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Research Article

PHASE I DRUG METABOLISM STUDY OF THE STANDARDISED EXTRACT OF *EURYCOMA LONGIFOLIA* (TAF-273) IN RAT HEPATOCYTES

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ABSTRACT

Eurycoma longifolia is widely used as an alternative medicine by society. Some patients may use a herb-drug treatment while taking prescribed medication. It is possible that one substance may alter the bioavailability of other substances by inducing the phase I or phase II hepatic enzymes system and may also affect the therapeutic effects. The objective of this study is to examine the effect of the standardised extract of *Eurycoma longifolia* (TAF-273) on phase I hepatic drug metabolising enzymes in normal and diabetic Sprague-Dawley (SD) rat hepatocytes. The male and female SD rats used in this study were divided into two main groups (normal and diabetic). Each group was divided into three sub-groups: young, adult and old males and females (n=6). Isolated hepatocytes from these groups were prepared by using the collagenase perfusion technique. Then, the aminopyrine N-demethylase assay was done. Aminopyrine N-demethylase activity was determined by measuring the quantity of formaldehyde formed at 415 nm by using microplate reader. Based on the results obtained, a significant effect on aminopyrine metabolism by TAF-273 was observed in the normal and diabetic rats in both the male and female groups ($p<0.05$), especially in the adult, normal male and female groups. The effect of TAF-273 in the increasing of amynopyrine metabolism in male and female rat hepatocytes was dose-independent.

Keywords*: Eurycoma longifolia* Jack (Simaroubaceae), Phase I metabolism, Rat hepatocytes

INTRODUCTION

There is an increasing use of alternative medicine by the public with a variety of plant-derived drugs or plant-based supplement which contains active compounds over the counter. The belief among many users and suppliers of herb-drugs that these preparations are natural and so that 'safe' is one of the reasons for the popularity of herbal remedies[1,2]. This, however, is a misconception because there are possibilities where patients use herbalist treatment while taking prescribed medication. It is possible that one substance may alter the bioavailability of another substance by inducing the phase I or phase II hepatic enzymes system so that can affect the therapeutic effect of drugs. There are many reports of herb-drug interactions, adverse effect of herbs and their ability to influence the therapeutic effect of clinical drugs [3,4,5] and the interaction sometimes causes a serious clinical consequences[6,7,8]. Thus, study about the pharmacological profiles involving drug metabolism phase I or II, and pharmacokinetics of herbal medicines become an important evidence for rational herbal remedies[2].

Eurycoma longifolia Jack *(E. longifolia)* is one of the traditional medicines in Asia, is commonly known in Indonesia as "Pasak Bumi", in Malaysia as "Tongkat Ali" and in Vietnam as "Cay ba binh". In Malaysia, it is claimed to increase virility and sexual prowess so that has gained a reputation as an aphrodisiac. Currently, there are more than 200 Tongkat Ali products circulated in Malaysian market, many of these products focus on the aphrodisiac property [9,10,11]. The root of this plant is used as traditional medicine such as for healing of boils, wound ulcer and for fever [12]. Several classes of chemical constituents have been isolated and characterized from the root and some studies demonstrated that some constituents have different effects: cytotoxic effect, antimalarial, antipyretic, induce apoptosis in HepG2 cells and aphrodisiac property[13-16].

E. longfolia has many constituents and in some cases has to be consumed regularly over time. If the patient uses concurrently with other medicines, may occur an interaction and may also affect the drug therapeutic effects or even generate toxicity. The pharmacological profiles of the standardised extract of *E. longifolia* (TAF-273) if used concurrently with other drug needs to be conducted, because the herb-drug has been widely used by society. The present report examined the effect of TAF-273 on phase I hepatic drug metabolising enzymes in normal and diabetic SD (Sprague-Dawley) rat hepatocytes.

