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Effect of *Vernonia Amygdalina* Leaf on Cytochrome P450 2D6- and 3A4-Mediated Metabolism of Dextromethorphan in Healthy Nigerian Subjects

Mariam Olaide Oladepo[1], Adebanjo Jonathan Adegbola[1], Julius Olugbenga Soyinka[1*] and Cyprian Ogbona Onyeji[1]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Authors JOS and COO designed the study. Authors MOO, AJA and JOS performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MOO and AJA managed the collection of samples and analyses of the study. Authors MOO, AJA and JOS managed the literature searches. All authors participated in the final analysis of data, and they all read and approved the final manuscript.

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ABSTRACT

Back-ground and Objectives: The study's focus is to investigate the effects of *Vernonia amygdalina* on the metabolic activities of Cytochrome P_{450} 3A4 and 2D6 in vivo. The assessment was based on CYP2D6-mediated O-demethylation and CYP3A4-mediated N-demethylation of dextromethorphan (DEX) to Dextrorphan (DOR) and 3-methoxymorphinan (3-MM), respectively.
Methods: The clinical study followed a two-phase cross over study with two weeks washout period. Volunteers received a single oral dose of DEX 30 mg alone in phase 1 and along with last dose of *V. amygdalina* leaf powder in phase 2. 8-hour urine samples were collected in both phases post-administration of DEX and analyzed using HPLC-UV. The chromatographic separation of DEX, DOR, 3-MM, and Imatinib was achieved on a C18 column. The analytes were eluted with a gradient elution consisting of 50mM potassium dihydrogen phosphate (pH 5)-acetonitrile at a 1

**Corresponding author: E-mail: juliussoyinka@gmail.com;*

mL/min flow rate, and detected at 280 nm. Activities of the enzymes investigated were evaluated using the urinary metabolic ratios of DEX:DOR and DEX:3-MM.
Results: Median (interquartile range) values for the metabolic ratios of DEX:DOR was 0.032 (0.028-0.246) and 0.029 (0.018-0.061) for phases with and without *V. amygdalina* respectively, while the average median values for DEX:3MM was 5.087 (3.692-71.420) and 5.609 (3.093-19.197) for phases with and without *V. amygdalina* respectively. However, the differences between both phases were not significant for both isoenzymes.
Conclusion: *V. amygdalina* does not significantly affect the activities of CYP2D6 and CYP3A4 *In vivo*, which indicates that it has minimal potential to interact with the substrates of both isoenzymes.

Keywords: Vernonia amygdalina; cytochrome P450 2D6; cytochrome P450 3A4; dextromethorphan.

1. INTRODUCTION

It has been reported that approximately 80% of the African population use some form of herbal/conventional medicine with no doubt that its use is growing exponentially due to the common belief that herbs are safe [1]. This has generally increased interest in research on alternative and complementary medicines for chronic and acute conditions. These herbs have chemicals known as phytochemicals, with the possibility of interacting with drugs when co-administered. There are various known drug-herbal interactions, which could occur by interaction with metabolism which plays a vital role in the disposition of drugs (pharmacokinetics), and the therapeutic effects (pharmacodynamics) of the drugs [2]. Oxidative biotransformation, the most typical form of drug metabolisms, requires a reducing agent (NADPH), molecular oxygen, and a complex of microsomal enzymes known as cytochrome P450 (CYP) enzymes [3]. Although they can be classified into more than 50 isoenzymes, six of them, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A5, are responsible for the metabolism of 90 percent of drugs [4,5]. The two most significant enzymes being CYP3A4 and CYP2D6 metabolizing up to 50% and 25% therapeutically available drugs respectively [6]. Drugs may be metabolized by only one CYP enzyme (e.g., metoprolol by CYP2D6) or multiple enzymes (e.g., dextromethorphan by CYP2D6, and CYP3A4) [7–10]. Likewise, some xenobiotics induce or inhibit the expression or activities of these metabolizing enzymes and are referred to as either inducers or inhibitors.

