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Research Article

Sub Chronic Toxicity Study of Coumacines

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ABSTRACT

Coumacine is a brand-new heterocyclic molecular nucleus that was discovered in 2018. In addition to the unique heterocycle known as coumacine, the designer has developed two variants known as coumacine I and II. Coumacine derivatives had been evaluated for their antibacterial effects *in vitro* against a variety of aerobic and anaerobic bacteria using conventional bacterial strains, using ciprofloxacin and metronidazole as positive controls. The purpose of this research is to look into the relationship between the anticoagulant activity and hepatotoxicity of coumarin and coumacine because the former is a synthetic precursor of the latter and many natural and synthetic coumarins involving warfarin have anticoagulant activity. Thirty male mice were used in this study and exposed to a subchronic dose of 250 or 500 mg/kg of coumacine I or coumacine II. The results of histochemistry showed dramatic changes in hepatocellular morphology that were dose-dependent for both coumacine I and II. Traditionally, higher doses of Coumacine I and II resulted in a significant increase in liver enzymes. Coumacine I or II did no effect on bleeding time. In conclusion, coumacines at subchronic high doses might have hepatotoxic effects through a mechanism that does not affect the coagulation process.

Key words: Coumacine, Hepatotoxicity, Bleeding, Clotting.

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INTRODUCTION

Antibacterial agent development is one of the therapeutic development challenges. Antibacterial resistance against the present antibacterial is widespread which makes very limited choices to eradicate bacteria causing serious infections. In 2018, Mustafa YF[1] has been reported the discovery of a heterocyclic compound derived from coumarin and named it coumacine which has a potential antibacterial activity when evaluated *via* agar dilution method versus two commonly used antibacterial ciprofloxacin which has very good activity against gram-negative bacteria and metronidazole which has excellent antibacterial activity against most anaerobic Bacteroides as positive controls. The latter study showed that coumacine has a dramatic broad-spectrum antimicrobial activity against the tested microorganisms.

Coumarins induce an elevation in liver enzymes in patients treated for malignancy had been reported.[2] Another clinical report had been highlight the effect of coumarin when used to treat lymphoedema, which can cause an increase in hepatic enzymes by 30 -100 times the normal level in two ladies using coumarin.[3-5] One of the earliest studies investigating *in vivo*/ *in vitro* hepatotoxicity of the coumarin mechanism was by Lake and his co-workers (1989) who reported the increase in liver weight, and depletion of glutathione starts after two hours of exposure to 125mg/kg of coumarin. Also, they reported that coumarin hepatoxicity is both dose and time-dependent as after 24 hr it causes hepatic centrilobular necrosis.[6] Coumarin can cause various hepatic damage depending on the frequency of exposure. Hepatocellular necrosis developed after a single high dose while frequent doses lead to hepatocyte degeneration.[7] The latter may stemmed from a different mechanism of toxicity as for single dose lead to an increase in mitochondrial number and size while a decrease in their function, on the other hand, repeated exposure does not affect mitochondrial function nevertheless CYP2E1 protein is present outside the mitochondria.[8] Another study showed that coumarin hepatotoxicity is associated with cytolytic hepatitis or cholestatic jaundice in very rare cases in oral anticoagulant therapy.[9] Furthermore, warfarin is the anticoagulant coumarin had been reported to associate with elevated liver enzymes and even liver damage in rare cases.[10-12] Hepatotoxicity induced by coumarin derivatives other than warfarin phenprocoumon had been observed using a retrospective analysis of patients treated for the liver disorder.[13] Recently, another coumarin derivative bergapten (a natural furocoumarin with very intriguing medicinal potential as neuroprotective, anticancer and in addition to its anti-inflammatory and antimicrobial effect, Figure 1) showed a complex effect on hepatocyte morphology and hepatic enzymes levels.[14,15] Although the mechanism of bergapten-induced liver injury is not fully understood, Zhao and his colleagues (2017) study found that bergapten may interfere with P-glycoprotein-mediated phospholipids efflux and maintain bile acid homeostasis which leads to may lead to cholestatic liver disorder.[16,17]

As mentioned before, coumacine (CM) I and II chemical structures are related to coumarin (as shown in Figure 1) and cross-reactions between coumarins have been reported. Accordingly, we in this study investigate the hepatotoxicity potential of coumacine when given in high doses and sub-chronic exposure. In addition, we report the effect of coumacine on bleeding time *in vivo*.

