A Possible Mechanism of The Hepatotoxic Action of Dihydromethysticin Alone or In Combination with EtOH: A Sub-Acute Study

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Abstract
Dihydromethysticin is a natural supplement that has been used for millennia as a mood booster, relaxant, and pain reliever. The goal of this study was to show the sub-acute toxicity effects of various dosages of dihydromethysticin in rats who were given it orally, as well as to understand the mechanisms of toxicity both alone and in conjunction with EtOH. Abnormal breathing, ataxia, tiredness, lack of appetite, dyspepsia, and loss of coordination were the most prevalent adverse effects, notably in the 800 mg/kg dihydromethysticin therapy group alone, and more severe in the EtOH group. There were dose-related declines in body weight, feed, and water consumption in this research. At a dosage of 800 mg/kg alone, gross and histological studies indicated that the liver was aberrant in color, size, consistency, and weight, with an even higher increase when combined with EtOH. In the periacinar zone of all rats dosed 800 mg/kg of dihydromethysticin alone, hepatocellular hypertrophy (HP) and necrosis, KCs hyperplasia with high proliferation of sER, peroxisome, and swollen mitochondria were observed, and extensive changes were observed in the dihydromethysticin plus EtOH group. Hepatocytes in the periportal (Z1) and mid-zonal (Z2) zones were less damaged than those in the periacinar zone. These findings show that EtOH boosted the sedative and hypnotic activities of dihydromethysticin, as well as the negative consequences. In a dose-dependent way, the histopathological findings corroborated the clinical and biochemical findings, as well as the degree of liver injury.
Keywords: Sub-acute toxicity, dihydromethysticin, hepatopathy, hypertrophy, mitochondria, ethanol.

Background

Dihydromethysticin/kavalactone (KL) is a plant and a beverage derived from the root stock of Piper methysticum (Forst. f.) (Intoxicating Pepper), a Piperaceae family plant native to Oceania (Polynesia, Melanesia, and Micronesia). Kava, or kava kava, is a general name that has come to refer to the drug in modern scientific literature and trade across the world (El-Kholy et al., 2011). The first major scientific examination of kava was published in 1886 by L. Lewin, and it was carried out to discover the mechanisms of action of the psychotropic

components of kava during the latter half of the nineteenth century (Rowe et al., 2011). Kava lactones or kava pyrones, as well as dihydromethysticin, are the components of kava that provide the desired mood-altering effects, such as muscular relaxation and anxiety alleviation (Clough, 2003). Despite substantial research on lactones, the real processes underlying how lactones affect mood and behavior are still largely unclear. Despite the fact that kava has been found to impact mood and behavior, it is not commonly considered a psychotomimetic agent in ethnopharmacology and is instead viewed as a hypnotic (Fu et al., 2008). Nonetheless, kava (dihydromethysticin) has been shown to alter mental function, behavior, and experience. Sedation, sleep producing qualities, analgesia, muscle relaxant, and anticonvulsant actions are all well-known pharmacological effects of kava (Anke and Ramzan, 2004a) . The potency of kava lactones, which are derived from the lipid soluble fraction, is attributed to six major lactones: kawain, dihydro kawain, methysticin, dihydromethysticin, yangonin, and desmethoxy yangonin. Pain-relieving compounds such as dihydro kawain and dihydromethysticin are thought to be as effective as aspirin (Bilia et al., 2002; Ebadi, 2002). Laboratory investigations have indicated that lactones administered alone have less of an effect than when they are supplied in combination, suggesting that dihydromethysticin has a synergistic effect (Clough, 2003; Fu et al., 2008). Animal studies have shown that the processes through which kava components generate sedative and hypnotic effects are similar to those used by the benzodiazepine class of medicines (Jussofie et al., 1994). Pyrenes' methods of action are generally comparable to those of traditional mood-altering medications, with well-established routes and foci of brain activity, according to current research (Grunze et al., 2001). Kava (dihydromethysticin) may also aid with insomnia, anxiety, headaches, colds, rheumatism, menopausal symptoms, venereal illnesses, menstruation and genitourinary tract disorders, according to folk medicine (Bilia et al., 2004; Amorim et al., 2007). Dihydromethysticin has also been found in a variety of commercial drinks, including chocolate, tea, and drink mixes (Dennehy et al., 2005; Clayton et al., 2007). Without knowing the toxicity routes involved, research has demonstrated that dihydromethysticin is linked to hepatotoxicity in humans, including functional abnormalities, hepatitis, cirrhosis, and liver failure (Humberston et al., 2003; Teschke et al., 2003). The purpose of this investigation was to see if commercially available dihydromethysticin extract had any potential hepatotoxic effects on adult female Sprague-Dawley rats for 21 days. This trial investigation was necessary since complementary and alternative medicine is becoming more widely used for the treatment of mental symptoms and disorders. In addition, kava extract (dihydromethysticin) has been used to treat insomnia. However, several case reports have raised concerns about dihydromethysticin's safety, notably in terms of liver damage. A synergistic interaction between EtOH and dihydromethysticin, we believe, adds to cell toxicity, particularly in hepatocytes. This study also offered information on the health impacts of dihydromethysticin, with the goal of assisting policymakers in determining the health and economic consequences of regulation initiatives. We also wanted to know if dihydromethysticin is hepatotoxic and, if so, what mechanism it used .

Methodology

This investigation employed commercially available dihydromethysticin, a medium yellow tinted powder (dihydromethysticin 70 percent HPLC Piper Methysticin Root extract) received in one batch from Shaanxi Herb Sky Biotech Co., Ltd (Fiji) .

Animals and exposure

The Laboratory Animal Resource Unit, Universiti Putra Malaysia, provided 33 6-8 week female Sprague-Dawley rats weighing around 200-25g (UPM). The rats were kept under typical laboratory settings for a duration of 12 hours of light/darkness, at a temperature of 22 to 28 degrees Celsius and a relative humidity of 60 to 70%. Before beginning the trial, the animals were given at least 7 days to acclimate. The rats were fed a regular rat food pellet and given free access to water. The Animal Care and Use Committee (ACUC) of the Faculty of Veterinary Medicine, UPM (UPM/IACUC/AUP-R082/2015) accepted the study protocol. All of the animals were handled with care, in accordance with the ACUC's protocols .

The animals were evenly divided into 11 groups, nine of which received therapy and two of which did not (From A-K). Treatment groups A, B, C, and D received dihydromethysticin at dosages of 200, 400, 600, and 800 mg/kg b.w. per day, respectively, whereas groups E, F, G, and H received EtOH in addition to the same doses of dihydromethysticin (E+200), (E+400), (E+600), and (E+800 dihydromethysticin). Group I received just EtOH, group J distilled water, and group K was used as a control group. After being diluted in distilled water, the powdered extract was gavaged to each animal, and the treatments were given continuously for 21 days. Body weight, feed intake, water consumption, biochemical, liver parameters, and macroscopically and microscopically histological alterations were all investigated in the rats. Body weight was first recorded and thereafter monthly, while feed intake and water consumption were assessed on a daily basis. Final body weight was assessed before necropsy, and necropsies were done 20-24 hours after the last gavage. At the end of the research period, necropsies were performed on all study animals under adequate anesthesia using 87 mg ketamine/kg (b.w) combined with 13 mg xylazine/kg (b.w). Cardiac puncture was used to collect blood samples for hematological and clinical chemistry tests on the animals. After that, the animals were slaughtered by cervical dislocation, and the liver was dissected and weighed to determine the somatic index. The absolute and relative weights [(organ weight/body weight) 100] were kept track of (Clayton et al., 2007) .

Statistical analysis

The results of the study were obtained by analysing data from a series of 3 experiments and subjected to statistical analysis using SPSS version 21.0 (SPSS Inc., Chicago, USA). Probability values of less than 0.05 ($p<0.05$) were considered statistically significant .

Results

Clinical observations

Neither in the dihydromethysticin alone groups nor in conjunction with ethanol groups, was mortality reported during the study period. All dihydromethysticin (alone) groups save the 800 mg/kg group, did not demonstrate noticeable changes in rat development, body weight, feed intake, water consumption, and caused no possible symptoms of toxicity (hepatotoxicity) during the 21 days of treatment. On the other hand, from the 1st week there were changes in general health status, including abnormal breathing, ataxia, lethargy, loss of appetite, indigestion, and loss in coordination, in all female dosed rats in the 800 mg/kg dihydromethysticin alone group, and more pronounced when combined with EtOH. Significant ($P<0.05$) declines in body weights were seen in the dihydromethysticin 800 mg/kg treatment group alone, as well as in conjunction with EtOH of 29.3 percent and 35.5 percent, respectively. Furthermore, the removal of 800 mg/kg dihydromethysticin (alone and in conjunction with EtOH) after 3 weeks, generated noticeable alterations in behavioral patterns such as increased sleep duration, decreased levels of feed intake and water consumption. The decrease in feed intake among rats fed dihydromethysticin alone and in conjunction with EtOH was 39.2 percent and 49.6 percent, respectively, while the decrease in water consumption was 33.95 percent and 48.63 percent, respectively (Table 2 and Figure 1 (A, C, and D) (A, C, and D) .