Dextromethorphan (DEX) is a commonly used marker for major metabolizing enzymes, CYP2D6 and CYP3A4, for in vivo studies in healthy human subjects [11–15]. DEX is a well-tolerated antitussive associated with a few side effects, and it is known to be metabolized to three major metabolites - dextrorphan (DOR), 3-hydroxymorphinan (3HM) and 3-methoxymorphinan (3MM) shown in Fig. 1 [10,16,17]. CYP2D6 is the prime isoform catalyzing O-demethylation of DEX to DOR, and CYP3A4 is the main enzyme mediating DEX N-demethylation to 3-MM [18]. The metabolic ratio (MR) of DEX:3MM in urine samples at any of the following; 0–2 h, 0–4 h, 0–6 h, 0–8 h, 0–12 h, is useful in determining the CYP3A4 activity as they correlate significantly with the MR of 0-24 h urine collection [10,19]. The metabolic ratio of DEX to DOR can also be used as an index to phenotype CYP2D6 with poor metabolizers having MR >0.3 [20–22].

Over time the research on herb-drug interaction has gone beyond just the hypothetic studies to identify the mechanism of interactions. Recent studies further verify how exactly these plants affect the metabolism of drugs by interacting with humans' cytochrome enzyme activities. Aqueous extracts of *Acacia catechu, Andrographis paniculata, Arctium lappa, Areca catechu, Bupleurum marginatum, Chrysanthemum indicum, Dysosma versipellis,* and *Spatholobus suberectus,* as well as grapefruit and seville orange juice have been reported to inhibit CYP3A4 [23–25]. *Curcuma longa* and *Catha edulis* have shown great potential to inhibit the metabolic activities of CYP2D6. In contrast, black seed, St john's wort, and honey, commonly regarded as safe, have been reported to induce CYP3A4 enzyme activity [11,12,26–29]. All these directed studies have been used to provide a generalized informed effect of these plants on a series of drugs when used concomitantly, once the primary enzyme responsible for metabolism is known.

Vernonia amygdalina, commonly called bitter leaf, is a typical stable vegetable amongst the West Africans. It contains phytochemicals such as saponins, alkaloids, terpenes, steroids,

coumarins, flavonoids, phenolic acids, lignans, xanthones, anthraquinones, and edotides [30–34]. The leaf's' ethnobotanical uses include an appetite stimulant, antipyretic and herbal drug for uncomplicated malaria in Nigeria and some other African countries [34–36]. The use of its aqueous extract in treatment of uncomplicated malaria has been further proven in a clinical trial conducted in Uganda [36]. Many naturopathic doctors have recommended the aqueous extracts of *V. amygdalina* for their patients as a treatment for emesis, nausea, diabetes, loss of appetite, dysentery, and other gastrointestinal tract problems as an anthelmintic, laxative/purgative, enema, expectorant, worm expeller and fertility inducer. It is also known to be a chemo therapeutic agent as *V. amygdalina* extracts have been reported to render cancerous cells to be more sensitive to chemotherapy [30,34,37–43].

Bitter leaf contains numerous phytochemicals, any of which may also undergo various interactions with drugs. It has been proven to affect the metabolic parameters of nifedipine, dihydroartemisinin, and chlorpropamide [44–46]. Therefore, these studies give a lead for future investigations on the potential of *V. amygdalina* as a significant plant in herb-drug interaction research, hence this study. This study focuses on evaluating the modulating potential of *V. amygdalina* on major metabolizing enzymes, CYP3A4 and CYP2D6, as reported in previous researches on various herbs using an average of 5 healthy volunteers [13–15,47].

2. MATERIALS AND METHODS

Dextromethorphan syrup (Benylin® dry cough syrup (7.5mg/5mL) was purchased from a retail pharmacy in Ibadan, Oyo State, Nigeria. Reference standards of; dextrorphan and dextromethorphan were purchased from Sigma Aldrich® (MO, USA), while 3- methoxymorphinan was purchased from Clearsynth® chemicals; (Mumbai, India), and imatinib from AK Scientific® (CA, USA). HPLC mobile phase, acetonitrile, was HPLC grade and purchased from Fischer Scientific (MA, USA) while the other solvents; potassium dihydrogen phosphate (KH_2PO_4) from Burgoyne Burbidges & Co (Mumbai, India); Sodium hydroxide (NaOH) from LOBA Chemie (Mumbai, India); orthophosphoric acid (H_3PO_4), hydrochloric acid (HCl), sodium carbonate (Na_2CO_3) and potassium hydroxide (KOH) from Merck (Darmstadt, Germany) were analytical grade.