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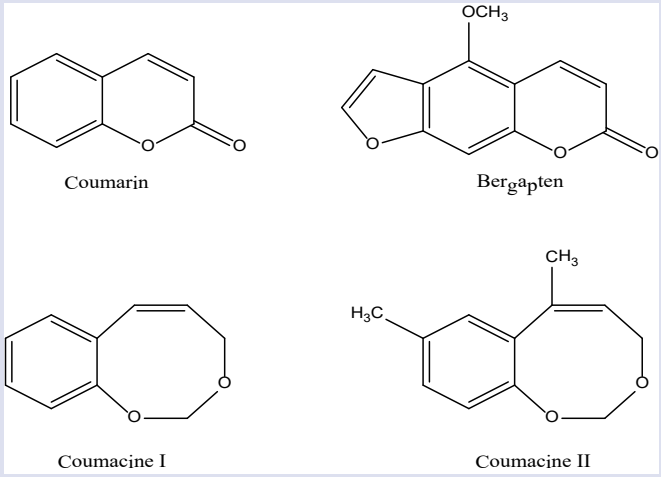

Figure 1: Chemical backbones of coumarin chemical nucleus, bergapten, coumacine I, and coumacine II.

MATERIALS AND METHODS

Chemicals

Coumacine I and II have been synthesized according to the previous report by Mustafa YF.[1] Coumacine I and II have been prepared freshly in a 10% solution of hydroxypropyl beta cyclodextrin (HPBCD) to enhance their solubility.

Animal treatment

The subjects of the study were Thirty Male Albino mice. Mice were housed in cages under a controlled environment (temperature was 23-25 ºC and humidity of 50-55%. The animals have been socially housed 2-3 mice per cage and they had access to water and food ad libitum. The tested animals were treated with either coumacine I (250, or 500 mg/kg) or coumacine II (250, or 500 mg/kg) dissolved in 10% of HPBCD or 10% HPBCD (10ml/kg) by IP every day for five days.

Tail bleeding assay

This procedure was done according to Liu's report.[18] All mice before sacrifice had subjected to tail tip amputation then their tail was immersed in a normal saline bath at 37 ºC. The bleeding volume had been calculated based on the change in body weight.

Alanine aminotransferase (GOT) and aspartate aminotransferase (GPT) measurements

Blood was collected immediately after mouse beheading using a sharp big scissor for the determination of serum alanine aminotransferase (GOT) and aspartate aminotransferase (GPT) activity. Trunk Blood was allowed to coagulate at room temperature and then centrifuged for 10 min at 3000g for serum separation. Serum GPT and GOT activity was determined by using the Colorimetric Assay of Reitman and Frankel method.[19]

Hematoxylin and eosin (H and E) staining

Immediately after mouse decapitation, liver tissue was harvested and postfixed for 24 hr in 4 % neutral formalin and then perform paraffin-embedded. Then, paraffin blocks were sectioned at 5 µm and stained with H and E pathologists who were blinded to the treatment groups and their doses, and examined the sections using a light microscope X400.[20]

Statistics

Analysis of GPT and GOT activity levels in the five different treatment groups was carried out using a one-way ANOVA with Bonferroni tests as a post hoc test to determine variations among groups. Analysis was performed using SPSS software. All data are presented as Mean ±standard error deviation. Using a power of 80% or greater to determine sample sizes and alpha error level ((P) in all experiments was ≤ 0.05 to be statistically significant.

