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Biosafety, Antioxidant and Antidiarrhoeal Potentials of *Afzelia africana* Seed *n*-Hexane Extract

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ABSTRACT: This study evaluated the biosafety, phytochemicals, antioxidant activity, acute toxicological profile and antidiarrhoeal property of *n*-hexane extract of *Afzelia africana* seeds. Results from the phytochemical profiling (using gas chromatography - mass spectrometry technique) elicited the presence of hexadecanoic acid (34.97 %), 9, 12, 15-octadecatrien-1-ol (16.27%) and oleic acid (6.71%) as major compounds associated with antioxidant and antidiarrhoeal effects. The study revealed that *A. africana* seed possesses antioxidant potentials across four models at 100 μg/ml (39.6%, 59.6%, 48.11% and 0.765 for 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, ferrous ion chelating, hydrogen peroxide scavenging, and reducing power assays respectively). No adverse effect or lethality was recorded at ≤5000 mg of extract per kg body weight of tested animals after 14 days observation. In castor oil induced diarrhoea, a significant ($p<0.05$) increase in delayed onset of stooling and percentage inhibition (up to 92.4%) with decrease in defecation frequency were observed across the treated groups when compared with the control. Similarly, the extract showed a significant ($p<0.05$) dose dependent decrease in gastrointestinal motility with increase in percentage inhibition (up to 91.9%) in charcoal meal test. In conclusion, *Afzelia africana* seed antidiarrhoeal property has been validated and its folklore benefit affirmed by this study.

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Diarrhoea is a gastrointestinal disorder that is characterized by an alteration in a normal bowel movement associated with increase in the water content, volume or frequency of stools (Camilleri and Murray, 2011). It is mostly associated with pain, urgency, perianal discomfort, and incontinence. It is a sign or symptom, not a disease by itself, and can be caused by numerous conditions (Camilleri and Murray, 2011). Diarrhoea has been reported as the second leading cause of mortality among children under five years of age next to respiratory infections and kills more young children than AIDS, malaria, and measles combined. To remedy this disease traditional medicine practitioners in African countries have used different plants (with varying degrees of medicinal properties); including *Calpurnia aurea*, *Osyris quadripartite*, *Lantana camara*, *Croton marcostachyus* and *Echinops kebercho* whose antidiarrhoeal activities have been scientifically evaluated (Meseret *et al.*, 2019; Edlam *et al.*, 2017), also *Afzelia africana* amongst many others which has not been scientifically evaluated. Diarrhoea is usually a symptom of diseases in the intestinal tract which can be caused by a variety of bacteria (*Escherichia coli*, *Vibrio cholerae* and *Shigella* species), virus (*Rota virus*, *Noro virus* and *Cytomegalo virus*) and parasitic organisms (protozoa and helminths). Ethnopharmacological studies revealed that *Afzelia africana* has been proven to have activities including antibacterial (Edlam *et al.*, 2017) antifungal (Chisom *et al.*, 2018), anti-inflammatory and analgesic activities (Akah *et al.*, 2007). This study investigated the biosafety, phytochemicals, antioxidant potential, acute toxicological profile and antidiarrhoeal property of *n*-hexane extract of *Afzelia africana* seed.

MATERIALS AND METHODS

Reagents: 2,2-Diphenyl-1-picrylhydrazyl, ferrous sulphate, ferrozine, iron (III) chloride, potassium ferricyanide, trichloroacetic acid, hydrogen peroxide

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and phosphate buffer used in this research were products of Sigma-Aldrich. Other chemicals used were methanol, vitamin C, *n*-hexane, loperamide (Vixa Pharmaceutical Co. Ltd), castor oil (Bell, Sons & Co (Druggists) Ltd), atropine sulphate injection (RHR Medicare PVT LTD), activated charcoal (Puritan's Pride) and gum acacia.

