

Biological effect of aqueous extract of *Heinsia crinita* on lipid peroxidation and angiotensin-1-converting enzyme *in vitro*

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ABSTRACT

Heinsia crinita; a common vegetable in the south-eastern part of Nigeria with the local name "Atama" have been used as a component of various herbal portions in ethnomedicine. The plant part has been previously used in the treatment of umbilical hernia and skin rashes. There is dearth of information on the scientific rationale behind the use of this plant. This study was designed to investigate the pharmacological potentials of aqueous extracts of *Heinsia crinita* for the management/prevention of hypertension. Fresh, matured green leaves of *Heinsia crinita* were harvested from a local farm in Cross River State, Nigeria. The total phenol, total flavonoids, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging ability and ability of the extract to chelate Fe^{2+} as well as inhibition of Fe^{2+} -induced lipid peroxidation in rat penile homogenate arginase and angiotensin-1-converting enzyme inhibitory activity were assessed. The results revealed that the extract had high total phenol and total flavonoid content. The extract also exhibited inhibitory effect on Arginase ($\text{EC}_{50} = 3.93 \text{ mg/mL}$) and angiotensin-1-converting enzyme (ACE) ($\text{EC}_{50} = 0.77 \text{ mg/mL}$). Furthermore, the extract exhibited strong antioxidant capacity as typified by DPPH scavenging and Fe^{2+} chelating abilities coupled with the inhibition of Fe^{2+} -induced lipid peroxidation in rat penile homogenate *in vitro*. The bioactive constituents with beneficial medicinal properties coupled with the antioxidant activities and its inhibitory effect on key enzymes linked with hypertension may be the mechanism by which the extract manage and/or prevent hypertension and other cardiovascular diseases.

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1. INTRODUCTION

Hypertension is a multifactorial genetic-related disease that precedes cardiovascular mortality and morbidity in developed and developing countries [1]. Renin produces angiotensin I from angiotensinogen, after which it is converted to a potent vasoconstrictor, angiotensin II, by angiotensin-1-converting enzyme (ACE). ACE also inactivates bradykinin, which has vasodilating action and promotes the secretion of aldosterone. As such, inhibition of ACE activity will yield major antihypertension benefits and this have been considered a useful therapeutic approach in the management and/or

treatment of high blood pressure [2]. Dysfunction of the endothelial tissues plays a major role in the development of erectile dysfunction (ED) and this endothelium dysfunction is impaired by increased oxidative stress and inflammatory conditions [3]. Arginase expressed in the human corpus cavernosum tissue and which catalyse the conversion of L-arginine to ornithine plus urea have been implicated in ED [4] and arginase activity have been reported to increase with hyperglycemia and aging [5]. Increased arginase can also provide ornithine for synthesis of polyamines via ornithine decarboxylase (ODC) and proline/collagen via ornithine aminotransferase

(OAT), leading to vascular cell proliferation and collagen formation, limiting its activity can prevent these changes.

Flavonoids and phenolic acids; which are classes of polyphenols can act as antioxidants in a number of potential pathways [6]. Plants have been shown to contain large amounts of antioxidants molecules which act by neutralizing and absorbing free radicals, decomposing peroxides and quenching single and triplet oxygen [7]. Free radical damage and oxidative stress which interferes with nitric oxide signaling and is toxic to the endothelium have been reported to be a major cause for the endothelial dysfunction in ED [8].

Heinsia crinita with the local name "Atama" is a common south-eastern Nigerian vegetable which belongs to the family known as Rubiaceae [9]. The leaf is widely consumed and the leaf extract have been reportedly used in tradomedicine in the treatment of skin rashes, umbilical hernia and various ailments such as cough, catarrh, sore throat, hypertension among others [10]. Currently, attention is shifting from the use of modern pharmaceuticals to the use of polyphenol-rich medicinal plants which have been engaged in the management of different diseases and pathologies [11]. This study was designed to investigate the pharmacological potentials of aqueous extracts of *Heinsia crinita* for the management of hypertension and infertility through inhibition of the enzymes involved such as angiotension-1-converting enzymes and anginase.

