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In vitro and *in vivo* cytochrome P450 3A enzyme inhibition by *Aframomum melegueta* and *Dennettia tripetala* extracts

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ABSTRACT

Objective: To evaluate the *in vitro* and *in vivo* inhibitory effects of two commonly used herbs, *Aframomum melegueta* (*A. melengueta*) and *Dennettia tripetala* (*D. tripetala*) on CYP 3A enzymes.

Methods: *In vitro* inhibition of the enzymes were assessed with microsomes extracted from female albino rats using erythromycin-N-demethylation assay (EMND) method while their *in vivo* effects were measured by estimating simvastatin plasma concentrations in rats. Pharmacokinetic parameters were determined using non-compartmental analysis as implemented in WinNonlin pharmacokinetic program.

Results: EMND assay with intestinal microsomes indicated that aqueous extracts of *D. tripetala* and *A. melengueta* significantly ($P < 0.05$) inhibited intestinal CYP 3A activity at both 50 μg and 100 μg concentrations. Petroleum ether extract of *D. tripetala* and ethanol extracts of *A. melengueta* inhibited intestinal CYP3A activity at 100 μg but not at 50 μg concentrations. All the extracts showed an *in vitro* dose dependent CYP 3A inhibition with liver microsomes. *In vivo* analysis showed that pre-treatment with the extracts enhanced systemic absorption of simvastatin with reductions in metabolizing enzymes activity as indicated in significant increases in maximal concentration, area under curve, area under moment curve and mean resident time of simvastatin ($P < 0.05$).

Conclusions: Herbal preparations containing these plants' extracts should be used with caution especially in patients on CYP450 3A substrate medications.

1. Introduction

Cytochrome P450 enzymes play major roles in the metabolism of endogenous compounds and biotransformation of xenobiotics [1]. They are found in different locations in the body with higher concentrations in the liver [2]. These enzymes are endogenously synthesized and their syntheses are influenced by both genetic and non-genetic factors. Substances such as drugs, food, herbs and other agents capable of inducing or inhibiting these enzymes could lead to changes in their metabolic capacity. Inhibition of these enzymes is of interest in research and practice because such inhibition is an immediate phenomenon that occurs with the first dose of the inhibitor agent [3].

Enzyme inhibition in pharmacotherapy is a major cause of clinically significant drug interactions [4] and, can either lead to an increase in the bioavailability of a parent drug with extensive first-pass metabolism, or lead to a decreased elimination of compounds with high systemic clearance resulting in substance accumulation and toxicity. Among the identified families of these enzymes involved in metabolism and biotransformation in humans, CYP 3A is the most important. This family is frequently implicated in most drug interactions involving the enzymes because they are highly inducible and can be inhibited by numerous agents including herbs and herbal products [5]. Many enzyme modulators of herbal origin have been found in practice [6–10].

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The prevalence of concurrent use of herbs and herbal preparations with conventional medications is on the increase globally, and some observed cases of herb-drug interactions call for the need to challenge some CYP 450 enzymes with commonly used herbs. Among the plants commonly used in ethnomedicine are *Aframomum melegueta* (*A. melengueta*) and *Dennettia tripetala* (*D. tripetala*). *A. melengueta* is claimed to be very effective in the treatment of infectious diseases like tuberculosis, small pox, chicken pox and catarrh including worm infestations and gastrointestinal problems [11,12]. It has also been shown to possess antidiabetic, antihypertensive, aphrodisiac and testosterone-boosting properties [12,13]. *D. tripetala* G. Baker fruit is a common plant used in most communities for cold, fever, typhoid, cough, worm infestation, vomiting, stomach upset and as an appetite enhancer [14]. Scientifically, this plant has shown strong antinociceptic effect comparable to potent opioid agonists and non-steroidal anti-inflammatory drugs [14].

Therefore, in line with the multiplicity of claims and use of most plants including their possible concurrent use with conventional medications, this study evaluated the inhibitory effect of two commonly used herbs, *A. melengueta* and *D. tripetala* on CYP 3A enzymes using *in vitro* and *in vivo* models.

