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Effects of *Vernonia amygdalina* Aqueous Leaf Extract on the Pharmacokinetics of Nifedipine in Rabbits

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A number of diabetic/hypertensive patients in Nigeria, use a flavonoid containing antidiabetic herb, *Vernonia amygdalina*; alongside nifedipine which is readily prescribed; and concomitant administration of herbal preparations with conventional drugs can modified the pharmacokinetic profile of a drug. Therefore we investigated possible influence of the aqueous extract of this herb on the pharmacokinetic profile of nifedipine using animal model. The animals were treated with or without *V. amygdalina* extract, blood samples were collected at different time interval and assayed for the concentration of nifedipine. The C_{max} , $t_{1/2el}$, $AUC_{0-\infty}$ and F were significantly higher ($P \leq 0.05$) while Cl was lower in the treated group than the control. No significance ($P \geq 0.05$) was seen in the T_{max} between the two groups. We conclude that concomitant use of *V. amygdalina* reduced the metabolism of nifedipine evident by decrease in clearance and increased F and AUC thus altering its pharmacokinetic profile.

Keyword: Nifedipine, Diazepam, Herb-Drug Interaction, Pharmacokinetics, Pharmacokinetics

1. Introduction

A growing percentage of the population use herbal products for preventive and therapeutic purposes. These herbal products contain numerous pharmacological active constituent – essential oil, tannins, alkaloid, saponins, glycosides, anthraquinones and flavonoids all of which may participate in herb-drug interaction. A study of interaction between herbal preparations and drug products will help in the understanding of the safety of concomitant use of these drugs.

Herbal-drug interactions can cause significant side effects, which occurs when the pharmacokinetic profile of a drug is modified by prior concomitant administration with herbal preparation^[1,2,3]. This interaction can impact strongly on pharmacotherapy which includes:- increasing the side effect, decreasing the

therapeutic effect, modifying the action or enhancing the therapeutic effect of drugs. These effects may consequence in toxicity, treatment failure or loss of the drug therapeutic effects, complication or overdose^[4,5].

Hypertension which is defined as a condition characterized by sustained elevated arterial blood pressure greater than 140/90 mmHg is in the increase. In 2000, 972 million people had hypertension with a prevalence rate of 26.4 %. This is projected to increase to 1.54 billion affected individuals and a prevalence rate of 29.2 % in 2025^[6]. This non-communicable disease has a prevalence of 28.3 % in Accra, Ghana, 32.8 % in Nigeria, 16.9% in Cameroon and 32.6% among the African Americans^[7-10]. Hypertension is frequent in patients with diabetes compared with

patients without the disease. Studies have suggested that hypertensive persons are more predisposed to the development of diabetes than are normotensive persons and presence of hypertension and diabetes in a patient are major risk factors in the development of coronary heart disease^[11,12]. The prevalence of coexisting hypertension and diabetes appears to be increasing in industrialized nations because populations are aging and both hypertension and NIDDM incidence increases with age^[13]. The WHO statistics 2012 has reported that one in three adults worldwide has raised blood pressure with one in every ten adults hypertensive patients being diabetic^[14]. The coexistence of diabetes and hypertension in the same patient is devastating to the cardiovascular system and blood pressure control in these patients is a great challenge. In our populace, high cost of available synthetic drugs for the treatment of these ailments has led most of these patients to resort to the use of herbal preparations as an alternative to the treatment of one of the ailments with little or no scientific prove on their possible interaction. It is thought that concomitant use of herbal preparation and an orthodox antihypertensive agent may result in untold consequences.

A host number of diabetic patient in Nigeria, have found good use in *Vernonia amygdalina* (Linn), a herb used in lowering blood glucose in diabetic condition^[15] also nifedipine is readily prescribed for significant number of hypertensive patients. Given widespread use of *Vernonia amygdalina* and in the light of the consideration that her-drug interaction is an important safety concern, we provide here the possible effect if any of the extract of the leaves of *Vernonia amygdalina* on the pharmacokinetic profile of nifedipine with a view of rendering correct advise on their uses.

