEMENT: The staff of Chemical Pathology, Pharmaceutical and Medicinal Chemistry, Olabisi Onabanjo University, Sagamu are highly appreciated for their contribution to the success of this work.

REFERENCES

Ashok Shenoy, K., S.N. Somayaji and K.L. Bairy, (2001) Hepatoprotective effects of *Ginkgo biloba* against carbon tetrachloride induced hepatic injury in rats. *Indian J. Pharmacol*., 33, 260-266.

Babu, B.H., B.S. Shylesh and J. Padikkala,(2001). Antiioxidant and hepatoprotective effect of *Acanthus ilicifolius. Fitoterapia*, 72, 272-277.

Dwivedi. Y., R. Rastogi, R. Chander, S.K. Sharma, N.K. Kapoor, N.K. Garg and B.N. Dhawan: (1990) Hepatoprotective activity of picroliv against carbon tetrachloride induced liver damage in rats. *Indian J. Med. Res*., 92, 195-200.

Drury, RAB.,Wallington,E.A., Cameron, R.(1976).Carliton histological techniques.4th.ed. Oxford University Press, NY. U.S.A . pp279-280

Eisenberg DM, Kessler RC, Van Rompay MI, Kaptchuk TJ, Wilkey SA, Appel S, Davis RB. Perceptions about complementary therapies relative to conventional therapies among adults who use both: results from a national survey. Ann Intern Med 2001;135(5):344-351.

Ellis, G.,Goldberg, D.M.,Spooner, R.J.,(1978). Serum enzyme test in diseases of liver and biliary tree. *Am. J. Clin. Pathol*., 70: 248-258.

Faulkner, W.R. and J.W. King (1976): Renal Function. Fundamentals in Clinical Chemistry (Ed: N. Teitz). W.B. Saunders, Philadelphia, USA. pp. 975-1014.

Giannini,G., Testa, R. and Savarina, V.,(2005). Liver enzyme alteration: A guide for clinicians. *Can. Med. Assoc. J*, 172 : 1-7.

Gopinath, C. and Ford E.J.H.: (1975). The role of microsomal hydroxylases in the modification of chloroform hepatotoxicity in rats. *Br. J. Exp. Pathol*., 56, 412-422.

Harborne JB (1973). Phytochenical methods, Chapman and Hall Ltd; London, 89 – 120

Hawk C, Ndetan H, Evans MW, Jr. Potential role of complementary and alternative health care providers in chronic disease prevention and health promotion: an analysis of National Health Interview Survey data. Prev Med 2012;54(1):18-22.

Kataria, M. and Singh L.N.: (1997). Hepatoprotective effect of Liv-52 and kumaryasava on carbon tetrachloride induced hepatic damage in rats. *Indian J. Exp. Biol.*, 35, 655-657.

Korsrud, G.O. and H.C. Grice (1972): Sensitivity of several serum enzymes in detecting carbon tetrachloride induced liver damage in rats. *Toxicol. App. Pharmacol.*, 22,474-483.

Lai Sam Aru K., Murthy Balakrishma P. and Pillai Sadasivan K., (2007). Screening of hepatotoxicity in Swiss albino mice. *J. of Envir. Biol.*28(2), 201-207.

Miura, K. Goldstein ., R.S and Morgan D.G.: (1987). Age related differences in susceptibility to renal ischemia in rats. *Toxicol. Appl. Pharmacol*., 87, 284-296.

McLean, E.K., A.E.M McLean and P.M. Sutton (1969). Perez: Instant Cirrhosis. An improved method of producing cirrhosis of the liver in rats by simultaneous administration of carbon tetrachloride and phenobarbitone. *Br. J. Exp.Pathol.*, 50, 502-506.

Perez-Tomayo, P.: (1983). Is cirrhosis of the liver experimentally produced by CCl4 an adequate model of human cirrhosis? *Gastroenterology*, 3, 112-120.

Reichling, J.J. and Kaplan, M.M., (1988). Clinical use of serum enzymes in liver diseases.*Dg. Dis. Sci*. 33: 1601-1614.

Singh, V.K., C.X. George, K.P. Gupta and B.M. (1991). Gupta: Antiviral activity of plant extract Liv 52 in mice experimentally infected with Semliki Forest Encephatitisvirus. *Science and Cult ure*, 49, 354.

Timbrell, J.A. (1991). Principles of biochemical toxicology. Taylor and Francis, London. pp. 297-312.

Zhao, Z.S. and 'Brien P.J. O (1996): The prevention of CCl_4 induced liver necrosis in mice by naturally occurring methylenedioxybenzenes. *Toxicol.Appl. Pharmacol.*, 140, 411-421.