2. Materials and methods

2.1. Drugs

Simvastatin tablets, Teva® (Teva, United Kingdom) and dexamethasone injection, Ecnudexa injection® (Yanzhou, China) were obtained from a registered community pharmacy in Awka, Anambra state, Nigeria. Ketoconazole USP and pure erythromycin samples were respectively purchased from Aarti Drugs Ltd. and Century Pharmaceuticals Ltd. India.

2.2. Animals

Female albino rats of weight range 127–220 g obtained from the Faculty of Veterinary Medicine, University of Nigeria, Nsukka were used for this study. Acclimatization of these animals in the new environment lasted for 14 d prior to the commencement of the experiment with free access to feed and water. All animal experiments were conducted in line with NIH guide on the use and care of laboratory animals.

2.3. Plant material

A. melengueta pods and *D. tripetala* fruits were purchased from Onitsha main market, Onitsha, Anambra State, Nigeria. The collected samples were identified and authenticated by a taxonomist, Mr. Alfred Ozioko of Bioresource Development and Conservation Project Center, Nsukka, Enugu State, Nigeria and voucher specimens deposited in the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka.

Fruits of *A. melengueta* were obtained from the pods and air dried in the laboratory under room temperature. The air dried fruits were weighed, crushed with a blender and then divided into two equal parts. One part (250 g) was extracted with water (AAM) and concentrated using freeze drier while the other part (250 g) was extracted with 90 percent ethanol (EAM). The resulting solution was filtered and concentrated *in vacuo* using rotary evaporator at 40 °C.

D. tripetala fruits were air dried also in the laboratory under room temperature and divided into two parts. First part (592 g) was extracted with water (ADT) and concentrated using freeze drier while the second part (657 g) was extracted with petroleum ether (PEDT) by cold maceration for 7 d with intermittent shaking [15]. The extract was filtered using 24 cm Whatman filter paper and was concentrated using rotary evaporator at 40 °C.

2.4. In vitro effect of extracts on cytochrome P450 3A isoenzyme

The method of Wrighton *et al* [16] was used in this assay. The method was based on the principle of demethylation of erythromycin to des-N-methyl-erythromycin and formaldehyde by cytochrome P450 3A microsomal enzymes. Intestinal and liver microsomes used were prepared from liver and intestine excised from female rats and they were prepared using the modified methods described by Umathe *et al* [17]. Microsomal protein concentrations were determined by standard Biuret method using bovine serum albumin as standard.

2.5. Erythromycin-N-demethylation assay

The microsomal erythromycin N-demethylation activity was performed as previously described [18]. CYP3A4 activity was expressed as nM of formaldehyde obtained per milligram of protein per min calculated from the equation [19],

$$\text{CYP 3A Activity} = \textit{Amount of CHO produced}(n/mol) \times \frac{1}{25\ mg\ Protein} \times \frac{1}{10\ min}$$

2.6. In vivo cytochrome P450 3A activity assay

A modified method of Kanazu *et al*, [20] was used for this study in rat using simvastatin as the probe substrate of choice [21,22]. Female rats were divided into seven groups, A to G of 5 animals per group. Six groups (A–F) received dexamethasone (80 mg/kg ip) daily in three divided doses for three consecutive days, while group G received vehicle and served as dexamethasone untreated control group. After 24 h of last dose of dexamethasone and vehicle treatment, 400 mg/kg dose AAM, EAM, ADT and PEDT was orally administered to groups A–D respectively; ketoconazole, 5 mg/kg (positive control) was administered to group E; the control groups F and G received vehicle (1% Tween 20). Simvastatin (20 mg/kg po) was administered across the groups 1 h post extracts administration and blood samples collected in heparinised tubes at different time intervals. Blood plasma were obtained from the samples through centrifugation at 3 000 rpm for 15 min. The obtained plasma were stored at −20 °C prior to analysis.

