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Lipid-lowering effect of *Vernonia amygdalina* leaf extracts on Triton WR 1339 – induced hyperlipidaemia in the rats

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ABSTRACT

Vernonia amygdalina is a common plant reported to have several health benefits. This study investigated the dose dependent hypolipidemic potentials of the leaf extracts, following Triton WR 1339 induced hyperlipidemia. Adult male albino rats (average weight 185 ± 0.7 g) were pretreated with the ethyl acetate extract at 0, 100, 200 and 400 mg/kg body weight for seven (7) days after which the hyperlipidemia was induced with Triton WR 1339 (200 mg/kg body weight). The serum and liver of the animals were then assayed for; total cholesterol, triglycerides, HDL (high density lipoprotein) and LDL (low density lipoprotein). Histopathological investigation was carried out in the liver of all experimental animals. The result showed that pretreatment with ethyl acetate extract of *V. amygdalina* elicited a significant dose dependent ($P<0.05$) decline in Triton WR 1339 invoked increase in Total cholesterol and triglycerides concentration. Similarly, there was a significant dose dependent reduction in the levels of LDL in the pretreated group when compared to Triton WR 1339 control group. Conversely, significant ($P<0.05$) dose dependent increase was observed in HDL levels in the pretreated group when compared to the Triton group. It could be inferred from the results that *V. amygdalina* reduces the risk of diseases caused by hyperlipidemia.

Keywords: Hyperlipidemia, *Vernonia amygdalina*, Lipoproteins, Cholesterol, Triton WR 1339.

INTRODUCTION

Vernonia amygdalina is a shrub that grows mostly in the sub-Saharan Africa. Leaves from this plant serve as culinary herb in soup and food vegetables. Anecdotal evidences suggest the use of V. amygdalina in the treatment of feverish condition, hyperglycemia, cough, constipation, hypertension and allied diseases, as well as hyperlipidemia [1,2]. Phytochemical screening of this plant leaves extracts showed the presence of Saponins, riboflavin, polyphenols, sesquiterpene and flavonoids [3,4]. Strong antioxidant activities involving flavonoids extracted from *V. amygdalina* and its saponins have been reported to elicit anti-tumoral activities in leukemia cells [5]. In addition, peptides from *V. amygdalina* are known to be potent inhibitor of mitogen activated protein kinase (MAPK) which are involved in the regulation and growth of breast tumor [6]. Furthermore, this plant has been reported to possess hypoglycemic and anti-hyperlipidemic property [1,7]. Its anti-hyperlipidemic potential is demonstrated by the low cholesterol and triglycerides level in rats placed on high fat diet followed by *V. amygdalina* treatment [4].

Hyperlipidemia is the presence of raised or abnormal levels of lipids, largely cholesterol and triglycerides in the blood. These lipids are transported in a protein capsule, and the density of the lipids and the type of protein determines the fate of the particle and its influence on metabolism [8-10]. This fat-protein complexes in the blood is best-known as lipoproteins, and they are either LDL (low density lipoprotein), or HDL (high density lipoprotein) [11,13].

Excess LDL cholesterol contributes to the blockage of arteries, which eventually leads to heart attack. Hence, it is referred to as "bad" cholesterol. In contrast, HDL commonly referred to as the "good" cholesterol prevents atherosclerosis [14,15]. For a healthy person, the ideal LDL/HDL ratio is 3.5 [16]. Low HDL cholesterol levels are typically accompanied by an increase in blood triglyceride levels, and high triglyceride levels are associated with an increased risk of coronary heart disease [17]. Du-bios and co-workers have shown a trend as regards the attenuation of cholesterol and lipoprotein following treatment of *V. amygdalina* [18].

In addition to *V. amygdalina*, plants such as *Occimum sactum*, Neem (*Azerdiraclta indica*) and Gallic (*Allium salivium*), are reputed to have lipid-lowering properties, in that they attenuate cholesterol level

in individuals with dyslipidemia [19]. However, not many of these plants have been subjected to rigorous experimental and clinical investigation with a view of giving a scientific validation of the claims. Thus, *V. amygdalina* was used to evaluate the possible lipid-lowering activity of a detergent (Triton WR 1339) induced Hyperlipidemia. Triton WR 1339 is a surfactant commonly used for inducing hyperlipidemia acutely in the rat and mice [20-22].

