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Journal of Acute Disease

journal homepage: www.jadweb.org

Biological research http://dx.doi.org/10.1016/j.joad.2015.04.010

Evaluation of the safety profile and antioxidant activity of fatty hydroxamic acid from underutilized seed oil of *Cyperus esculentus*

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ARTICLE INFO

Article history:
Received 9 Apr 2015
Received in revised form 15 Apr 2015
Accepted 19 Apr 2015
Available online 9 Jul 2015

Keywords:
Antioxidants
Cyperus esculentus
Fatty hydroxamic acid
Hepatotoxicity
Serum chemistry

ABSTRACT

Objective: To evaluate the safety profile and antioxidant activity of fatty hydroxamic acid (FHA) from the seed oil of *Cyperus esculentus* (*C. esculentus*).
Methods: FHA was synthesized from the seed oil of *C. esculentus* via a two-step reaction system. The FHA was later subjected to antioxidant activity test using 2, 2-diphenyl-1-picyrl hydrazyl assay. Additionally, adult male Wistar rats were randomly assigned to four groups of five rats each and were orally administered with FHA at 0, 5, 15 and 50 mg/kg for seven days. Clinical observations and serum biochemical parameters were assessed to monitor treatment–related adverse effects in the rats.
Results: Fourier transform infrared spectra showed that FHA was synthesised from the seed oil of *C. esculentus*. The antioxidant property of FHA increased as the concentration reduced below 0.05 μg/mL. Result of oral administration of FHA revealed no adverse effect levels at the dose of 5 mg/kg/day. However, the adverse effects seen in rats receiving 15 mg/kg/day (the least observed adverse effect level) were significant increase in alkaline phosphatase activity, triglycerides, and creatinine levels. Moderate hyperalbuminemia and hypoalbuminemia resulted in an increased albumin/globulin ratio. These effects might be the result of a physiological response to exposure to a very high level of FHA which is not part of the normal diet, and are most likely not toxicologically relevant.
Conclusions: *C. esculentus* has been presented as a potential source of feed stock for the synthesis of a relatively cheap and non toxic FHA which has antioxidant and free-radical scavenging activity.

1. Introduction

Hydroxamic acid is known as an important family of organic bioligands which is considered to be derivatives of hydroxylamine and carboxylic acids as seen in Figure 1; one of its earlier physiological importances being associated with its use as siderophores[1]. Over the years, there has been increasing interest in its roles as potent and selective inhibitors of a range of enzymes[2–4]. It has also been reported as chemotherapeutic agents[5,6] while a number of its derivatives have been reported as pharmaceutics in treating cancer, cardiovascular diseases, hypertension, tuberculosis and fungal infections[7]. Specific derivatives of hydroxamic acid such as suberoylanilide hydroxamic acid has been reported to be a potent inhibitor of histone deacetylases, and possesses anticancer and apoptosis effect against several tumour types[8,9].

Chemically, hydroxamic acid and its derivatives have been shown to be nitric oxide donors by way of their chemical reactivity. Their biological activity has also been attributed to the strong metal ion chelating ability and the nitric oxide releasing properties[10]. Its ability to delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions suggests them as potential antioxidants[11]. It has the ability of reacting with single free radicals to form neutral molecules due to its ability to donate electrons and as potential antioxidants; they may be able to prevent cell and tissue damage as they act as scavenger[12].

Although hydroxamic acid and a number of its derivatives have been synthesised from simple fine chemicals, they have been shown to be expensive and may have environmental concerns[13–15]. There have been little reports on the use of biomass

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Peer review under responsibility of Hainan Medical College.