2.7. Sample preparation and HPLC analysis of simvastatin

Modified method of Eggadi *et al* [23] as previously described [18] was adopted.

2.8. Determination of pharmacokinetic parameters

Non-compartmental methods as implemented in WinNonLin pharmacokinetic program (version 5.0), (Pharsight Corporation, Mountain View, California) was used to determine the various pharmacokinetic parameters including, time taken for drugs to attain maximal plasma concentration (T_{max}), maximal drug plasma concentration (C_{max}); last measurable drug plasma concentration (C_{last}); area under curve from the time of dosing to the time of the last observation (AUC), area under moment curve from the time of dosing to the time of last measurable concentration (AUMC), mean residence time (MRT), terminal half-life ($t_{1/2}$); volume of distribution based on the terminal phase (V_z), and total body clearance (Cl).

2.9. Statistical analysis

Obtained results were expressed as mean ± SEM and were considered statistically significant at $P < 0.05$. Test of significance were carried out with SPSS using one-way analysis of variance (ANOVA) test while multiple comparisons where determined using post hoc Dunnett's multiple comparisons test.

3. Results

3.1. Protein concentration assay

The concentration of microsomal protein was higher in the intestinal microsomes (616.94 mg protein) when compared with the liver microsomes (534.68 mg protein).

Table 1
Effect of herbal extracts on intestinal and liver microsomes (nM/mg protein/min).

Plant extracts	Concentration (μg)	Intestinal enzyme activity	Liver enzyme activity
ADT	50	21.2 ± 0.40	42.9 ± 0.02*
	100	15.4 ± 0.01*	40.3 ± 0.01*
PEDT	50	20.3 ± 0.00	36.2 ± 0.96
	100	17.9 ± 0.11	31.7 ± 0.03*
AAM	50	14.9 ± 0.01*	36.3 ± 0.02*
	100	16.1 ± 0.02*	35.0 ± 0.01*
EAM	50	20.2 ± 0.71	38.4 ± 0.34*
	100	20.0 ± 0.53	34.8 ± 0.32*
Ketoconazole	5 (μM)	14.4 ± 0.01*	34.2 ± 0.02*
Tween 20	1%	21.3 ± 0.51	101.7 ± 0.35

*$P < 0.05$ compared to Tween 20. ADT: aqueous extract of *D. tripetala*; PEDT: petroleum ether extract of *D. tripetala*; AAM: aqueous extract of *A. melengueta*; EAM: ethanolic extract of *A. melengueta*.

3.2. Effect of herbal extracts on intestinal and liver microsomes

The results of the *in vitro* effects of the extracts on both liver and intestinal microsomes as presented in Table 1 showed that the extracts produced variable effects on the intestinal microsomes. ADT significantly inhibited intestinal CYP 3A activity at 100 μg ($P = 0.01$) concentrations but not at 50 μg ($P = 0.40$) compared to the negative control. Similarly, intestinal CYP 3A activity was significantly inhibited by both concentrations of AAM ($P < 0.020$). However, 50 μg concentrations achieved more inhibition compared to the inhibition achieved with the 100 μg concentration. Both PEDT and EAM could not produce any significant reductions in intestinal CYP 3A activity at the used concentrations compared to the control group. On the other hand, all the extracts showed a direct dose dependent liver CYP 3A inhibition with the 100 μg of each of the extracts inhibiting the liver microsomal activity more than their corresponding 50 μg concentrations. While one way ANOVA showed significance difference in all the extracts, post hoc analysis indicated that aqueous extract of *D. tripetala* was significant ($P < 0.05$) at the two concentrations used compared to the negative control, the PEDT was not significant at 50 μg concentration. AAM and EAM were both significant at the two concentrations. PEDT showed more microsomal activity reductions than the positive control at 100 μg and was significant.