2. Materials and Method

2.1 Plant Collection and Extraction

Leaves of *Vernonia amygdalina* Linn. (Voucher number: FHI 104046) previously authenticated in Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria by Mr. G. Ighanesebhor was harvested from Ikotun area of Lagos State,

Nigeria. The leaves were dried at room temperature in a light/dark cycle (12:12 h) for 5 days. The dried leaves were milled into powder and extracted by exhaustive soxhlet extraction into water for 30 hours (pH 5.65). The aqueous extract was lyophilized using freeze dryer to obtain a yield of 72.62 g (32.28 % w/w) from 225 g of the powdered plant material. The crude extract residue was stored in impervious amber bottle at 4°C until used.

2.2 Animals

Fourteen healthy rabbits of either sex weighing between 1.6 and 2.1 kg were purchased from a local market in Lagos State, Nigeria. The rabbits were housed at room temperature in a well-ventilated Laboratory Animal Centre of the College of Medicine of the University of Lagos, Nigeria under standard laboratory condition (12:12 h dark/light cycle). They were cared for and used in accordance with the Institute of Laboratory Animal Research (ILAR) guidelines for care and use of animals in experimental studies^[16]. The animals were allowed to acclimatize for 21 days before the commencement of the study during which they had free access to standard animal chow (Pfizer Feeds, Ibadan, Nigeria, Plc.) and water *ad libitum*.

2.3 Chemicals and Reagents

All chemicals were of analytical reagent grade, purchased from sigma chemical company (St. Louis, MO) unless otherwise stated. HPLC grade acetonitrile and methanol were from British Drug Houses (BDH Chemicals Poole, UK). Tablets of nifedipine were locally purchased. The internal standard, diazepam was a gift from Swipha Pharma Nigeria limited Lagos, Nigeria.

2.4 Chromatographic System and Conditions

Hewlett-Packard high performance liquid chromatographic system (Agilent 1200 series, Brookside PKwy Alpharetta, USA) equipped with solvent delivery module (Serial No. DE 43630403, Product No. G1311A; Hewlett-Packard, Germany), UV-Visible spectrophotometer detector (Serial No. JP

43826101, Product No. G1314A; Japan), Rheodyne 7725i (with 20 µl capacity loop) injection Port and C18 RP hypersil (ODS) Column (stainless steel column of 250 mm x 4.6 mm i.d., packed with 5 µm particle size) protected by a Lichrospher^R Si 60 guard column, 30 mm x 4.6 mm i.d., placed between the Injector and the analytical column, the degasser (Serial No.JP40720373, Product No. G1379A; Japan) was used to remove gasses from the mobile phase. The component samples were eluted with mobile phase consisting of acetonitrile: methanol: water (35:17:48 v/v) adjusted to pH 4.0 with phosphoric acid and pumped isocratically at a flow rate of 1.0 ml/min at ambient temperature. The effluent was monitored by ultraviolet detection wavelength set at 237 nm.

2.5 Experimental Protocol and Blood Sample Collection

All the procedures of drug preparation, dosing and blood collection were performed under subdued light to prevent photodecomposition of nifedipine. After an overnight fast of 12 h, at 9.00 a.m., each rabbit was given 20 mg/kg body weight of nifedipine by gastric probe. Whole blood, 1 ml, was withdrawn from the retro orbital plexus of each animal before (0) and at 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8 and 10 hrs post dosing into heparinized eppendorff bottles; centrifuged immediately at 3000 g for 15 min. The plasma was carefully collected using Pasteur pipette and stored frozen at -20 °C until assayed. In another study, after a four week washout period, the rabbits were dosed orally with aqueous leaf extract of *Vernonia amygdalina*, 500 mg/kg body weight followed an hour after with nifedipine, 20 mg/kg body weight. Blood samples were withdrawn and treated as in the first study. In both studies, animals were not allowed food till 4 h post dose. Drug-free plasma was also frozen at -20 °C until assayed while protected from light. The assays were performed within one week of blood collection. Calibration, recovery and precision measurements were subsequently made using the frozen drug-free plasma sample.