MATERIALS AND METHODS

Plant material and chemicals

TAF-273 extract was made and standardised in Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Universiti Sains Malaysia following the protocol described previously and a voucher specimen of the plant was deposited, with Reference No. 785-117, at the Penang Botanical Garden[9]. Aminopyrine, acetyl acetone, collagenase type IV, streptozotocin, and trypan blue were purcashed from Sigma Chemicals Co, USA while glucose monohydrate and calcium chloride were obtained from Riedel-deHaen, France. Diethyl ether, magnesium sulphate and magnesium chloride were purcashed from BDH Laboratory Supplies, UK. Ammonium acetate, disodium hydrogen phosphate, barium hydroxide and zinc sulphate heptahydrate were obtained from R & M Chemicals, UK and formaldehyde solution 37 percent from Merck, Germany.

Experimental animals

In this study, male and female SD rats from different age were used (young = 6-8 weeks, adult = 12-16 weeks and old = 20-24 weeks). The rats were bred in the animal house of the Universiti Sains Malaysia, Penang, and caged according to their body weight (young male and female = 120±10 and 100±10 g; adult male and female = 220±30 and 170±10 g; old male and female 350±50 and 250±50 g). All the rats had free access to tap water and standard food pellets (Gold Coin®, Penang, Malaysia) *ad libitum.* The study protocol was approved by the Animal Ethics Committee, Universiti Sains Malaysia, Penang, Malaysia.

Induction of type I diabetes mellitus on animal by streptozotocin (STZ)

Streptozotocin (STZ) was used to induce diabetic in SD rats by intravenous injection at doses 60 mg/kg b.w. (body weight). The STZ was dissolved in normal saline (0.9 percent NaCl) and injected immediately into rat's tail vein under ether anesthesia. The blood glucose level was measured from the STZ-induced diabetic rat after three days of STZ injection. Only SD rat with glucose level higher

than 15.6 mmol/L at fasting state was considered as type I diabetic rat.

Formaldehyde standard curve

A stock solution was prepared by added 0.7 ml of 37 percent w/w formaldehyde into 100 ml volumetric flask and made up to 100 ml by adding distilled water. 0.25 ml of the solution was taken out and made up to 250 ml by distilled water. Then, some solutions were made at concentrations 0.5; 1; 1.5; 2; 2.5 µg/ml by serial dilution of the stock solution and 1 ml was transferred into different test tubes. In the control test tubes, the 1 ml stock solution was replaced with 1 ml of distilled water. The Nash reagent (2 ml) was added in to each test tube and was capped. All the tubes were incubated in a shaking water bath at 60 ^{0}C for 30 minutes. Then, 0.20 ml aliquot from each tube was transferred to 96 wells plate. Absorbances for all samples were read at 415 nm by using a microplate reader (Power wave X-340®, Biotek). The standard curve was constructed by plotting the value of absorbance versus concentration of the formaldehyde, µg/ml.

Effect of TAF-273 on phase I hepatic drug metabolising enzyme (N-demethylase assay)

SD rats used in this study were divided into two main groups (normal and diabetic). Each group was divided into three sub-groups: young, adult and old males and females (n=6). Isolated hepatocytes from these groups were prepared by using the collagenase perfusion technique [17]. Equal volume (1.0 ml) of serial dilutions of TAF-273 (0.001 to 100 µg/ml) in distilled water were added into petri dishes containing aminopyrine (25 mM), freshly isolated hepatocytes (3.75x10^3 cells) and incubation medium. For the control dishes essentially contain all of the above with the exception of the herbal preparation, being replaced with distilled water. The petri dishes were then incubated for twelve minutes at room temperature on a table top shaker (Belly Dancer®). The reaction was terminated by adding of $ZnSO_4$ (25 percent w/v; 0.5 ml) and was followed by the addition of saturated $Ba(OH)_2$ (0.5 ml) after five minutes.

The samples were then centrifuged by centrifuge machine (Eppendorf®) at 1000 rpm for ten minutes. 1 ml of supernatant was taken out and added to 2 ml of Nash reagent and incubated at 60 ^{0}C for 30 minutes in a waterbath shaker. Aminopyrine N-demethylase activity was determinated by measuring the quantity of formaldehyde formed at 415 nm by microplate reader (Powerwave X-340®; Biotek) according to the colourimetric method of Nash (1953)[18].