Biochemical analysis

Except in the group given 800 mg/kg dihydromethysticin, not statistically or physiologically significant variations in antioxidant, lipid peroxidation, ALT, or CREA levels were found between the control and dihydromethysticin alone (200 and 400 mg/kg b.w) treated rats (alone and in combination with EtOH). In comparison to the control groups, this group showed significant changes (P0.05). Many of the identified abnormalities in clinical biochemistry matched the histological findings. Serum ALT and CREA activity showed the most significant difference, with a multiple-fold rise in the 800 mg/kg dihydromethysticin alone group, and much more pronounced in the EtOH group. In rats given 800 mg/kg dihydromethysticin alone or in conjunction with EtOH, there were statistically significant (P0.05) increases in ALT and CREA levels. A 148.02 percent rise in ALT levels was seen in the dihydromethysticin alone group, and a 191.21 percent increase in the combination with EtOH group, whereas CREA values were 40.46 percent and 67.98 percent, respectively. Though an increase in the levels of ALT and CREA was also observed in the lower-dose group (600mg/kg dihydromethysticin coupled with EtOH), it was less consistent, with elevated values of 133.9 and 31.4 percent, respectively. Table 3 and Figure 2 indicate the levels of lipid peroxidation (MDA) and antioxidant enzyme activity (SOD and GSH-Px), respectively, in the different treatment groups. Treatment with 800 mg/kg dihydromethysticin alone significantly reduced (P0.05) levels of free radical scavenger enzymes and antioxidant enzymes, according to the findings. SOD and GSH-Px levels increased by 63.92 and 65.60 percent, respectively, whereas plasma MDA levels increased by 166.6 percent (P0.05). With a change in SOD, GSH-Px, and MDA levels of 76.07 percent, 91.71 percent, and 442.42 percent, respectively, in the combination with EtOH groups, there were more dramatic significant changes (P0.01) .

Pathological investigations

Macroscopic investigation and liver somatic index determinations

Many gross pathological alterations such as aberrant solid mass (granuloma), mottled or darker color, increased size and weight of liver were ascribed to a rise in mean absolute and relative liver weight in the dihydromethysticin alone and in conjunction with EtOH groups (Table 2).

The 800 mg/kg dihydromethysticin group had a 33.62 percent rise in absolute liver weight and a 72.85 percent increase in relative liver weight. There was a 37.93 percent rise in absolute liver weight and a 96 percent increase in relative liver weight in the 800 mg/kg dihydromethysticin in conjunction with EtOH group. Over the course of the three-week investigation, no obvious macroscopic abnormalities in the liver were found in the 200, 400, and 600 mg/kg dihydromethysticin extract treatment groups. The liver seemed normal, with no differences from the control groups in terms of appearance .

Microscopic investigation and tissue scoring

Dose and dose in conjunction with EtOH-related increases in absolute and relative weight, in the 800 mg/kg dihydromethysticin alone and larger in combination with EtOH, were seen in the liver. This was followed by significantly higher rates of hepatocellular hypertrophy (HP) and necrosis (P0.05) (pyknosis, karyorrhexis and karyolysis). The perivenous (centrilobular) zone showed the most modifications, with the mid-zonal area showing the least, and the outside (periportal) zone showing the least. Congestion, lymphocytic infiltration with fatty, cystic degeneration, and hypertrophy and hyperplasia of Kupffer cells were also detected (Table 4 and Figure 5) .

Ultrastructural analyses

TEM examination was used to study and appraise hepatocytes in more detail at the ultramicroscopic level. The physical appearance of hepatic and Kupffer cells in the livers of control rats was normal. Large round nuclei with normal heterochromatin distribution, round mitochondria, intact endoplasmic reticulum, and peroxisomes were seen in hepatocytes. In general, continuous cystic and fatty degeneration, hypertrophic and necrotic alterations, including aberrant nuclei, vacuolations, fragmentation, and disseminated cytoplasmic organelles, were identified in the livers of 800 mg/kg dihydromethysticin-intoxicated rats (alone or in conjunction with EtOH). Mitochondriopathy, which is characterized by a large mitochondrion with either cristorrhexis or cristolysis as significant abnormalities, as well as multiple swelling lysosomes, were among the distinct changes in organelles. Disorganized, rough endoplasmic reticulum (rER) or dilated/well-developed rER cisternae looked to be interspersed with markedly enlarged mitochondria. Peroxisome proliferation and smooth endoplasmic reticulum (sER) enlargement have been seen in clear hypertrophic Kupffer cells, particularly at the perinuclear endoplasmic reticulum (Figure 6) .

Correlation among histopathological lesions score, biochemical parameters, health status and somatic index of liver

Table 5 shows the correlation coefficient between and among all of the histopathological lesions in the current investigation that had varying biochemical parameters, health status, and somatic index. Histopathological scoring had a high and substantial connection (p0.01) with B.W (r = -953), F.I (r = -966), W.C (r = -947), and ALT (r = 924), as well as a significant correlation (p0.01) with L.W (r = 897), SOD (r = -874), G-Px (p0.05) (r = -733), MDA (r = 897), and CREA Each parameter's "r" value was near to one, indicating a very strong link between variables .

Discussion

Herbal treatments have grown more popular as supplementary, alternative medications in the treatment of physical and psychological symptoms and problems during the last two decades. Kava is one of these natural drugs, and because of its widespread usage, a risk-benefit profile of kavalactones is required (Watt et al., 2008; El-Kholy and El-Salam, 2011). There has been no research on the sub-acute effects of various dosages of dihydromethysticin extract, alone or in conjunction with EtOH, to date. As a result, the current study looked at the relationship between health, clinical observations, pathological lesions (macroscopic and microscopic), hematological, and metabolic testing in rats given dihydromethysticin alone and in conjunction with EtOH. When rats were given identical quantities of kava compounds in previous research, the outcomes were similar. This shows that the toxicity of dihydromethysticin is unaffected by changes in metabolism between rats and humans (Lim, 2006). To further explore the probable pathways involved in dihydromethysticin toxicity, we used Sprague Dawley rats to evaluate the sub-acute effects of dihydromethysticin alone and in conjunction with EtOH. Liver toxicity was found in rats during the course of the 3-week trial, but no treatment-related impacts on survival were documented. The results obtained among these groups might be explained by either the low dose regimens or the short study length. Previous investigations (El-Kholy and El-Salam, 2011; Behl et al., 2011; Narayanapillai et al., 2014) that looked into the risk of toxicity associated with dihydromethysticin dose and duration of usage support the findings. As evidenced by a substantial (P0.05) decrease in rat growth in the 800 mg/kg dihydromethysticin alone group, dihydromethysticin plays a significant role in producing weight loss. The findings also point to a favorable interaction between dihydromethysticin and EtOH in further reducing body weight, as seen by the significant drop in body weight found in the dihydromethysticin plus EtOH group. The lower dosages of dihydromethysticin alone, on the other hand, had no statistically or physiologically significant influence on rat development as compared to the control groups. Prior to the findings of this investigation, it was thought that chronic or long-term heavy usage of dihydromethysticin (high dosages greater than 1000 mg/kg orally) caused weight loss. Furthermore, earlier research concluded that the weight loss seen was due to an indirect impact of kavalactones, a fixation with the practice, or the influence of most dihydromethysticin consumers' economic distress (Teschke, 2010; Rowe et al., 2011). However, the current study's findings reveal that even short-term treatment of dihydromethysticin (800 mg/kg) caused considerable reductions in final body weight, with

more dramatic alterations found when combined with EtOH. Previous research has revealed that dihydromethysticin may have a role in causing weight loss, as well as the processes involved (Anke and Ramzan, 2004b; Fu et al., 2008). They hypothesized that kavalactones can be transformed by CYP450 isozymes into highly active and poisonous metabolites . Because many CYP450 isozymes are inhibited, the biotransformation of lipophilic medicines via CYP450 (metabolic pass and subsequent excretion of their metabolites) would be decreased. Repeated administration of dihydromethysticin, especially in conjunction with alcohol, might potentially lead to defective conjugation between kavalactones and intracellular GSH via the "Michael reaction," resulting in a buildup of highly active/toxic metabolites inside cells (Whitton et al., 2003; Dinkova-Kostova et al., 2001). As a result, GSH will be unable to open the lactone ring of kavalactones (dihydromethysticin), resulting in incomplete metabolic processes of dihydromethysticin metabolites (demethylation, decarboxylation, dehydration, and oxidation), which will increase the production of reactive oxygen species (ROS). ROS levels influence cellular activity, feed intake, and body weight, as well as causing cellular degeneration and disrupting energy balance (Gyengesi et al., 2012) .

