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The antihyperglycemic, antioxidant and antimicrobial activities of *Ehretia cymosa*

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Abstract
Ehretia cymosa Thon. (Family Boraginaceae) is a shrub used by inhabitants of several communities in Ghana to treat diabetes mellitus and treatment of diarrhoea in indigenous poultry species including Guinea fowls (*Numida meleagris*). In this study, the antihyperglycaemic, antioxidant and antimicrobial activities of *Ehretia cymosa* was investigated. A 70% ethanolic extract of the whole plant of *Ehretia cymosa* was assessed for its *in vitro* anti-oxidant and antimicrobial activities by antioxidant and agar diffusion assays respectively. Antihyperglycaemic activity was investigated in vivo in Sprague Dawley rats while modulatory effects on intestinal absorption of glucose were also determined *ex vivo*. The extract showed a significant dose independent reduction of the fasting blood glucose level and the rat intestinal sacs had a significant decrease in glucose absorption. The extract showed strong antioxidant activity and also exhibited inhibitory activity against *P. aeruginosa*, *E. coli*, *B. subtilis* and *S. aureus*. These findings justify the folkloric use of the extract of *E. cymosa* as an antidiabetic medicine and as well as antibacterial agents for susceptible species.

Keywords: Diabetes mellitus, Antihyperglycaemia, Antioxidant, Antimicrobial, *Ehretia cymosa*, Streptozotocin**.**

Abbreviations: streptozotosin (STZ), ethanolic extract of *Ehretia cymosa* (EEEC), fasting blood glucose level (FBGL), butylated hydroxytoluene (BHT),Gallic acid equivalents (GAE), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid (AA), minimum inhibitory concentration (MIC)

1. Introduction
The surge of non-communicable diseases in Africa cannot be overemphasized. It is expected that within the next two decades, non-communicable diseases together with diabetes mellitus will overtake infectious diseases as the leading cause of death in Africa [1]. Diabetes is a chronic disease, which occurs when the pancreas does not produce enough insulin (Type 1 or childhood-onset diabetes), or when the body cannot effectively use the insulin it produces (Type 2 or non-insulin-dependent diabetes). This leads to an increased concentration of glucose in the blood (hyperglycaemia) [2]. The presence of diabetes is associated with a high incidence of hypertension, hyperlipidaemia, nephropathy, and retinopathy and these comorbidities account for increased risk for vascular complications [3]. It is estimated that 347 million people globally have diabetes. In Sub-Saharan Africa, about 8 % of the population above 25 years has diabetes [4].
The cost of management of diabetes per patient in Ghana increased from $180 – $420 in 2001 to $1276 -$7660 in 2007 while the economic cost of controlling diabetes using orthodox drugs worldwide is estimated to be $232 billion and could rise to $302.5 billion by 2025 [5-6]. This chronic disease takes an ever-increasing proportion of national healthcare budget, as the number of people with the disease increases. Immediate action is needed to stem the tide of diabetes mellitus and introduce cost effective treatment strategies to reverse this trend [7]. Ethnomedical management of chronic diseases has been encouraged by the World Health Organization as an alternative treatment to orthodox medicine [8]. As such, further research into affordable, available and effective antidiabetic agents is widely encouraged.
Accumulated evidence indicates that the generation of reactive oxygen species may play a key role in the etiology of diabetic complications [9]. Many biochemical pathways associated with hyperglycemia such as glucose autoxidation, polyol pathway, prostanoid synthesis and protein glycation can increase the production of free radicals which may quench essential endogenous

biochemicals such as nitric oxide [10]. The deleterious effects of free oxygen radicals may be delayed or reversed by antioxidants and this led to the suggestion that antioxidants limit susceptibility of microvascular and macrovascular complications arising from diabetes [10].

Patients afflicted with diabetes mellitus are also easily susceptible to infections [11] with increased severity and higher risks of complications. In diabetes mellitus, several aspects of cellular immunity are adversely altered including polymorphonuclear leukocyte function, chemotaxis and phagocytosis [12]. Glycaemic control therefore improves immune function [12]. Another alternative to controlling infection in this group of immunocompromised individuals is using antidiabetic agents with antimicrobial properties. This reasoning justifies further research into medicinal plants which have medicinal constituents capable of possessing multipurpose for the treatment of various disease states and their associated comorbid conditions. Ethnobotanical information suggests that about 800 plants may possess antidiabetic potential [13]. This justified the research into the antihyperglycaemic, antioxidative and antimicrobial activities of the ethanolic extract of *Ehretia cymosa*. Apart from its documented biological activities, there have been anecdotal reports of the use of the leaves of this plant for its antidiabetic activity.

