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HEPATOPROTECTIVE ACTIVITY OF TRITERPENOIDS: NOVEL PHYTOCHEMICALS ISOLATED FROM A WILD MEDICINAL PLANT

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ABSTRACT

Hepatic tissue damage causes impairment in liver function which has harmful effect on human health. Management of liver diseases is still a challenge in developing world. The present study has been carried out to evaluate *in vitro* and *in vivo* hepatoprotective activity of triterpenoids isolated from *Zizyphus mauritiana* leaves. *Zizyphus mauritiana* ethyl acetate (ZMEA) extract was further purified on silica gel named as *Z. mauritiana* triterpenoids (ZMT) and tested for its hepatoprotective potential *in vitro* by using HepG2 cell line and *in vivo* by using Wistar albino rats. GC-MS chromatogram shows that ZMT contains triterpenoids. *In vitro* MTT assay depicts the protective activity of ZMT on CCl_4 intoxicated HepG2 cells. The results of biochemical parameter showed that ZMT has an efficacy to restore the liver function *in vivo*. The restoration property of hepatic cells by ZMT shows comparable results with standard sylimarin, which was also observed in liver histological examination of Wistar albino rat. Results of this study strongly demonstrate ZMT has been found to have promising hepatoprotective potential.

Keywords: *Zizyphus,* triterpenoids, Phytocompounds, hepatoprotection.

INTRODUCTION

The liver is the most important organ involved in the metabolism. It has a key role in detoxification and excretion of a variety of substances including xenobiotics. This detoxification process results in the production of highly reactive free radicals and cytotoxicants causing injury leading to liver cirrhosis and cancer (Osadebe *et al.,* 2012). Hepatic tissue damage causes impairment in liver function with harmful effect on human health (Jha *et al.,* 2009).

At present, the chemically synthesized drugs, available in market do not have satisfactory results in hepatoprotection, and

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some of them have many side effects, too. In contrast, herbal drugs are acknowledged to have protective effect against liver damage (Chaudhari and Mahajan, 2016). The majority of the herbal medicines have been reported to speed up the natural healing process of liver with almost negligible adverse effects (Kshirsagar *et al.,* 2011).

In India, many medicinal plants are in practice for treatment of hepatic disorders, since ancient time (Mahajan and Chaudhari, 2012). It has been proven that plants contain essential components responsible to cure several diseases. The *Zizyphus* genus, a Chinese date (Pushto), has been reported to have large number of medicinal properties. The bark, leaves and fruits of several species have been used as laxatives, curing jaundice, used to prevent malaria (Gul *et al.,* 2009), proven to be antibacterial, with curative properties (Ahmad *et al.,* 2011, Schomburg *et al.,* 2001, Kumar *et al.,* 2009). This plant has been used in the treatment of liver diseases from an ancient time by tribal and rural community (Morton, 1987). Taking in to consideration above mentioned properties of *Zizyphus* genus, the present study was aimed to evaluate the efficacy of the active phytochemicals present in leaves of *Z. mauritiana* on carbon tetrachloride induced liver damage. To identify hepatoprotective potential of *Zizyphus mauritiana*, HepG2 cell line was used for *in vitro* experiment while Wistar albino rats were used as animal model for *in vivo* studies.

MATERIALS AND METHODS

Materials

Plant material was collected from the forest of Gadchiroli district, MS, India, and authenticated by the taxonomist of the region (Voucher No. 9848). HepG2 cell line obtained from Human liver was procured from National Centre for Cell Science, Pune, India. Wistar albino rats, as experimental animal model, were sanctioned from Institutional Animal Ethics Committee, Department of Biochemistry, RTM

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Nagpur University Nagpur (MS) and obtained from National Centre for Laboratory Animal Sciences, Hyderabad, India.

All chemicals and cell culture media were obtained from SD Fine Chemicals and Himedia, Mumbai, India. Silymarin, and carbon tetrachloride were purchased from Sigma Chemical Co., St. Loius, MO, USA. All analytical kits for estimation of biochemical parameters were procured from Abcam, Cambridge, UK.