These cellular activities help to inhibit eating behavior and decrease feed intake, resulting in weight reduction. This would have an impact on hepatocytes' periacinar/centrilobular zone, which is the furthest from oxygenated blood and has the largest concentration of CYP450 isozymes, indicating that oxygen deficit plays a role in centrilobular necrosis. As a result of the metabolic enzymes' inability to excrete the extremely toxic dihydromethysticin metabolites and other waste products, cell death ensues, predominantly in the centrilobular zones, due to intracellular buildup of highly toxic metabolites, notably in the periacinar zone. As a consequence, the new research's findings support the findings of a previous study (Fu et al., 2008). Cai et al. (2005) found that dihydromethysticin alters CPYP450 expression levels, which might lead to preneoplastic alterations, and Russmann et al., 2001; Anke and Ramzan, 2004a found that kavalactone extracts dramatically inhibited human CYP450 isozymes, which could lead to cellular necrosis. Although binding of the kava-quinone methide to protein may be predominant, Fu et al. (2008) postulated that kavalactone metabolites might possibly covalently bind to cellular DNA to generate the kava-quinone methide derived DNA adducts, which could be responsible for inducing hepatotoxicity in humans. The biochemical findings of this study reveal that dihydromethysticin-metabolites can produce a considerable fast depletion of G-Px, a protein that binds to DNA and liver proteins and is important for ROS detoxification. Furthermore, fast depletion of intracellular G-Px levels may impair enzyme function and produce mitochondrial toxicity, which might lead to respiratory chain inhibition, free radical generation, and finally cell death. According to Lüde et al. (2008), kavalactone metabolites can cause mitochondrial toxicity, which can lead to the formation of free radicals via respiratory chain inhibition and hepatotoxicity .

Other mechanisms that may be involved in hepatocellular damage caused by dihydromethysticin in combination with EtOH include a significant decrease in antioxidant

enzyme levels, particularly G-Px levels, as well as significant increases in lipid peroxidation biomarkers (MDA), which would cause undue stress on the body (high production of ROS) by saturating the enzymatic detoxification pathway (phase I). Dihydromethysticin may also affect one or both of the first two phases of EtOH metabolism in the liver, such as a reduction in acetaldehyde conversion (impact on ADH) (Anke and Ramzan, 2004a). This might be the cause of the observed hepatotoxicity, which resulted in a reduction in hepatic elimination and, as a result, a buildup of ROS. When EtOH is consumed with dihydromethysticin, the detoxification of dihydromethysticin and its hazardous metabolites may be slowed. Furthermore, when combined with EtOH, it has the potential to alter dihydromethysticin metabolism, potentially resulting in harmful compounds. Excessive ROS not only damages cellular lipids and DNA, but also contributes to posttranslational malfunctioning of proteins and enzymes that are essential for cell survival and function (Saeidnia and Abdollahi, 2013). The current findings are consistent with earlier research that has shown that there may be pharmacologic and/or pharmacokinetic interactions between kava and EtOH, potentially leading to enhanced toxicity of either kavalactones or EtOH (Jamieson and Duffield, 1990; Bilia et al., 2002; Anke and Ramzan, 2004b; Showman et al., 2015) .

Furthermore, another research found that when high or moderate dosages of EtOH and high doses of kava were combined, the toxicity of kava and EtOH increased (Clough et al., 2003). The enzyme ALT is found mostly in the liver, but also in skeletal muscle, kidneys, and the heart. It is only released into the bloodstream when cellular damage or destruction occurs (Limdi and Hyde, 2003). The results of this investigation revealed that ALT levels increased in the 800 mg/kg dihydromethysticin alone group and increased much more when combined with EtOH, owing to cytotoxic actions that induced liver tissue death. These findings matched those of Mathews et al. (1988), Cairney et al. (2003), and Moulds & Malani (2003). The biochemical and pathological findings of this investigation further reinforced the idea that dihydromethysticin can cause cytotoxicity in certain concentrations when used alone, and that the negative effects are amplified when combined with EtOH. Our findings contradict those of several prior research, which found no negative or toxic effects of kavalactones in animal models, even when given for lengthy periods of time and with ALT levels in the normal range (Sorrentino et al., 2006; Narayanapillai et al., 2014). The existence of several morphological and histological abnormalities corroborated the clinical findings, as well as hematological and biochemical changes. We discovered that dihydromethysticin caused degeneration and hepatocellular necrosis in this experiment. While dose-related liver weight increases revealed hepatocellular and Kupffer cell hypertrophy, these alterations were consistent with earlier research findings (Amacher et al., 1998; Clayton et al., 2007; El-Kholy and El-Salam, 2011). It has been claimed that hepatocellular enlargement in kava-intoxicated rats is connected to intrahepatic protein and fat accumulation due to the stimulation of drug-metabolizing enzymes by kava treatment. Furthermore, after consuming kavalactones, protein catabolism is slowed, particularly those intracellular proteins destroyed in lysosomes by autophagy, especially when

combined with EtOH (Clayton et al., 2007; Singh et al., 2009). The stimulation of drug-metabolizing enzymes by kava treatment has been linked to increased liver weight, hepatomegaly, and other adverse consequences in rats' livers in a number of prior investigations (Guo et al., 2009; Behl et al., 2011) .

Conclusion

Dihydromethysticin consumption of 800 mg/kg resulted in an increased incidence of non-neoplastic lesions in the rat liver, according to the findings of this study. Furthermore, when combined with dihydromethysticin, ethanol significantly exacerbated toxicity and hepatopathy lesions. Long-term administration of commercially available dihydromethysticin aqueous solutions at a concentration of 800 mg/kg was linked to apparent liver dysfunction and numerous morphological changes, as observed by light and electron microscopy .