2. MATERIALS AND METHODS

2.1 Chemicals and equipment

Thiobarbituric acid (TBA), Trichloroacetic acid (TCA) were obtained from Sigma (St. Louis, MO USA), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 1, 10- phenanthroline were obtained from Fluka Chemie and Merck (Germany). Acetic acid was procured from BDH Chemical Ltd., (Poole, England). All other chemicals were obtained from standard chemical suppliers and were of analytical grade. A JENWAY UV-visible spectrophotometer (Model 6305; Jenway, Barloworld Scientific, Dunmow, United Kingdom) was used to measure absorbance throughout the experiment. While the water used was glass distilled water.

2.2 Sample collection and preparation of extract

Fresh, matured green leaves of *Heinsia crinita* were harvested from a local farm in Cross river state, Nigeria. The leaves were identified to be *Heinsia crinita* and authenticated at Crop Soil and Pest Department of the school of Agriculture and Agricultural Technology, Federal University of Technology Akure, Nigeria. The leaves were washed and air-dried in the laboratory at 20-25 °C. The dried leaves were manually grinded into powder form and the 0.5g

of the sample was soaked in 10mL of distilled water for about 24 h. The mixture was then filtered and the filtrate was centrifuged to obtain a clear supernatant liquid, which was subsequently used for the various assays. All tests and analyses were performed in triplicate and results were averaged.

2.2.1 Determination of total phenol content

The total phenol content of the extract was determined using the method reported [12]. Appropriate dilutions of the extract was oxidized with 2.5 mL of 10% Folin-Ciocalteu's reagent (v/v) and neutralized by 2.0 mL of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45 °C and the absorbance was measured at 765nm. The total phenol content was subsequently calculated using Gallic acid as standard.

2.2.2 Determination of total flavonoid content

The total flavonoid content of the extract was determined using a slightly modified method reported [13]. Briefly, 0.5 mL of appropriately diluted sample was mixed with 0.5 mL methanol, 50 μ L of 10% AlCl_3 , 50 μ L of 1mol L⁻¹ potassium acetate and 1.4 mL water, and allowed to incubate at room temperature for 30 min. Thereafter, the absorbance of the reaction mixture was subsequently measured at 415 nm. The total flavonoid was calculated using Quercetin as standard.

2.2.3 DPPH free radical scavenging ability

The free radical scavenging ability of the extract against DPPH (1,1-diphenyl-2 picrylhydrazyl) free radical was evaluated as described by [14]. Briefly, an appropriate dilution of the extracts (1 mL) was mixed with 1 mL of 0.4 mmol L⁻¹ methanolic solution containing DPPH radicals. The mixture was left in the dark for 30 min and the absorbance was measured at 516 nm. The DPPH free radical scavenging ability was subsequently calculated with respect to the reference (which contains all the reagents without the test sample).

2.2.4 Determination of ferric reducing antioxidant activity

The reducing power of the extracts was determined by assessing the ability of the extract to reduce FeCl_3 solution as described [15]. Briefly, appropriate dilution of the extract (2.5 mL) was mixed with 2.5 mL 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min and then 2.5 mL 10% trichloroacetic acid was added. This mixture was centrifuged at 353 x g for 10 min. five milliliters of the supernatant was mixed with an equal volume of water and 1 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm. The ferric reducing antioxidant power was expressed as mg ascorbic acid equivalent/g of the sample.

2.2.5 Fe²⁺ chelation assay

The Fe²⁺ chelating ability of the extracts were determined using a modified method of [16] with a slight modification [17]. Freshly prepared 500 µmol L⁻¹ FeSO₄ (150 µL) was added to a reaction mixture containing 168 µL of 0.1 mol L⁻¹ Tris-HCl (pH 7.4), 218 µL saline and the extracts (0 – 100 µL). The reaction mixture was incubated for 5 min, before the addition of 13 µL of 0.25% 1,10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in a spectrophotometer. The Fe²⁺ chelating ability was subsequently calculated.