MATERIAL AND METHOD

Reagents: Triton WR 1339 (Tyloxapol) was purchased from sigma chemical company, St. Louis, Missouri, USA. Diagnistic kits for cholesterol, Triglycerides, and HDL, were purchased from Randox, UK. All other reagents used were of analytical grade.

Plant identification and extraction

The leaves of *Vernonia amygdalina* were collected from the Staff Quarters, Kogi State University, Nigeria.The fresh leaves were oven dried at 40 ºC, powdered and extracted with ethanol by soxhlet apparatus. Ethanol extracts was concentrated to dryness under vacuo at 40 ºC in a rotary evaporator. The methanolic extract was then suspended in 100 ml of distilled water and subsequently partitioned with ethyl acetate (3x100 ml). The ethyl acetate fraction was mostly used for the study.

In vivo experimental design

Adult male albino rats, bred from the same colony, with average weight of 185 ± 0.7g were obtained from the Animal House, Faculty of Natural Sciences, Kogi State University. The animals were left to acclimatize to laboratory conditions for at least two weeks before the start of the experiment. They were then randomly allotted into six (6) groups of six animals; three groups for control; Triton treated (200 mg/kg BW), *V. amygdalina* treated (400 mg/kg BW) and Normal saline treated. The other three groups were; *V. amygdalina* treated (400 mg/kg BW) +Triton (200 mg/kg BW), *V. amygdalina* treated (200 mg/kg) + triton treated and *V. amygdalina* treated (100 mg/kg) +Triton (200 mg/kg BW). Rats were fed with pelletised rat chow (growers' mash, Ladokun Livestock feeds, Ibadan) and water *ad libitum*. The treatments lasted seven (7) days. Animals were sacrificed on the eight (8) day, blood samples were collected into EDTA sample containers and serum was prepared by centrifugation at 3000 x g for 15 minutes. Tissue homogenates were prepared in ice cold 0.25 M sucrose and stored at -20 ºC for further assays.

Biochemical assays

Biochemical assays were carried out using Randox Diagnostics kits according manufacturer's instructions. Total cholesterol was determined according to method of Richmond (1973). The triglycerides are determined after enzymatic hydrolysis with lipases followed spectrophotometrically at wavelength of 500 nm. Low density lipoprotein was calculated using Friedewald formula:

LDL Cholesterol (mg/dl) = Total Cholesterol – (Triglycerides/5 + HDL Cholesterol).

Protein concentration was determined using the Folin-Cicocalteau colour reagent measured against Bovine Serum Albumin as standard.

Histopathological Investigation

For histopathological examination, liver tissues from control and test rats were fixed in 10 % buffered formalin (pH 7.3), dehydrated, embedded in paraffin, cleared with xylene and stained with hematoxylin and eosin prior to examination with a zeiss EM light microscope.

Statistical Analysis: The results are expressed as mean ± SEM using Graph Pad Prism Graphical-Statistical Package version 5. The difference between groups was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test with 5 % level of significance ($P<0.05$).

RESULTS

Percentage Yields

The percentage yields of crude methanolic extract, ethyl acetate fraction and aqueous fraction from solvent-solvent partitioning was 8.95 %, 3.01 % and 4.67 % respectively.

Effect of *V. amygdalina* fractions on plasma and liver of Triton WR 1339-induced hyperlipidemic rats

The crude methanolic extract and the fractions (aqueous and ethyl acetate fractions) were used to evaluate the ex *vivo* hypolipidemic properties of *V. amydalina* (100 mg/kg body weight) in the plasma and liver of rats induced with acute hyperlipidemia. Excitingly, Table 1 showed blunted level of plasma triglycerides in rats pretreated with ethyl acetate fraction compared to the Triton WR 1339-induced rats. Similarly, ethyl acetate fraction blunted plasma cholesterol as opposed to the crude extract and the aqueous fraction. The values were compared to Triton control rats. Moreover, ethyl acetate fraction lowered liver triglycerides and cholesterol than the crude and aqueous fraction, when compared to both Triton and normal saline control rats ($P<0.05$; Table 1). This suggests that the ethyl acetate fraction is more potent and was adopted in subsequent experiments to further evaluate the hypolipidemic properties of *V. amygdalina ex vivo.*