Plate 1: *E. cymosa* Thon. 1 × Magnification

The plant under study, *E. cymosa* is a shrub that is commonly located in the Savanna and secondary jungle of West Africa. This medicinal plant is used in the management of venereal diseases, epilepsy, dry cough, tonsillitiss, typhoid, malaria, asthma, wounds and as an aphrodisiac, along with several others conditions [14]. The leaves are used as febrifuge, laxative, pain-killer, and in paralysis, epilepsy, convulsions and spasm and toothache [15]. The sap from the fresh leaves is used as a mild laxative for children. A decoction of the bark is used to regulate menstrual cycle [16]. Leaf and root decoctions are used in the treatment of hyperthermia, stiffness, and dysentery and tetanus. Bark decoctions are topically applied for the prevention against skin diseases. Ghanaians use the leaf poultices by applying them on fractured bones to promote healing. Leafy twigs of *E. cymosa* are combined with other plant parts in the treatment of gastric ulcers. In Ethiopia, stomach complaints are managed by crushing the roots in water and taking this infusion orally. The people of Maasei use the roots in the treatment of brucellosis [17]. Within the Akans and Ewe ethnic groups in Ghana, there have been anecdotal reports of the use of *E. cymosa* in the management of diabetes. Beside its use in treatment of human diseases, a survey conducted by the team in Volta region of Ghana into use of ethnomedicine in treatment of diseases affecting Guinea fowls *(Numida meleagris*) revealed that *E. cymosa* was one of the key plants ranking third according to frequency of citations. Participating farmers indicated the use of infusion from root and leaves of *E. cymosa* in treatment of diarrhea and loss of appetite (unpublished). In the backdrop of reported ethnomedical and ethnoveterinary use of *E.cymosa* this study aimed at studying antihyperglycaemic, antioxidant and antimicrobial activities of *E. cymosa.*

2. Materials and methods

2.1. Plant Material

The whole plant material of *E. cymosa* (Fig 1) was collected in August, 2014 from Tsawula in the Volta region of Ghana. Mr. E. A. Blagoghi, a botanist at Centre for Plant Medicine Research (CPMR) assisted in the identification of the plant. Subsequently, the plant was authenticated at the herbarium in Plant Development Department at Mampong (CPMR) and given voucher specimen number of DPHM/001/04/15. The plant material was air dried for two weeks and pulverised to a coarse powder.

2.2 Extraction by cold maceration

The extraction was done in September, 2014. Five liters of 70% ethanol was used to extract 500 g of the coarsely powdered plant material for 3 days with intermittent shaking. The extract was concentrated using a rotary evaporation at 40 ^{o}C. The concentrated extract was pooled together and further evaporated under vacuum to obtain a solvent free semi-solid mass with an extract yield of 2.2097% w/w per dried plant material. The extract was stored in a desiccator at room temperature.

2.3 Phytochemical assay

Phytochemical analysis was carried out on the dried 70% ethanol extract of *E. cymosa* according to the method described by [18].

2.4 Antimicrobial Assays

Stock solutions of the plant extract were prepared at concentrations of 20 mg/mL using 10% Tween 60. The stock solutions were sterile filtered and subsequently diluted to 10, 5 and 1 mg/mL with sterile water. Molten agar was stabilize at 45 ^{o} C and seeded with 0.1 mL inocula of a 24 hour nutrient broth cultured of the test organism. It was rolled in the palm to ensure uniform mixing of the agar and the test organism. This was aseptically poured into a Petri dish and allowed to set. A 10 mm cork borer was used to bore four holes in the agar equidistant from each other. The holes were filled with 200 µL of the respective concentration of the test extract. The Petri dishes were pre-incubated for 30 min and incubated at 37 ^{o}C for 24 h. Diameters of the clear zones of inhibition were

measured in millimeters. Gentamicin at a concentration of 10 µg/mL was used as the standard drug for the positive control. This experiment was repeated in triplicate for *Pseudomonas aeruginosa (P. aeruginosa)*, *Escherichia coli* (*E. coli)* NCTC1351, *Staphylococcus aureus* (*S. aureus)* NCTC6571 and *Bacillus subtilis (B. subtilis)*.

2.5 Antioxidant assays

2.5.1 DPPH Free Radical Scavenging Activity

To evaluate the antioxidant activity of the extract, the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activity was performed as described by [19], with few modifications. Various concentrations of 5, 2.5, 1.25, 0.625, 0.3125, 0.1563, 0.0781, and 0.0391 mg/mL of 70% ethanolic extract of *E. cymosa* (EEEC) and a stock solution of 0.5 mM DPPH in methanol were prepared. A volume of 100 µL of each extract concentration was added to 100 µL of DPPH solution in a 96 well microtiter plate in increasing concentrations of the extract. The plate was shaken to uniformly mix and incubated for 30 min in darkness, at room temperature. The absorbance was read at λ 517 nm using Infinite M200Pro microtiter plate reader (Tecan, Austria). Each sample was done in triplicate. BHT 0.0391, 0.0781, 0.1563, 0.3125, 0.625, 1.25, 2.5, 5.0) mg/mL and Ascorbic acid (0.000781, 0.001562, 0.003125, 0.006250, 0.012500, 0.02500, 0.0500, 0.1000) mg/mL were used as positive controls and methanol as the blank.