Plant Collection and Preparation: Afzelia africana seeds were purchased from a market in Port Harcourt, Nigeria. The seeds were identified in the Department of Plant Science and Biotechnology, Rivers State University, Nigeria. The seeds were handpicked with its aril removed, washed to remove debris, cleaned and dried. Dry seeds of *A. africana* (600 g) were pulverized and extracted with *n*-hexane using a Soxhlet apparatus at 50 ℃ (Ndukwe et al., 2016). The resulting crude extract (HEAAS) was then weighed and stored in the refrigerator at 4 ℃; while extraction yield was calculated.

Characterization of n-Hexane Extract of A. africana Seed ((HEAAS): Gas chromatography - mass spectrometry (GC-MS) was used to analyze HEAAS. Components of the extract were identified based on comparison of their retention indices and mass spectra with those of standards and National Institute of Standards and Technology (NIST) Standard Reference Database 69. Gas Chromatography used for the analysis was Varian 3800 gas chromatograph equipped with an Agilent MS capillary column (30 m × 0.25 mm) connected to a Varian 4000 mass spectrometer operating in the EI mode (70 eV; m/z 1-1000; source temperature 230 °C and a quadruple temperature 150 °C). The column temperature was initially maintained at 200 °C for 2 min, increased to 300 °C at 4 °C/min, and maintained for 20 min at 300 °C. The carrier gas was nitrogen at a flow rate of 1.0 ml/min. The inlet temperature was maintained at 300 °C with a split ratio of 50:1. A sample volume of 1 µl was injected using a split mode, with the split ratio of 50:1. The mass spectrometer was set to scan in the range of m/z 10-650 with electron impact (EI) mode of ionization, runtime was 60 minutes.

Determination of In vitro Antioxidant Activities of HEAAS: DPPH Antioxidant Assay: Radical scavenging activity of HEAAS against DPPH was determined using UV Spectrophotometer (Wincom company LTD, Model: 752 D) at 517 nm. Aliquots of 1 ml of methanol solution containing each concentration of extract and vitamin C (20 - 100 µg/ml) were added to 2 ml of 0.1 mM DPPH in methanol solution. The mixtures were shaken vigorously and allowed to stand at room temperature in the dark. The reduction of the DPPH radical was determined by measuring the absorptions at 517 nm after 30 minutes. Absorbance of the blank was also measured (Ita and Ndukwe, 2017). Tests were performed in triplicate while percentage of inhibition (% inhibition) was calculated using equation 1.

$$\% \ inhibition = \frac{A_0 - A_1}{Ao} \times 100 \qquad 1$$

Where A_O is the absorption of the blank and A_I is the absorption of HEAAS.

Ferrous ion Chelating Assay: The chelating of ferrous ions by HEAAS was estimated using reported protocol (Ita and Ndukwe, 2017). Different concentrations of HEAAS (20 - 100 µg/ml) were separately mixed with 100 µl of 2 mM ferrous sulphate solution and 300 µl of 5 mM ferrozine. The mixtures were incubated at room temperature for 10 minutes. Absorbance of the solutions were measured at 562 nm. Vitamin C was used as standard. All the tests were performed in triplicate and percentage of ferrous ion chelating activity was calculated using equation 2.

$$Chelating\ ability\ (\%) = \frac{A_0 - A_1}{Ao} \times 100 \qquad 2$$

Where A_O is the absorption of the blank sample and A_I is the absorption of HEAAS.

Hydrogen Peroxide Scavenging Assay: Antioxidant activity of HEAAS was also evaluated using the hydrogen peroxide scavenging activity method as reported by Ndukwe *et al.* (2020). Briefly, five concentrations (20, 40, 60, 80, 100 µl) of HEAAS were prepared and mixed with 0.3 ml of 4 mM solution prepared in phosphate buffer (0.1 M, pH 7.4) and incubated at room temperature for 10 minutes. Absorbances of the solutions were taken at 230 nm against blank solution containing HEAAS without H_2O_2. Vitamin C was used as the standard. All the tests were done in triplicate and average of the three observations was considered. The scavenging effect was then calculated according to equation 3.

$$H_2O_2\ scavenging\ activity\ (\%) = 1 - \frac{A_1}{A_C} \times 100 \qquad 3$$

Where A_c is the absorption of the control and A_I is the absorption of HEAAS.