RESULTS

Dose dependence of coumacine alterations in hepatocellular morphology

To examine if the coumacines causing changes in hepatocyte structure are affected differentially by the dose of coumarins, mice were administered either a placebo, 250, or 500 mg/kg IP once daily for 5 days. The histopathological results revealed that Coumacine induces alterations in hepatocellular morphology after 5 days of continuous exposure, interestingly, the changes were dose-dependent, as shown in Figure 2. The reduction in hepatocytes cytoplasmic stain was dose-dependent as increases in novel coumacine exposure amount induce hepatocellular alterations.

Bleeding time

To examine if sub-chronic dose coumacine I or II induce alteration in bleeding time, a tail bleeding assay was performed and the results showed

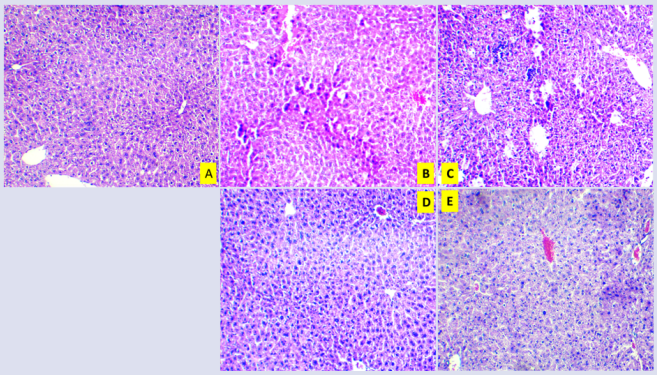

Figure 2: Effect of 5 days after coumacine I or coumacine II administration on alterations in hepatocellular morphology. mice treated with either compound for 5 days IP. (A) hepatocellular morphology of the control group. (B) hepatocellular morphology of coumacinel 250mg/kg. (C) hepatocellular morphology of coumacine II 250mg/kg. (D) hepatocellular morphology changes and intracellular collagen accumulation after exposure to coumacine I 5000mg/kg. (E) hepatocellular morphological changes after exposure to coumacine I 500mg/kg (400X magnification).

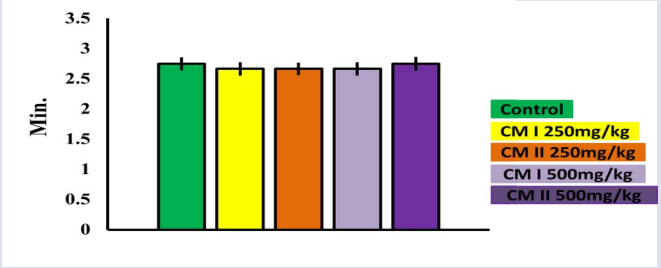

Figure 3: Bleeding time comparison between different treatment groups showed no difference in bleeding time due to coumacine exposure (all data presented as Mean ±SEM).

no differences between the HPBCD treated group and coumacine I and II treated groups in both 250 and 500 mg/kg ($F(4,29)=2.1$, $P>0.05$), as shown in Figure 3.

Coumacine I and II effect on hepatocellular morphology

In order to investigate if the alterations observed in hepatocellular morphology after coumacines exposure are evident of hepatocellular injury, serum enzyme activity levels of hepatocellular GPT and GOT were measured after daily administration of coumacines 5 days are indicative of cellular damage.[21] Serum activity of the liver cellular GPT enzyme was 39 ±2.7 IU/L in the control group. Mice which received coumacine I and II at a dose of 500 mg/kg had higher GPT levels of 60±2.5 IU/L and 58±2 IU/L respectively, while mice that received coumacine I and II at a dose of 250 mg/kg had GOT activity levels of 40±3 IU/L and 38±2.3 IU/L respectively. the results of one way ANOVA test showed statistically significant differences between the five treatment groups ($F(4,28)$ 13.3, $P<0.05$) (22). A post hoc Bonferroni test showed significant differences in serum GPT activity in both coumacine I at 10% HPBCD solution and 500 mg/kg dose groups ($q=5.4$, $P<0.05$) and significant differences in serum GPT activity in both coumacine II at 10% HPBCD solution and 500 mg/kg dose groups ($q=5.4$, $P<0.05$); however, there were no statistically significant differences in serum GPT in HPBCD-treated group versus either coumacine I and II-treated groups (Figure 4).