2.2.6 Lipid peroxidation assay and thiobarbituric acid reactions

Male albino rats were decapitated via cervical dislocation and the reproductive tissue (whole penis) was rapidly dissected, placed in normal saline (0.9 g NaCl in 100mL distilled water) on ice and weighed and later homogenized with phosphate buffer pH 7.4 (1:5 w/v), with about 10-up and down strokes at approximately 1,200 rev/min in a Teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 3,000 g to yield a pellet that was discarded and the supernatant was used for lipid peroxidation assay [18]. The lipid peroxidation assay was carried out using a modified method as described [19]. Briefly, 100µL of the tissue supernatant was mixed with a reaction mixture containing 30 µL of 0.1 M Tris-HCl buffer (pH 7.4), quinine solution (0.001–13 µM) and 30 µL of 250 µM freshly prepared FeSO₄. The volume was made up to 300 µL with distilled water before incubation at 37 °C for 2 hours. Subsequently, 300µL of 8.1 % sodium dodecyl sulphate (SDS), 500 µL of acetic acid/HCl buffer (pH 3.4) and 500 µL of 0.8% thiobarbituric acid (TBA) were added to the reacting mixture. This mixture was incubated at 100 °C for 1 h and Thiobarbituric acid reactive species (TBARS) produced were measured at 532 nm using a spectrophotometer. Malondialdehyde (MDA) was used as standard and TBARS produced was reported as MDA equivalent.

2.2.6.1 Arginase activity assay

Arginase activity was determined by the measurement of urea produced by the reaction of Ehrlich's reagent according to the modified method [20]. The reaction mixture contained in final concentration 1.0 mM Tris-HCl buffer, pH 9.5 containing 1.0 mM MnCl₂, 0.1 M arginine solution and 50 µL of the enzyme preparation in a final volume of 1.0 mL. The mixture was incubated for 10 min at 37 °C. The reaction was terminated by the addition of 2.5 mL Ehrlich reagent (2.0 g of p-dimethylaminobenzaldehyde in 20.0 mL of concentrated hydrochloric acid and made up to 100 mL with distilled water). The optical density reading was taken after 20 min at 450 nm. The control experiment was performed without the test sample and the arginase inhibitory activity was expressed as

percentage inhibition.

$$\% \text{ Inhibition} = \frac{[(\text{AbsControl} - \text{AbsSample})/\text{AbsControl}] \times 100}{1}$$

Where AbsControl = absorbance of control, and AbsSample = absorbance of sample

2.2.6.2 Angiotensin-1-converting enzyme (ACE) Assay

Appropriate dilution of the aqueous extract (0-25 µL) and ACE solution (50 µL, 4 mU) was incubated at 30 °C for 15 min. The enzymatic reaction initiated by adding 150 µL of 8.33 mM of the substrate Bz-Gly-His-leu in 125 mM Tris-HCl buffer (pH 8.3) to the mixture. After incubation for 30 min at 37°C, the reaction was arrested by adding 250 µL of 1 M HCL. The Bz-Gly produced by the enzymatic reaction was extracted with 1.5 mL ethyl acetate. Thereafter the mixture was centrifuged to separate the ethyl acetate layer; then 1mL of the ethyl acetate layer was transferred to a clean test tube and evaporated. The residue was re-dissolved with 1mL of distilled water and its absorbance was measured at 228 nm [21]. The ACE inhibitory activity was also expressed as percentage (%) inhibition.

2.7 Data analysis

The results of the replicate readings were pooled and expressed as means ± standard deviation. Student *t*-test was performed and significance was accepted at *P* ≤ 0.05 [22]. EC₅₀ (concentration of extract that will cause 50% concentration activity) was determined using linear regression analysis. Microsoft Excel 2003 and Origin 6.1 version software was used.