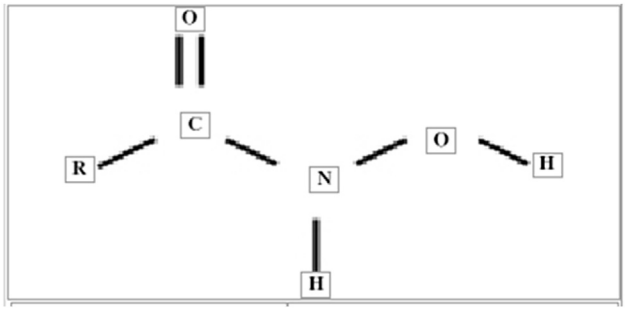

Figure 1. The general structure of hydroxamic acid.

as feed stock for the synthesis of hydroxamic acid. Use of biomass such as seed oils as feed stock in oleochemicals is important in replacing petrochemicals; such biomass will lead to a product which is cost effective, biodegradable and environmentally friendly. Underutilized seed oil such as tiger nut [*Cyperus esculentus* (*C. esculentus*)] oil is an example of biomass feed stock which can be used to achieve this purpose.

C. esculentus commonly called tiger nut belongs to the Cyperaceae family of plant species. The tubers are about the size of peanuts and are commonly found in Nigeria[16]. Research studies have shown that 100 g tiger nuts contain 386 kcal (1 635 kj) as 7% proteins, 36% fats (oils), 31% starch, 21% glucose, 26% fiber of which 14% is non-soluble and 12% soluble, mineral and vitamin E and C indicating the possibility of *C. esculentus* tuber being exploited[17,18]. The nut was found to be rich in myristic acid, oleic acid and linoleic acid[19]. It has been reported to reduce the risk of colon cancer with positive effect on cholesterol level due to high content of vitamin E[20]. Since the seed contains about 36% oil, it is considered as a good source of seed oil. The fatty acid composition of the oil has been reported to be mainly oleic, linoleic and palmitic acids[21–23].

Aside the synthesis from biomass, the establishment of the toxicity level of such bio-based hydroxamic acid is crucial. In response to this, the present work has evaluated the synthesis of fatty hydroxamic acid (FHA) from underutilized seed oil of *C. esculentus*. The antioxidant properties of the FHA and its toxicity profile in blood clinical parameters were also examined.

2. Materials and methods

2.1. Materials

The seeds of *C. esculentus* were purchased from Festac Town market in Lagos State, Nigeria and identified at the Herbarium Unit, Botany Department University of Ibadan. They were air dried and subsequently ground in a laboratory mill. The ground seeds of *C. esculentus* were later extracted with *n*-hexane for 10 h using a soxhlet extractor[23].

2.2. Methyl esters from the seed oil of C. esculentus

Oil of *C. esculentus* was converted to methyl esters as previously described by Adewuyi *et al*[24]. Briefly, the oil was firstly pre-treated using 2% sulphuric acid in methanol at 70 °C for 2 h to convert the free fatty acid content of the oil to methyl esters. The resultant product was extracted with ethyl acetate, washed with water until free of acid, passed over sodium sulphate and concentrated using a rotary evaporator. The pre-treated oil was finally transesterified using 1% KOH in methanol at 70 °C. The methyl esters formed were extracted with ethyl acetate, washed with water until free of KOH and dried over sodium sulphate while ethyl acetate was removed using a rotary evaporator.

2.3. Synthesis of hydroxamic acid

Hydroxamic acid was synthesised by adding 2.75 g (0.04 moL) sample of hydroxylamine hydrochloride to 16.50 mL of water in a 250 mL Erlenmeyer flask. This was gently warmed after which 11 mL of potassium hydroxide (0.05 moL) and 1.1 g of fatty methyl esters of *C. esculentus* oil were added followed by ethanol (15 mL) to give a clear solution. The mixture was warmed in a water bath for 15 min after which it was cooled in an ice bath. The resulting solid was filtered and dried to give hydroxamic acid. The progress of the reaction was monitored using Fourier transform infrared spectroscopy (FTIR) spectrophotometer (Schimadzu, 8 400 s).