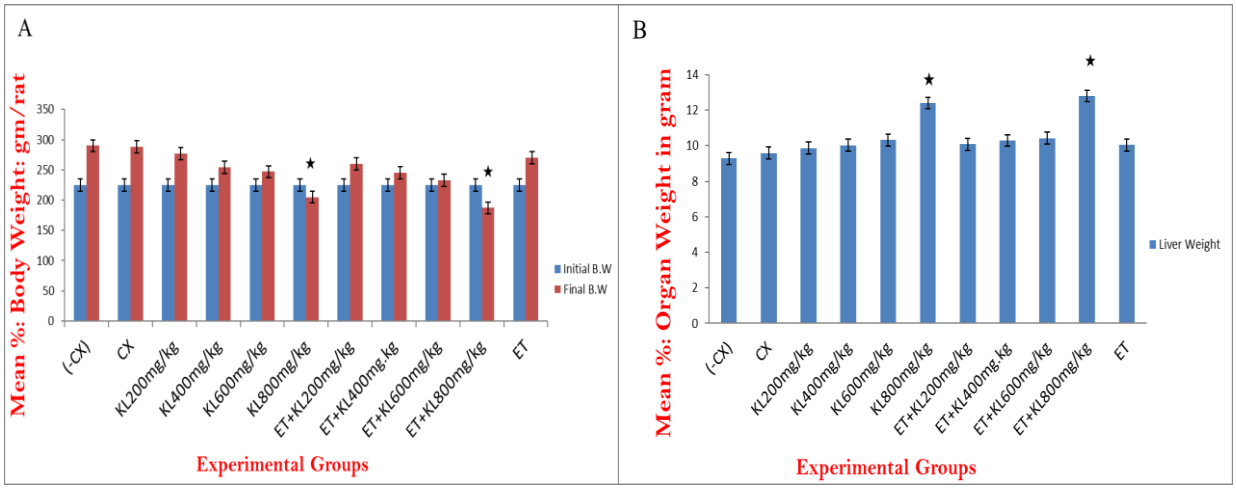
References

1. Allen, J., & Bradley, R. D. (2011). Effects of oral glutathione supplementation on systemic oxidative stress biomarkers in human volunteers. *The Journal of Alternative and Complementary Medicine*, *17*(9), 827–833.
2. Amacher, D.E., Schomaker, S. J., & Burkhardt, J. E. (1998). The relationship among microsomal enzyme induction, liver weight and histological change in rat toxicology studies. *Food and Chemical Toxicology*, *36*(9–10), 831–839.
3. Amorim, M. F. D., Diniz, M. F. F. M., Araújo, M. S. T., Pita, J. C. L. R., Dantas, J. G., Ramalho, J. A., et al. (2007). The controvertible role of kava (Piper methysticum G. Foster) an anxiolytic herb, on toxic hepatitis. *Revista Brasileira de Farmacognosia*, *17*(3), 448–454.
4. Anke, J., & Ramzan, I. (2004a). Kava Hepatotoxicity: Are we any closer to the truth? *Planta Medica*, *70*(3), 193–196.
5. Anke, J., & Ramzan, I. (2004b). Pharmacokinetic and pharmacodynamic drug interactions with Kava (Piper methysticum Forst. f.). *Journal of Ethnopharmacology*, *93*(2–3), 153–160.
6. Behl, M., Nyska, A., Chhabra, R. S., Travlos, G. S., Fomby, L. M., Sparrow, B. R., et al. (2011). Liver toxicity and carcinogenicity in F344/N rats and B6C3F1 mice exposed to Kava Kava. *Food and Chemical Toxicology*, *49*(11), 2820–2829.
7. Bilia, A. R., Gallori, S., & Vincieri, F. F. (2002). Kava-kava and anxiety: growing knowledge about the efficacy and safety. *Life Sciences*, *70*(22), 2581–2597.
8. Bilia, A. R., Scalise, L., Bergonzi, M. C., & Vincieri, F. F. (2004). Analysis of kavalactones from Piper methysticum (kava-kava). *Journal of Chromatography B*, *812*(1–2), 203–214.
9. Cai, Y., Wang, H., Xiao, Y., Ren, J., et al. (2005). Altered expression of cytochrome P450 and possible correlation with preneo plastic changes in early stage of rat hepatocarcinogenesis. *Acta Pharmacologica Sinica*, *26*(6), 737–744.
10. Cairney, S., Clough, A. R., Maruff, P., Collie, A., Currie, B. J., & Currie, J. (2003). Saccade and cognitive function in chronic kava users. *Neuropsychopharmacology*, *28*(2), 389.
11. Clayton, N. P., Yoshizawa, K., Kissling, G. E., Burka, L. T., Chan, P.C., & Nyska, A. (2007). Immunohistochemical analysis of expressions of hepatic cytochrome P450 in F344 rats

following oral treatment with kava extract. *Experimental and Toxicologic Pathology*, *58*(4), 223–236.

12. Clough, A. R. (2003). Health effects of heavy kava use on indigenous Australians.
13. Clough, A. R., Bailie, R. S., & Currie, B. (2003). Liver function test abnormalities in users of aqueous kava extracts. *Journal of Toxicology: Clinical Toxicology*, *41*(6), 821–829.
14. Dennehy, C. E., Tsourounis, C., & Miller, A. E. (2005). Evaluation of herbal dietary supplements marketed on the internet for recreational use. *Annals of Pharmacotherapy*, *39*(10), 1634–1639.
15. Dinkova-Kostova, A. T., Massiah, M. A., Bozak, R. E., Hicks, R. J., & Talalay, P. (2001). Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. *Proceedings of the National Academy of Sciences*, *98*(6), 3404–3409.
16. Ebadi, M. S. (2002). Pharmacodynamic Basis of Herbal Medicine, 145. CRC Press, Florida.
17. El-Kholy, S. M. S., & El-Salam, S. A. (2011). Kava extract drug: what is the actual risk of hepatotoxicity in adult male albino rats? *Egyptian Journal of Histology*, *34*(4), 697–704.
18. Fu, P. P., Xia, Q., Guo, L., Yu, H., & Chan, P.C. (2008). Toxicity of kava kava. *Journal of Environmental Science and Health Part C*, *26*(1), 89–112.
19. Grunze, H., Langosch, J., Schirrmacher, K., Bingmann, D., von Wegerer, J., & Walden, J. (2001). Kava pyrones exert effects on neuronal transmission and transmembraneous cation currents similar to established mood stabilizers-a review. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *25*(8), 1555–1570.
20. Guo, L., Li, Q., Xia, Q., Dial, S., Chan, P.-C., & Fu, P. (2009). Analysis of gene expression changes of drug metabolizing enzymes in the livers of F344 rats following oral treatment with kava extract. *Food and Chemical Toxicology*, *47*(2), 433–442.
21. Gyengesi, E., Paxinos, G., & B Andrews, Z. (2012). Oxidative stress in the hypothalamus: the importance of calcium signaling and mitochondrial ROS in body weight regulation. *Current Neuropharmacology*, *10*(4), 344–353.
22. Humberston, C. L., Akhtar, J., & Krenzelok, E. P. (2003). Acute hepatitis induced by kava kava. *Journal of Toxicology: Clinical Toxicology*, *41*(2), 109–113.
23. Jamieson, D. D., & Duffield, P. H. (1990). The antinociceptive actions of kava components in mice. *Clinical and Experimental Pharmacology and Physiology*, *17*(7), 495–507.
24. Jussofie, A., Schmiz, A., & Hiemke, C. (1994). Kavapyrone enriched extract fromPiper methysticum as modulator of the GABA binding site in different regions of rat brain. *Psychopharmacology*, *116*(4), 469–474.
25. Lim, S. T. S. (2006). *Kava-associated Hepatotoxicity*. University of Hawaii at Manoa.
26. Limdi, J. K., & Hyde, G. M. (2003). Evaluation of abnormal liver function tests. *Postgraduate Medical Journal*, *79*(932), 307–312.

27. Lüde, S., Török, M., Dieterle, S., Jäggi, R., Büter, K. B., & Krähenbühl, S. (2008). Hepatocellular toxicity of kava leaf and root extracts. *Phytomedicine*, *15*(1–2), 120–131. https://doi.org/10.1016/j.phymed.2007.11.003
28. Mathews, J. D., Riley, M. D., Fejo, L., Munoz, E., Milns, N. R., Gardner, I. D., et al. (1988). Effects of the heavy usage of kava on physical health: summary of a pilot survey in an aboriginal community. *The Medical Journal of Australia*, *148*(11), 548–555.
29. Moulds, R. F. W., & Malani, J. (2003). Kava: herbal panacea or liver poison? *The Medical Journal of Australia*, *178*(9), 451–453.
30. Narayanapillai, S. C., Leitzman, P., O'Sullivan, M. G., & Xing, C. (2014). Flavokawains A and B in kava, not dihydromethysticin, potentiate acetaminophen-induced hepatotoxicity in C57BL/6 mice. *Chemical Research in Toxicology*, *27*(10), 1871–1876.
31. Rowe, A., Zhang, L. Y., & Ramzan, I. (2011). Toxicokinetics of kava. *Advances in Pharmacological Sciences*, *2011*.
32. Showman, A. F., Baker, J. D., Linares, C., Naeole, C. K., Borris, R., Johnston, E., et al. (2015). Contemporary Pacific and Western perspectives onawa (Piper methysticum) toxicology. *Fitoterapia*, *100*, 56–67.
33. Singh, R., Kaushik, S., Wang, Y., Xiang, Y., Novak, I., Komatsu, M., et al. (2009). Autophagy regulates lipid metabolism. *Nature*, *458*(7242), 1131.
34. Sorrentino, L., Capasso, A., & Schmidt, M. (2006). Safety of ethanolic kava extract: Results of a study of chronic toxicity in rats. *Phytomedicine*, *13*(8), 542–549.
35. Teschke, R. (2010). Kava hepatotoxicity–a clinical review. *Ann Hepatol*, *9*(3), 251–265.
36. van der Watt, G., Laugharne, J., & Janca, A. (2008). Complementary and alternative medicine in the treatment of anxiety and depression. *Current Opinion in Psychiatry*, *21*(1), 37–42.
37. Whitton, P. A., Lau, A., Salisbury, A., Whitehouse, J., & Evans, C. S. (2003). Kava lactones and the kava-kava controversy. *Phytochemistry*, *64*(3), 673–679.