2.6 Preparation of Standard Stock and Working Solutions

Reference standards of nifedipine and diazepam (I.S.) were made of 1000 µg/ml and 500 µg/ml in methanol respectively. Nifedipine standard solution was further diluted to give standard solution of 100 µg/ml. Different working standard solutions of nifedipine were prepared by serial dilution with equal volume of acetonitrile: water (1:1). All solutions were stored at -20 °C in amber bottle protected from light.

2.7 Analysis of Nifedipine

The nifedipine concentrations in rabbit plasma were determined by means of the HPLC-UV method reported by Debbas *et al.*, 1986^[17] and Abou-Auda *et al.*, 2000^[18] with slight modification. One hundred microlitre of 500 µg/ml diazepam (I.S.) was added to 0.5 ml aliquot each of the rabbit plasma and vortexed. The mixture was alkalinized by addition of 500 µl of 1 M NaOH; 3 ml of hexane:diethyl ether (1:1) was added to the mixture and vortexed. The precipitated proteins were centrifuged at 5,000 g for 10 min. The organic supernatant phase was separated and concentrated to dryness at room temperature protected from light. The residue was reconstituted in 100 µl of mobile phase. Twenty microlitre of the reconstituted residue was injected into the HPLC system for separation. The concentration of nifedipine in the plasma, determined by measurement of the peak area ratio of nifedipine to diazepam was extrapolated from the calibration graph.

2.8 Method Validation Calibration Procedure

All assays were performed under subdued light. The calibration points were obtained by spiking drug-free plasma with working standard solution of nifedipine to yield final concentrations of 0.01, 0.05, 0.1, 1, 5 and 10 µg/ml. To each concentration of nifedipine, 100 µl of 500 µg/ml diazepam; 500 µl of 1 M NaOH and 3 ml of hexane:diethyl ether (1:1) were added and vortexed. The remaining extraction procedure was as described above. The peak area ratios of

nifedipine to diazepam obtained for the different concentrations of nifedipine were plotted against the corresponding concentrations. Linearity, slope, intercept and correlation coefficient (r) was determined and used to calculate the unknown concentration of nifedipine in the plasma samples.

2.9 Accuracy, Precision and Recovery

The intra and inter-day accuracy and precision curves were obtained in 10 replicate assays at different concentrations of 1, 5, 10, and 40 $\mu\text{g/ml}$. The relative standard deviation (RSD) or coefficient of variation was calculated and used for the assessment of accuracy and precision. Accuracy was also determined based on the extraction efficiency recovery of nifedipine by comparing the peak area ratios of the spiked drug-free plasma to the peak area ratios obtained by direct injection of the standard solutions of the same concentration of nifedipine. Working standards were made fresh each day of the assay.

2.10 Stability of Nifedipine

The stability of nifedipine in the mobile phase or spiked plasma was determined in seven weeks. The drug solution, 40 $\mu\text{g/ml}$ was stored in the dark at room temperature and assayed once each week. In another experiment, the concentration of nifedipine in spiked plasma, 40 $\mu\text{g/ml}$ was determined on the day of preparation and the remainder of the plasma was stored frozen at -20°C . The spiked plasma was thawed, re-assayed and refrozen over a period of seven weeks. In each experiment, the concentrations of nifedipine were extrapolated from a calibration curve obtained from freshly prepared standard stock solution.

2.11 Pharmacokinetic Analysis of Data

The pharmacokinetic parameters were estimated for each subject by a standard non-compartmental pharmacokinetic model.^[19] The pharmacokinetic parameters included, the areas under the plasma concentration-time curves ($\text{AUC}_{0 \rightarrow \infty}$) measured by the trapezoidal rule and extrapolated to infinity. The peak plasma concentration (C_{max}) and the time to reach C_{max}

(T_{max}) were determined from the data obtained after administration. The apparent total body clearance (CL/F) calculated as $\text{dose}/\text{AUC}_{0 \rightarrow \infty}$, the absorption constant (k_a) was obtained from the absorption phase of the plasma concentration time curve. The terminal elimination rate constant (K_{el}) was calculated as the slope of the terminal log-linear phase of the plasma concentration-time curve. The corresponding elimination half-life ($t_{1/2}$), was calculated by dividing 0.693 by K_{el} , volume of distribution (V_d) was calculated as dose/C_0 , where C_0 is initial concentration of nifedipine and bioavailability (F).

