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MODULATION OF RAT HEPATIC AND INTESTINAL CYP3A4 ACTIVITY BY *CNIDOSCOLUS ACONITIFOLIUS* LEAF ETHANOLEXTRACTS

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ABSTRACT
Background: CYP3A4 is a major cytochrome P450 enzymes involved in the metabolism of several clinically prescribed drugs that have been implicated in herb-drug interactions.
Aim: Thisstudy investigated the *in vitro* effect of ethanol extract of *Cnidoscolus aconitifolius* Leaf on CYP3A4 activity in rat liver and intestinal microsomes.
Method: The hepatic and intestinal microsomes were obtained through differential centrifugation method. The concentration of formaldehyde obtained from the erythromycin N-demethylation reaction catalyzed by CYP3A4 was determined using spectrophotometer.
Results: Based on the results obtained, *C. aconitifolius*extract at 0.25, 0.5, and 1 mg/mL significantly reduced metabolism of erythromycin by inhibiting CYP3A4 activity in rat liver and intestinal microsomes when compared to the control group.
Conclusion: The findings from this study suggest that ethanol extract of *C. aconitifolius* leaf may interact with the metabolism of erythromycin in both rat liver and intestinal microsomes. Thus, this plant should be used with caution for drugs metabolized by CYP3A4 enzymes.
Keywords: *Cnidoscolus aconitifolius*, Rat liver microsomes; CYP3A4; Herb-drug inhibitions

INTRODUCTION
There is an upsurge in the use of medicinal plants as a form of complementary and alternative medicine (CAM) worldwide [1]. Thus, simultaneous use of medicinal plants and drugs is inevitable especially among patients with chronic disease such as hypertension, diabetes, and HIV infection [2]. Consequently, the likelihood of herb-drug interactions (HDIs) increases due to the simultaneous use medicinal plants with the orthodox drug.*C. aconitifolius* belongs to the family of Euphorbiaceae, locally known as "*Iyana ipaja*" in the western part of Nigeria and it is commonly known as "Chaya and tree spinach" in many countries [3,4]. This plant is known for its medicinal and nutritional values; very rich in proteins, vitamins, minerals, and dietary fibers [5]. Thus, it is commonly used locally in anemia and malnutrition as a form of diet or herbal preparations [6]. In southwestern Nigeria, *C. aconitifolius* leaves are used to prepare infusion to treat high blood sugar, iron-deficiency anemia, indigestion, and peptic ulcer [7-9]. Flavonoids such as quercetin, kaempferol, and rutin have been reported to be present in the extracts of *C.aconitifolius*

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leaf [10]. Alkaloids, phenols, tannins, and glycosides have also been reported to be present in the extract of *C. aconitifolius* leaf [7,8]. Phytochemicals such as flavonoids are also substrate for CYP3A4, capable of modulating drug metabolizing enzymes activity. Thus, we hypothesized that ethanol extracts of *C. aconitifolius* leaf could affect drug metabolism through inhibition of CYP3A4 activity. The broad objective of this study was to investigate the *in vitro* effects of *C. aconitifolius*leaves extracts on the metabolism of erythromycin, a CYP3A4 substrate in rat hepatic and intestinal microsomes.

MATERIALS AND METHOD

Chemicals and Dugs

Ketoconazole (J94064 with 95% purity) and Erythromycin (J10493 with 98% purity) produced by AK Scientific, USA were used for this study.Ethanol, sucrose and calcium chloride ($CaCl_2$), tween 20, glycerol, potassium chloride (KCl), trichloroacetic acid, formaldehyde and Nash reagent (ammonium acetate, glacial acetic acid and acetyl acetone), magnesium Chloride ($MgCl_2$), and NADPHwere purchased from a local supplier in Ibadan, Nigeria. All the chemicals were at analytical standard grade.