Data analysis

The unknown concentration of formaldehyde formed (the metabolite after N-demethylation) in the rat hepatocytes was calculated by using a linier regression equation. The standard curve equation was Y = 0.0624X - 0.0009, R2 = 0.9996 (Y = absorbance; X= formaldehyde concentration in µg/ml). The means and standard deviation were calculated and results were compared with the control. The results were analysed using Anova (analysis of variance) and the Tukey Test and the level of significance was set at $p<0.05$. A univariate analysis was performed to evaluate the effect of age, disease, gender and TAF-273 concentrations on formaldehyde formed.

RESULTS AND DISCUSSION

Metabolism enzymes can be affected by many factors such as age, disease, and gender for endogenous factor and drug, or other substances that consumed by people for exogenous factor[19]. An alteration may occur in phase I or II of drug metabolism. There are many reactions in phase I metabolism such as hydroxilation, reduction and dealkylation that divided by N-, O- or S-dealkylation. This experiment was performed to observe effects of gender, age and disease factors on drug metabolism through N-dealkylation (in this case N-demethylation).

Effect of TAF-273 on phase I hepatic drug metabolising enzyme (N-demethylase assay) in normal and diabetic male SD rat hepatocytes

In general, histograms in Fig. 1 and 2 have shown that TAF-273 increased the N-demethylase activity, confirmed by increasing the formaldehyde formed as compared to the positive control, even though some concentrations decreased it. There was a significant difference ($p<0.05$) in young and old, normal male rats in the presence of TAF-273 at concentration of 10 and 100 µg/ml, as compared to the positive control aminopyrine, respectively. A significant elevation was found in all the tested concentrations (0.001 to 100 µg/ml) of TAF-273 in adult, normal male group (Fig. 1). In diabetic male groups, the increasing of formaldehyde formed was significant difference ($p<0.05$) in young and adult groups, was only found in the presence of TAF-273 at the highest concentrations (100 µg/ml) and there was no significant difference ($p>0.05$) in the old group (Fig. 2).

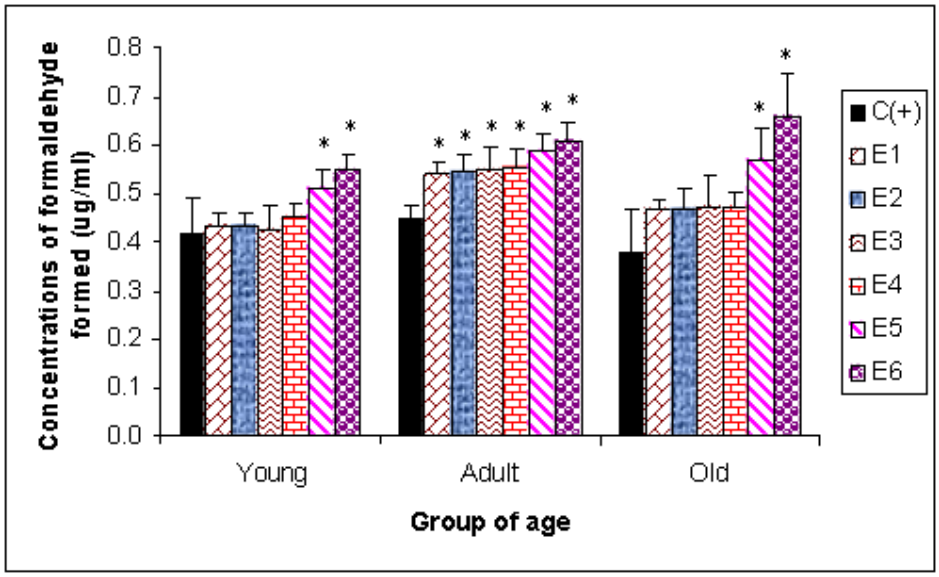


Fig. 1: TAF-273 effect on N-demethylase activity in young, adult and old normal male rat hepatocytes (positive control group of aminopyrine [C(+)], the treated group with TAF-273 at concentrations: 0.001 to 100 µg/ml [E1-E6]; (mean ± SD; n=6; *p<0.05)