3. RESULTS AND DISCUSSION

The results of the total phenol and flavonoid content in the aqueous extract of *H. crinita* are presented in Fig. 1. The total phenolic content reported as gallic acid equivalent was 14.47 mgGAE/g, while the total flavonoid content reported as quercetin equivalent was 8.77 mgQUE/g. Furthermore, the extract dose-dependently scavenged DPPH radical as revealed in Fig. 2 with EC₅₀ values of 17.16 mg/mL Table 1 and the extract also able to chelated Fe²⁺ in dose dependent manner with EC₅₀ value of 7.34 mg/mL (Fig. 3 and Table 1).

The incubation of rat penile tissue homogenates in the presence of Fe²⁺ caused a significant increase (*P* < 0.05) in the malondialdehyde (MDA) content (108.96%) as shown in Fig. 4. However, introduction of *H. crinita* (Atama) aqueous extract inhibit MDA content in a dose-dependent manner with EC₅₀ values of 3.06 mg/mL (Table 2). Furthermore, the study revealed that the leaf extract inhibit arginase and angiotensin-1-converting enzyme activities in a dose-dependent manner as shown in Figs. 5 and 6 with their EC₅₀ values in Table 2.

Table 1. The IC₅₀ Value of DPPH* Scavenging and Fe²⁺ chelating Abilities of aqueous extracts from *Heinsia crinita* (mg/mL).

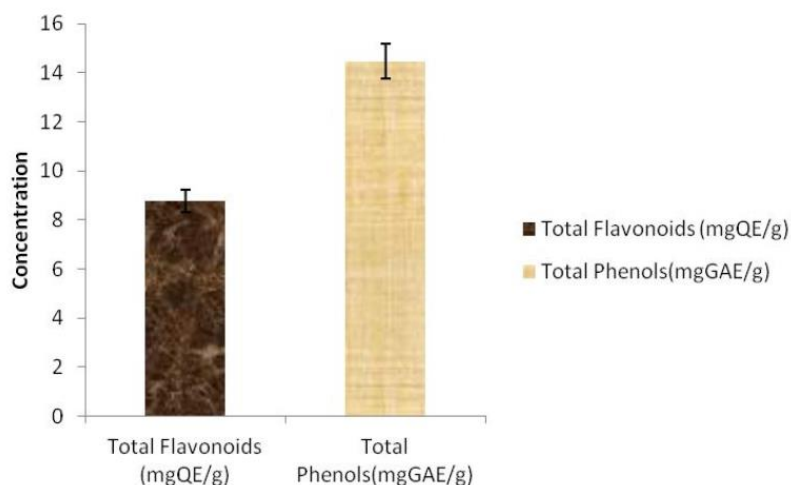
Parameters	EC ₅₀ (mg/mL)
DPPH free radical scavenging ability	17.16±1.00
Fe ²⁺ chelating ability	7.34±0.70

Values represent means ± Standard deviation of triplicate readings.

Table 2. IC₅₀ of enzyme inhibitory activities of *Heinsia crinita* extract (mg/mL).

Aqueous <i>Heinsia crinita</i> extract	EC ₅₀ (mg/mL)
Inhibition of Fe-induced Lipid peroxidation	3.06±1.06
Inhibition of Arginase activity	3.93±1.47
Angiotensin-1-Converting enzyme	0.77±1.38

Values represent means ± Standard deviation of triplicate readings

**Fig. 1.** Total phenol and total flavonoid content of *Heinsia crinita* (Atama) aqueous extract. Values represent mean ± standard deviation, n = 3.