Effect of the ethyl acetate fraction of *V. amygdalina* treatments on Plasma Triglycerides and Total Cholesterol Levels

To further ascertain the effect of ethyl acetate fraction on the hyperlipidemia, varied concentration (100, 200, 400 mg/kg) was used to pre-treat hyperlipidemia-stimulated rats. As shown in table 2, the collected plasma displayed increased triglycerides and cholesterol in Triton WR 1339-injected animals, which were used as control rats. Upon treatment with the varied concentration of *V. amygdalina*, there was a dose-dependent reduction of triglyceride and cholesterol levels; as treatment with 100 mg/kg caused 50.67 ± 11.02 and 13.56 ± 8.91% reduction in triglycerides and total cholesterol, respectively. Moreover, treatment with 200 mg/kg of *V. amygdalina* caused 64.02 ± 9.99 and 58.33 ± 14.30 % in triglycerides and cholesterols, correspondingly. While treatment with 400 mg/kg of *V. amygdalina* revealed 83.77 ± 12.21 change in total cholesterol compared to the control littermates, and 64.18 ± 14.30 % change in triglycerides compared to Triton WR1339 control (Table 2). This suggests that the ethyl acetate fraction pretreatment reduced triglycerides and cholesterol in a dose-dependent manner in Triton WR 1339-induced hyperlipidemia.

Table 1: A pilot study showing the effect of the crude extract and the fractions of *V. amygdalina* on induced hyperlipidemia

Treatments (100 mg/kg body weight)	Plasma triglycerides (mg/dl)	Plasma cholesterol (mg/dl)	Liver triglycerides (mg/dl)	Liver cholesterol (mg/dl)
Triton (200 mg/kg)	210.45±5.76	170.43±6.98	287.93±11.46	171.32±7.11
Saline	208.55±4.25	166.46±3.91	268.63±10.22	173.13±6.72
Crude extract	199.87±10.26	140.56±13.38	200.11±5.37	153.59±9.21
Ethyl acetate fraction	147.41±7.22*	100.45±8.32*	134.48±14.09*	120.31±12.66*
Aqueous fraction	200.02±12.22	134.44±9.29	189.67±10.68	169.28±7.87

All values are expressed as mean ± SEM. The levels of cholesterol and triglycerides following pre-treatment with the ethyl acetate fraction of the extract in the plasma and liver compared to the Triton (control), was significantly different (*P<0.05; 5 littermates were used in each group).

Table 2: A pilot study showing the effect of the crude extract and the fractions of *V. amygdalina* on induced hyperlipidemia

Treatments	Plasma triglycerides		Plasma cholesterol	
	mg/dl	% change	mg/dl	% change
Control (normal saline)	69.25±4.76	-	75.11±4.53	-
V. amygdalina (400mg/kg)	68.66±7.12[d]	0.85±2.33	69.84±10.81[d]	7.55±7.86
Triton (200 mg/kg)	222.11±6.13[a]	-	176.24±9.02[a]	-
Triton+*V. amygdalina* (100mg/kg)	147.41±7.22[c]	50.67±11.02	155.19±2.50	13.56±8.91
Triton+*V. amygdalina* (200mg/kg)	135.42±5.25[b]	64.02±9.99	111.31±8.88[c]	58.33±14.30
Triton+*V. amygdalina* (400mg/kg)	120.86±12.56[b]	83.77±12.21[b]	107.34±6.35[b]	64.18±9.17[b]

[a] Triton WR 1339 (200 mg/kg; iv); % change of *V. amygdalina group* was calculated relative to the control (normal saline) group; % changes of TRI+*V. amygdalina* treatments versus TRI group; [c] P<0.05 and [b] P<0.001 when compared to TRI. [d] P>0.05 when compared with Normal saline; n= 5 rats per group; All values are expressed as mean±SEM.