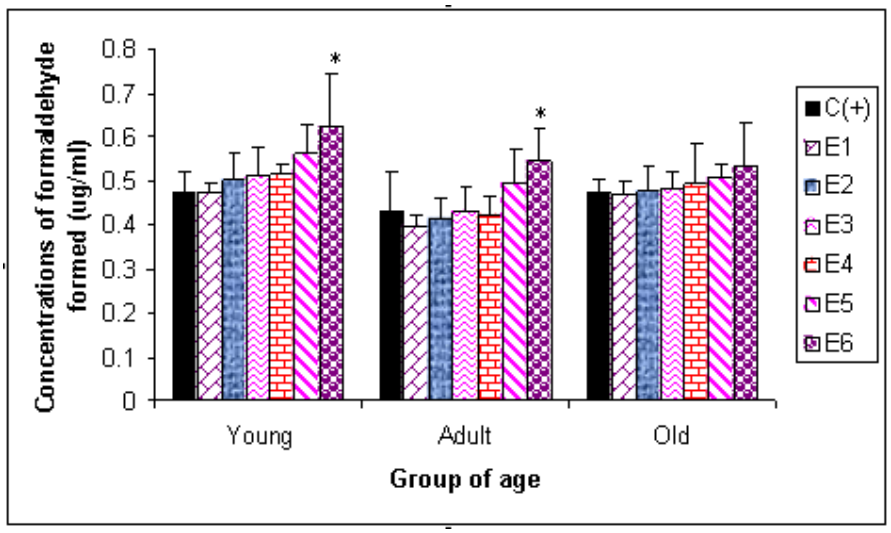


Fig. 2*:* TAF-273 effect on N-demethylase activity in young, adult and old diabetic male rat hepatocytes (positive control group of aminopyrine [C(+)], the treated group with TAF-273 at concentrations: 0.001 to 100 µg/ml [E1-E6]; (mean ± SD; n=6; *p<0.05)

Effect of TAF-273 on phase I hepatic drug metabolising enzyme (N-demethylase assay) in normal and diabetic female rat hepatocytes

Effect of TAF-273 on the formaldehyde formed in normal and diabetic female groups are shown at Fig. 3 and 4. TAF-273 increased the aminopyrine metabolism significantly in adult normal rats ($p<0.05$) at concentrations of 0.1 to 100 µg/ml and not significantly different ($p>0.05$) in the old normal group. While in the young normal group, a significant elevation was only found at the highest concentrations of TAF-273 tested (100 µg/ml) (Fig. 3). The increase of formaldehyde formed was significantly different ($p<0.05$) in young and old diabetic rat groups, was only altered by TAF-273 in the highest concentrations but not significantly different in the adult diabetic rats ($p>0.05$) as compared to the positive control group respectively.