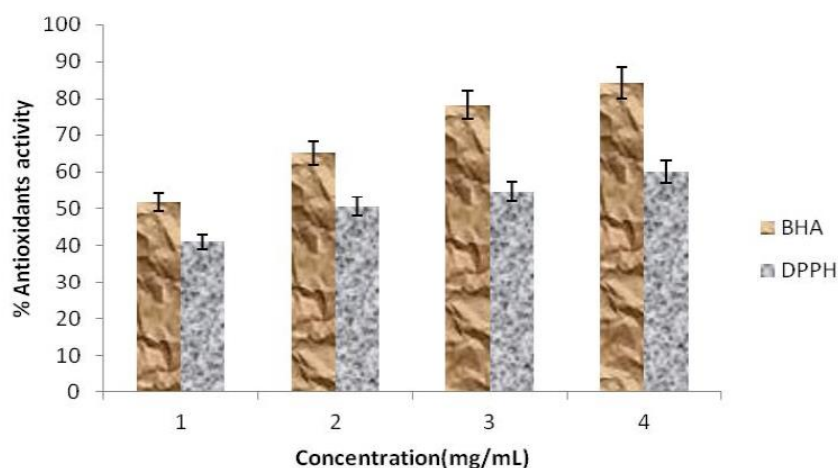


Fig. 2. DPPH scavenging ability of *Heinsia crinita* (Atama) aqueous extract. Values represent mean \pm standard deviation, $n = 3$.

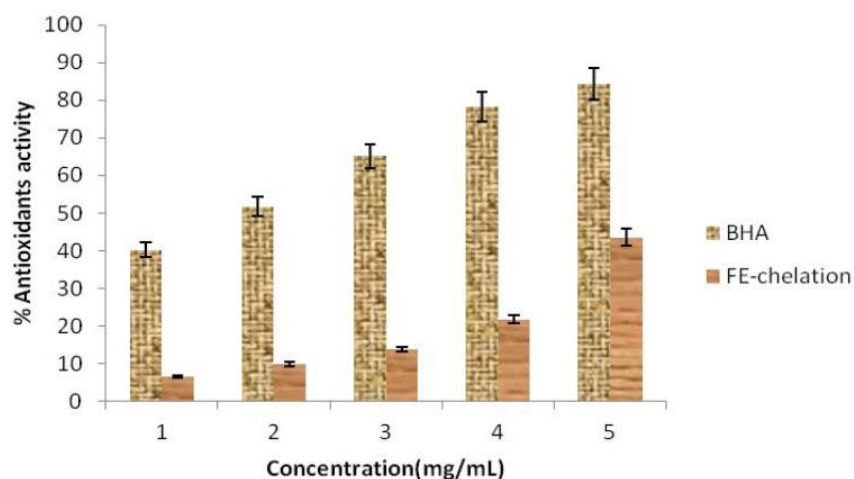


Fig. 3. Fe^{2+} -chelating ability of *Heinsia crinita* (Atama) aqueous extract when compare to control drug butylated hydroxyanisole (BHA). Values represent mean \pm standard deviation, $n = 3$.