2.12 Statistical Analysis

Data were expressed as Mean \pm SEM (standard error of mean). The statistical difference of the pharmacokinetic parameters for the groups was accessed by means of Student t- test using SPSS 15 statistical package with the level of statistical significance, P set at 0.05.

3. Result

3.1 Method validation

The method used gave a good linearity and a regression equation of $Y = 0.1084X + 0.0168$ with $r^2 = 0.9997$ ($n = 10$, $X =$ concentration of nifedipine and $Y =$ peak area ratio of nifedipine). The plasma concentrations of nifedipine were extrapolated from the regression equation. The intra- and inter-day precision, measured as relative standard deviation were less than 10 % over the range of concentration studied, thus indicating that 10 % of our results were equal to the mean. From our values, the accuracy, expressed as percentage bias was not significant evident from the fact that there is no obvious difference between the mean concentration and nominal concentration nifedipine. Accuracy as assessed by the percentage extraction recovery of nifedipine in the plasma for the intra- and inter-day assay ranged from 96.10 ± 6.77 % to 98.96 ± 4.35 % and 97.22 ± 4.87 % to 99.10 ± 4.56 % respectively (Table 1). The percentage recovery of nifedipine in the freezed plasma or direct injection were very stable as shown in table 2.

Table 1: Intra-day and Inter-day precision and accuracy determination of nifedipine in rabbit plasma.

Initial Conc (µg/ml)	Observed Conc (µg/ml)	% R.S.D. Precision	% Bias Accuracy	% Recovery
Intra-day				
1	0.96 ± 0.07	7.29	-4.00	96.10 ± 6.77
5	4.90 ± 0.24	4.90	-2.00	98.04 ± 4.81
10	9.75 ± 0.39	4.00	-2.50	97.47 ± 3.93
40	39.58 ± 1.74	4.40	-1.05	98.96 ± 4.35
Inter-day				
1	0.99 ± 0.05	5.05	-1.00	99.10 ± 4.56
5	4.86 ± 0.24	4.75	-2.80	97.22 ± 4.87
10	9.81 ± 0.44	4.49	-1.90	98.07 ± 4.37
40	39.14 ± 1.17	2.99	-2.15	97.84 ± 2.91

Values are expressed as Mean ± SD. N = 10

Table 2: Stability of Nifedipine in plasma at room temperature and at -20 °C

Periods (weeks)	Initial Concentration (40 µg/ml)			
	Room Temperature		-20 °C	
	Final Conc. (µg/ml)	% R.S.D. Precision	Final Conc. (µg/ml)	% R.S.D. Precision
0	40.01 ± 1.96	4.90	39.98 ± 2.48	6.19
1	39.99 ± 2.24	5.60	40.12 ± 2.77	6.90
2	39.77 ± 2.01	5.05	40.04 ± 1.97	4.92
3	40.11 ± 1.72	4.29	39.92 ± 1.41	3.54
4	39.01 ± 1.83	5.71	39.86 ± 2.12	5.32
5	39.49 ± 2.25	4.24	39.74 ± 2.36	5.94
6	39.51 ± 1.68	4.25	39.66 ± 2.22	5.59
7	39.85 ± 2.47	6.20	39.84 ± 1.19	2.99

Values are expressed as Mean ± SD. N = 7

3.2 Pharmacokinetic Analysis

A typical chromatogram of the standard solutions of nifedipine and diazepam (IS) is shown in figure 1. The chromatogram of the plasma concentration of nifedipine without or with the administration of *V. amygdalina* are shown in figures 2a and 2b respectively. The plasma concentration-time curve of the oral administration of nifedipine, 20 mg/kg body weight (Mean ± SEM, n=14) with or without simultaneous administration of *V. amygdalina*, 500 mg/kg body weight are shown in figure 3.

The corresponding pharmacokinetic parameters are summarized in table 3.