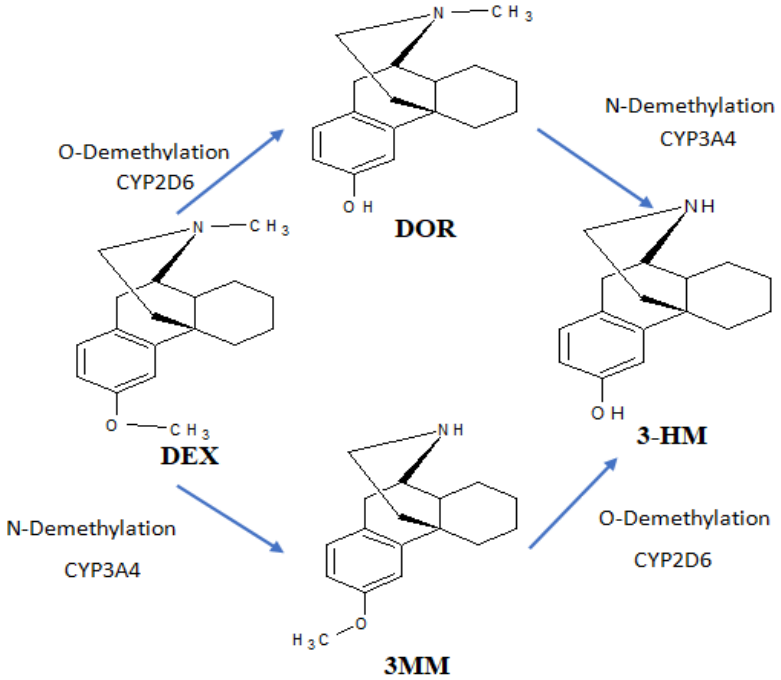

Fig. 1. Dextromethorphan O- and N-demethylation pathways catalyzed by CYP2D6 and CYP3A4/5

2.1 Collection and Processing of *V. amygdalina* Leaves

The leaves of *V. amygdalina* Delile were harvested on a farm at Ibadan, Nigeria. The plant's authenticity was verified at the Department of Botany, Obafemi Awolowo University, Ile Ife, Osun State, Nigeria (voucher number IFE17743). The leaves were washed with 0.9% sodium chloride solution and distilled water to remove dirt and debris without squeezing the leaves. The leaves were then air-dried under a shed for three weeks and the dried leaves were grounded with a local grinding machine into a coarse powder (≤ mesh 35). The powders were then uniformly capsulated into size 0 capsule shells using a semi-automatic capsule filling with each capsule containing 370mg of the *Vernonia amygdalina* powder. The capsules were then stored in an airtight amber coloured container until administered to the volunteers.

2.2 Study Design

Ethical approval (reference number: IPH/OAU/12/1215) was obtained from the Institute of Public Health, College of Health Sciences, Obafemi Awolowo University prior to commencement of the study. This was a two-phase, cross-over study with a two-weeks wash out period conducted among healthy volunteers. The study participants were with no diagnosed medical condition and were between the age of 18 and 45. The volunteers were asked to abstain from any conventional drug, herbal medicines, grapefruit juice, at least two weeks before and during the study period. They were also asked to avoid caffeine consumption at least 24 hours before and during the study. Smokers, people hypersensitive to DEX, as well as pregnant or lactating women were excluded from the study. Twenty healthy-10 males and 10 female volunteers with acceptable eligibility for the research were recruited from the community of the Obafemi Awolowo University, Ile- Ife, Osun State, Nigeria. The volunteers were taken through the research procedures and were then given a fully detailed subject information sheet and informed consent form to sign. The volunteers' information was recorded anonymously to maintain confidentiality.