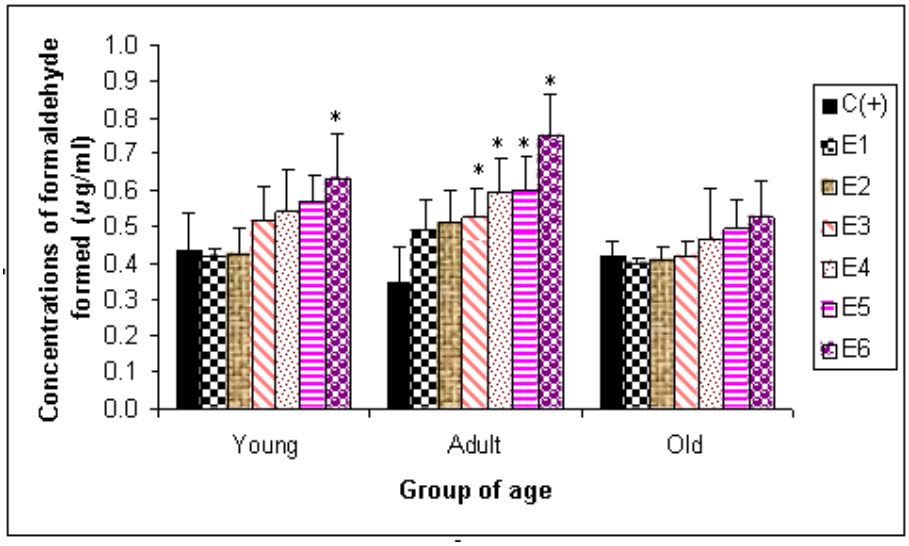


Fig. 3: TAF-273 effect on N-demethylase activity in young, adult and old normal female rat hepatocytes (positive control group of aminopyrine [C(+)], the treated group with TAF-273 at concentrations: 0.001 to 100 µg/ml [E1-E6]; (mean ± SD; n=6; *p<0.05)

The univariate analysis results showed that the increasing effect of TAF-273 on phase I hepatic drug metabolising enzyme (N-demethylase assay) in rat hepatocytes was affected by age and gender of the rats, also by concentrations of TAF-273 ($p<0.05$), but not altered by diseases ($p>0.05$). The effect of TAF-273 at different concentrations in aminopyrine metabolism that affected by age and gender of the rats are shown in Fig.5 to 7. In the young groups, the rate of aminopyrine metabolism was higher on the normal female rats, while in diabetic groups was similar for both male and female groups (Fig.5). The percentage of formaldehyde formed was higher on female rats for both adult normal and diabetic groups (Fig.6). Different with the results before, on Fig.7 is shown that in the old normal rats, formaldehyde was formed rapidly in male group while in diabetic rats occurred on the contrary.

Age has an important effect on drug metabolism. In young animal, hepatic enzymes for drug metabolism is not well developed until adulthood, and on the old animal the susceptibility may be due to decreased blood flow to liver, plasma protein binding and its small active liver mass [20,21]. So in general, the young and old of many animals are more susceptible to certain drug action as compared to adult animal. Aging animals show different responses to inducing agents[21]. Some enzyme systems decline with age but sometimes it is limited to the species and gender.