Effect of treatment with ethyl acetate fraction of *V. amygdalina* hepatic triglycerides and cholesterol levels

Liver cholesterol and Liver triglycerides levels were significantly increased in Triton WR 1339-injected animals as compared to control rats (normal saline and *V. amygdalina* 400 mg/kg treated). However, treatment with *V. amygdalina* (100 mg/kg) caused 26.55 ± 6.88 and 38.27 ± 10.65 % reduction in liver triglycerides and liver cholesterol, respectively. Treatment with (200 and 400 mg/kg) of *V. amygdalina* caused a dose dependent change in triglycerides and total cholesterol (Table 3). The percentage change at 400 mg/kg body weight dosage for triglycerides and cholesterol showed a significant reduction compared to Triton WR 1339 treated-control (P<0.0001). However, control rats treated with *V. amygdalina* showed no significant change in liver cholesterol and triglycerides (P>0.05). This suggests that *V. amygdalina* treatments lowered liver triglycerides and cholesterol in Triton WR 1339-induced hyperlipidemia.

Table 3: Effect of treatment with ethylacetate fraction of *V. amygdalina* on hepatic triglycerides and cholesterol levels following Triton WR-1339 induced hyperlipidemia

Treatments	Liver triglycerides		Liver cholesterol	
	mg/dl	% change	mg/dl	% change
Control (normal saline)	116.68±5.33	-	66.22±4.48	-
V. amygdalina (400mg/ml)	104.11±4.88[d]	12.07±7.66	64.86±3.99[d]	2.09±5.12
Triton (200 mg/kg)	299.01±7.51	-	177.12±15.03	-
Triton+*V. amygdalina* (100mg/kg)	236.28±11.24[c]	26.55±6.88	128.10±3.93[c]	38.27±10.65[b]
Triton+*V. amygdalina* (200mg/kg)	179.24±7.11[b]	66.82±3.31	100.27±5.97	76.64±9.94[b]
Triton+*V. amygdalina* (400mg/kg)	171.28±7.77[b]	74.57±5.23	89.92±8.35[b]	96.97±13.13

[a] Triton WR 1339 (200 mg/kg; iv). All values are expressed as mean±SEM. [c] P<0.05 and [b] P<0.001 when compared to TRI. [d] P>0.05 when compared with Normal saline. n= 5 in each group.

Plasma lipoproteins levels in response to treatment with ethyl acetate fraction *V. amygdalina* in hyperlipidemic rats

In order to investigate the effect of the varying levels of triglycerides and cholesterol on the lipoproteins, the levels of plasma HDL and LDL were quantified as shown Table 4. The plasma HDL levels rose relatively to the dosage (100, 200, 400 mg/kg body weight) when compared to the control littermates. Whereas, the detergent Triton increased the levels of LDL in the control group, however, as expected, *V. amygdalina* blunted LDL levels in a dose-dependent manner. This strengthens the hypolipidemic effect of the plant as earlier exhibited in Tables 1 and 2. There was no change in HDL and LDL levels in normal saline treated rats, implying that the variations in fatty acid and lipoprotein levels were entirely due to Triton WR 1339 induced as well as the *V. amygdalina* provoked-treatment. Furthermore, to establish the healthy and diet state of the rats, it was

germane to quantify the blood and liver protein levels following Triton-stimulation and pre-treatments. Table 4 shows a more or less constant concentration of protein in the blood and liver. Although there was slight variation, but not statistically powerful to reveal any change in contrast to the controls. The nearly constant protein concentration in the blood and liver suggest that the animals were in a good state of diet.

Table 4: Effect of *V. amygdalina* on plasma lipoproteins and protein levels following Triton WR 1339-induced hyperlipidemia

Treatments	Plasma lipoproteins		Protein levels	
	HDL (mg/dl)	LDL (mg/dl)	Plasma (mg/ml)	Liver (mg/ml)
Control (normal saline)	76.44±7.11	139.45±10.48	15.33±1.23	15.78±2.15
V. amygdalina (400mg/kg)	80.34±11.46	120.98 ±17.33	14.99±0.54	13.71±1.99
Triton (200 mg/kg)	58.22±5.78	180.43 ±15.37	10.23±2.89	12.56±1.33
Triton+*V. amygdalina* (100mg/kg)	60.23±10.15	178.66±14.21	12.44±4.03	16.10±2.01
Triton+*V. amygdalina* (200mg/kg)	64.56±4.88	100.56±13.44	15.89±2.12	13.31±1.64
Triton+*V. amygdalina* (400mg/kg)	70.52±8.45^{c}	97.87±9.88^{c}	14.19±0.98	15.02±1.93

All values are expressed as mean ± SEM. ^{c} P<0.05 when compared to Triton WR 1399. n= 5 in each group.