2.4. In vitro anti-oxidant assay

The antioxidant activity of the *C. esculentus* FHA was measured using 2,2-diphenyl-1-picyrl hydrazyl (DPPH) assay as described in our previous study[25]. This spectrophotometric assay uses the stable radical DPPH as a reagent. The DPPH free radical was prepared at a 0.1 mmol/L concentration (2.5 mg/L) in methanol while radical was prepared, protected from light and refrigerated[26]. The sample stock solution (10 mg/mL) was diluted to final concentrations of 0.1–0.05 mg/mL. The dilutions were prepared in triplicate and 1 mL of DPPH was added to each concentration of triplicate which was incubated in the dark for 30 min while absorbance was taken at 518 nm. Control experiment was carried out to determine the absorbance of DPPH before interacting with the hydroxamic acid. Blank experiment was also carried out to determine the absorbance of the sample without DPPH. Absorbance was recorded to check the stability of the radical throughout the time of analysis. The total antioxidant activity was calculated using the following equation[27]:

$$\text{Total antioxidant activity} = 100 \times \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}$$

2.5. Animals and treatment

Twenty male adult albino rats of the Wistar strain, weighing between 270 and 300 g were used in this study. Animals were obtained from the primate colony of the Department of Veterinary Anatomy, University of Ibadan. Rats were fed on commercial pelleted diet (Ladokun Feeds Ibadan, Nigeria) and drinking water *ad libitum*, maintained under standard laboratory conditions and subjected to natural photoperiod of 12 h light/12 h dark cycle. Rats were randomly assigned into four groups of five rats each. Rats in Group A served as controls and received distilled water. Group B rats were treated with FHA at the dose of 5 mg/kg body weight/day and Rats in Groups C and D were treated with FHA at a dose of 15 mg/kg and 50 mg/kg body weight/day, respectively. These doses represent low, medium and high doses. All treatments were given, orally, once daily for seven days. During the experimental period, all animals were observed daily for clinical signs and symptoms of toxicity. The animals were sacrificed 24 h after the last treatment. All

experiments conformed to guidelines governing the handling of laboratory animals as outlined by the Redeemer's University Committee on Ethics for Scientific Research.

2.5.1. Preparation of samples

Rats were sacrificed by cervical dislocation and blood samples were collected by cardiac puncture into clean plain bottles and centrifuged at 4000 g for 10 min (Heraeus Labofuge 300, Thermo Scientific, Hampshire UK). Serum was carefully separated out and stored frozen until required for analysis.

2.5.2. Serum biochemical analyses

Biochemical analyses were carried out to determine the serum concentrations of alanine and aspartate aminotransferases, alkaline phosphatase, lactate dehydrogenase, gamma glutamate transferase, bilirubin, albumin, total cholesterol, triglyceride and creatinine using diagnostic kits (Randox Laboratories Limited, Crumlin, United Kingdom) as reported in our previous studies[28].

2.6. Data analysis

All data were expressed as mean ± SEM. Differences between the groups were determined by One-way ANOVA and *post hoc* testing was performed using Dunnett's test (Graph Pad Prism 3 software). Values were regarded as significantly different at $P < 0.05$.

3. Results

3.1. Synthesis

Light golden coloured oil was obtained from the seed of *C. esculentus* which was first converted to methyl esters and finally FHA via simple reaction steps. The FTIR results provided clear and convincing evidence regarding the chemical structure, mainly from amide-type carbonyl, NH bending, and OH/NH stretching bands. Certain peaks found in the spectrum of the oil before the reactions were not seen in that of FHA establishing that chemical reaction has taken place.

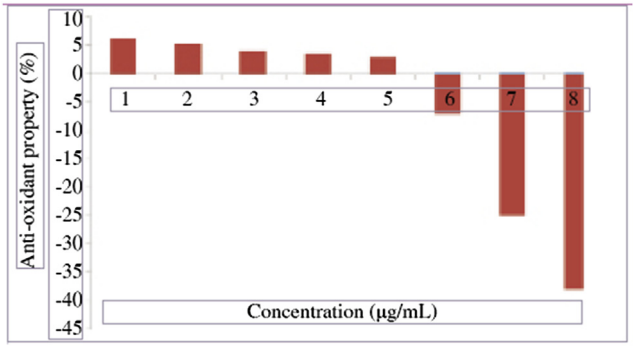

Figure 2. Anti-oxidant capacity of FHA from *C. esculentus*.