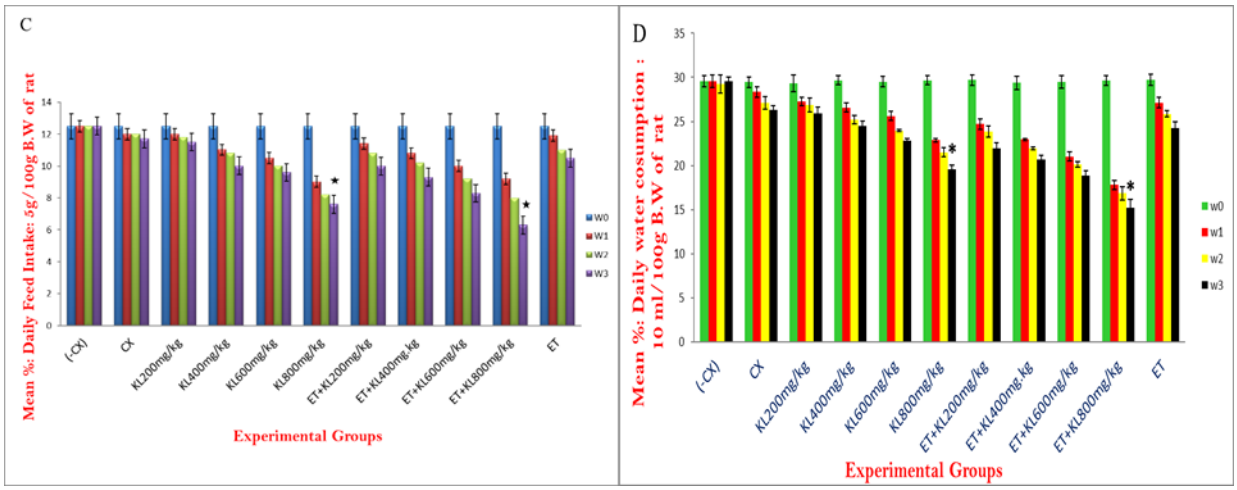
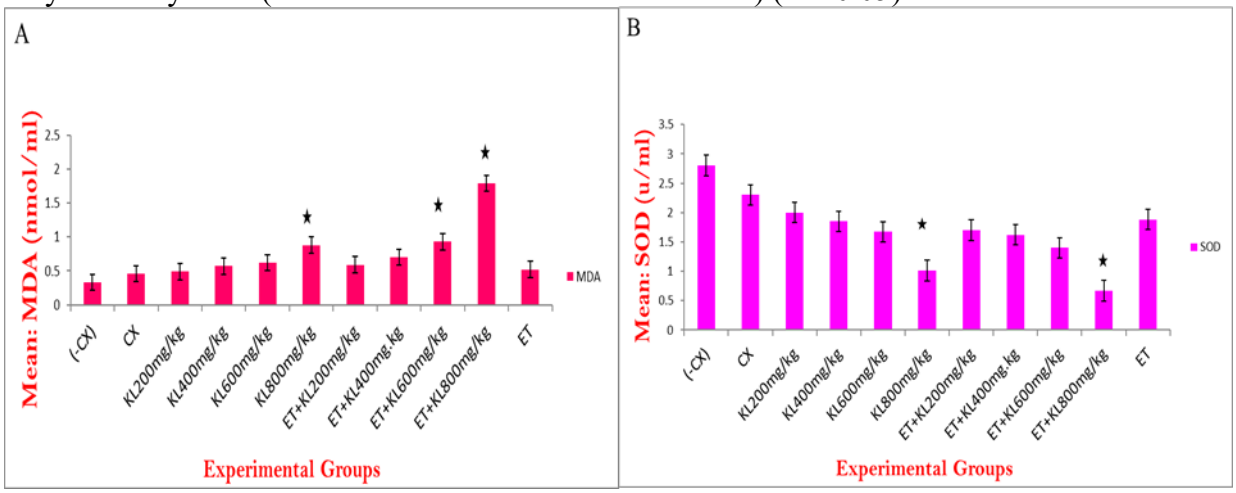


Figure 1. Bar graphs of effects dihydromethysticin on **A)** Body Weight **B)** Liver Weight **C)** Feed Intake **D)** Water consumption, after 3 weeks of treatment with different doses of dihydromethysticin (alone and in combination with ETOH) (*P<0.05)



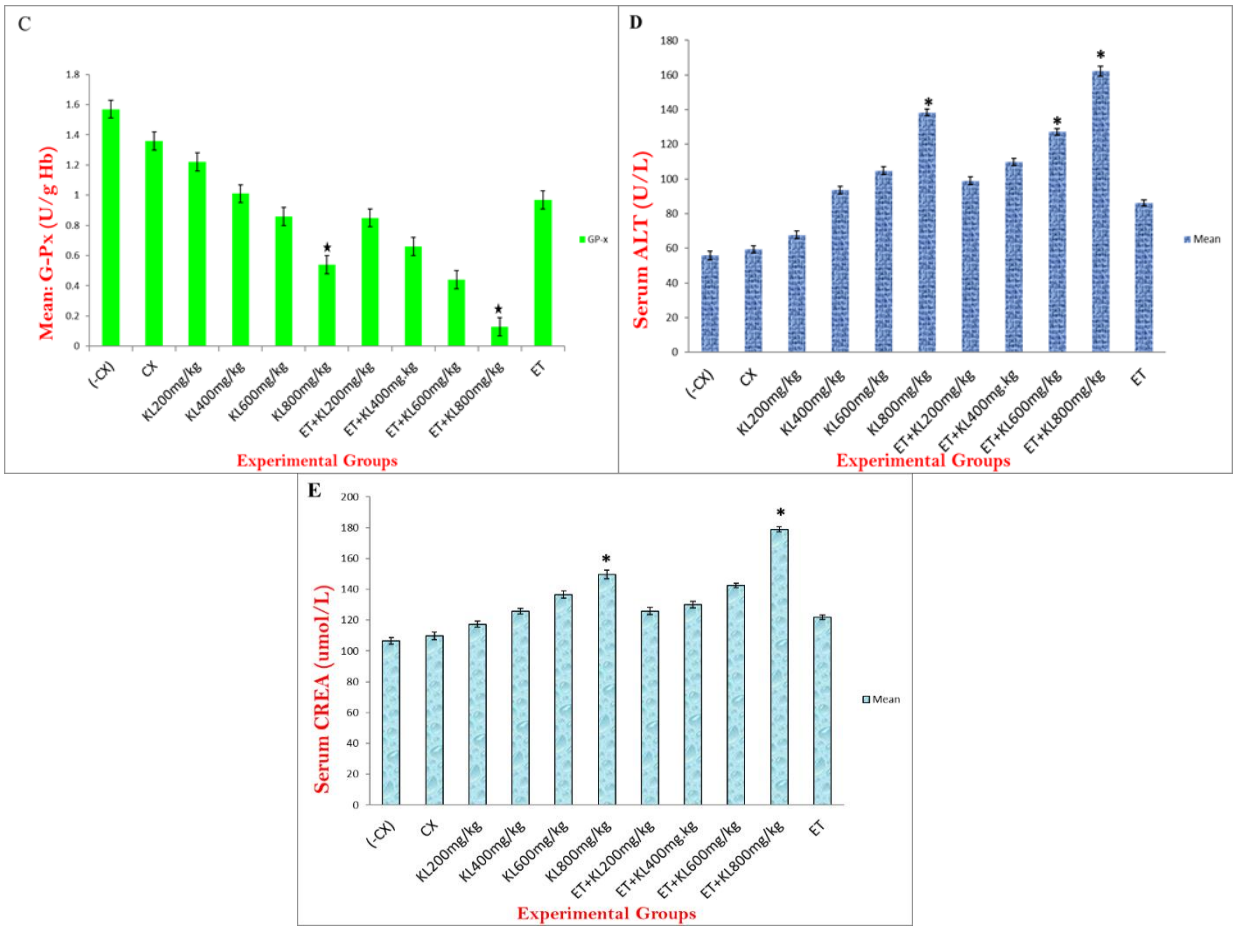


Figure 2. A) Dihydromethysticin effects on Malondialdehyde formation, **B)** SOD enzyme level, **C)** GSHP-x enzyme level, **D)** ALT and **E)** CREA after 3 weeks of treatment with different doses of dihydromethysticin (alone and in combination with EtOH)