2.2.1 Drug administration and collection of urine samples

The administration of drugs and sample collection followed the pattern shown in Fig. 2. During the first phase, the volunteers were given a 30 mg single dose of dextromethorphan hydrobromide (20 mL of Benylin® syrup). The volunteers were asked to empty their bladder pre-dosing with dextromethorphan, and urine samples were collected over 8 hours post-dosing. After two weeks wash-out period, phase two of the study was carried out. The same volunteers were given a daily dose of 5.55 g (15 capsules) of encapsulated powder of *V. amygdalina* in divided doses every 8 hours for seven days. On the 7th day, the volunteers were given 30 mg dextromethorphan hydrobromide alongside the last dose of the capsules, and urine samples were collected as was done in phase I. The urine samples were collected in a 1-liter collection bottle, total volume of the urine voided was measured, recorded, and an aliquot of 10 ml was collected in a urine sample collection tubes and samples were stored at -70^{o}C till analysis.

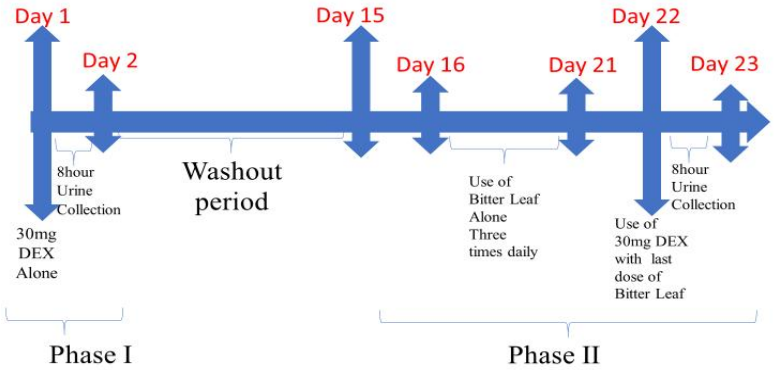

Fig. 2. Depiction of the study design showing time of drug use and sample collection

2.2.2 Extraction of dextromethorphan and its metabolites from urine samples

To 2mL urine, 880µL 10N hydrochloric acid was added and heated at 85°C for 1 hour to hydrolyse the conjugated DEX metabolites according to a method described by *Daali et al.* [48] After hydrolysis, 50uL of internal standard (imatinib 2µg/ml) was added and alkalinized with 1.1mL 10N KOH and 1mL Na_2CO_3 to achieve a pH of 10±0.5. This was then extracted with 6mL Diethyl ether/Hexane 95:5 v/v, mixed for 20 seconds and vortex mixed for 1 minute and then centrifuged at 14500G for 20 minutes. The organic layer extract was transferred into another centrifuge tube and then vigorously mixed gently with 100 μL of 50mM orthophosphoric acid and centrifuged at 14500G for 20 minutes at room temperature. The aqueous layer was collected into Eppendorf tubes and injected into HPLC for detection.

2.3 HPLC-UV Detection

HPLC-UV method was used for analysis. The liquid chromatographic system was an Agilent 1100 series instrument, made up of quaternary pumps, a gradient mixer, a diode array (DAD) and detection was done at the wavelength 280nm. The injection was by a Rheodyne fitted with a 20µL loop and an on-line vacuum degasser. The column used was a reversed phase (C_{-18}) silica with 5µm particle size and 150 x 4.6 mm I.D -Agilent, Santa Clara, (CA, USA). A gradient system which consisted of mobile phase A of 50mM potassium dihydrogen phosphate and mobile phase B of acetonitrile with a run time of 14.0min was pumped through the column at a flow rate of 1 mL/min. The aqueous mobile phase's pH was adjusted to 6 with concentrated sodium hydroxide and the analytical run was performed at ambient temperature. 80% of the mobile phase A was run for the first 4min, then reduced to 65% of mobile phase A and maintained for 8 min before returning to initial concentration of 80% of mobile phase A till 14minutes.