3.2. In vitro anti-oxidant assay

As presented in Figure 2, the antioxidant property of the synthesized FHA increased as the concentration reduced. On the other hand, on increasing the concentration of the FHA above 0.05 µg/mL, the anti-oxidant capacity FHA reduces and tends toward being a pro-oxidant. The half maximal IC_{50} was found to be 185.10 ± 0.04 µg/mL. This measures the effectiveness of FHA in scavenging for free radicals.

3.3. Clinical chemistry studies

3.3.1. Effect of FHA on body weight, liver and kidney weights of rats

Table 1 showed the effect of FHA on body and organ weight. FHA administered to rats at the doses of 5, 15 and 50 mg/kg did not alter body, absolute/relative liver and kidney weights in these animals when compared with control.

The results of the total cholesterol and triglycerides levels are also presented in Table 2. Administration of FHA at all treatment doses did not significantly affect the total cholesterol levels when compared with the control, however, the triglyceride levels were significantly elevated upon treatment at 15 and 50 mg/kg. This contradicted an earlier study by Izydore *et al.* which reported the hypolipidemic activities of benzohydroxamic acid and dibenzohydroxamic acids in mice and rats at a dose of 20 mg/kg/day[29]. Increase in serum triglyceride level, as observed in this present study, may indicate impairment of lipid metabolism in the animals. Serum level of creatinine is an indicator of renal function. The result of this study showed that per os administration of FHA increased the level of serum creatinine at all treatment doses. This is an indication that FHA may adversely impair the glomerular filtration function of the kidney. This may also be possibly due to increased catabolic state in the rats from prolonged appetite[30].

3.3.2. Effect of FHA on some blood clinical chemistry indices

Animals treated with FHA at a dose of 15 mg/kg showed a statistically significant decrease ($P < 0.05$) in mean total protein level when compared with control. This may be due to decrease in the constitutive globulin fraction. Also, a non-significant increase in albumin level was also observed in all treatment groups in comparison with controls indicating that the synthesis of the protein by the liver was not adversely affected by the ingestion of FHA.

Table 1
Effect of FHA on body weight, liver and kidney weights of rats.

Variables	Control	5 mg/kg	15 mg/kg	50 mg/kg
Final body weight (g)	302.50 ± 8.50	302.50 ± 6.30	315.00 ± 6.50	310.00 ± 5.80
Liver weight (g)	7.90 ± 0.22	7.10 ± 0.41	8.10 ± 0.57	6.70 ± 0.41
L/B (%)	2.61	2.35	2.58	2.16
Kidney weight (g)	1.40 ± 0.06	1.40 ± 0.04	1.40 ± 0.06	1.30 ± 0.13
K/B (%)	0.47	0.46	0.46	0.42

Values are expressed as mean ± SEM. B: Final body weight; L: Liver weight; K: Kidney weight.

Table 2
Effect of FHA on some serum constituents in rats.

Variables	Control	5 mg/kg	15 mg/kg	50 mg/kg
Total protein (g/dL)	20.40 ± 0.58	18.60 ± 1.37	16.30 ± 0.28[a]	16.60 ± 0.88
Albumin (g/dL)	10.70 ± 1.25	11.80 ± 2.00	12.40 ± 1.27	12.4 ± 2.0
Globulin (g/dL)	9.70 ± 0.71	6.80 ± 0.31	3.90 ± 0.28	4.20 ± 0.92
A/G ratio	1.10 ± 0.08	1.80 ± 0.50	3.30 ± 0.26[b]	2.60 ± 0.42[a]
Bilirubin (mg/dL)	0.19 ± 0.01	0.17 ± 0.01	0.14 ± 0.02	0.25 ± 0.01[a]
Cholesterol (mg/dL)	284.90 ± 7.38	253.70 ± 11.19	256.20 ± 14.21	276.40 ± 6.64
Triglyceride (mg/dL)	385.60 ± 8.57	383.20 ± 8.20	338.40 ± 11.01[a]	334.80 ± 11.26[b]
Creatinine (mg/dL)	36.90 ± 9.52	82.00 ± 10.26[a]	112.80 ± 5.91[b]	102.60 ± 10.34[b]

Values are expressed as mean ± SEM of five animals per group. [a]: Significantly different from control ($P < 0.05$); [b]: Significantly different from control ($P < 0.01$); A: Albumin; G: Globulin.