Reducing Power Assay: Reducing power of HEAAS was determined according to established protocol (Ndukwe *et al.*, 2020). Various concentrations (20, 40, 60, 80, 100 µl) of HEAAS in distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% of potassium ferricyanide aqueous solution (2.5 ml, $K_3[Fe(CN)_6]$). The mixtures were incubated at

50 ℃ for 20 min. Aliquots of trichloroacetic acid (2.5 ml, 10% aqueous solution) were added to each of the mixtures which were then centrifuged at 3000 rpm for 10 min. The supernatants (2.5 ml) were each mixed with distilled water (2.5 ml) and freshly prepared $FeCl_3$ solution (0.5 ml, 0.1%). Absorbances were measured at 700 nm as indicative of increased reducing power. All experiments were performed in triplicate and a graph was plotted using the mean values.

Experimental Animals: Adult Wistar rats (twenty-five) and thirty-seven Adult Swiss mice weighing 180-200 g and 28-32 g respectively of either sex were obtained and housed in plastic cages (five per cage) and maintained under controlled room temperature (25±1 °C) with relative humidity of 45-55% under 12:12 hour light and dark cycle for one week with free access to food and water *ad libitum* within the animal house facility. The ethical committee of Life Sciences University of Benin, Nigeria, gave approval for the use of animals with ethical number LS19019.

Acute Toxicity Test: HEAAS was used for pharmacological screening on male Swiss mice. Acute toxicity test was conducted according to Lorke's method (Lorke, 1983). This was done in two phases.

Phase 1: Three Swiss mice were allotted into three groups (one animal each). Each was administered orally a dose of 1000 mg/kg of HEAAS per body weight. The mice were kept under close observation for 24 hours to monitor their behavior as well as occurrence of mortality.

Phase 2: Nine Swiss mice were allotted into three groups (three animals each). The mice were administered higher doses (1600, 2900 and 5000 mg/kg for group 1, 2 and 3 respectively) of HEAAS per body weight of the mice and were observed for 24 hours (special attention given to the first 4 hours) for signs of toxicity which include paw-licking, change in skin color, changes in fur, eye lacrimation, nostril discharge, salivation, diarrhoea, tremor, convulsion and death; and then subsequently observed for 14 days. LD_{50} was calculated using equation 4.

$$LD_{50} = \sqrt{(D_0 \times D_{100})} \qquad 4$$

D_0 = Highest dose that gave no mortality; D_{100} = Lowest dose that produced mortality

Antidiarrhoeal Assay: Castor Oil-induced Diarrhoea in Mice: Twenty-five mice were fasted for 18 hours and randomly divided into five groups (group 1, 2 3, reference and control). HEAAS at graded doses (25, 50, and 100 mg/kg) were administered orally to treated groups 1, 2 and 3, respectively. The control group received 0.2 ml/kg body weight of distilled water, and reference group received 3 mg/kg body weight loperamide. After 30 minutes, all the animals were predisposed to 0.2 ml/rat of castor oil orally via gavage. They were kept in a separate transparent plastic container with plain filter paper at the base (Awouters *et al.,* 1978). The onset and severity of diarrhoea was evaluated for 4 hours. Total number of faeces (diarrhoea and non-diarrhoea) expelled were compared with that of the control group. Total score of diarrhoea faeces for control group was measured as 100%. The results were presented as percentage inhibition of diarrhoea and was calculated with equation 5.

$$\% \text{ Inhibition of Antidiarrhoeal} = \frac{A_0 - A_1}{A_0} \times 100 \qquad 5$$

A_0 - Mean number of wet defecations of control; A_1 - Mean number of wet defecations of test

Gastrointestinal Motility Test in Rats: Wistar rats (25) were randomly divided into five groups (5 per group) and fasted for 18 hours prior to the study with free access to water. Control group received distilled water orally (0.2 ml/kg body weight); treated groups (1, 2 and 3 respectively) were given HEAAS in doses of 25, 50 and 100 mg/kg body weight orally. The reference group received standard drug (5 mg/kg body weight of atropine sulphate). An hour later, each animal was administered with 1 ml castor oil. Thereafter charcoal meal (10% activated charcoal in 5% gum acacia) of 1 ml via oral route was given an hour later. All animals were sacrificed an hour thereafter, and distance travelled by charcoal meal in the intestine, from the pylorus to the caecum was measured and evaluated as percentage of distance moved (Pazhani, 2001). The results were presented as percentage inhibition of mobility and was calculated using equation 6.