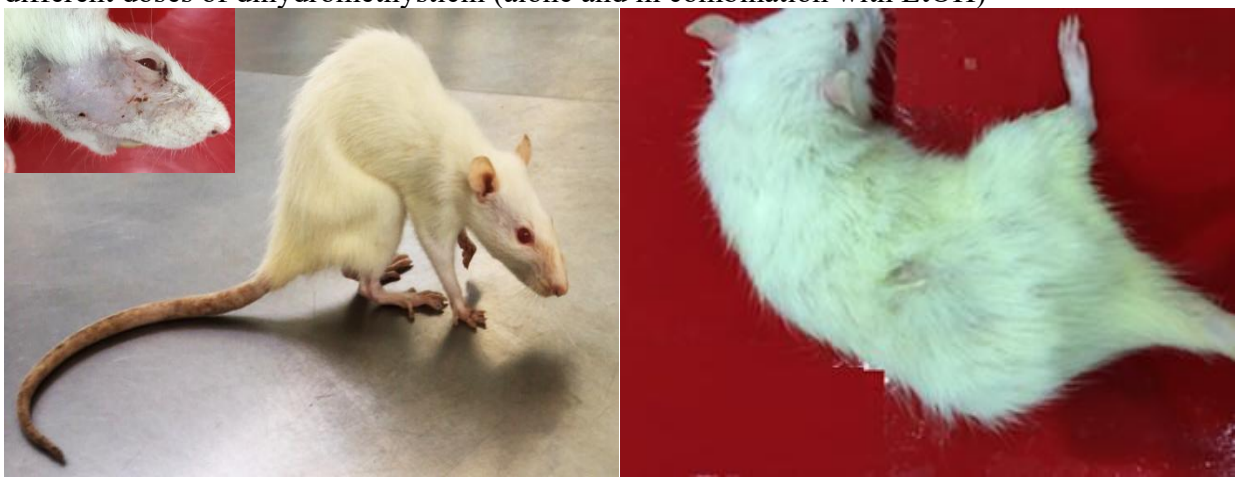


Figure 3. Photographs of the treated rats with EtOH+dihydromethysticin (800mg/kg), suffered from emaciation, ataxia, lethargy, ruffled and falling out hair (Kani Kani lesion), erythema and severe weight loss with severe arching back

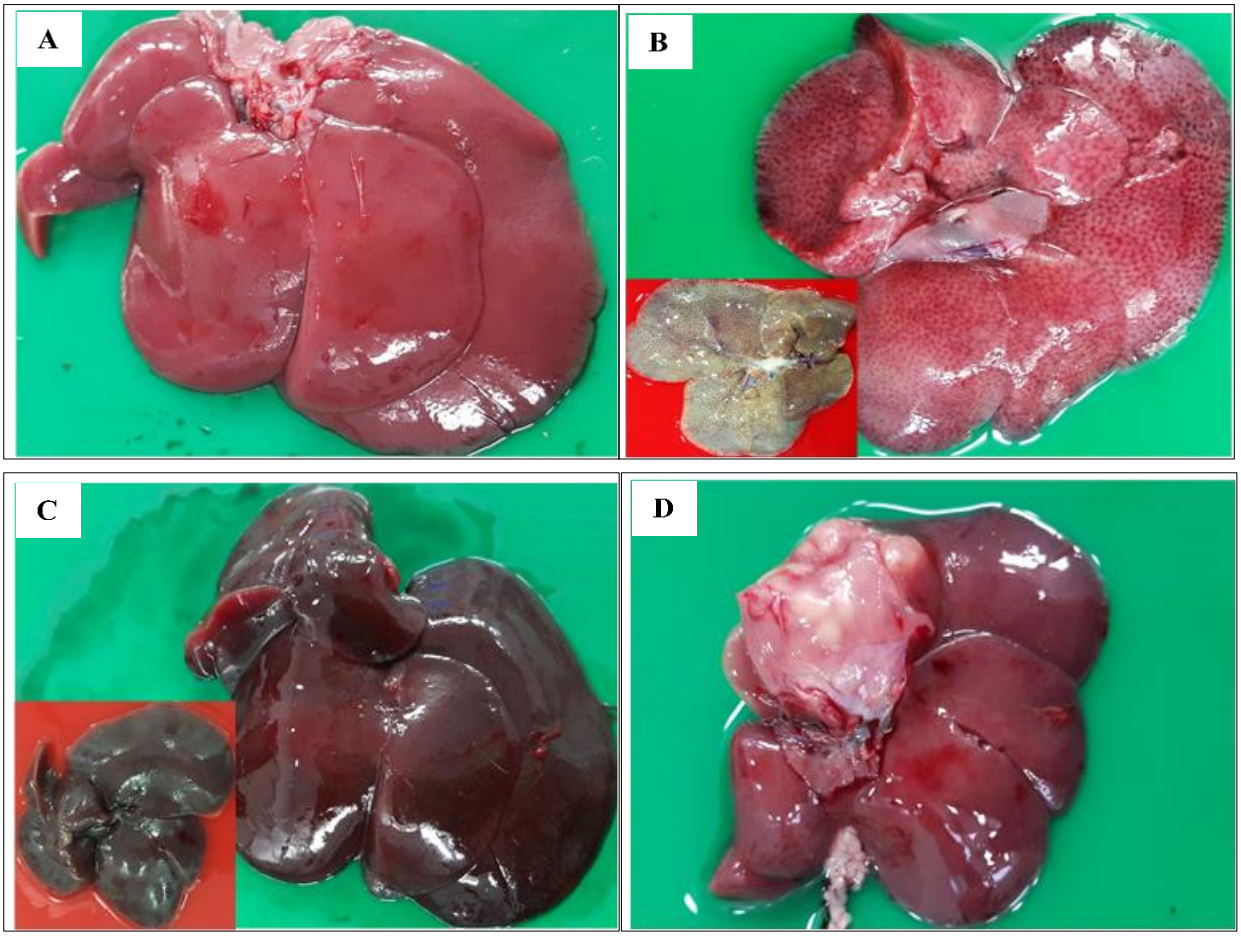
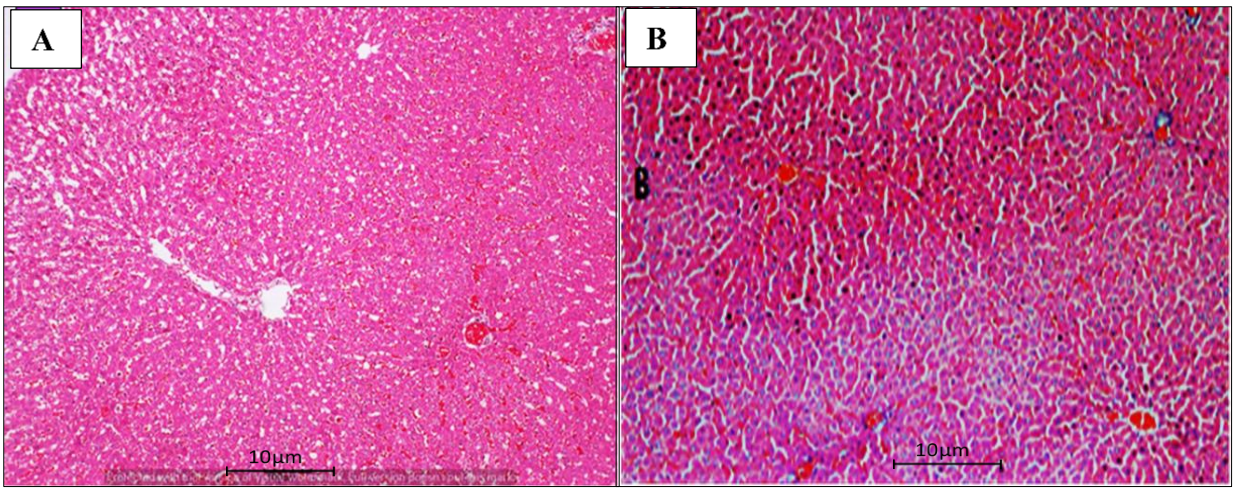


Figure 4. A healthy control liver, B) Dihydromethysticin (800mg/kg)-affected liver, abnormal mottled liver, moderate to severe fatty alterations with necrotic black area delimited from unaffected region and rounded borders. C&D) ETOH+dihydromethysticin (800mg/kg), significant darkening, necrotic black region with rounded borders, and rise in size and weight with aberrant solid-bulk