Methods

Extraction and purification of triterpenoids from *Z. mauritiana*: Bioactive compounds from the leaves of *ZM* were extracted in ethyl acetate solvent (ZMEA) (Cordeiro *et al.,* 1999). Solvent was evaporated by rotary vacuum evaporator (Superfit™ DB-3135S) and dried in hot air oven at 60°C. ZMEA extract was column chromatographed over silica gel (60-120 mesh size) and eluted with Benzene:Ethyl acetate solvent mixture) in 2:1 solvent mixture ratio, to obtain *Z. mauritiana* triterpenoids (ZMT), as mentioned by Kundu *et al.* (1989).

Gas Chromatography – Mass Spectrophotometry: A Varian 4500 GC coupled with Varian MS240 ion trap mass spectrometer (Varian, Walnut Creek, USA) was employed for determination of analytes in most potent partially purified ZMT using electron ionization (EI) mode, keeping all other modes at standard.

MTT assay for *in vitro* hepatoprotective activity on HepG2 cell line: Screening of ZMT for hepatoprotective activity was performed using HepG2 cells by MTT assay (Hu *et al.,* 1999). Triterpenoid fraction greater than 80 µg/ml and standard drug Silymarin greater than 250 µg/ml were found to be cytotoxic to the cells; therefore, the concentrations in the range of 40–80 µg/ml were used for the ZMT fraction and 250 µg/ml were used for the standard Silymarin. At the end of incubation period, cytotoxicity was assessed by estimating the viability of the HepG2 cells by the MTT reduction

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assay as per the instructions given by the manufacturer.

***In vivo* hepatoprotective activity on CCl_4 induced rat model:** Wistar adult albino rats (200–250 g) of either sex were used for the present investigations. All the animals were maintained under standard laboratory conditions with food and water *ad libitum*. The animals were divided into four groups of six animals in each group. Liver damage in rats was induced by administration of 30% CCl_4 suspended in olive oil (1 ml/kg body weight, i.p). Acute toxicity studies were performed and the dose was fixed at 60 mg/kg body weight (b.w.) and 50 mg/kg b.w. for ZMT and standard Silymarin, respectively. After 24 h of intoxication, on the 8 day, blood was collected in sterile centrifuge tubes. Serum was separated and used for the estimation of Asparate transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), triglycerides (TGL), total proteins, albumin, total bilirubin and lactate dehydrogenase (LDH) using Abcam diagnostic kits, Cambridge, UK (Kiso *et al.,* 1983; Lowry *et al.,* 1951).

Histological Examination of rat liver: Liver was removed, fixed overnight in 10% buffered formalin, and paraffin-embedded. The sections were stained with hematoxylin and eosin (H and E) for histological evaluation and examined under light microscope. (Vasanth *et al.,* 2010).

Statistical Analysis: The results have been presented as Mean ± SEM. The statistical analysis was carried out by one way analysis of variance (ANOVA). Comparison of different groups treated with different dose of ZMT and positive control with normal was performed by Turkey's Multiple Comparison Test. P values set at < 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Incredible time-honoured traditional medicinal usage to cure several disorders and ailments during ancient time was not truly based on the knowledge of its chemical constituents (Kumari and

Pandey, 2012). Later on literature of scientific reports divulged the occurrence of bioactive phyto-chemicals called secondary metabolites like alkaloids, terpenoids, flavonoids, glycosides, waxes and fatty acids in medicinal plants, documented to be responsible for their medicinal and pharmacological actions (Kalimuthu *et al.,* 2014).

Extraction and purification of triterpenoids from Z. mauritiana: GC-MS analysis conducted on the effective ZMT fraction revealed to identify four chemical compounds in figure 1 (A, B, C, D), disclosing the presence of compounds belonging to triterpenoid group. Reports have already been declared with triterpenoids from medicinal plants possessing enormous anticancer activity, with ability to induce apoptosis and also capable of inhibiting tumour angiogenesis (Mu *et al.,* 2012).