Preparation of *Cnidoscolusaconitifolius* Extract

Fresh leaves of *Cnidoscolusaconitifolius* were collected from a garden located in Oshogbo, Osun State, Nigeria. The plant's identity was confirmed by a taxonomist at the Department of Botany, Obafemi Awolowo University, Ife, Osun State, Nigeria. A specimen was registered with the herbarium number IFE18274. The collected leaves were cleaned, dried at ambient temperature, and ground into a powder. 256.11g of the powder underwent cold maceration in aqueous ethanol (>99.7%) for 72 hours, with periodic stirring. The mixture was then filtered to remove insoluble particles and concentrated using rotary evaporator (Buchi, Switzerland) at 40°C. The concentrated crude extract was freeze-dried and stored in the desiccator until further usage.

Preparation of Rat Liver and Intestinal Microsomes

All the experiments for animal studies were performed in compliance with theethical guidelines by the Ladoke Akintola University ethical review committee and NIH guides on the use and care of laboratory animals. Rat liver and intestinal microsomes were obtained from six (6) healthy male Wistar rats. Rat liver microsomes (RLMs) and rat intestinal microsomes (RIMs) were prepared by sequential centrifugation following previously described methods [11].

RLMs were prepared by mincing and homogenizing isolated liver in a solution containing 0.25M sucrose and 10mM Tris-HCl (pH 7.4). This homogenate was centrifuged twice: once at 600 x g for 5 minutes and again at 12,000 x g for 10 minutes, resulting in a post-mitochondrial supernatant. Solid $CaCl_2$ was added to the supernatant to reach an 8mM concentration. The mixture was then centrifuged at 17,800 x g for 20 minutes. The resultant pellets were resuspended in a 150mM KCl-10mM Tris-HCl solution and centrifuged at 17,800 x g for 22 minutes. This final step resulted in

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a pink-colored microsomal pellet, that was suspended in 0.5ml of 0.1M potassium phosphate buffer with 20% glycerol. The prepared microsomes were stored at -20℃ until needed.

RIMs were prepared by scraping of intestinal mucosa with a light plastic slip, which was then combined and centrifuged successively using cold centrifuge (TGL-16R, China) in ice-cold sucrose buffer pH 7.0 to obtain a supernatant. The supernatants were mixed with 52mM CaCl2 (0.2mL of 52mM to 1mL of the supernatant), and this was allowed to stand for 20mins to precipitate the microsomes, followed by centrifugation at 17,800xg for 17mins to obtain the microsomal pellets, which were suspended in 0.5mL of 0.1M potassium Phosphate buffer containing 20% glycerol and stored at -20°C until needed.

In vitro Determination of CYP34 Activity

Spectrophotometer method as previously by [11] was used to determine rat CYP3A4 activity by measuring the amount of formaldehyde formed from N-demethylation of its substrate erythromycin. Erythromycin was used as the probe substrate, ketoconazole was used as positive control (PC, 5 µM), and tween 20 was used as negative control (NC, 1%) in this study. Incubation assay consisted of either RLMs or RIMs (0.1 mL, 25%), erythromycin (0.1mL, 10mM), magnesium chloride (0.1mL, 150Mm) and potassium phosphate buffer (0.6mL, 50mM, pH 7.25), and ethanol extract of *C. aconitifolius* leaf at different concentrations (7.8125 µ/mL - 1000 µ/mL). The positive and negative control were used to replace the test plant, incubation was initiated with the addition of NADPH (0.1mL, 10mM), and terminated after 10mins by adding ice-cold trichloroacetic acid (0.5mL, 12.5% w/v) solution. At the end of reaction, the tubes were centrifuged at 1740 x g for 10min to remove proteins. To 1mL of the supernatant, 1mL of freshly prepared Nash reagent (2M ammonium acetate (30g), 0.05M glacial acetic acid (0.4mL and 0.02M acetylacetone (0,6ml) were added and then heated in a water bath at 50°C with intermittent shaking for 30min. After cooling, their absorbances were read at 412nm. The amount of formaldehyde formed from CYP3A4 mediated N-demethylation of erythromycin was calculated from the plotted standard curve of formaldehyde. The CYP3A4 activity was expressed in nM formaldehyde/minute/mg protein.