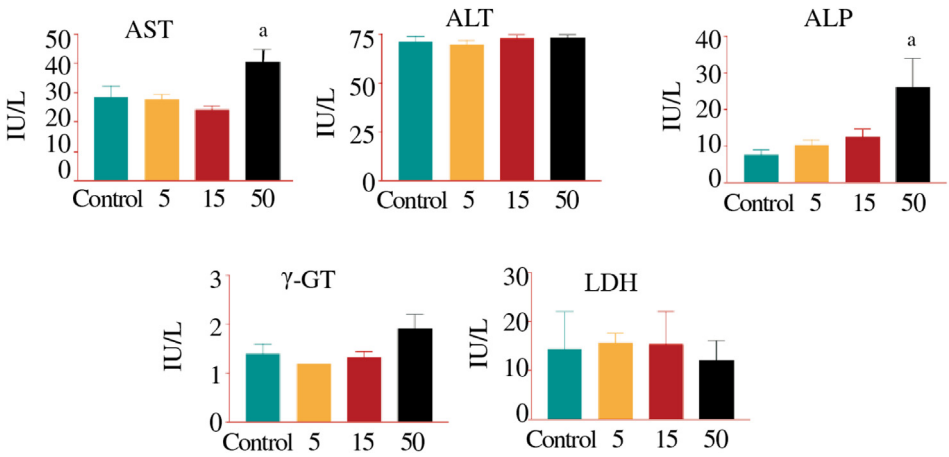

Figure 3. Effect of FHA from *C. esculentus* on activities of serum enzyme markers, AST, ALT, ALP, γ-GT and LDH in rats (mg/kg).
[a]: Significantly different from control ($P < 0.05$); Enzyme activity is quantified as IU/L.

3.3.3. Effect of FHA on serum marker enzymes

In our study (Figure 3), FHA administered at the different doses did not cause any significant change in alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and γ-glutamyl transferase (γ-GT) activities, whereas aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities were significantly elevated ($P < 0.05$) when compared with control at a 50 mg/kg dose of FHA. γ-GT activity was not adversely affected just as there was no disproportionate elevation of LDH.

4. Discussion

Both spectral of oil and FHA have peaks at 3007 cm^{-1}, 2922 cm^{-1} and 2852 cm^{-1} corresponding to the vibrational frequencies of unsaturated carbon bonds (CH=CH), methyl alkane (CH_3) and the methylene groups (CH_2). The absorption band for the ester functional group was found at 1745 cm^{-1} in the oil which disappeared after the reaction with the appearance of characteristic bands at 1560 cm^{-1}(C–N–C) and 1653 cm^{-1} (C=O). A peak was also found at 3387 cm^{-1} which can be attributed to the OH functional group in FHA; these peaks suggest the formation of FHA[31,32]. Peaks found at 1425 and 1464 cm^{-1} in FHA were also accounted for as being the C–N absorption bands while the N–O vibrational frequency appeared at 923 cm^{-1}. The design and synthesis of oleochemicals from biomass for industrial applications in fields such as Biomedical Sciences has been of great importance[33]. FHA falls into this group of oleochemicals with tremendous industrial applications which may be attributed to its expected reactivity, low toxicity and wide spectrum of activities in biological systems[34,35]. From the present study, FHA was synthesized from *C. esculentus* seed oil with the yield of FHA being 94%. This high yield of 94% suggests *C. esculentus* seed oil as a good source of FHA.

