

Original Research Article

***In vitro* antidiabetic, aphrodisiac and antimicrobial properties of para-propoxybenzoic acid isolated from *Acacia auriculiformis* A. Cunn Ex. Benth (Fabaceae) stem bark**

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Abstract

Purpose: To investigate the *in vitro* antidiabetic, aphrodisiac and antimicrobial properties of para-propoxybenzoic acid isolated from *Acacia auriculiformis* stem bark.

Methods: Powdered dried stem bark of *A. auriculiformis* was extracted with 70 % ethanol in water and the dried extract obtained was suspended in water and partitioned with ethyl acetate and *n*-butanol to give their respective soluble fractions. The ethyl acetate fraction being more active, in terms of *in-vitro* aphrodisiac, antidiabetic and antimicrobial properties, was subjected to chromatographic separation to obtain C1 identified as paraproxybenzoic acid, which was also evaluated for *in-vitro* properties using standard procedures.

Results: 4-Propoxybenzoic acid showed a weak aphrodisiac property which was not significantly different ($p < 0.05$) from that of control (normal saline) for all the sexual parameters tested. The compound exhibited antidiabetic activity by dose-dependently inhibiting the actions of α -amylase with half-maximal concentration (IC_{50}) value of $50 \pm 0.45 \mu\text{g/mL}$. In this regard, it was comparable to that of the standard, acarbose ($30 \pm 0.18 \mu\text{g/mL}$). In the antimicrobial study, a concentration of $200 \mu\text{g/mL}$ of the compound gave the highest zone of inhibition of $18 \pm 0.23 \text{ mm}$ against *Vibrio cholerae* (NCTC 5438)

Conclusion: The results indicate that the isolated compound, 4-propoxybenzoic, exhibits good antidiabetic and anti-microbial but weak aphrodisiac properties.

Keywords: *Acacia auriculiformis*, Aphrodisiac, Antidiabetic, Antimicrobial, Para-propoxybenzoic acid, Antidiabetic, Acarbose, Erythromycin, Fluconazole

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INTRODUCTION

Phytochemicals are natural chemical compounds produced by plants. They are non-nutritive but

are needed by plants for purposes such as disease and pathogen defense and control [1]. Studies have shown that phytochemicals are essential in human health maintenance. This is

because they display different good health promoting properties by acting as antioxidant, anti-inflammatory, anticancer antimicrobial agents, etc [2]. Plant-derived substances have recently become of great interest due to their broad applications [3]. Medicinal plants are the richest biosource of drugs in traditional systems of medicine, modern medicine, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [4]. It has been estimated that 14-28% of higher plant species are used medicinally, and that 74 % of pharmacologically-active plant-derived components were discovered after following up on ethnomedicinal uses of the plants [5].

A number of interesting outcomes have been found with the use of mixture of natural products to treat diseases; most notably are the synergistic effects and polypharmacological applications of plant extracts [6]. The production of pharmaceuticals starts with the identification of bio-active compounds, detailed biological assay and dosage formulations, followed by clinical studies to establish safety, efficacy and pharmacokinetic profile of the new drug [7]. This is also true for therapeutic agents from plants. Thorough biological evaluation of plant extracts is vital for ensuring their efficacy and safety. These factors i.e., efficacy and safety, are important to qualify the plant extracts as valid medicinal agents [8].

Natural products play an important role in the field of new drug research and development because of their low toxicity, ready availability and cost effectiveness [9]. *A. auriculiformis* is a medicinal plant known for several potent pharmacological activities including anthelmintic, antifilarial and microbicidal properties [10,11].

An extract produced from the root of the plant is known to be useful in the treatment of sore eyes and various aches. The Aborigines of Australia are known to use the bark extracts of this plant to treat rheumatism [12]. Various extracts of this plant have demonstrated antioxidant benefit [13]. The seeds of this plant are reported to produce triterpenoid saponins namely: acaciaside A (Ac-A) and acaciaside B (Ac-B); and earlier investigations showed that Ac-B possessed spermicidal activity even at significantly low concentrations [14]. Experiments by Kabir *et al* [14] showed that such extract can also inhibit HIV transmission without any mutagenic effect.

A recent study has revealed that an ointment prepared from ethanol and aqueous extracts of stem bark of *A. auriculiformis* evaluated for

wound healing property showed extensive healing activity by enhancing wound contraction, shortening epithelization period with increased tensile strength [15]. The pesticidal action of the stem bark extracts of the plant has also been reported [16]. Studies have shown that *A. auriculiformis* has many pharmacological properties but the information available on this plant indicates that attention is devoted to studying mostly the leaf and the seed parts. This research work was designed to investigate the pharmacological effect of the ethanol stem bark extract of *A. auriculiformis* with the aim of establishing the presence or lack of aphrodisiac, antidiabetic and antimicrobial potential as well as isolating and identifying the compounds responsible for the properties.