3.3. Assessment of in vivo CYP 3A activity

The pharmacokinetic parameters of simvastatin administered to albino rats with aqueous and non-aqueous extracts to both dexamethasone and vehicle pretreated rats and then, to simvastatin control groups were presented in Tables 2 and 3. The plasma concentration–time graphs used for the estimation of the pharmacokinetic parameters using WinNonlin pharmacokinetic program (Pharsight Corporation, Mountain View California) were presented in Figure 1. One way analysis of variance showed significant difference in all the parameters except $t_{1/2}$. However, further subgroup analysis (using LSD) indicated that pretreatment with dexamethasone and vehicle resulted in a significant decrease in C_{max} ($P = 0.005$), AUC ($P = 0.001$), and AUMC ($P = 0.005$) while $t_{1/2}$, V_z ($P = 0.001$) of simvastatin and Ke were increased compared to the non-dexamethasone treated control group.

Pretreatment with ADT significantly increased C_{max}, AUC, AUMC and MRT of simvastatin while clearance and volume of distribution were significantly reduced compared to the

Table 2
Comparative pharmacokinetic parameters of simvastatin (20 mg/kg) administered alone and with the aqueous extracts to dexamethasone and vehicle pretreated rats.

Parameter	Vehicle + SIMVA	DX + ADT + SIMVA	DX + AAM + SIMVA	DX + KETO + SIMVA	DX + Vehicle + SIMVA
T_{max} (h)	8.000 ± 1.000	3.000 ± 1.000*	4.500 ± 4.950	4.000 ± 0.000*	1.000 ± 0.000
C_{max} (μg/mL)	5.890 ± 0.010	4.390 ± 0.150*	3.430 ± 0.010	5.840 ± 0.010*	2.220 ± 0.020#
AUC (μg/ml/h)	83.300 ± 0.490	73.100 ± 0.720*	69.600 ± 0.790*	58.400 ± 0.120*	29.500 ± 0.350#
$t_{1/2}$ (h)	11.000 ± 1.170	74.000 ± 20.040*	73.000 ± 26.900*	10.000 ± 0.550*	26.000 ± 2.380#
Vz (mL/kg)	2.750 ± 0.360	5.770 ± 0.500*	6.040 ± 0.010*	3.750 ± 0.810*	11.840 ± 2.070#
Cl (mL/kg/h)	0.170 ± 0.010	0.060 ± 0.000*	0.062 ± 0.000*	0.260 ± 0.020	0.310 ± 0.050
AUMC (μg/mL/h^2)	955.300 ± 4.150	839.900 ± 5.630*	807.200 ± 9.350*	534.200 ± 6.030*	311.700 ± 4.880#
MRT (h)	11.470 ± 0.110	11.530 ± 0.360	11.610 ± 0.010	9.150 ± 0.180	10.540 ± 0.420
Ke (1/h)	0.044 ± 0.000	0.010 ± 0.000*	0.011 ± 0.000*	0.070 ± 0.000*	0.027 ± 0.000

*$P < 0.05$ compared to DX + Vehicle + SIMVA group; #$P < 0.05$ compared to vehicle + simva group ($n = 5$).

Table 3
Comparative pharmacokinetic parameters of simvastatin (20 mg/kg) administered alone and with the non-aqueous extracts to dexamethasone and vehicle pretreated rats.