Molecular oxygen and its reaction products such as superoxide radical and hydrogen peroxide generate free radicals that can cause injury to biological organisms; such damage may result in protooncogene activation and/or suppressor gene inactivation[36]. Antioxidants are required to scavenge these free radicals from biological systems in order to avert or cancel out the cell-damaging effects of free radicals. This present study has used DPPH as a stable free radical that can easily accept an electron or hydrogen radical to become a stable diamagnetic molecule; which is a typical means of evaluating the antioxidant potential of substances[36,37]. From the results obtained, antioxidant property of the synthesized FHA increased with a reduction in its concentration while on increasing its concentration above 0.05 μg/mL, the antioxidant capacity reduces and tends toward being a pro-oxidant. This observation had also been previously reported that aside from antioxidants being reducing agents they can also act as pro-oxidants[37–41].

Analysis of organ weight in toxicology studies is an important endpoint for identification of potentially harmful effects of chemicals. The measured body weight and organs did not change when compared with control. This observation correlated with another study reported that administration of suberoylanilide hydroxamic acid to rats at doses of 15 and 50 mg/kg/day for 14 days did not elicit any treatment-related decreases in body weight[42].

However, there were significant increases in the A/G ratio at the doses of 15 and 50 mg/kg, but these increments were not dose dependent. Based on clinical interpretations, the phenomenon of increased A/G ratio is not clinically significant. Bilirubin is a yellow pigment produced when heme is catabolized. In this study, we observed a one fold increase in serum total bilirubin levels in the FHA-treated (50 mg/kg) rats compared with the control rats. This consequent hyperbilirubinemia may result from ineffective erythropoiesis or an impaired ability of the liver to excrete normal amounts of bilirubin[43].

Liver function tests are commonly used in clinical practice to screen for liver disease, monitor the progression of known disease, and monitor the effects of potentially hepatotoxic drugs. The most common liver function tests include the serum aminotransferases, alkaline phosphatase, bilirubin, albumin, and prothrombin time. Aminotransferases, such as ALT and AST, measure the concentration of intracellular hepatic enzymes that have leaked into the circulation and serve as a marker of hepatocyte injury. Alkaline phosphatase, γ-GT and bilirubin act as markers of biliary function and cholestasis. Albumin and prothrombin reflect liver synthetic function[44]. There was no significant change in ALT, LDH, and γ-GT activities. Yeboah and others have studied the phytosterols and the vitamin E content of *C. esculentus*[45]. They reported the presence of total vitamin E content (120.1 μg/g of oil) and high amounts of phytosterol such as β-sitosterol, stigmasterol and campesterol. This moderately high content of phytosterols and Vitamin E could protect the liver from oxidative damage by preventing free radical formation or scavenging already-formed free radicals. The ability of FHA to scavenge the DPPH radical also corroborates this protective mechanism. On the contrary, AST and ALP activities were significantly elevated ($P < 0.05$) relative to control following administration of FHA at a dose of 50 mg/kg. But because of the cellular distribution of ALP, increased serum activity may be caused by a wide variety of disorders. Consequently, ALP values are interpreted in tandem with other clinical indices. In our study, there was no disproportionate elevation of LDH which suggested the absence of multi-organ system disease. Likewise γ-GT activity was not adversely affected in all treatment groups, so this precluded the hepatobiliary system as the source of the elevated ALP. This observed increase in ALP and AST could be more of physiological than pathological as a result of the high fatty acid content of the FHA[46].

FTIR spectroscopy showed that FHA based on *C. esculentus* seed oil was successfully prepared. Advantages of this synthesis from the seed oil include ease of preparation from the seed oil of *C. esculentus* which is a cheap source and moderate reaction conditions consistent with green chemistry principles. Also in this paper we showed that FHA has antioxidant and free-radical scavenging activity. It was also observed that FHA was not overly toxic to the liver, although care must be exercised during formulation to avoid target organ toxicity.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors are grateful to the Department of Chemical Sciences, Redeemer's University for the provision of chemicals, reagents and laboratory space towards the successful completion of this work.

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