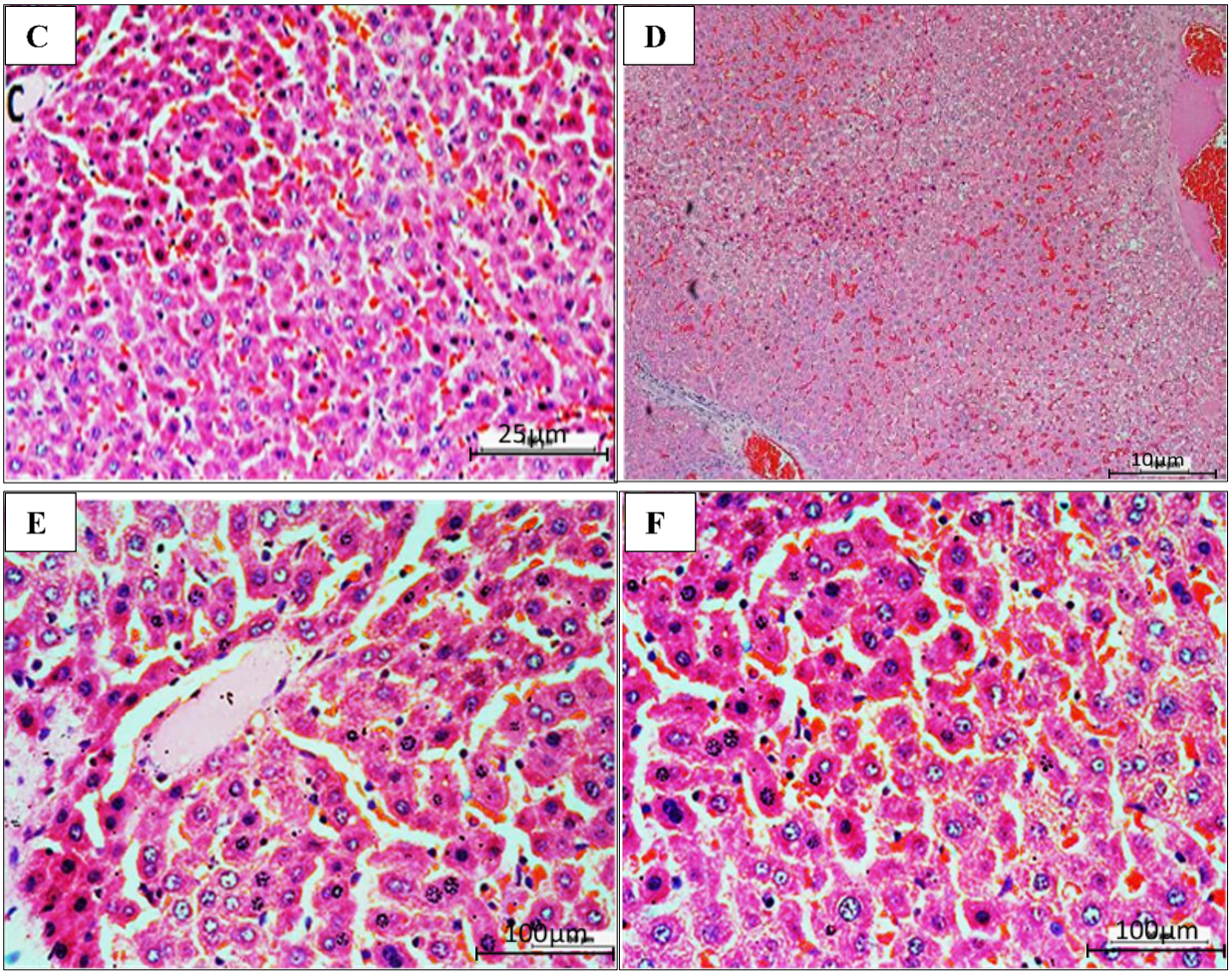


Figure 5. Photomicrographs of the liver from the A) control group, demonstrating a normal hepatocellular architecture with a central portal vein, bile duct, and radiating hepatocytes. Dihydromethysticin (800mg/kg) group, centrilobular necrosis, minor hepatocellular enlargement, and congestion of hepatic vein with minimal infiltration of inflammatory cells disturbing radiating and hepatic cord architecture (H&E, X10), B and C D) EtOH+Dihydromethysticin (800mg/kg), localized hepatocellular necrosis with hypertrophic cells, fatty degeneration, angiectasis, and congested blood vessels (H&E, X10 and 20). (H&E, X10). ETOH+Dihydromethysticin, severe centrilobular necrosis with severe centrilobular enlargement and hypertrophy of Kupffer cells disturbing hepatocellular architecture (H&E, X40)

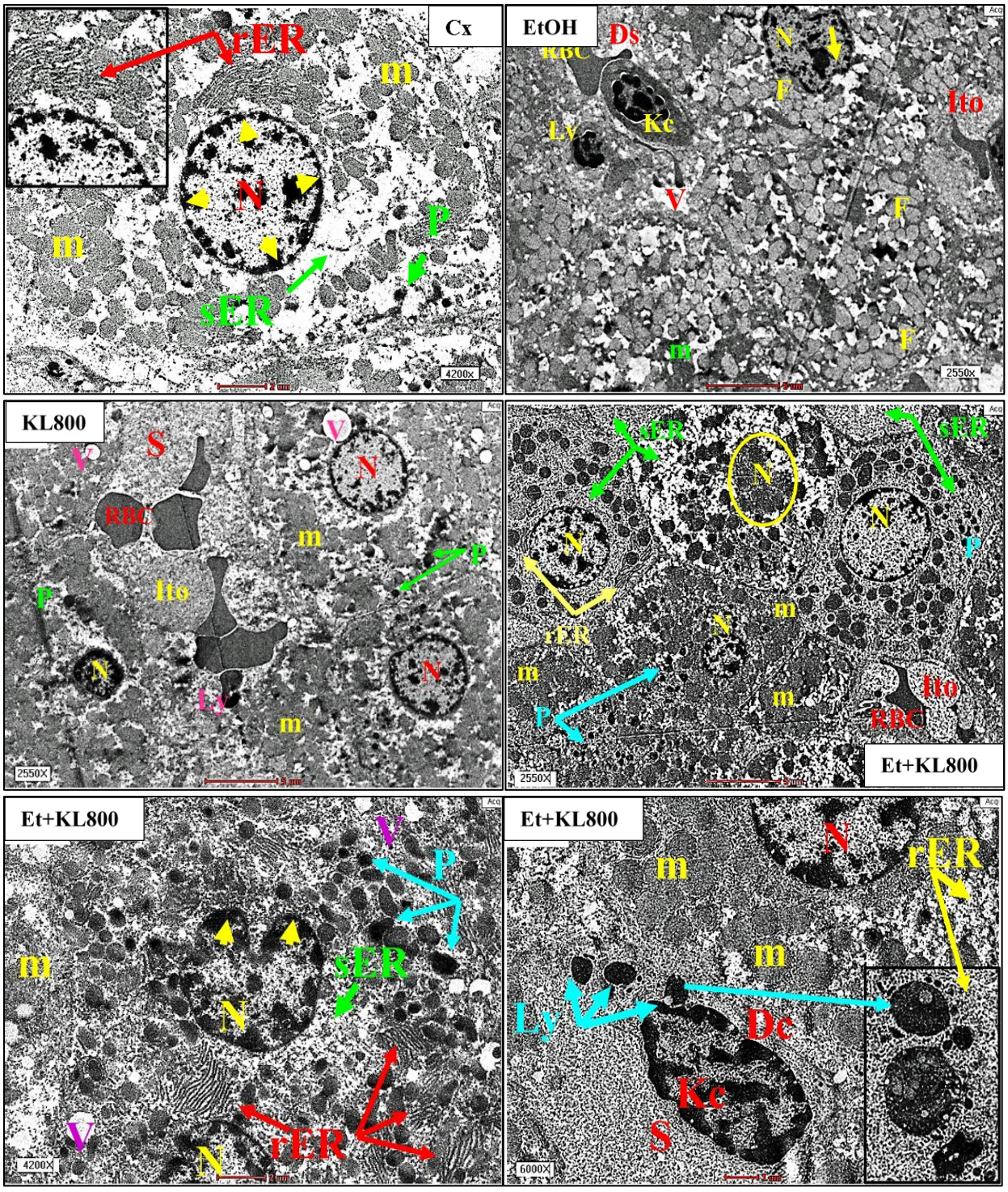


Figure 6. The cytoplasm contains normal mitochondria (m), an array of rough endoplasmic reticulum (rER) and smooth endoplasmic reticulum (sER), and normal peroxisome in a transmission electron micrograph from a control hepatocyte with a euchromatic nucleus (N) and developed nuclear membrane (yellow head arrow). Hepatocytes with intracytoplasmic vacuolization (V), organelle clumping ("steatosis liver"), large lipid droplets ("steatosis

liver"), degenerative hepatic nuclei (N) surrounded by a shattered irregular nuclear membrane, EtOH rat
while hepatocytes from Dihydromethysticin-treated rats (alone and in combination with EtOH) showed intracytoplasmic vacuolization (V), swollen mitochondria (m), dilated blood sinusoids (S) with severe hypertrophic Kupffer cells (KC), severe dilated smooth endoplasmic reticulum (sER) and highly proliferation of peroxisome (P), debris of necrotic nuclei, a lot of lysosomes

Table 1. Criteria for Grading the Severity of Hepatopathy changes

Severity of Hepatopathy	Grade	Description
Minimal	1	0% of organ affected (normal histological appearance of hepatocytes).
Mild	2	1–39% of organ affected (congestion with slight sinusoidal space and central vein dilatation).
Moderate	3	40–79% of organ affected (cystic and fatty degeneration, hepatocellular hypertrophy and necrosis), and dilatation with congestion of sinusoids and central vein.
Marked (severe)	4	80%–100% of organ affected. Greater severity of changes noted in grade 3 with Kupffer cells hyperplasia and hypertrophy and possible pathological evidence of liver damage/fibrosis.