Serum activity of the liver cellular GOT enzyme was 41 ±1.7 IU/L in the control group. Mice which received Coumacine I and II at a dose of 500 mg/kg had higher GOT levels of 59±1.5 IU/L and 58±1.7 IU/L respectively, while mice that received Coumacine I and II at a dose of 250 mg/kg had GOT activity levels of 39±3 IU/L and 38±2.3 IU/L respectively. A one-way ANOVA showed significant differences between the five groups ($F(4,28)$ 10.3, $P<0.05$). the post hoc Bonferroni test showed significant differences in serum GOT activity in both Coumacine I at 10% HPBCD solution and 500 mg/kg dose groups ($q=5.4$, $P<0.05$) and significant differences in serum GOT activity in both Coumacine II at 10% HPBCD solution and 500 mg/kg dose groups ($q=6.4$, $P<0.05$); however, there were no statistically significant differences in serum GOT in HPBCD-treated group versus either Coumacine I and II-treated groups (Figure 4).

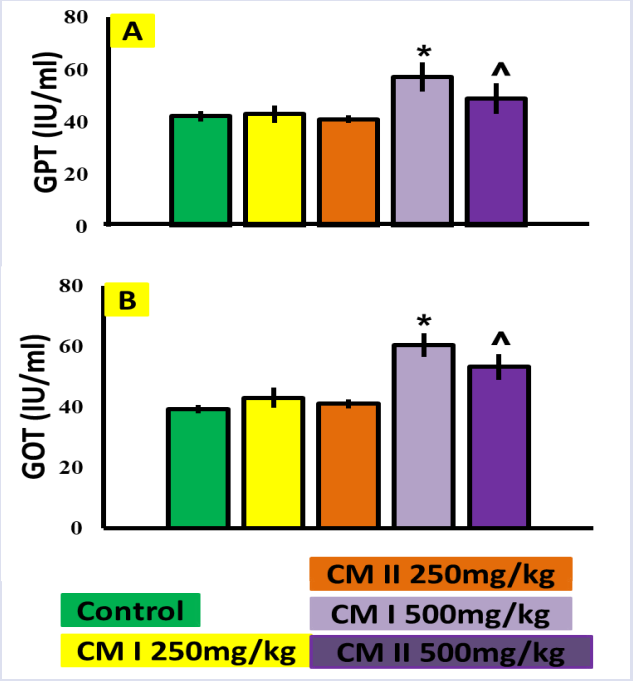

Figure 4: Liver enzyme activity levels (A) showed a significant elevation of aspartate aminotransferase activity in both mice administered 500mg/kg IP of both coumacine I or II in comparison with the control group. (B) showed) showed a significant elevation of alanine aminotransferase activity in both mice administered 500mg/kg IP of both coumacine I or II in comparison with the control group (all data presented as Mean ±SEM).

DISCUSSION

In this study, we investigated the potential hepatotoxicity of the novel coumacine and its derivative after repeated doses for 5 days. The results showed that 250 mg/kg dose for both coumacine I and II have no detectable hepatoxicity evident by liver enzyme or hepatocellular morphology. On the other hand, repeated doses of 500mg/kg of both coumacine I and II cause hepatocellular morphological changes and an increase in liver enzymes.[23,24]