$$\% \text{ Inhibition of Motility} = \frac{A_0 - A_1}{A_0} \times 100 \qquad 6$$

A_0 - Mean distance traveled by the charcoal meal of control; A_1 - Mean distance traveled by the charcoal meal of test

Statistical Analysis: Experimental results were analyzed using Graph Pad Prism, version 8.0 software. Results are expressed as mean±standard error of the mean (SEM), and statistical analyses were carried out by employing one-way analysis of variance (ANOVA), followed by Dunnets post hoc test to

compare results with controls. In all cases, statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Plant Extract and Phytochemicals: Extraction process of pulverized *A. africana* seeds yielded 195 g of *n*-hexane extract (HEAAS) corresponding to 32.5%. Table 1 shows the phytochemical profile of HEAAS. Seventeen compounds comprising hexadecanoic acid (34.97%), 9,12,15-octadecatrien-1-ol (16.27%), oleic acid (6.71%), 4,6-dimethyldodecane (5.91%), squalene (4.82%), 4-methylheptadecane (4.6%) and other minor compounds were identified in HEAAS. Gas chromatography combined with mass spectrometry has been greatly used for qualitative analysis due to its ability to provide precise information about a sample (Cong *et al.*, 2007). GC-MS has been adopted in conducting several phytochemical screening studies in various parts of the world. Thus, there is a growing awareness in correlating the phytochemical compounds with their biological activities (Duke, 2012). The most abundant compound in HEAAS is hexadecanoic acid (34.97%) commonly known as palmitic acid. Previous studies have confirmed that palmitic acid is associated with antioxidant, anti-androgenic, hemolytic, 5-alpha reductase inhibitor, antipsychotic, antidiarrhoeal, hypocholesterolemic (which involves the decrease in cholesterol level and reduction of high blood pressure in the body) activities (Duke, 2012; Ogukwe *et al.*, 2018). This means that the seeds of *A. africana* can possibly assist in managing high blood pressure in those who consume it.

Table 1: Phytochemical Profile of HEAAS

S/N	Compound	Rt	% Composition
1	Hexadecanoic acid	43.26	34.97
2	9,12,15-Octadecatriene-1-ol	36.48	16.27
3	Oleic acid	44.00	6.71
4	4,6-Dimethyldodecane	40.49	5.91
5	Squalene	56.25	4.82
6	4-Methylheptadecane	45.43	4.60
7	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	51.06	4.18
8	(Z,Z)-9,12-Octadecadienoic acid	48.22	4.00
9	*n*-Hexadecenoic acid	48.57	3.62
10	(E)-Pentatriacont-17-ene	43.85	3.42
11	Eicosanoic acid	46.81	3.22
12	(E)-Hexadec-9-enoic acid	40.62	2.75
13	(Z,Z,Z)-4,6,9-Nonadecatriene	52.00	2.39
14	Linalool	25.21	1.95
15	3,5-Ditert-butylphenol	17.48	1.18
16	Zymosterol	27.73	0.42
17	5-Methyl-2-furfuraldehyde	34.00	0.39

In vitro Antioxidant Activity: HEAAS showed low activity in the DPPH scavenging assay. It showed 39.61% inhibition at the highest concentration (100 μg/ml) which was less than the activity of vitamin C (Figure 1a). The result of ferrous ion chelating assay indicated that HEAAS showed antioxidant activity of 59.6 % at 100 μg/ml.