Table 2. The correlations between all selected histopathological lesions at variable biochemical parameters, health status and somatic index at sub-acute intoxicated trials

Correlations	B. W	L.W	F. I	W.C	SOD	G-Px	MDA	ALT	CREA	SCORING
B. W	1									
L.V	-.918**	1								
F. I	.947**	-.930**	1							
W.C	.982**	-.964**	.973**	1						
SOD	.849**	-.766*	.926**	.859**	1					
G-Px	.781*	-0.601	.783*	.760*	.922**	1				
MDA	-.878**	.806**	-.958**	-.899**	-.985**	-.900**	1			
ALT	-.847**	.817**	-.953**	-.880**	-.959**	-.822**	.963**	1		
CREA	-.798**	.737*	-.917**	-.834**	-.951**	-.854**	.970**	.975**	1	
SCORING	-.953**	.897**	-.966**	-.947**	-.874**	-.733*	.897**	.924**	.860**	1

B.W: body weight, L.W: Liver weight, F.I: Feed intake, W.C: Water consumption. ** Correlation is significant at the 0.01 level. * Correlation is significant at the 0.05 level .

Table 3. Changes (%) in body weight, absolute and relative liver weights, feed intake and water consumption (mean)±SE of Dihydromethysticin 800 (mg/kg b.w/day) alone and in combination with EtOH groups compared to the (NCx) negative control group

Parameters (mean)±SE	NO of rats at terminal of study	Body Weight		Liver Weight (g)		Feed Intake (g)		Water consumption (ml)	
Groups		**Initial**	**Terminal**	**Absolute**	**Relative**	**Initial**	**Terminal**	**Initial**	**Terminal**
Control	3	225.0±1.47	290±1.62	9.28±0.03	3.5±0.01	12.5±0.13	12.5±0.13	29.57±0.66	29.53±0.50
KL (800mg/kg)	3	226.0±0.66 **(NS)**	205.0±0.96*	12.40±0.02*	6.05±0.02*	12.6±0.13 **(NS)**	7.6±0.08*	29.66±0.53 **(NS)**	19.59±0.49*
Changes (%)	-	0.29↓	29.3↓	33.6↑	72.8↑	0.8↑	39.2↓	0.30↑	33.9↓
KL (800mg/kg) +ETOH	3	225.7±2.48 **(NS)**	187.0±1.32**	12.80±0.09**	6.86±0.07**	12.6±0.10 **(NS)**	6.3±0.17**	29.56±0.55 **(NS)**	15.23±0.90**
Changes (%)	-	0.45↓	35.5↓	37.93↑	96.0↑	1.6↑	49.6↓	0.03↓	48.6↓

NS: non-significant deference compared with control. ** Significant difference at the 0.01 level. * Significant difference at the 0.05 level. (%) compared with the control

Table 4. Changes (%) in Lipid Peroxidation Enzyme (MDA), Antioxidant enzymes (SOD and G-Px), Liver function enzyme (ALT) and Kidney function enzyme (CREA) of (only) Dihydromethysticin 800 (mg/kg b.w/day) alone and in combination with ETOH groups compared with the (NCx) negative control group

Parameters (mean)±SE	Lipid peroxidation Enzyme	Antioxidant enzymes		Liver function enzyme	Kidney function enzyme
Groups	**MDA**	**SOD**	**G-Px**	**ALT**	**CREA**
Control	0.33±0.03	2.8±0.17	1.57±0.06	55.8±2.48	106.5±2.02
KL (800mg/kg)	0.88±0.08*	1.01±0.03*	0.54±0.04*	138.4±1.82*	149.6±2.74*
Changes (%)	166.6↑	63.9↓	65.6↓	148.02↑	40.46↑
KL (800mg/kg) +ETOH	1.79±0.06**	0.67±0.06**	0.13±0.05**	162.2±2.88**	178.9±1.59**
Changes (%)	442.4↑	76.0↓	91.7↓	191.2↑	67.98↑

NS: non-significant deference compared with control. ** Significant difference at the 0.01 level. * Significant difference at the 0.05 level. (%) compared with the control

Table 5. Sub-acute lesion scoring for liver tissue obtained from treated groups

Liver	Lesions	NCx & PCx	EtOH	EtOH+KL (200mg/kg)	EtOH+KL (400mg/kg)	EtOH+KL (600mg/kg)	KL (800mg/kg)	EtOH+KL (800mg/kg)
Perivenous zone	Congestion	0.00	0.16±.0.01	0.13±0.01	0.09±0.00	0.16±0.02	0.23±0.01	0.33±0.01*
	SSD	0.00	0.36±0.01*	0.23±0.01	0.33±0.01*	0.35±0.01*	0.47±0.01*	0.65±0.01**
	HHP	0.00	0.23±0.01	0.20±0.01	0.16±0.01	0.25±0.01	0.45±0.01*	0.57±0.01**
	HN	0.00	0.23±0.01	0.21±0.01	0.11±0.01	0.33±0.02*	0.45±0.01*	0.68±0.01**
	C& FD	0.00	0.37±0.01*	0.00±0.00	0.22±0.01	0.15±0.01	0.05±0.01	0.33±0.02*
	KCPA	0.00	0.26±0.02	0.19±0.01	0.12±0.01	0.25±0.02	0.3±0.02*	0.63±0.02**
	Total Score of Zone	**0.00**	**1.61**	**0.96**	**1.03**	**1.50**	**1.96**	**3.20**
Midzonal	Congestion	0.00	0.11±.0.01	0.10±0.00	0.07±0.01	0.12±0.02	0.17±0.01	0.24±0.01
	SSD	0.00	0.41±.0.01*	0.21±0.01	0.23±0.01	0.28±0.01	0.42±0.01*	0.58±0.01**
	HHP	0.00	0.16±.0.01	0.09±0.00	0.11±0.01	0.16±0.01	0.21±0.01	0.36±0.01*
	HN	0.00	0.11±.0.00	0.12±0.01	0.07±0.00	0.14±0.01	0.23±0.01	0.39±0.0
	C& FD	0.00	0.21±.0.01	0.00±0.00	0.10±0.00	0.10±0.01	0.01±0.00	0.21±0.02
	KCPA	0.00	0.17±.0.01	0.10±0.00	0.09±0.00	0.15±0.01	0.18±0.01	0.36±0.02*
	Total Score of Zone	**0.00**	**1.16**	**0.62**	**0.68**	**0.95**	**1.22**	**2.14**
Periportal zone	Congestion	0.00	0.11±.0.01	0.10±0.00	0.09±0.00	0.12±0.01	0.11±.0.01	0.20±0.01
	SSD	0.00	0.31±0.01*	0.19±0.01	0.17±0.01	0.20±0.01	0.34±0.01*	0.41±0.01*
	HHP	0.00	0.14±0.01	0.10±0.01	0.09±0.00	0.11±0.01	0.26±0.01	0.33±0.01*
	HN	0.00	0.11±.0.01	0.09±0.00	0.06±0.01	0.09±0.00	0.18±0.00	0.22±0.00
	C& FD	0.00	0.21±0.01	0.01±0.00	0.10±0.01	0.12±0.01	0.02±0.00	0.33±0.01*
	KCPA	0.00	0.16±.0.01	0.09±0.00	0.10±0.00	0.13±0.01	0.19±0.00	0.30±0.01*
	Total Score of Zone	**0.00**	**1.04**	**0.57**	**0.61**	**0.78**	**1.10**	**1.80**
Liver Total Score		**0.00**	**3.81**	**2.15**	**2.32**	**3.23**	**4.29**	**7.14**

SSD: Sinusoidal space dilation, NH: Hepatocellular necrosis, HHP: Hepatocellular hypertrophy, C&FD: Cystic and fatty degeneration, KCPA: Kupffer cells phagocytic activity. Data are expressed as the (mean±SE). ** Significant difference at the 0.01 level. * Significant difference at the 0.05 level