Statistical Analysis

Data were presented as mean ± standard deviation (n=3, SD). Data were analyzed by GraphPad Prism 8 software using analysis of variance (ANOVA) following Dunnett's post hoc test to examine the significant differences when the test and PC group was compared with the NC group. p value lesser than 0.05 ($p<0.05$) was considered as significant when the test and PC group was compared to the NC group.

RESULTS

Figure 1 showed the result of *in vitro* effect of *C. aconitifolius* extract on rat intestinal CYP3A4 activity. From the result, 0.5 mg/mL and 1 mg/mL of the extractsignificantly decreasedrat intestinal CYP3A4 activitywhen compared with the negative control. However, a significant decrease in hepatic CYP3A4 activity was

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seen in the rat liver microsomes treated with the extract at concentrations of 0.25, 0.5 and 1 mg/mL (Figure 2.).

DISCUSSION

Erythromycin, a macrolide antibiotic, is a known probe substrate for the determination of CYP3A4 enzyme activity in both *in vitro* and *in vivo* experiments [12]. It undergoes N-demethylation reaction by CYP3A4 to form N-demethylated metabolites and formaldehyde, which was measured by spectrophotometry method[12].

The combination of herbal preparations with the conventional drugs has brought to the fore the potential risk of interactions between these treatments. The consequence of such interactions could be harmful or beneficial when it involves cytochrome P450 enzymes, which play a crucial role in drug metabolism [2]. The CYP3A4 enzyme is of special interest due to its involvement in the metabolism of a wide range of clinically prescribed drugs [13]. CYP3A4 is a major enzyme that is in the liver and intestine; thus, modulation of this enzyme can lead to first pass effect [14]. Drug metabolizing enzymes mediated pharmacokinetic interactions have been postulated as one of the mechanisms involved in herb-drug interactions. Since, plant extracts contain phytochemicals such as alkaloids, flavonoids, and other secondary metabolites. They are capable of modulating CYP3A4 activity as well as other drug metabolizing enzymes [15]. Several studies have reported that various plant extracts are capable of inhibiting or inducing CYP3A4 activity [17]. For example, aqueous extract of *Acacia catechu* inhibits CYP3A4 activity and *in silico* study of this plant revealed two polyphenol phytochemicals that may be responsible for the inhibition of CYP3A4 activity [18].

The inhibitory potential of *C. aconitifolius*observed in this study may indicate interactions between one or more components of this plant with CYP3A4 enzymes. The study found that *C. aconifolius* inhibits CYP3A4 activity in rat liver and intestine microsomes in a concentration-dependent fashion, suggesting that it may cause metabolism mediated interactions with drugs that are metabolized by CYP3A4 enzyme [19]. The most well investigated mechanism of pharmacokinetic drug interactions is drug-mediated inhibition of cytochrome P450 enzymes. For instance, the mechanism underlying *Artemisiaannua*extract interaction with drugs was irreversible inhibition of CYP3A4 and CYP2B6 enzymes [20]. Inhibition of *C. aconitifolius* may result in dangerous concentration of a concurrently administered drug or its metabolite in the blood, leading to delayed clearance and potential toxicity. Thus, there is likelihood of herb-drug interactions involving *C. aconitifolius*through its inhibitory effect on CYP3A4 enzymes in humans. This plant should be used with caution especially when using drugs that could serve as substrate of CYP3A4 enzyme.

Further study is required to identify and isolate the *C. aconitifolius* constituent that is responsible for the observed inhibitory property. Also, this study may serve as basis for *in vivo* interaction study.