Nifedipine was rapidly absorbed and was detectable in the plasma within 8 min of administration; the absorption being more rapid in the rabbit model that consumed *V. amygdalina* (0.11 h ± 0.29) than in the rabbit that did not consumed *V. amygdalina* (0.25 h ± 0.17). Absorption rate constant, K_a was (1.94 ± 0.11/ h, control group and 3.92 ± 0.09 /h, treated group) with mean absorption half-life, ($t_{1/2ab}$) of 0.35 ± 0.13 h and 0.18 ± 0.02 h for the control and

treated groups respectively. There was no significant difference ($p \geq 0.05$) in the T_{max} (0.73 ± 0.15 h and 0.62 ± 0.23 h) between the control and the treated groups respectively. The C_{max} which was significantly ($p \leq 0.05$) higher in the treated group than the control group was achieved at ($1.41 \pm 0.21 \mu\text{g/ml}$) and ($3.89 \pm 0.28 \mu\text{g/ml}$) respectively. The C_{max} was followed by a concentration decay that could be fitted to a

straight line on semilogarithmic scale, indicating first order mode of elimination. The significantly high plasma concentration of nifedipine resulted in significant ($p \leq 0.05$) increase AUC, F and elimination half-life ($t_{1/2el}$) in the treated group compared to the control. The therapeutic range of nifedipine (15-35 ng/ml) was achieved in all the animal models within 10 min after dosing maintained for more than 8 h.

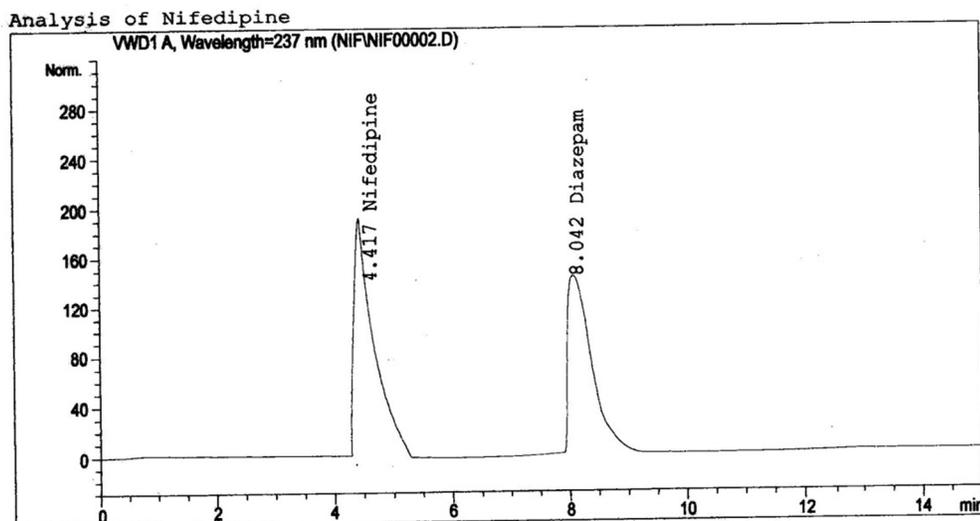


Fig 1: A typical chromatogram of standard solutions of nifedipine and diazepam, internal standard.