Parameter	Vehicle + SIMVA	DX + PEDT + SIMVA	DX + EAM + SIMVA	DX + KETO + SIMVA	DX + Vehicle + SIMVA
T_{max} (h)	8.000 ± 1.000	4.000 ± 0.000	3.000 ± 1.000*	4.000 ± 0.000*	1.000 ± 0.000
C_{max} (μg/mL)	5.920 ± 0.010	5.540 ± 0.040*	3.370 ± 0.010	5.420 ± 0.010*	2.220 ± 0.020#
AUC (μg/mL/h)	83.300 ± 0.490	69.600 ± 0.600*	49.900 ± 0.220*	58.400 ± 0.120*	29.500 ± 0.350#
$t_{1/2}$ (h)	11.000 ± 1.170	18.000 ± 5.740	30.000 ± 3.040	10.000 ± 0.550	26.000 ± 2.380
Vz (mL/kg)	2.750 ± 0.360	3.920 ± 1.260*	7.100 ± 3.400	3.750 ± 0.810*	11.840 ± 2.070#
Cl (mL/kg/h)	0.170 ± 0.010	0.160 ± 0.070	0.170 ± 0.030	0.260 ± 0.020	0.310 ± 0.050
AUMC (μg/mL/h^2)	955.300 ± 4.150	746.500 ± 11.370*	541.400 ± 2.610*	534.200 ± 6.030*	311.700 ± 4.880#
MRT (h)	11.470 ± 0.110	10.660 ± 0.710	10.840 ± 0.040	9.150 ± 0.180	10.540 ± 0.420
Ke (1/h)	0.044 ± 0.000	0.042 ± 0.010	0.024 ± 0.000	0.070 ± 0.000*	0.0270 ± 0.000

*$P < 0.05$ compared to DX + Vehicle + SIMVA group; #$P < 0.05$ compared to vehicle + simva group ($n = 5$).

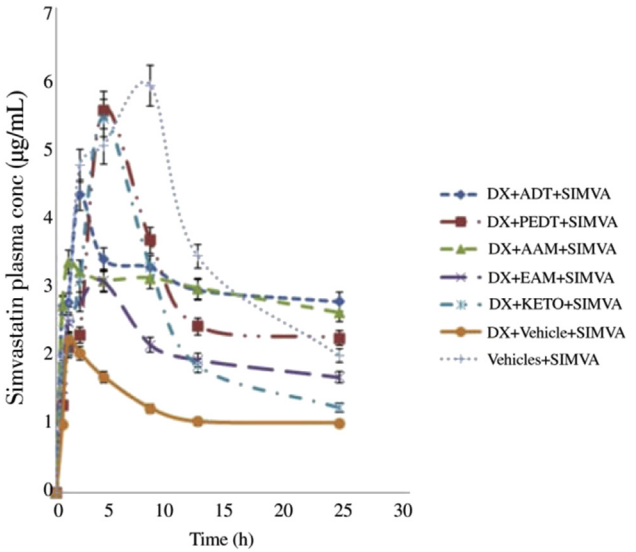

Figure 1. Plasma concentration–time curve of simvastatin (20 mg/kg po) administered with extracts to dexamethasone/vehicle pretreated rats.
DX: dexamethasone; KETO: ketoconazole; SIMVA: Simvastatin.

dexamethasone and vehicle treated group. Similarly, pretreatment with AAM led to significant increase in AUC and AUMC with a non-significant increase in C_{max} and T_{max} ($P > 0.05$). PEDT and EAM showed significant increase in C_{max}, AUC, AUMC and significant decrease in volume of distribution with almost no change in MRT. Furthermore, though clearance decreased with PEDT and EAM pre-administration, they were not significant.

4. Discussion

The study assessed the *in vitro* and *in vivo* effects of *D. tripetala* and *A. melengueta* extracts on CYP450 3A isoenzyme. The result indicated inhibitory activity of the extracts on the enzyme. Some constituents of herbal extracts have been shown to alter CYP450 enzymes causing potential drug interactions [24].

The small intestine, in addition to its primary role of absorption, has the ability to metabolize drugs and other xenobiotics through phase 1 and phase 11 metabolisms [25] since almost all the cytochrome P450 enzymes present in the liver are also present in the small intestine although, in lower concentrations. The higher microsomal protein detected in the intestinal microsome in this study may not necessarily indicate higher cytochrome P450 content than the liver fraction.