Histopathological investigation

To evaluate the cellular hypolipidemic properties of *V. amygdalina*, liver sections were evaluated for focal area of fatty change, perinuclear vaculation, as well as apoptotic bodies. As shown by the photomicrograph Figure 1, the control normal saline treated rats as well as non-induced *V. amygdalina* (400 mg/kg body weight) treated rats showed normal hepatocytes. However, as expected, Triton treated group showed numerous apoptotic bodies and marked fatty change. Subsequently, at 100 mg/kg body weight treatment, the fatty change was moderate, and at an increased dosage of 200 mg/kg body weight, the liver damage was reduced as well as diffuse change in fat nodules within the hepatocytes. Interestingly, at 400 mg/kg body weight, the hepatocytes became essentially normal. Consistent with Tables 1 and 2, *V. amygdalina* reverses Triton-induced hyperlipidemia in a dose-dependent manner.

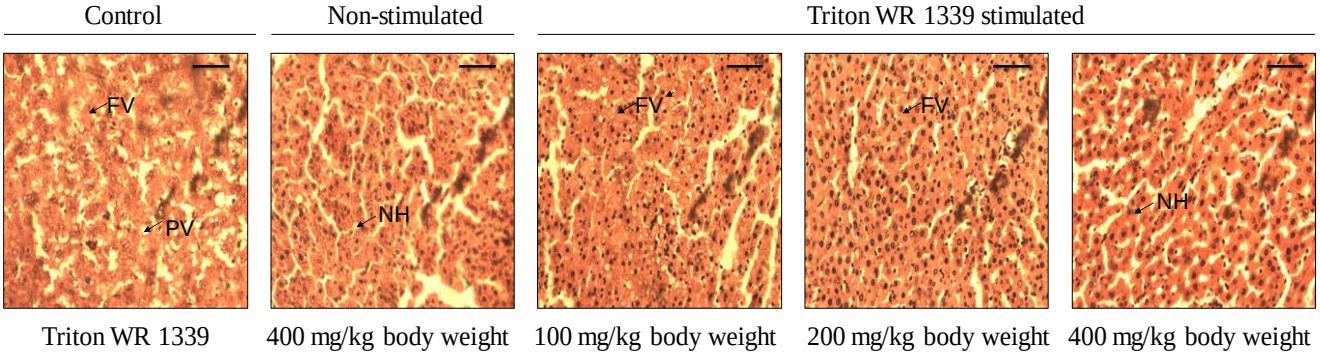

Figure 1: Varied dosage of *V. amygdalina* following Triton-induction blunted hyperlipidemia phenotype in a dose-dependent manner. At 100 mg/kg the hepatocytes displayed diffuse fatty change with fat droplets. This fatty change was reduced to mild diffuse fatty change following treatment at 200 mg/kg body weight. Interestingly, the highest dosage, 400mg/kg reversed the insulted hepatocytes to normal. *Magnification 10 x, H/E, PV*; Perinuclear vaculation, FV; Fat vacuole, NH; Normal hepatocyte.