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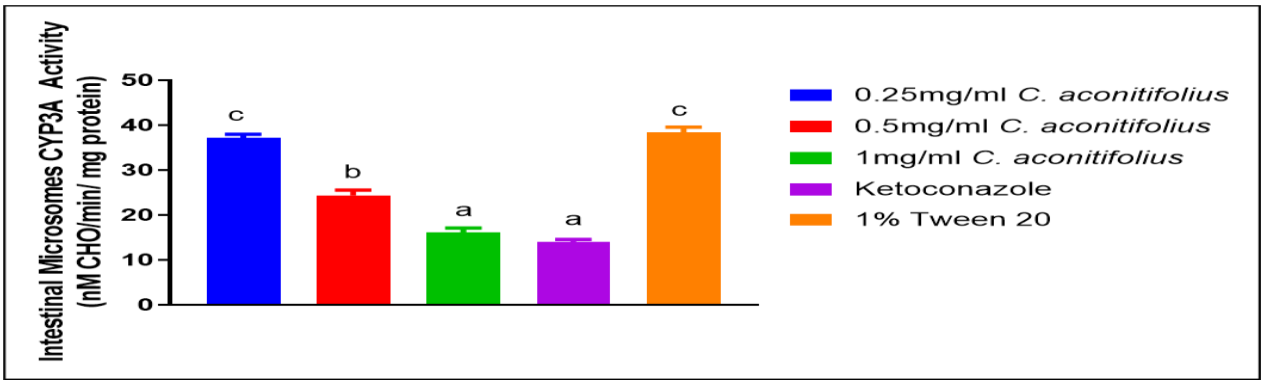

Figure 1. *In vitro*effect of C. *aconitifolius* extract on the rat intestinal CYP3A4 activity. Mean±SD, (n=3), different alphabet from that of negative control (Tween 20) indicate statistically significant.

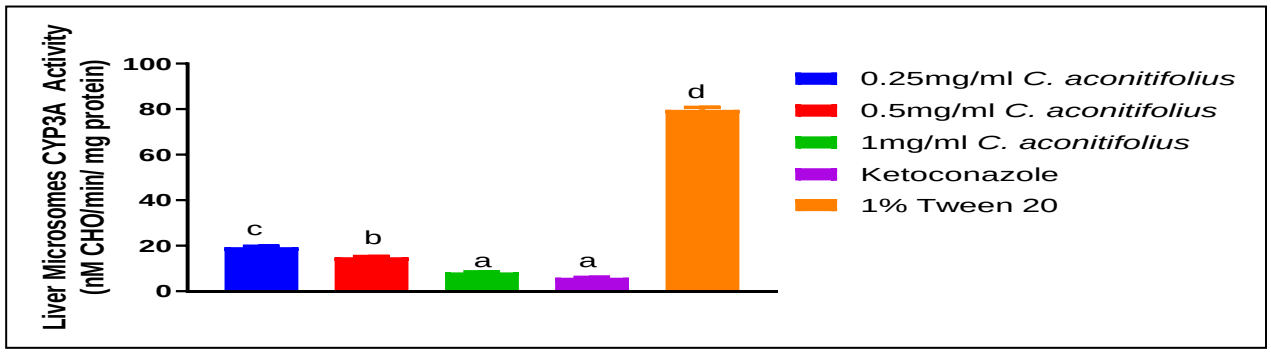

Figure 2. *In vitro*effect of *C. aconitifolius* extract on the rat liver CYP3A4 activity. Mean±SD, (n=3), different alphabet from that of negative control (Tween 20) indicate statistically significant.

CONCLUSION

This study evaluatedthe *in vitro* inhibition of CYP3A4 activity in rat liver and intestine microsomes by different concentrations of*C. aconitifolius* extract. This shows that co-administration of this plant extract with conventional drugs that are substrates of this enzyme may lead to significant interactions.

RECOMMENDATIONS

These findings give discernment to the field of herbal medicine. Future investigations should focus on confirming these results through *in vivo* studies, assessing dose-response relationships and taking genetic variability into account. Such research will be essential to ensuring the safe and effective integration of *C. aconitifolius* into clinical practice, particularly when used in conjunction with conventional medications.

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Conflict of interest: The authors declare no conflict of interest.

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