Constructed on the principle that CYP3A converts erythromycin to N-demethyl erythromycin and formaldehyde which determines CYP 3A activity based on the quantity of formaldehyde formed, the result of the study on intestinal microsomes indicated that ADT and AAM significantly inhibited intestinal CYP 3A activity when compared to the negative control. However, the greater inhibition achieved with the 50 μg concentration of AAM compared to the 100 μg concentration may be due to saturable inhibition that may result in saturable first pass metabolism with the rate of inhibition being concentration dependent. This saturable inhibition has been shown to occur more with intestinal enzymes [26]. The inhibitions observed with the 100 μg concentrations of PEDT and EAM with no inhibitions with their 50 μg concentrations was indicative of dose dependent activity. Similarly, the result of this study on liver microsomes indicated that all the extracts exhibited a direct dose dependent liver CYP 3A inhibition with the 100 μg of each of the extracts inhibiting the liver microsomal activity more than their corresponding 50 μg concentrations.

The greater inhibition of the intestinal microsomal CYP 3A activity compared to that of the liver similar to other studies [17] may be due to higher concentrations of CYP enzymes in liver microsomes. Hence, higher metabolic clearance is seen in the liver preparations than intestinal preparations [26,27].

The effect of the extracts on CYP 3A was further assessed using the *in vivo* method. Using simvastatin as the substrate of choice [21,22], the *in vivo* assay method was built on the principle that inhibition of a major metabolising enzyme responsible for the metabolism of an orally administered agent will lead to an enhanced plasma concentration of the agent. Simvastatin is majorly metabolised by CYP3A4 with minor contribution of CYP3A5 [28,29].

Since AUC and C_{max} are used to measure the extent of drugs bioavailability [30], the significant ($P < 0.05$) increases in AUC, AUMC and C_{max} of simvastatin observed in the extracts treated groups were indicative of enhanced absorption and bioavailability of the drug with possible suppression of CYP 3A enzyme responsible for the drug's metabolism. The significant increase in these parameters in the vehicle and simvastatin treated group compared to the dexamethasone, vehicle and simvastatin treated group is indicative of the role of dexamethasone in inducing CYP 3A enzymes in the rats. Dexamethasone is a potent CYP 3A inducer and is used in inducing this enzyme family in animal studies [31]. Although dexamethasone treated liver microsomes has been an accepted model for evaluating drug interactions involving CYP450 3A enzyme inhibition [20] as reported by Nduka *et al* [18]; findings

by Perloff *et al* [32] likewise indicated that dexamethasone also induces P-glycoprotein in rats in addition to CYP3A. Hence, possible effect of dexamethasone on P-glycoprotein may have also contributed to the observed differences in simvastatin dispositions in these two groups. On the other hand, ketoconazole is a potent and well-studied inhibitor of CYP 3A enzyme in humans and animals and often used for *in vitro* and *in vivo* studies as the CYP 3A inhibitor of choice [33]. Therefore, ketoconazole's use as a positive control in dexamethasone and simvastatin treated group showed C_{max}, AUC and AUMC significantly higher than the vehicle treated group showing its potent CYP 3A inhibition. This inhibition of the metabolizing enzyme family by the extracts was further shown by the decreased clearance of the drug in the extracts treated groups and their corresponding increased MRT though not all were significant. Hence, these suggested the *in vivo* cytochrome P450 3A inhibition by the extracts though, at different degrees. Furthermore, it is important to state that P-glycoprotein may have also been inhibited by these extracts because CYP 3A enzymes and P-glycoproteins have been reported to share similar modulators [34].

Since transporter proteins could be modulated by mechanisms similar to those of cytochrome P450 enzymes, the inability of this study to evaluate the possible effect of the extracts on transporter proteins especially, P-glycoproteins and their contributions to the observed results was one of the major limitations of this study. However, this forms the bases of our further research.

In conclusion, this study indicated that *D. tripetala* and *A. melengueta* extracts inhibited CYP 3A enzyme both *in vitro* and *in vivo*. Although these inhibitions may appear to be weak from the US FDA classification point of view, caution must be applied in the concurrent use of herbal preparations containing these extracts with conventional medications to avoid possible herb-drug interactions.

Conflict of interest statement

The authors declare no conflict of interest.

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