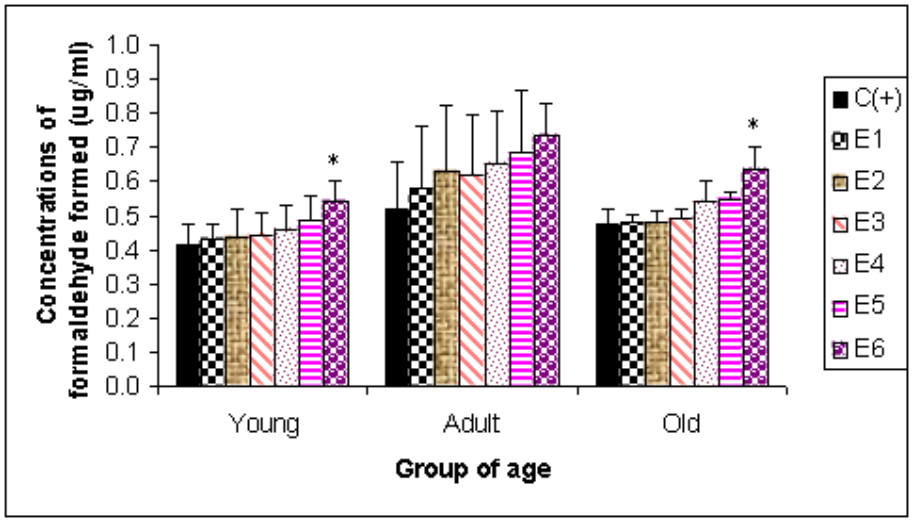
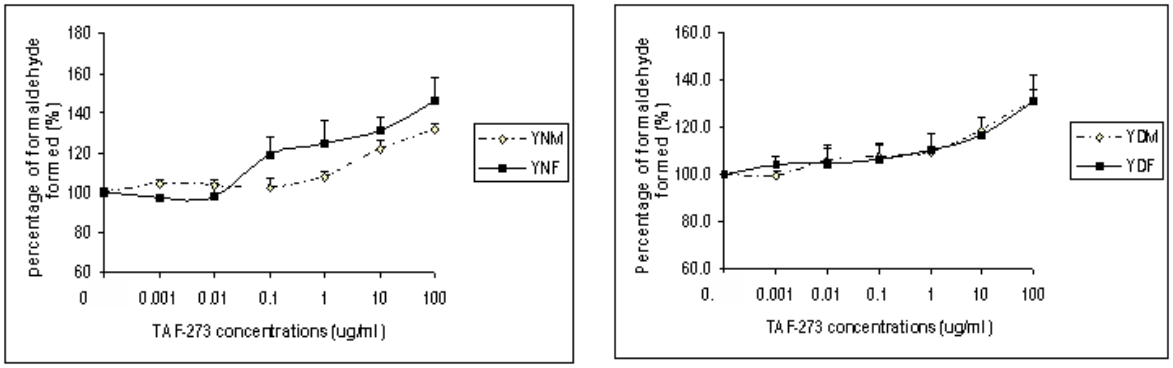
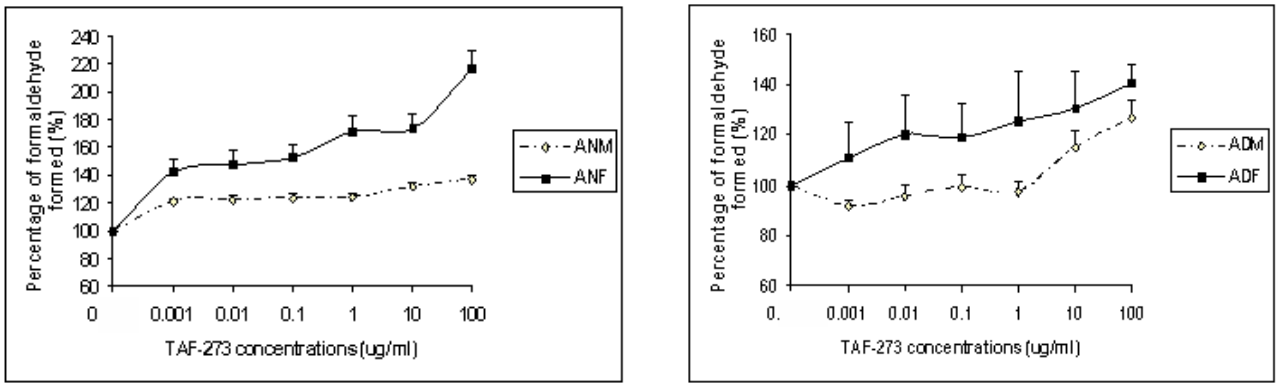


Fig. 4: TAF-273 effect on N-demethylase activity in young, adult and old diabetic female rat hepatocytes (positive control group of aminopyrine [C(+)], the treated group with TAF-273 at concentrations: 0.001 to 100 µg/ml [E1-E6]; (mean ± SD; n=6; *p<0.05)



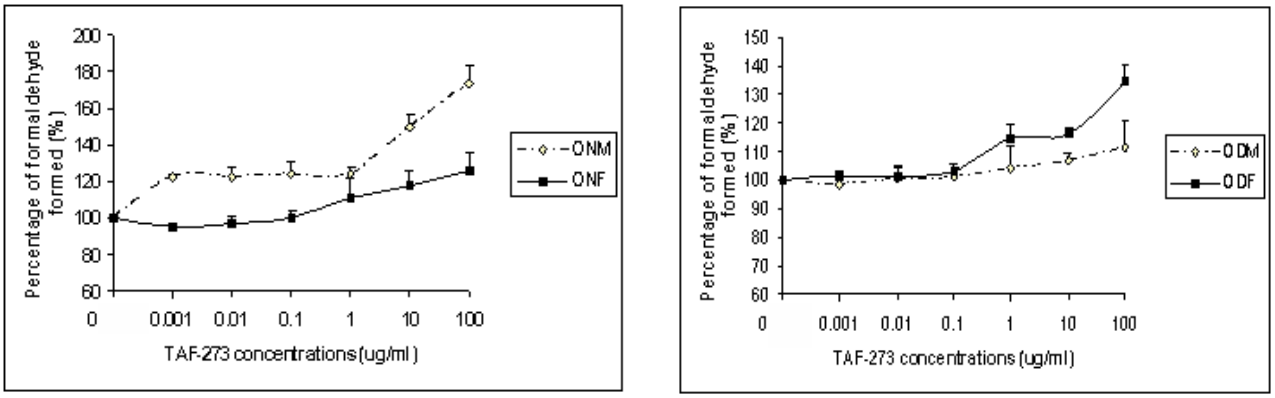
[A] [B]