2.5.2 Total Phenol Content

The total phenol content of the EEEC was determined by the Folin-Ciocalteau reagent method with some modifications [20]. A volume of 100 µL of 5% Folin-Ciocalteau reagent was added to 20 µL of various concentration of EEEC (5, 2.5, 1.25, 0.625, 0.3125, 0.1563, 0.0781, and 0.0391) mg/mL followed by 80 µL of 7.5 % Na_2CO_3 in a microtiter plate and the resulting solution was mixed. The solutions were incubated at room temperature in the darkness for 1 hour and the absorbance measured at λ 765 nm using the Infinite M200Pro microtiter plate reader. Gallic acid (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 µg/mL) was used to develop the standard calibration curve. The total phenol content was expressed in milligram of Gallic acid equivalents (GAE) per gram of the extract.

2.5.3 Determination of reducing power

The reducing power of the EEEC was determined using the reduction of Fe^{3+} to Fe^{2+} [21] with Gallic acid, BHT and ascorbic acid. A quantity of 0.5 mL of various concentration of EEEC (5, 2.5, 1.25, 0.625, 0.3125, 0.1563, 0.0781, and 0.0391) mg/mL was added to 0.5 mL of water and mixed with 1.25 mL of sodium phosphate buffer (0.2M, pH=6.6) and 1.25 mL of 1% potassium ferricyanide. After incubation at 50 °C for 20 min, 1.25 mL of 10% trichloroacetic acid was added and samples centrifuged at 3000 g for 10 min. A 100 µL of supernatant was mixed with 100 µL of distilled water and 100 µL of 0.1% $FeCl_3$. The absorbance of the resulting solutions was measured at λ 700 nm. The blank was prepared in a test tube by adding 0.5 mL of distilled water to 1.25 mL of sodium phosphate buffer and 1.25 mL of $FeCl_3$. The reducing power was expressed as mg of Gallic acid equivalents (GAE)/g of the extract.

2.6. Experimental animals

Thirty two (32) adult albino Sprague Dawley male rats, weighing within the range of 175-190 g were obtained from the Centre for Plant Medicine Research (CPMR) at Mampong, Ghana and housed in cages containing saw dust as bedding. Animals were housed under standard conditions of room temperature of 27 °C and supplied with standard pellet food (GAFCO, Ghana) and given tap water *ad libitum*. The animals were allowed three days acclimatization to this environment with a 12 hour light dark cycle. Handlings of the animals in this study were in accordance with the guidelines published by the National Institute of Health for the Care and Use of Laboratory Animals [22]. The research protocol was approved by the College of Health Sciences Ethics Committee, University of Ghana.

2.6.1. Assessment of Antihyperglycaemic effect of *E. cymosa*

Baseline fasting blood glucose level (FBGL) of the rats were taken a day before experimentation. The glucose levels of the individual animals were found to be within the range of <6 mmol/L. Diabetes was induced in the rats by a single intraperitoneal dose of streptozotocin (70 mg/kg) after an overnight fast. After a rest period of 48 hours, the fasting blood glucose levels of the rats were measured using an Accu-Chek® glucometer (Roche Diagnostics GmbH, Mannheim Germany). Animals with FBGL ≥ 10 mmol/L were considered as diabetic and included in the study.

To investigate the antihyperglycaemic effect of the plant extract, the rats were randomly divided into six groups of five animals each: Groups A, B, C, D, E and F. Group A was given distilled water and this group was used as the untreated control group. Groups B, C and D were given high dose, mid-dose and low dose respectively of 90, 60 and 30 mg/kg body weight of the plant extract respectively. The fasting blood glucose levels were determined and monitored hourly for six hours to ascertain the antihyperglycaemic activity of the plant extract. Group E and F were given intramuscular (IM) injections of insulin (1 unit/kg) and glibenclamide (5 mg/kg) dose respectively to serve as the positive control groups [23].

2.6.2. Preparation of intestinal sacs to determine glucose absorption modulation

Eighten (18) adult albino male Sprague Dawley rats (weighing175-195g) housed under similar conditions as in section 2.6 was used in this experiment. The animals were starved for 12 hours but given water *ad libitum*. Rats were sacrificed by cervical dislocation and the abdomen opened by midline incision. The entire small intestine was immediately removed by cutting across the upper end towards the duodenum and the lower end of the ileum. The mesenteries were stripped manually. The small intestine was then washed out with normal saline (0.9% w/v NaCl). A thread ligature was then tied around one end of the intestine to facilitate subsequent identification.

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