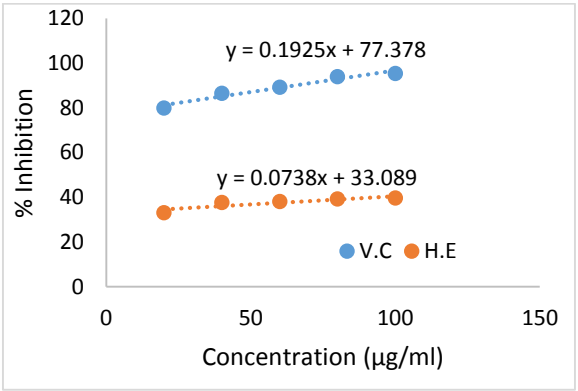

Fig 1a: DPPH Scavenging Activity of HEAAS

This percentage is also lower than that observed for vitamin C (Figure 1b). Likewise for hydrogen peroxide scavenging assay, vitamin C exhibited higher activity than HEAAS (48.11%) at 100 μg/ml (Figure 1c).

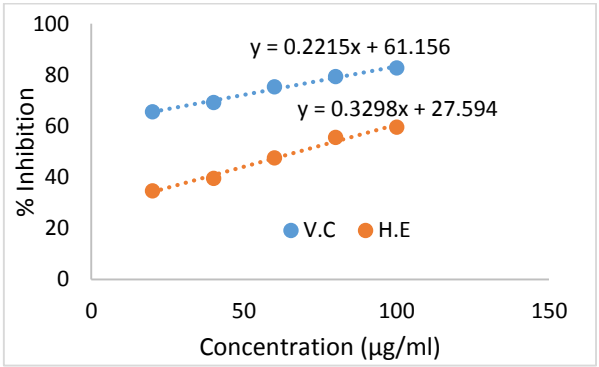

Fig 1b: Ferrous Ion Chelating Activity of HEAAS

However, reducing power assay result indicated that HEAAS exhibited high antioxidant activity. From the

result obtained, HEAAS showed an absorbance of 0.765 at 100 μg/ml. In this test, a higher absorbance indicates higher antioxidant activity (Figure 1d). *In vitro* antioxidant assay conducted on HEAAS revealed that HEAAS possesses moderate antioxidant potential compared with vitamin C (standard). The recorded antioxidant activity of HEAAS would logically arise from some of its phytochemicals such as hexadecanoic acid and squalene previously reported as possessing antioxidant activity (Duke, 2012). Other antioxidative substances directly or indirectly may have contributed to the inhibition or suppression of oxidation.

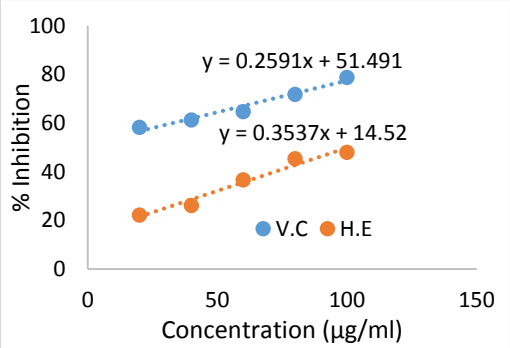

Fig 1c: Hydrogen Peroxide Scavenging Activity of HEAAS

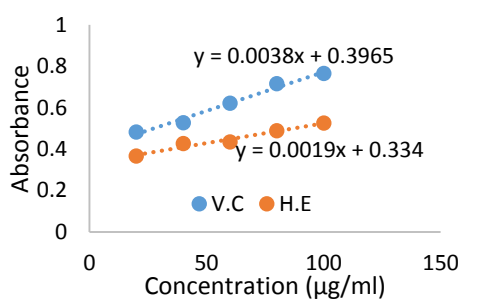

Fig 1d: Reducing Power of HEAAS

Toxicity of HEAAS: Oral administration of HEAAS did not produce any form of overt toxic signs nor death during the observation period of 14 days after a single administration of 5000 mg/kg (Table 2). In addition, neither food nor water intake was found to be reduced during the period. Acute toxicity study showed that HEAAS has a considerable good safety profile such that even 5000 mg/kg/day oral dose of HEAAS is still safe for experimental rats as previously reported (Igboeli *et al.*, 2015).