2.3.1 Estimation of metabolic ratio

The results of the concentrations of the dextromethorphan and its metabolites were compiled in an Excel 2016 file. The urinary metabolic ratio (equations 1 and 2) with and without *V. amygdalina* were estimated to indicate the metabolic activities of CYP2D6 and CYP3A4 respectively.

$$DEX{:}DOR\ MR = \frac{Total\ amount\ of\ DEX\ voided\ in\ 8hrs\ urine}{Total\ amount\ of\ DOR\ voided\ in\ 8hrs\ urine} \quad (1)$$

$$DEX{:}3MM\ MR = \frac{Total\ amount\ of\ DEX\ voided\ in\ 8hrs\ urine}{Total\ amount\ of\ 3MM\ voided\ in\ 8hrs\ urine} \quad (2)$$

2.4 Statistical Analysis

For the clinical study, the statistical analysis included data from the subjects who completed the study. The statistical comparison of the phases (DEX with or without *V. amygdalina*) was performed using the Student's paired t-test. The differences were considered statistically significant when P-values were <0.05. Statistical analysis was conducted and figures were constructed using Graph-Pad Prism5. Posthoc analysis was also conducted using G*Power 3.1, to determine the power of the data used in the study.

3. RESULTS

3.1 Subject Demographic Features

Twenty volunteers were recruited but 14 were able to complete both phases of the study between July 2019 and October 2019 of which 43% (n=6) were females and 57% (n=8) were males. One of the drop outs was due to the observation of adverse effect - nausea and vomiting while using *Vernonia amygdalina* in the phase II of the study, three volunteers had to commence a different treatment therapy during the study due to development of other clinical conditions, while two volunteers were dropped due to lack of continuous commitment.

3.1.1 Chromatographic analysis of DOR, 3MM and DEX

The retention time for DOR, 3MM, DEX and the internal standard IMB were 3.9, 8.3, 9.05 and 11.2 min respectively as shown in Fig. 3. Blank urine samples spiked with the standards solutions of the investigated drugs and the internal standard (IMB) also showed retention times for all compounds in the sample chromatograms corresponded to those obtained from the reference compounds when injected neatly.

3.2 Estimation of Metabolic Ratio

The urinary concentrations of DOR were successfully measured for all the study participants. However, data were insufficient to

estimate the metabolic ratio for CYP3A4 and CYP2D6 in some subjects as the urinary concentrations of DEX and 3MM were only quantifiable in seven and four subjects respectively as seen in Table 1. Fig. 4 shows the graphical change in the metabolic ratios between both phases for patients with complete data. There was no significant difference in the MR, DEX:DOR, irrespective of the presence of *V. amygdalina* with p value of 0.3601. Likewise, there was no significant difference in the MR, DEX:3MM, irrespective of the presence of *V. amygdalina* with p value of 0.3965.

4. DISCUSSION

This research is expected to be used to extrapolate the effect of *V. amygdalina* on drugs generally metabolized by the CYP2D6 and 3A4 in patients who plan to concomitantly use the herb with any of such medications or in patient whose stable food includes the bitter leaf. The encapsulation of the powder ensured constant dosing, with no deviation from standard by the British Pharmacopeia [49] as well as aid the compliance of study participants as the plant is known to be a bitter plant which would have otherwise discouraged its use. The cross-over study used in this research eliminated errors due to inter-individual variability regarding the expression of the metabolizing enzymes being investigated.

While DEX O-demethylation has been widely accepted to assess CYP2D6 metabolic activities both in vitro and in vivo, limitations of using DEX N-demethylation as a measure of human CYP3A4 in vivo activity have been reported [18,50]. Nevertheless, the use of DEX as a dual probe to phenotype both CYP2D6 and CYP3A4