DISCUSSION

The aim of this study is to evaluate the lipid-lowering ability of *V. amygdalina* leaf extracts in the Triton TWAR-1339 rat model. Hyperlipidemia and allied diseases have been a major cause of death in the world [8,23]. The drugs are very expensive, and with side-effects [24,25]. It is germane to look for a cheap source to tackling this problem, most especially in resource poor nations. Anecdoctal evidence suggests that medicinal plants, including *V. amygdalina* is useful in this regard. To investigate this, methanolic extraction was employed, followed by solvent-solvent partitioning between water and ethyl acetate, a less polar solvent, in order to selectively isolate secondary metabolites, which may be responsible for the health benefits. The results from the pilot study (Table 1) revealed that the ethylacetate fraction was more effective in lowering triglycerides and cholesterol levels in Triton induced hyperlipidemic rats. Thus, ethylacetate was selected for further investigation. This is in line with standard, routine laboratory practice; consistent with the work of Fatema and colleagues [26]. *V. amygdalina* is believed to be a rich in flavonoids, saponins, alkaloids, beta-carotene, terpenes, glycosides and tannins, amongst others [18,27,28] some of which have been reported to modulate lipid metabolism. have been reported to modulate lipid metabolism [29-32]. Trace minerals, such as sodium, potassium, calcium, zinc, iron, phosphate, and copper are contained in *V. amydalina* [27]. Some studies have focused on the anti-hyperlipidemic properties of *V. amygdalina* using the high fat-diet model [1,32,33]. Such studies revealed that *V. amygdalina* is effective in regulating lipid metabolism however, there is need to elucidate underlining biochemical. To this end in this work Triton WR-1339 a nonionic surfactant capable of causing dyslipidemia by destroying a major enzyme in lipid metabolism HMG CoA reductase was used to create an animal model. Triton is presumed to block the egress of triglycerides, as well as preventing the hydrolysis of plasma lipoproteins by cellular lipoprotein lipases [34]. Moreover, triton physically alters very low-density lipoproteins (VLDL) rendering them refractive to the action of lipolytic enzymes of blood and tissues,

delaying their removal from blood [35,36]. In confirmation, Yao and co-workers reported that the concentration of triglycerides in the plasma was increased following induction of Triton WR-1339 in the rats [37]. Consistent with this study, Touiss and colleagues reported that Triton WR-1339 administered at 200 mg/kg body weight increased serum triglycerides, phospholipids, and cholesterol levels [38]. Nonetheless, in this study, the result conforms to the rise in triglycerides and cholesterol and was found to be markedly suppressed in animals treated with *V. amygdalina*. This is also similar to another study, where *V. amydalina* blunted the provoked levels of cholesterol and triglycerides [39].

Although there was no positive control as regards to an antihypercholesterolaemic drug used, such as atorvastatin, however, the level of reduction expressed by the ethyl acetate fraction of *V. amygdalina* in this research, compared with the control used, can compete with any drug. Asante and colleagues reported similar reduction of LDL and a corresponding increase in HDL following treatment of *V. amygdalina* extract [18]. The alteration of HDL in a concentration-dependent manner by the detergent (Table 4), could be as a result of the displacement of apoA-I from the HDL surface to a state where all lipids are solubilized into the Triton WR-1339 micellar phase, thereby transferring the protein moiety [40]. The increase in HDL perhaps was directly provoked by the plant. This could be by mobilizing cholesterol from extra hepatic tissue, where it is catabolised. Hepatic HMG-CoA reductase is the rate-limiting enzyme in the cholesterol biosynthetic pathway and its inhibitors are very effective in lowering plasma cholesterol. With the significant decrease in cholesterol, *V. amygdalina* could possibly be an inhibitor of HMG-CoA reductase in rats. It is beyond the scope of this research to speculate in great detail on the mechanism involved. Moreover, the hypolipidemic effect of *V. amygdalina* administration could result from activation the rate limiting step in cholesterol catabolism, which is the conversion of cholesterol 7-α-hydroxylase to bile acid.

Histopathologically, the hepatocytes following treatments showed fatty infiltration, haemorrhages and mass of eosinophilic materials. The vacuoles coalesced to create clear space that displaced the nucleus to the periphery of the cell. There were many ruptured hepatocytes, with enclosed fat globules that coalesced to produce fatty cysts. This collaborates the ex vivo results (Tables 2 and 3). The variation in fatty change was in a dose-dependent manner. Littermates treated with Triton WR-1339 showed complete fatty change. This hyperlipemic state was associated with the appearance of fatty nodules in liver tissue. However, the hepatocyte was somewhat normal at the highest dosage of the fraction, as hepatic lobes were entirely preserved, hepatic plates and central lobular veins were normal without alterations. The visible nodules could be lipoproteins, which were collected in bags by liver cells.

CONCLUSION

Evidence from this study confirms the lipid-lowering effects of *V. amygdalina* in rats induced by Triton WR-1339. *V. amygdalina* was effective in blunting the levels of plasma and hepatic cholesterol, triglycerides, as well as LDL. Further studies are warranted to determine the exact active component in *V. amygdalina* responsible for the observed effect and, such component may be a candidate for use as a prophylactic agent against hyperlipidemia.

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