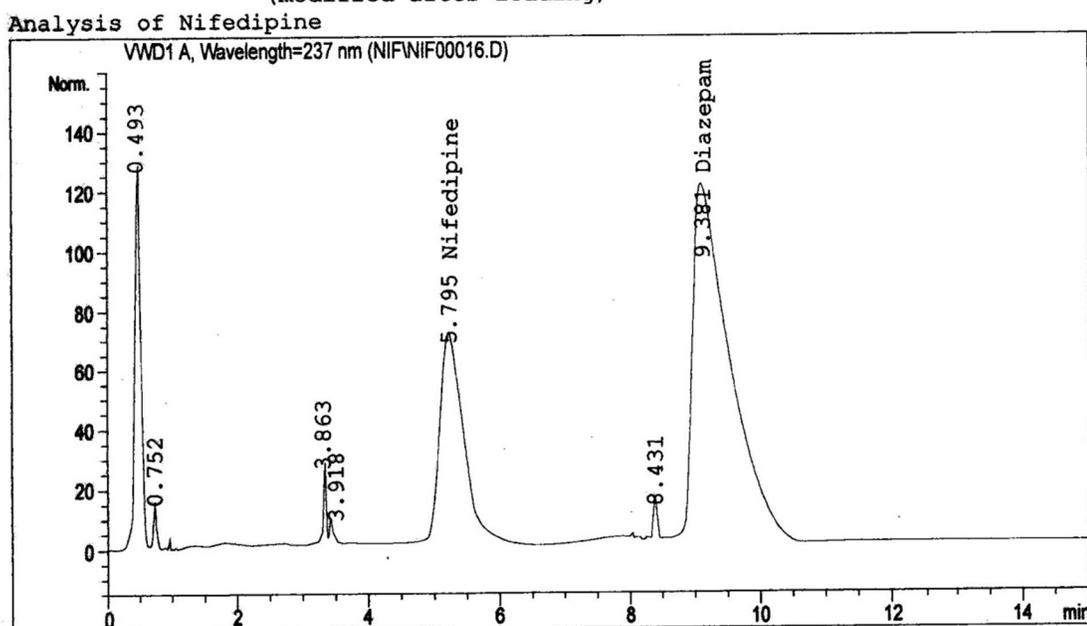


Fig 2a: Chromatogram of the plasma concentration of nifedipine without *Vernonia amygdalina*.

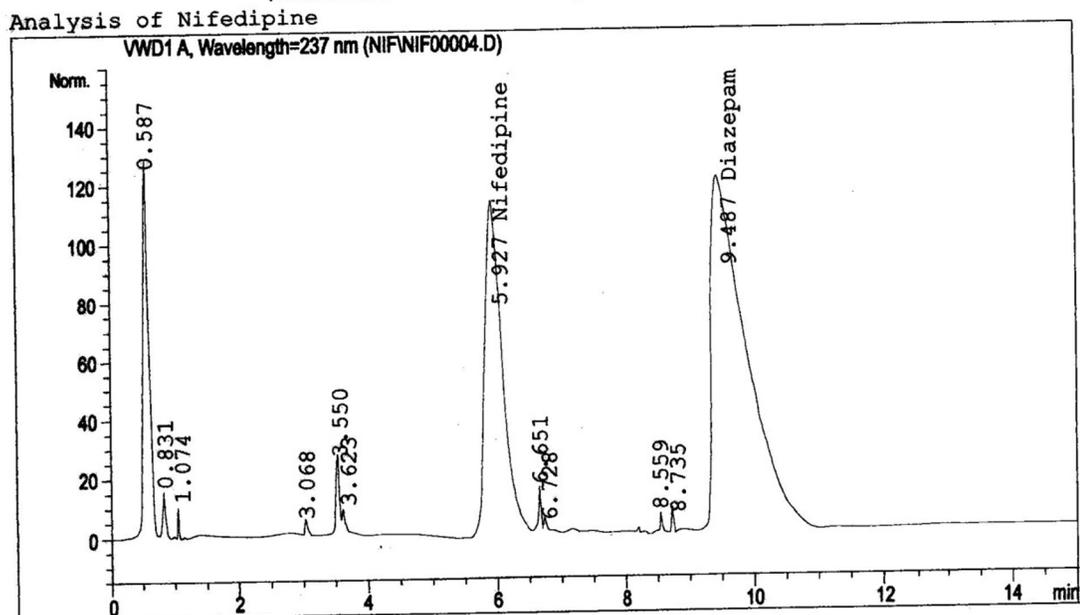


Fig 2b: Chromatogram of the plasma concentration of nifedipine with *Vernonia amygdalina*.