Fig. 5. Percentage of formaldehyde formed (%) in young normal [A] and diabetic [B] rat hepatocytes treated with TAF-273 at concentrations 0.001 to 100 µg/ml as compared to their respective positive control (mean ± SD; n=6; YNM = young normal male; YNF = young normal female; YDM = young diabetic male; YDF = young diabetic female)



[A] **[B]**

Fig. 6: Percentage of formaldehyde formed (%) in adult normal [A] and diabetic [B] rat hepatocytes treated with TAF-273 at concentrations 0.001 to 100 µg/ml as compared to their respective positive control (mean ± SD; n=6; ANM = adult normal male; ANF = adult normal female; ADM = adult diabetic male; ADF = adult diabetic female)



[A] [B]

Fig. 7: Percentage of formaldehyde formed (%) in old normal [A] and diabetic [B] rat hepatocytes treated with TAF-273 at concentrations 0.001 to 100 µg/ml as compared to their respective positive control (mean ± SD; n=6; ONM = old normal male; ODF = old normal female; ODM = old diabetic male; ODF = old diabetic female)

A few aspects like hormones of the pituitary and adrenal gland, sex organs have an important role in affecting drug metabolism. Sex differences in the rat follows a general pattern of the male metabolising faster than the female[19]. Some cytochrome P450 show a gender-specific expression such as CYP 2C11 is only expressed in male rats[22], but the expression some genes are different, sometimes is greater in female rats and on the other hand is higher in the male rats.

Diabetes mellitus causes marked changes in hepatic phase I and II metabolism. Studies using isolated hepatocytes have confirmed that in some cases, the effect of insulin is direct on the liver and in others it is secondary to the metabolic changes induced by diabetes[19]. Insulin deficiency in rats, mainly in males, seems to enhance some drug metabolizing reactions and to inhibit others[21].

Cytochrome P450 is a very important protein involved in the metabolism of drugs and xenobiotics[23]. It is responsible for the oxidation and reduction of phase I metabolism of wide range of drugs. Many herbs and their natural compounds are identified as inhibitors or inducers for cytochrome P450 system[7]. The induction and inhibition may occur through different pathways, depend on their mechanism when a substrate (such as drug or hormone) binds to the specific receptor on a target cell, such as phosphatidyl inositol pathway, cyclic adenosine monophosphate (cAMP) pathway or cyclic guanosine monophosphate (cGMP) pathway.

Scrugg (2008)[24] mentioned that *E. longifolia* is a testosterone booster (increased testosterone up to 440 percent). Increasing of testosterone may increase metabolic rates and oxidative phosphorylation. *E. longifolia* acts through the enhancement of testosterone and also c-GMP production. However, TAF-273 increased the aminopyrine N-demethylase activity and future experiments should be carried out to get the correlation of these results and the statement.

CONCLUSION

A significant effect on aminopyrine metabolism by TAF-273 was observed in the normal and diabetic rats for both the male and female groups ($p<0.05$), especially in the adult, normal male and female groups. The effect of TAF-273 in the increasing of amynopyrine metabolism was dose-independent since the increase of TAF-273 concentrations did not always produce an elevation of formaldehyde formed.

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