The worldwide emergence of multi-drug resistance against the standard antibacterial therapy renders previously effective antibacterial ineffective. In addition to stagnation in developing new efficient antibacterial agents causes huge health and economic issues.[25-27] Heterocyclic compounds are a very important source of drug development with several biological activities acting as antitumor[28,29] antifungal,[30,31] antiviral,[32,33] anti-inflammatory,[34,35] and anticonvulsant.[36-38] Novel coumacines have been developed by Mustafa YF (2018) and he reported their antimicrobial effect against several clinically significant aerobic bacteria (*E. coli*, *H. influenzae*, *K. Pneumonia*, and *P. aeruginosa*) and four anaerobic bacteria (*C.perfringens*, *B. fragilis*, *P. melaninogenica* and *F. necropharum*) using agar solution method and compare them against both ciprofloxacin and metronidazole solutions. Novel coumacines showed a potential for broad-spectrum antibacterial activity.[39,40]

As novel coumacine structures are related to coumarin, one of the most important preclinical studies for the development of these agents as antimicrobial medication is the study of their hepatoxicity potential. Although coumacines showed a sort of hepatoxicity further studies are needed as liver enzymes are modestly elevated and might return to their basic levels with continuous use. Selective toxicity is one of the most important criteria for developing novel antibacterial. Accordingly, this study reports the safety margin of novel coumacines.

CONCLUSION

Novel coumacines cause hepatotoxicity only when given in high repeated doses and do not affect bleeding time even after repeated doses. Novel coumacines might be the leading compound to the development of Novel broad-spectrum antibacterial agents. Furthermore, both *in vitro* and *in vivo* studies are needed with higher doses and chronic exposure to confirm the possible hepatic toxicity. In addition, renal and cardiac safety of coumacine is of utmost importance besides other preclinical studies before proceeding to clinical study.

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CONFLICTS OF INTEREST

No conflicting interests are disclosed by the authors.

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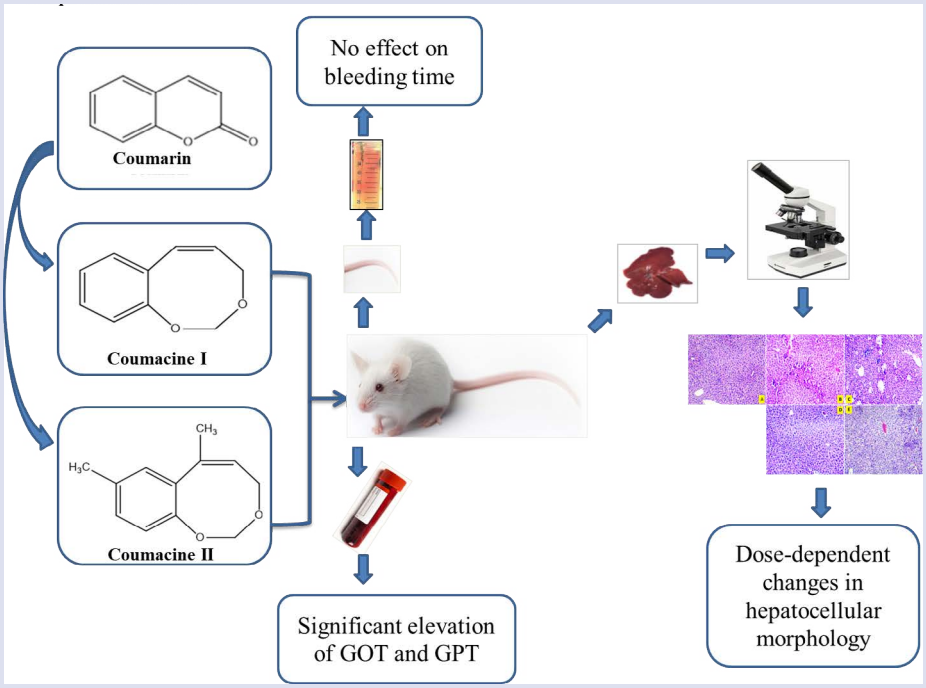
GRAPHICAL ABSTRACT



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