GC-MS chromatogram has identified the presence of four triterpenoids in ZMT fraction, which are probably, responsible for its bioactive properties. The triterpenoids of other *Zizyphus species* were also reported to have similar pharmacological activities. Betulin, a triterpenoid has already been evaluated with anticancer and antiangiogenic property in animal model (Dehelean *et al.,* 2013). Lupeol, is another promising bioactive agent that has been reported to exhibit antitumor, anti-inflammatory and antiangiogenic potential *in vivo* studies (Manimaran *et al.,* 2015).

***In vitro* hepatoprotective activity on HepG2 cell line:** The CCl4 exposed HepG2 cells exhibited a percentage viability of 17.65%. These results were highly significant ($P < 0.001$, when compared to CCl_4 intoxicated group). The percentage viability ranged between 72.51 - 93.52% at 80–40 µg/ml concentration of the ZMT (Figure 2). The increase in percentage viability of the HepG2 cells treated with ZMT at 80 and 70 µg/ml was significant ($P < 0.01$, when compared to standard Silymarin) and more potent than that produced by the standard

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Silymarin at 250 µg/ml. Treatment with the ZMT has significant antiproliferative activity with HepG2 cells, too. Similar *in vitro* results were also found by others with *Zizyphus* species using liver slices (Umamaheswari *et al.,* 2009).

***In vivo* hepatoprotective activity on Wistar albino rats:** It has been shown that CCl_4 gets accumulated in hepatic cells (parenchyma) and by activated cytochrome P450-dependent monooxygenases, it converts CCl_4 to a trichloromethyl radical (CCl3), which produces lipid peroxides, leading to liver damage (Bishayee *et al.,* 1995).

There is a significant decrease in content of total bilirubin, triglycerides and proteins in CCl_4 intoxicated rats in the present study (Table 1). Liver enzymes such as ALT, AST, ALP and LDH were also altered to normal level depicting re-establishment of liver function, to certain extent. Thus, treatment with the ZMT exhibited significant restoration of the altered biochemical parameters towards normal in CCl4 intoxicated rats. Its hepatoprotective effect with *in vivo* studies at 60 mg/kg body weight was comparable to the standard Silymarin at 50 mg/kg b.w., positively supported by the histopathological results, also (Figure 3). Similar results related to biochemical parameters of hepatoprotective activity were also observed with fruit extracts of other *Ziziphus* species (Dahiru *et al.,* 2010).

CONCLUSION

In recent years, researchers have focused attention on the identification of novel biomolecules from medicinal plants for treatment of various diseases. Phytochemicals have been proved to perform a significant role, amongst them triterpenoids, due to the broad range of exceptional bioactive properties, mostly receive great attention. In the present investigation, triterpenoids were isolated from leaves of *Zizyphus mauritiana* (ZMT) with proven medicinal properties. ZMT, in a very less concentration, has been found to have hepatoprotective

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potential as compared to standard drug silymarin, with HepG2 cells. Restoration of biochemical parameters by treatment, with ZMT on Wistar albino rats, reveals tremendous bioactivite properties of ZMT. All biochemical parameters and histological findings in this study provided evidence that the progression of the liver cirrhosis induced by CCl_4 in rats can be intervened using the ZMT as a natural drug. However, to authenticate these observations, many more experiments are required before recommendation for human trials.