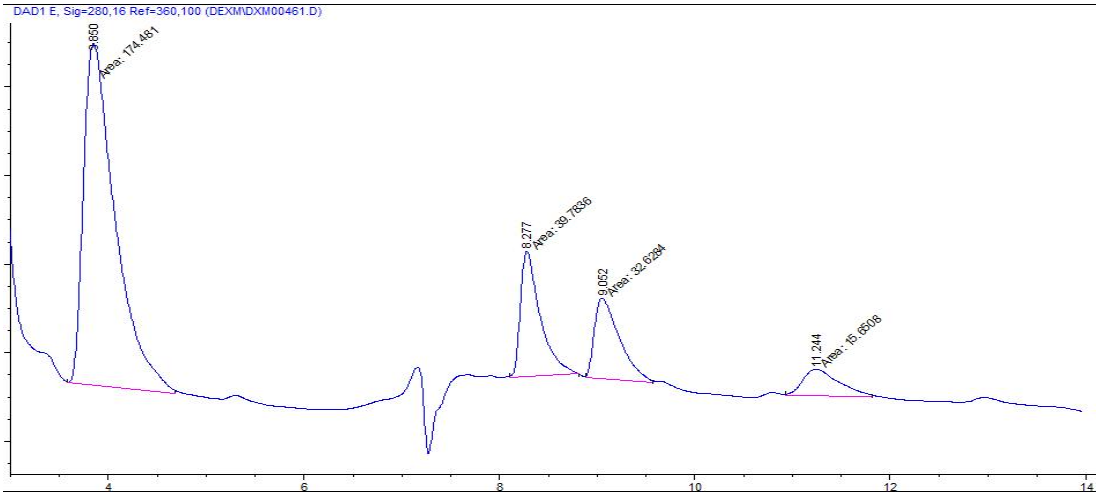

Fig. 3. HPLC chromatogram following direct injection of test samples containing DOR-8ug; 3MM- 4ug; DEX-4ug; IS- 1ug. The peak eluting at 3.9, 8.3, 9.05 and 11.2 represents DOR, 3MM, DEX and the internal standard IMB

Table 1. Urinary metabolic ratios estimating CYP3A4 (A) and CYP2D6 (B) with and without *V. amygdalina*

Code	DEX without VA	DEX with VA	DEX without VA	DEX with VA
	MR=$\left(\frac{DEX}{DOR}\right)$	MR=$\left(\frac{DEX}{DOR}\right)$	MR=$\left(\frac{DEX}{3MM}\right)$	MR=$\left(\frac{DEX}{3MM}\right)$
FA	0.032084	0.022221	*	*
FD	0.00499	0.010589	*	*
FE	4.302642	35.52744	0.036045	0.032374
MB	0.316334	0.059521	137.5774	31.289
MC	0.036273	0.061961	5.262275	7.104115
MH	0.02816	0.013445	4.911146	4.113267
MI	0.027787	0.028613	*	*
P value	0.3601		0.3965	

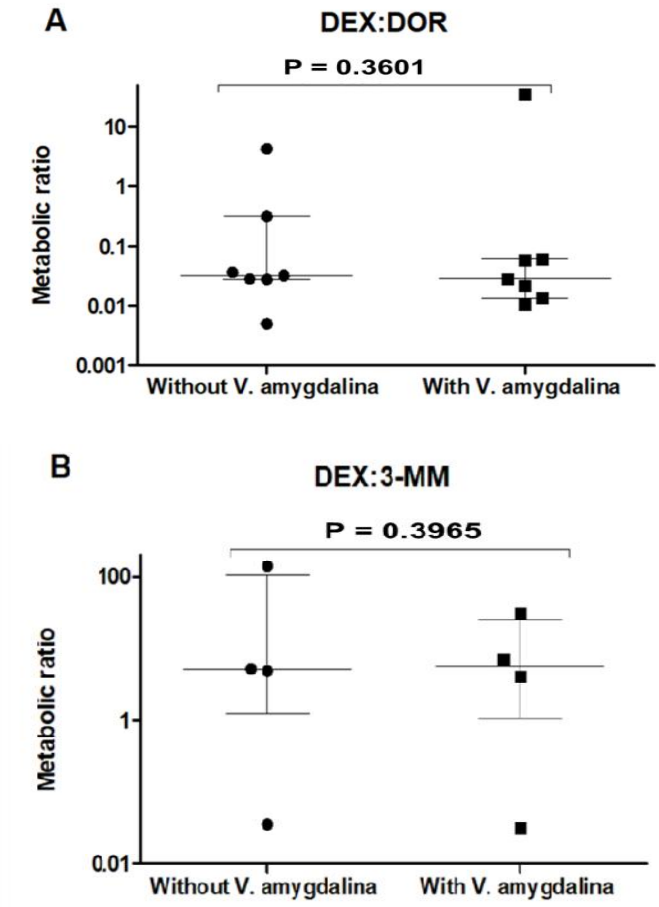

Fig. 4. Graphical representation of change in metabolic ratios estimating CYP3A4(A) and CYP2D6(B) with and without *V. amygdalina.*