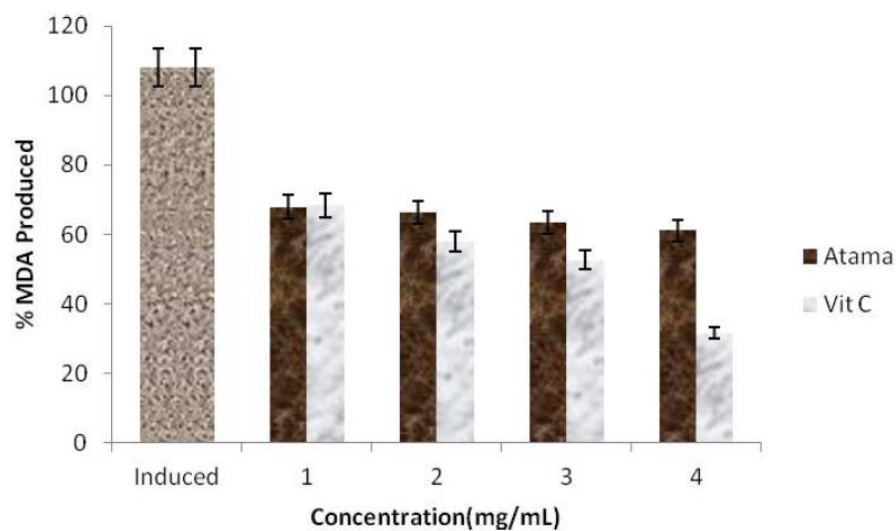


Fig. 4. Inhibition of Fe^{2+} -induced lipid peroxidation MDA produced by *Heinsia crinita* (Atama) aqueous extract compare to control drug Ascorbic acid (Vit C). Values represent mean \pm standard deviation, $n = 3$.

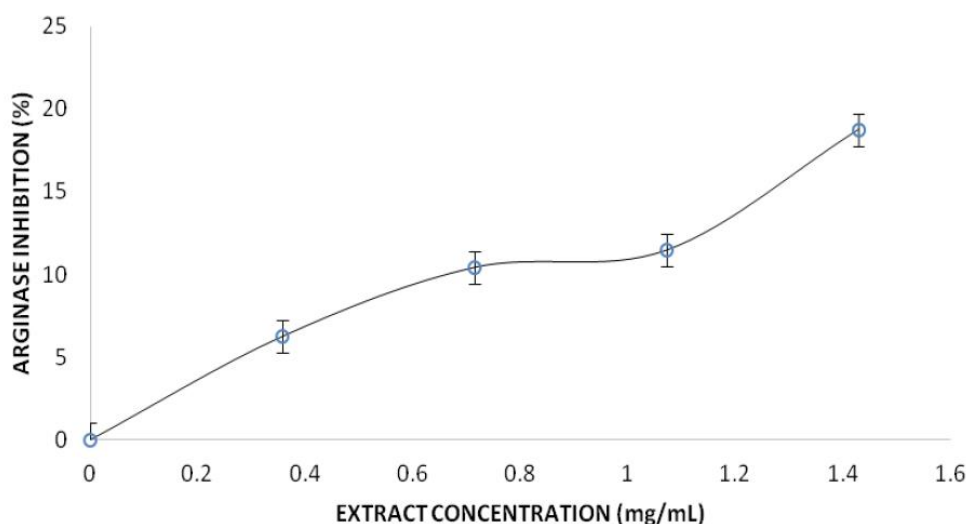


Fig. 5. Inhibition arginase by *Heinsia crinita* (Atama) aqueous extract. Values represent mean \pm standard deviation, $n = 3$.

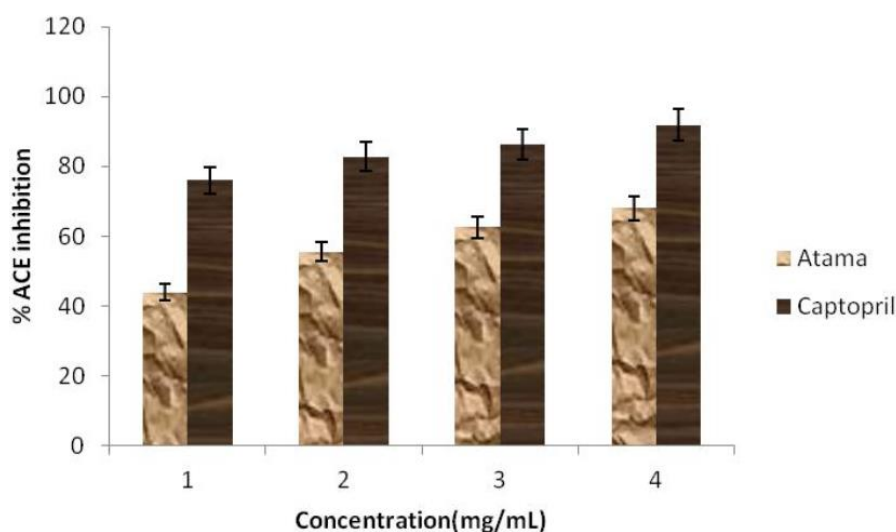


Fig. 6. Angiotensin-1-converting enzyme inhibition by *Heinsia crinita* (Atama) aqueous extract compare to control drug Captopril. Values represent mean \pm standard deviation, $n = 3$.