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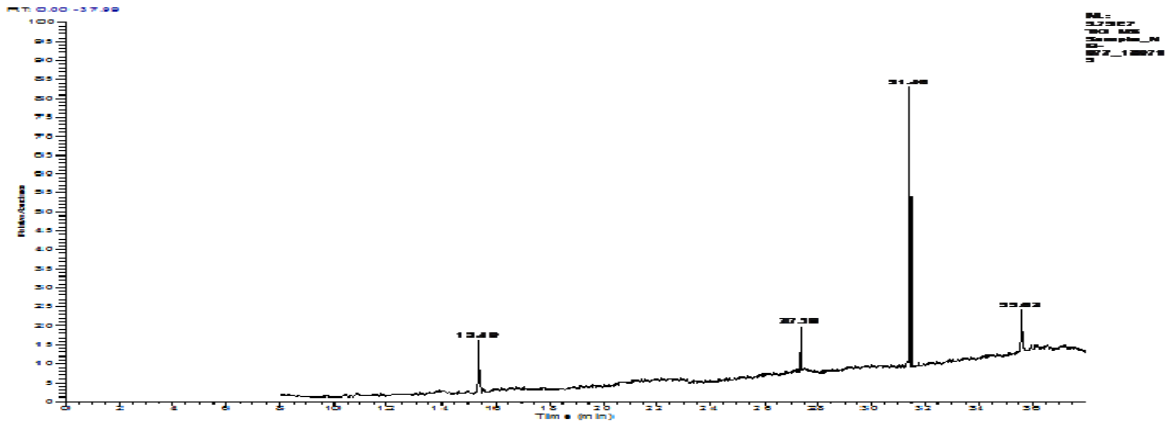

Figure 1 (A), GC-MS of ZXT

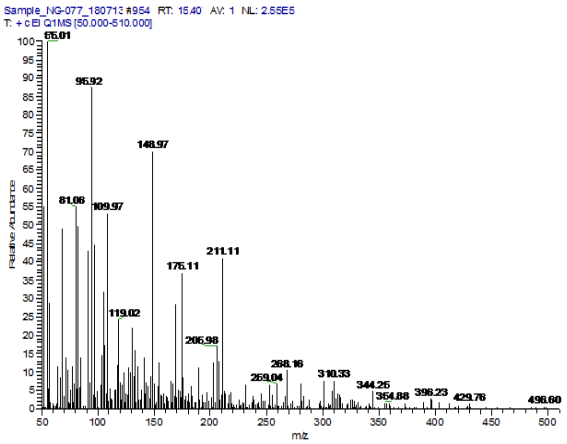

Figure 1 (B), MS of Lupeol

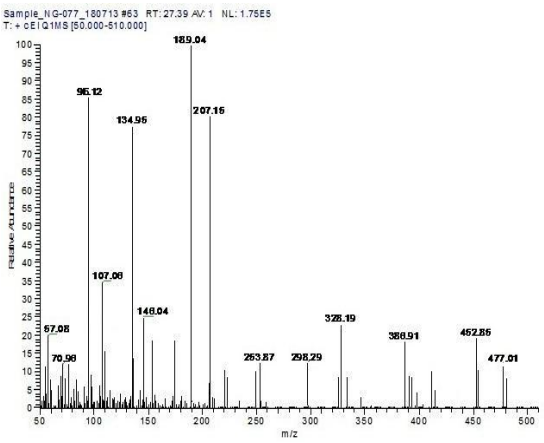

Figure 1 (C), MS of Betulin

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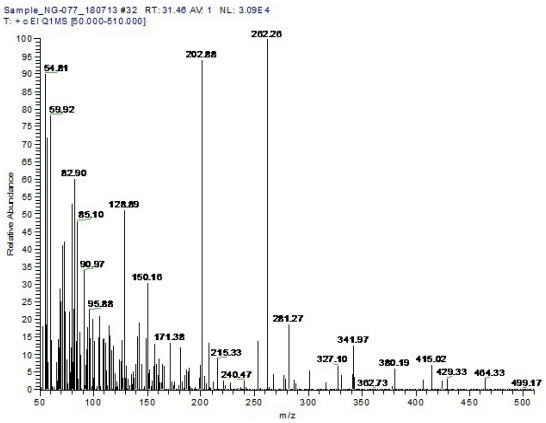