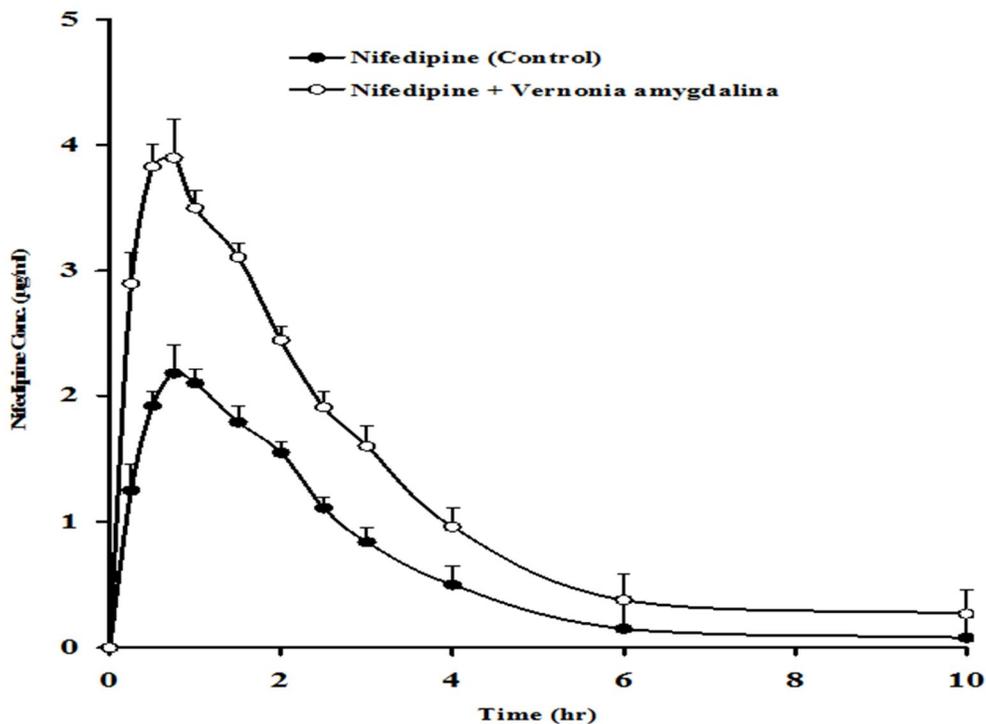


Fig 3: Mean plasma concentration-time curve of nifedipine following oral administration of 20 mg/kg body weight nifedipine with (○) or without (●) 500 mg/kg body weight *Vernonia amygdalina* aqueous crude extract. N = 14, Mean±SEM.

Table 3: Pharmacokinetic parameters of nifedipine after oral administration with and without the aqueous leaf extract of *Vernonia amygdalina*.

Pharmacokinetic parameters	Without <i>Vernonia amygdalina</i>	With <i>Vernonia amygdalina</i>
C _{max} (µg/ml)	1.41 ± 0.21	3.89 ± 0.28
T _{max} (hr)	0.73 ± 0.15	0.62 ± 0.23
T _{1/2el} (hr)	0.77 ± 0.25	1.43 ± 0.19
AUC _{0-∞} (µg.h ml ⁻¹)	3.03 ± 1.29	9.90 ± 2.87
F (%)	54.55 ± 1.09	87.41 ± 1.37
T _{1/2ab} (hr)	0.35 ± 0.13	0.18 ± 0.02
Cl (ml/h)	3.6 ± 1.13	1.62 ± 1.72

Values are expressed as Mean ± SD. N = 14

4. Discussion

This study examined the effect of simultaneous oral administration of *Vernonia amygdalina* on the pharmacokinetics of nifedipine. The method adopted in this study is simple, specific, accurate (Table 1) and capable of determining concentrations of nifedipine in small volumes of plasma. The mean recovery was good and the calibration graph was linear with a correlation coefficient, r^2 of 0.9997. Nifedipine stored frozen was stable during the seven week study period (Table 2) indicating that prolong storage of nifedipine had no interference with the results of this study.

Nifedipine is metabolized in the intestine and liver cells of man and rats to dehydronifedipine by well-known drug metabolizing enzymes, Cytochrome P₄₅₀ after intravenous or oral administrations^[20-23]. It is reported that following absorption, nifedipine has low bioavailability and high plasma clearance which has been speculated to be primarily due to extensive presystemic metabolism as well as hepatic extraction and metabolism^[20,24,25]. The administration of *V. amygdalina* resulted in significantly high ($P < 0.05$) bioavailability (F), elimination half-life ($t_{1/2}$), area under plasma-time concentration curve (AUC_{0-∞}) and maximum plasma concentration (C_{max}) in the *V. amygdalina* treated than the control groups. This study showed no significant difference ($P \geq 0.05$) in T_{max}, in the treated group compared to the control. (Table 3, Figure 1).