Side effects and cost are some of the problems encountered with the use of modern pharmaceuticals in the treatment of different pathologies prompting a shift to the use of polyphenol-rich medicinal plants [11]. *H. crinita* is a widely consumed vegetable in the Southern part of Nigeria which have been reportedly used in folklore medicine [10]. Phenolic phytochemicals in plants have been shown to have positive health effects based on the abilities to counter negative effects of free radicals [23]. Flavonoids which are widely distributed phytochemicals with antioxidant activities [6]. Flavonoids possess the ability to scavenge hydroxyl radicals, superoxide anions and lipid peroxy radicals [24]. The results of the total phenol and flavonoid contents of the aqueous *H. crinita* extract are as presented in Fig. 1 as gallic acid (GAE) and quercetin (QE) equivalent respectively. Phenol and phenolic

compounds have been reported to possess anti-microbial and disinfecting abilities [25]. Flavonoids whose detoxification and antioxidant activities have been well established have also been reported to modify the body's reaction in response to virus, allergens and carcinogens [26,27].

The presence of phenolic compounds in the aqueous extract of *H. crinita* could be an explanation for the strong antioxidant activities as typified by the DPPH scavenging and Fe^{2+} chelating abilities displayed by the studied plant. These polyphenols are capable of donating hydrogen atoms to free radicals, thus inhibiting oxidation process [28]. Phytochemicals present in plants complexes with the metal as extract chelate Fe^{2+} , thereby aiding its excretion from the body.

Lipid peroxidation of cell membranes causes damage which reduces antioxidant which reduces antioxidant enzyme and antioxidant substrates to induce oxidative stress which contributes to the causation of injuries in the penile tissues [29]. Oxidative damage can occur in DNA during the peroxidative breakdown of membrane polyunsaturated fatty acids [28]. DNA damage affects homeostasis of various cells leading to cell damage and death [8]. However, the inhibition of Malondialdehyde production brought about by the introduction of aqueous *H. crinita* extract could be attributed to the ability of the extract to inhibit its production, thus prevent initiation of oxidative damage [30]. Dose-dependent inhibition of arginase activity by the aqueous *Heinsia crinita* extract could be of immense importance in the management of hypertension as this would help to reduced elevated diastolic and systolic blood pressure. Moreover, [31] reported that elevated arginase activity is involved in Angiotensin -II-induced arterial thickening, fibrosis, and stiffness and controlling it will reduced the elevated blood pressure. Polyphenols present in aqueous various plant extracts have been reported to possess inhibitory effects on arginase activity [32,33,34]. Moreso, studies have shown [35] that inhibition of angiotensin-1-converting enzyme (ACE) activity could be used in the management of hypertension. In the same vein, polyphenols constituents of the aqueous *H. crinita* extracts inhibited Angiotensin-1-converting enzyme and arginase activities in-vitro in a concentration-dependent manner.

4. CONCLUSION

The potent antioxidant properties, Arginase and Angiotensin-1-converting enzyme inhibitory effect of the aqueous *Heinsia crinita* extract suggest it could be promising in the management of hypertension and infertility. However, this health promoting effect is suggested to be a function of it phenolic and flavonoid content. More studies on the characterization of the bioactive compounds are encouraged.

AUTHOR CONTRIBUTIONS

EEN designed the study, GO wrote the protocol, BCA managed the analyses of the study, TMA wrote the first draft, IE managed the literature searches. All authors read and approved the final manuscript.

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