activities has been continually established to be practicable and acceptable [10–14,18,51]. The gradient elution used for the HPLC-UV analysis reduced the run time due to the difference in lipophilicity between the analytes with log P=3.46, 3.86, 4.11, 4.38 for DOR, 3MM, DEX and Imatinib respectively.

From the 14 subjects who were able to complete the study, there was enough data to phenotype 10 of them, of which 40% (N=4) were females and 60% (N=6) were males. Based on their CYP2D6., it showed that 20% (N=2) were poor metabolizers with MR>0.3 [22]. The remaining 80% (N=8) were phenotypes as extensive metabolizers with MR falling below 0.3. However, there was no significant difference ($p > 0.05$) between MRs with and without *V. amygdalina*, inferring that it has no significant effect on the metabolic activity of CYP2D6 metabolism of dextromethorphan. The same trend was observed for the metabolic activity of CYP3A4.

Our findings contrast the conclusion of an in vivo animal model which revealed that the extract of *V. amygdalina* altered the pharmacokinetics on nifedipine. The area under the curve (AUC), maximum plasma concentration (C_{max}), elimination half-life, and bioavailability were affected in rabbits [44]. As CYP3A4 mainly metabolizes nifedipine, the study concluded that *V. amygdalina* modulated the activity of CYP3A4. This contradiction might have arisen from the use of rabbits to determine the CYP3A isoenzyme activities as it is not easy forthright to extrapolate or replicate findings in the animal model to another species [52]. It could also be as a result of the higher dose of extract of 500mg/kg used in the animal model suggesting that a higher dose of the herb could modulate the CYP3A4 activity. To the best of our knowledge, no human study

has been done to suggest the effect of *V. amygdalina* on the activities of CYP2D6 or conducted to suggest the effect of *V. amygdalina* on the activities of CYP enzymes, unlike it has been done to other plants.

Additionally, some plants such as fenugreek, rosemary, garlic and asafetida have also been established to have an insignificant effect on drug metabolism by CYP2D6 [13,14,53]. Similarly, fenugreek, and Curcuma did not affect the activity of CYP3A4 significantly [12,14]. However, the present study does not completely eliminate the possibility of a significant herb-drug interaction as some plants have been established to affect other minor pharmacokinetic routes by influencing drug-transporter proteins such as p- glycoproteins [53–58].

The present study suggests that no dose adjustment is necessary for patients who regularly use *V. amygdalina* alongside their conventional medicines known to be metabolized by CYP2D6 and CYP3A4. However, the post hoc analysis done produced a power lower than desired, that is; <0.8 [59,60] showing that there is a high probability of incorrectly rejecting H0 when is in fact true and vice versa.

5. CONCLUSION

In conclusion, further studies are needed to clarify whether or not *V. amygdalina* has any significant effect on the activities of both CYP2D6 and CYP3A4.

6. LIMITATION

The encapsulation despite being an advantage for compliance could pose as a limitation in the study. This is due to the uncertainty of the effect of capsulation on the absorption of the *V. amygdalina* as dosage form has been reported to have an effect on the absorption on drug [9,61,62].

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

The volunteers were taken through the research procedures and were then given a fully detailed subject information sheet and written consent form to sign. The volunteers' information was recorded anonymously to maintain confidentiality.

ETHICAL APPROVAL

Ethical approval (reference number: IPH/OAU/ 12/1215) was obtained from the Institute of Public Health, College of Health Sciences, Obafemi Awolowo University prior to commencement of the study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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