Table 2: Acute Toxicity Effect of HEAAS

Dose of HEAAS (mg/kg)	Number of animals used	Number of deaths
1000	3	0
1600	3	0
2900	3	0
5000	3	0

Antidiarrhoeal Potential HEAAS: The ethno-medical and traditional claim of antidiarrhoeal activity of HEAAS was evaluated on diarrhoea induced rats and mice using two antidiarrhoeal activity testing models; castor oil-induced diarrhoea and castor oil-induced gastrointestinal motility in rats and mice. With these models, diarrhoea was induced by administering castor oil to each of the mice. Castor oil induces diarrhoea in animal species because of the liberation of ricinoleic acid (its active metabolite) by the action of lipases in the upper part of the small intestine (Kulkami and Pandit, 2005). The release of ricinoleic acid results in the irritation and inflammation of the intestinal mucosa causing the release of prostaglandins, which stimulate secretion, thereby preventing the reabsorption of sodium chloride and water. Castor oil therefore causes both secretory and motility diarrhoea. Inhibitors of prostaglandin synthesis have been reported to delay diarrhoea induced by castor oil (Igboeli *et al.*, 2015). According to Igboeli *et al* (2015), loperamide is an opioid-receptor agonist that acts on the µ-opioid receptors in the mesenteric plexus of the large intestine by decreasing its activity, which in turn decreases the tone of the longitudinal and circular smooth muscles of the intestinal wall.

Table 3: Effect of HEAAS on Castor Oil-induced Diarrhoea

Treatment	Dose mg/kg	Onset of Stool (sec) Mean ± SEM	Total Number of Stool (Mean ± SEM)	Number of Wet Stool (Mean ± SEM)	Weight of Stool (g) (Mean ± SEM)	% Inhibition of Diarrhoea
Control	DW	2640±993.8	6.00±2.00	4.33±2.40	0.50±0.00	-
Loperamide	3	2880±14.15	3.33±0.67	1.00±1.00	0.41±0.35	76.9
HEAAS	25	1080±124.9	5.00±1.00	3.00±0.58	0.37±0.12	30.72
HEAAS	50	4180±2773	5.00±2.52	2.67±2.19	0.37±0.20	38.3
HEAAS	100	4300±2116	1.67±0.67	0.33±0.33	0.17±0.12	92.4

P-value < 0.05, showed the level, DW---- distilled water, HEAAS --- *n*-Hexane extract of *A. africana* seed

Diarrhoea is induced by castor oil within 1 to 2 hours just after administration of 0.1 to 0.3 ml castor oil for mice as other researchers have previously reported (Tadesse *et al.*, 2014). In castor oil-induced diarrhoea model (Table 3), all tested doses of HEAAS were found to be effective (P<0.05) in terms of reduction of frequency of defecation and number of wet faeces (consistency of faeces). The results obtained from this

model are in line with reports elsewhere, where methanol extract of *Pterocarpus erinaceus* leaf (Ezeja *et al.*, 2012), methanol extract of *Osyris quadripartite* leaf (Meseret *et al.*, 2019), aqueous extract of *Lantana camara* stem (Edlam *et al.*, 2017), methanol extract of *Lophira lanceolata* Tiegh leaf (Igboeli *et al.*, 2015) and methanol extract of *Bombax buonopozense* leaf (Akuodor *et al.*, 2011) showed significant reduction of number of wet faeces.

Table 4: Effects of HEAAS on Gastrointestinal Motility

Treatment	Dose mg/kg	Total Length of Intestine (cm)	Length Travel by Charcoal Meal (cm)	% Peristalsis Index	% Inhibition
Control	DW	89.67±0.88	34.67±5.24	38.67±5.81	-
Atropine	5	100.20±6.41	23.33±3.84	23.03±2.48	40.5
HEAAS	25	95.67±2.85	2.83±2.83	2.95±2.95	91.9
HEAAS	50	79.67±5.90	22.00±2.08	28.33±4.97	26.7
HEAAS	100	96.50±1.89	19.33±4.67	20.08±4.91	48.1

p-value < 0.05 showed the level, DW---- distilled water, HEAAS --- *n*-Hexane extract of *A. africana* Seed