The metrics, C_{max} and T_{max} have important influence on drug responses. The time to peak (T_{max}) can control the onset of drug action, while C_{max} may determine if a dose is therapeutic or toxic^[26]. They also play important role in

evaluating the result of clinical pharmacokinetic studies including drug-drug interaction studies^[27]. From our study, the increase in C_{max} is consistent with increased in rate of absorption (K_a) and bioavailability which is in keeping with common sense that if bioavailability increases, the effective dose of the drug increases and thus C_{max} increases^[26,27]. This increase in effective dose may be attributed to i) reduced drug metabolism; ii) reduced systemic clearance or iii) increased rate of absorption, K_a, of nifedipine in the presence of *V. amygdalina*. However, increase in K_a may not be involved in increase effective dose as the T_{max} was unchanged. Nevertheless, longer elimination half-life could well explain the observed reduced clearance and high bioavailability.

The significantly high AUC values of nifedipine in the *V. amygdalina* treated group ($P \leq 0.05$) may indicate reduced systemic clearance of nifedipine which may due to reduced hepatic extraction and metabolism resulting in increased bioavailability. The significantly prolonged elimination half-life of nifedipine in the *V. amygdalina* treated group compared to the control is consistent with increased AUC which further explained reduced systemic clearance. Nifedipine is a high clearance drug and therefore a shorter half-life is expected^[25,28]. The longer half-life experienced in our study could indicate a decrease in metabolism and systemic clearance which is shown in significantly high AUC.

Genetic as well as environmental factors may be involved in the metabolism of nifedipine which may act at different sites^[21]. Environmental factors such as dietary component may inhibits Cytochrome P₄₅₀ enzymes which is known to

metabolize nifedipine to dihyronefidipine at the first step of the metabolic pathway in the intestinal tract and liver of rats as well as human.^[23] Consistent to this, it has been shown that citrus fruits: grapefruit juice increased the AUC of calcium antagonists: oral felodipine and nifedipine^[21,29] which was attributed to the inhibition of Cytochrome P₄₅₀ microsomal substrate oxidation in the intestine and or inhibition of phase II metabolic enzymes which are responsible for conjugation of drugs thus increasing their hydrophilicity by flavonoids found in the grapefruit juice^[21,30,31]. The study of Yoshioka h.^[23] has speculated that the effect of orally administered *ginklo bibloba* extract on the pharmacokinetic profile of nifedipine is due to the inhibition of CYP4503A.^[23] The authors attributed this to the fact that *ginklo bibloba* extract contains many kinds of flavonoids such as quercetin^[32,33], which has been reported to be potent CYP3A4 inhibitor and are able to increase the plasma concentration and AUC of drugs^[17].

Flavonoids have been reported to alter the pharmacokinetics profiles of concurrently administered drugs in some animal models^[34-37]. This action was explained to be due to the inhibition of various Cytochrome P₄₅₀ isoforms by flavonoids. Among the Cytochrome isoforms, are those belonging to the CYP3A subfamily: CYP3A4 and CYP3A5 found in the liver and intestinal tract and play prominent role in the metabolism of about 50 % of all prescribed drugs^[36,38,39]. In particular the flavonoids; quercetin, luteolin, kaempferol, apegenin, myricetin, chrysin, naringenin, diosmetin^[30, 40-42] have been shown to inhibit the metabolism of the calcium channel blockers - nifedipine and felodipine by interfering with the activity of CYP3A4 in the human liver. In our laboratory, phytochemical screening of our plant *V. amygdalina* revealed the presnce of flavonoid, cardiac glycoside. Also some worker have reported high concentration of flavonoid^[43] diterpene and triterpene alcohols^[44] in this plant. Thus the result of our study may well be attributed to the action of flavonoids on a number of Cytochrome P₄₅₀.

In summary, the result of this study indicate that concomitant administration with *V. amygdalina* with nifedipine may reduce the metabolism of nifedipine evident by decreased clearance resulting in increased bioavailability. Whether the flavonoid content or any other component of the plant is involved is subject to investigation. This study therefore gives a lead for future investigations using humans on the potential pharmacokinetic interactions between *V. amygdalina* and a variety of drugs extensively metabolized by Cytochrome P₄₅₀.

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