Figure 1 (D), MS of urs-12-en-28-oic acid, 3β- hydroxy-,methyl ester**,**

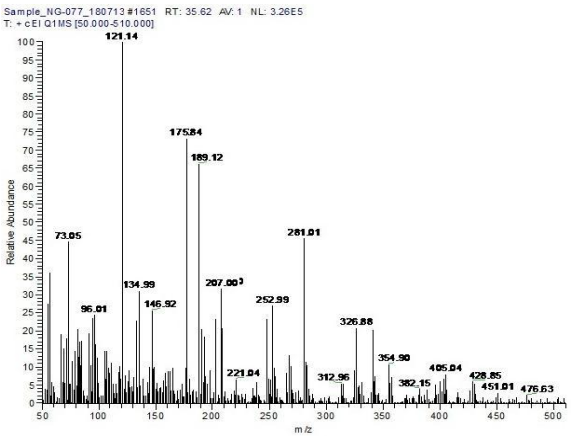

Figure 1 (E), MS of 2-Hydroxy-10-isopropenyl-3,3,5a,5b,12b-penta methyl octadecahydro dicyclopenta [a,i] phenanthrene-1,7a(1H)-dicarboxylic acid

Figure1. Gas Chromatography – Mass Spectrophotometry analysis of isolated terpenoides:

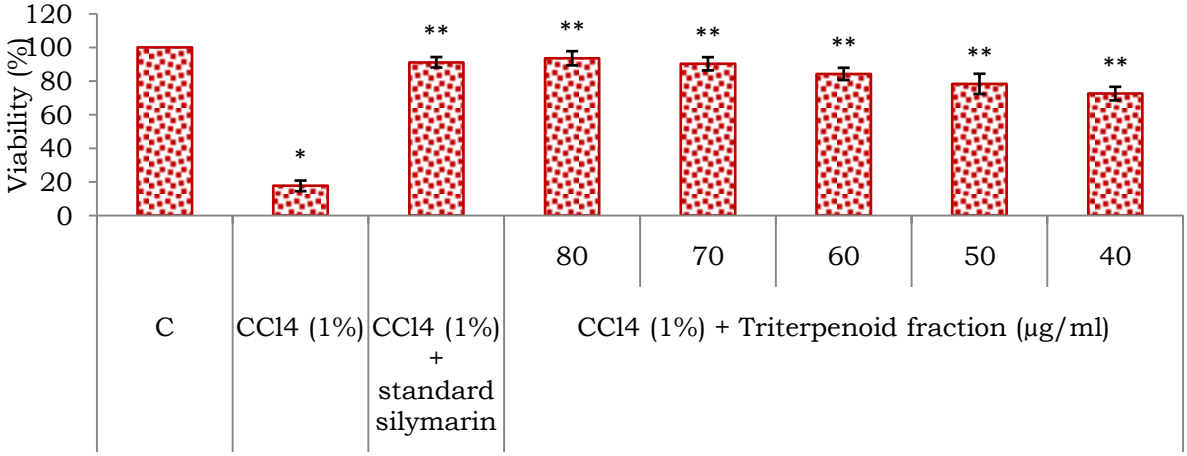

Figure 2 Hepatoprotective activity of the ZMT on CCl4 intoxicated HepG2 cells *(Average of 5 determinations (n=5); *= P < 0.001, when compared to the normal cells; **= P < 0.01, when compared to the CCl_4 intoxicated cells).*

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Table 1. Effects of treatment of ZMT on the biochemical parameters of CCl4 intoxicated rats.

Treatment	Concentration	AST U/L	ALT U/L	ALP U/L	Albumin g/L	Total bilirubin mg/dL	Total protein g/dL	TGL mg/dL	LDH U/L
Normal	--	87.68 ± 0.547	59.55 ± 13.47	329 ± 2.116	3.281 ± 0.379	0.45 ± 0.11	7.475 ± 3.11	75.4 ± 0.32	254.3 ± 0.141
CCl_4	1ml/kg body wt	169 ± 2.11^{x}	116.2 ± 13.16^{x}	588 ±4.69^{x}	1.5± 0.10x	0.99 ± 0.02^{x}	4.41 ± 1.3^{x}	29.3 ± 0.10^{x}	522.4 ± 0.10^{x}
CCl_4 (1%) + standard silymarin	50 mg/kg body wt	82.77 ± 1.57^{z}	60.2 ± 9.87^{z}	360 ± 4.41^{z}	3.412 ± 0.324^{z}	0.45 ± 0.01^{z}	6.58 ± 2.31^{z}	69.03 ± 0.09^{y}	304.72 ±0.02^{z}
CCl_4 (1ml/kg body wt) + triterpenoid fraction	60 mg/kg body wt	86.92 ± 1.22^{z}	64.55 ± 13.12^{z}	369.25± 2.284^{z}	3.318 ± 0.208^{y}	0.47 ± 0.011^{z}	6.30 ± 2.11^{z}	68.09 ± 0.1y	313 ± 0.14^{z}