In the gastrointestinal motility test (Table 4), all tested doses of the extract produced significant reduction ($P<0.05$) in percentage of intestinal motility. The result obtained from this model is in line with reports of other researchers; aqueous extract of *Lantana camara* stem (Edlam *et al., 2017*) and methanol extract of *Lophira lanceolata* Tiegh leaf (Igboeli *et al.*, 2015) produced significant reduction in percentage of intestinal motility also. The stimulation of cholinergic activity has been reported to cause diarrhoea by increasing gastrointestinal motility (Shaphiullah *et al.*, 2003) and anticholinergics like atropine sulfate prevent diarrhoea by blocking cholinergic stimulation. The significant inhibition of gastrointestinal motility by HEAAS suggests that HEAAS had good anticholinergic effect on intestinal mucosa. Atropine sulfate, the reference drug, showed significant ($P<0.05$) peristalsis index (% charcoal meal transit) compared to the control. This indicates that atropine sulfate prevented the movement of charcoal meal in the intestine significantly, confirming its antimotility effect as an anticholinergic drug. On the other hand, HEAAS at all doses did show strong significant ($P<0.05$) peristalsis index. Substances such as drugs or any plant extract that have the ability to inhibit intestinal transit in pathophysiological states have been reported to be remarkably effective in treating diarrhoea (Shaphiullah *et al.*, 2003). Results of the analysis showed that HEAAS significantly inhibited gastrointestinal transit in the pathophysiological state when compared with the control. Phytochemicals are deemed responsible for biological activities of medicinal plants and their presence in any plant extract provides for the extract's biological activities (Ogukwe *et al.*, 2018). The antidiarrhoeal activity of HEAAS may be attributed to the presence of some phytochemicals as identified in HEAAS such as hexadecanoic acid, oleic acid, *n*-hexadecenoic acid (Garba and Garba, 2017), 4,6-dimethyldodecane, squalene, 4-methylheptadecane amongst other compounds. Hexadecanoic acid, predominately present in HEAAS has been reported to exhibit antidiarrhoeal property (Duke, 2012; Ogukwe *et al.*, 2018).

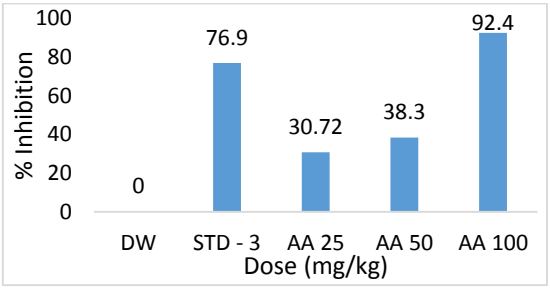

Fig 2a: Antidiarrhoeal Effect of HEAAS *(Key: DW – Distilled water, STD – Loperamide, AA – HEAAS)*

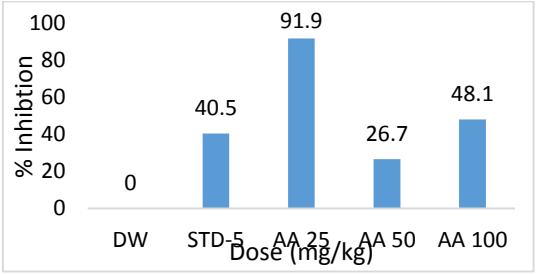

Fig 2b: Effect of HEAAS on Gastrointestinal Motility (*Key: DW – Distilled water, STD – Atropine sulphate, AA – HEAAS*)

Other phytochemicals such as oleic acid have been reported to be highly active against diarrhoea causing microbes (Garba and Garba, 2017). The possible mechanisms of antidiarrhoeal activity of HEAAS could be associated with inhibition of secretion, reduction of intraluminal fluid accumulation induced by castor oil or enhancing water and electrolyte absorption (Igboeli *et al.*, 2015; Shaphiullah *et al.*, 2003).

Conclusion: In conclusion, *Afzelia africana* seed extract possesses antidiarrhoeal property which validates its folklore use, as a potentially safe antidiarrhoeal. *Afzelia africana* seed can also be considered as a mild non-toxic antioxidant.

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