Number of animals (n=6). (^{x}= P < 0.001, when compared to the normal group; ^{y}= P < 0.01; ^{z}= P < 0.001, when compared to the CCl_4 group)

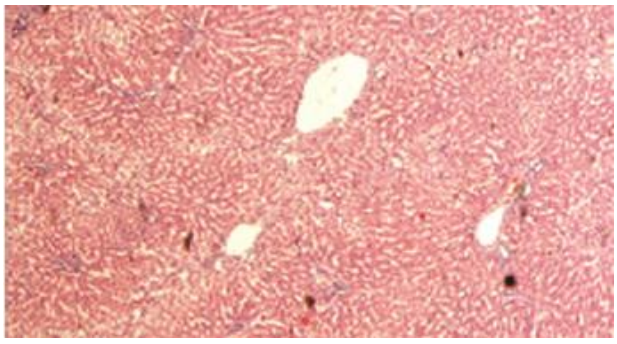

Figure 3 (A) Histopathology of liver having normal histological structures of hepatic lobules

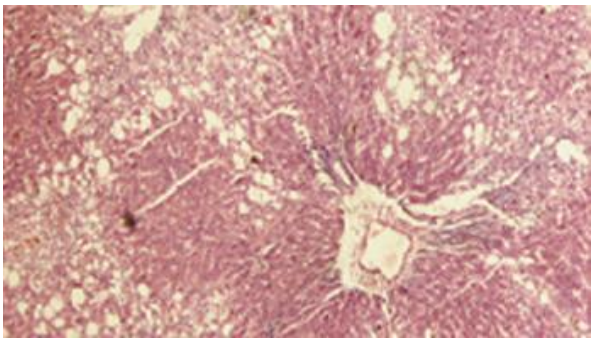

Figure 3 (B) Histopathology of toxicant-treated liver (CCl_4 1 ml/kg body wt) showing damage to hepatocytes with hepatocellular vacuolization, focal hepatic necrosis, and congestion of hepatic sinusoids

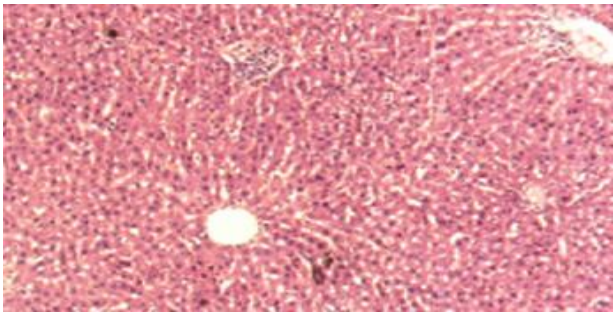

Figure 3 (C) Histopathology of standard silymarin drug-treated liver (50 mg/kg body wt) showing apparently normal hepatocytes

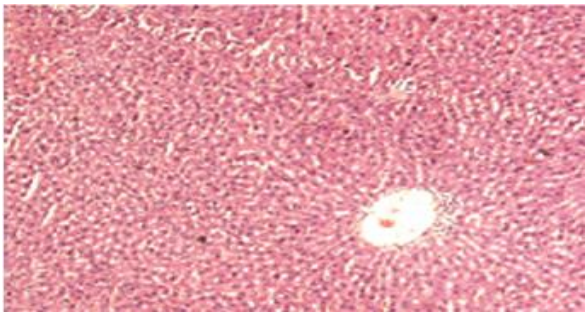

Figure 3 (D) Histopathology triterpenoid (ZMT) treated liver (60 mg/kg body wt) showing mildvacuolization

Figure 3 Histopathologcal examination of rat liver by H & E staining

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