EXPERIMENTAL

Equipment and reagents

Gradient elution column chromatography was performed on Silica Gel G 60-120 mesh (Loba Chimie, India); analytical and preparative thin layer chromatography were performed on Pre-coated silica gel G TLC plates (aluminium backed, 0.2 mm, and silica gel G, glass backed, 0.5mm). The melting point of the isolated compound was determined using the Scientific Instruments (India). The Nuclear Magnetic Resonance (NMR) spectroscopic analysis was performed using Agilent NMR-vnmrs 400 (400 MHz, USA) run on CD₃OD at 31 °C for ¹H and ¹³C NMR using TMS as internal standard with drops of CD₃OD previously added to dissolve the sample. The Fourier Transform Infra-Red (FTIR) spectroscopic analysis was done using transmittance method on Agilent FTIR (USA) on KBr disc and the signals measured in cm⁻¹. All solvents, 17 β -estradiol, progesterone, testosterone, sodium phosphate, enzyme, 3, 5-dinitrosalicylic acid, acarbose, erythromycin, fluconazole and Streptomycin were obtained from Sigma-Aldrich chemicals, (USA) and S.D. Fine Chemicals Limited (India).

Extraction and isolation of CI

The fresh stem bark plant materials of *Acacia auriculiformis* were collected in the month of June 2019, authenticated by the herbarium at the Department of Pharmacognosy, Faculty of Pharmacy, University of Uyo, dried under shade for some weeks and pounded to powder using mortar and pestle. A herbarium specimen with voucher number UUPH 14d was prepared and deposited in the Herbarium. The dried powder of the plant material (0.5 kg) was exhaustively extracted using 70 % ethanol (3 x 5L) at room

temperature (27 ± 2 °C) for 72 h. The resultant crude extract was filtered, concentrated in vacuo on a rotary evaporator (R-3, CH-9230, BuchiLab Switzerland), weighed and stored in a desiccator (Monsori, Scotland) prior to further use. The crude extract (50.0 g) was partitioned using ethyl acetate and butanol (4 x 500 mL each) to obtain ethyl acetate (EA) and n-butanol (BUOH) fractions, respectively. The EA fraction that had higher positive response to the pharmacological tests was subjected to chromatographic separation. Briefly, the EA fraction (2.0 g) was chromatographed on the silica gel in a column (3x50 cm, Pyrex, USA) and eluted with a gradient elution solvent system - chloroform: methanol in increasing polarity starting with the ratio 99:1. Eluates (5 mL each) were collected, monitored on silica TLC plates (Merck, Germany) in Acetone: toluene: Water (10:20:1) using 10% sulphuric acid in methanol and vanillin-sulphuric acid as spray reagents.

Fractions with similar TLC characteristics (R_f values, colour reaction with spray reagents) were bulked to give three semi-pure residues coded T1 - T3. T1 and T3 were difficult to purify further and were discarded. T2 was purified using preparative TLC; dried T2 (0.15 g) was carefully dissolved in methanol and applied across the coated silica gel plate (20 x 20 cm, 0.25 mm) using a micro-Pasteur pipette (Simax, India) 1 cm above the bottom edge of the plate. The plates were developed using n-hexane:dichloromethane:methanol (1:4:1) solvent system in a Chromatank (USA). The chromatogram obtained showed two distinctly resolved layers which were carefully scrapped, separated, filtered, recrystallized in methanol and concentrated *in vacuo*. Further TLC evaluations indicated a single spot in one of the layers. This was stripped of the solvent to give a light brown solid coded C1 with R_f of 0.49 (Acetone: Toluene: Water (10:20:1)). The chemical structure of C1 was elucidated using a combination of physical and spectroscopic analyses.

Determination of aphrodisiac activity

Twenty (20) sexually mature male Wistar albino rats weighing 150 - 250 g were randomly assigned into five groups of four animals each with a distinct identity. Groups 1-3 received 136.93 mg/kg, 273.86 and 410.79 mg/kg of the extract (representing low dose (LD), middle dose (MD) and high dose (HD) respectively) and 50 µg/mL of C1 interparietally (ip), while Group 4 received 10 ml/kg of normal saline as the negative control and group 5 received 1 mg/kg of testosterone, administered subcutaneously (sc) as the positive control. The duration of this

experiment was seven days. The experimental protocol was approved by the Faculty of Pharmacy, University of Uyo Research Animal Ethics Committee (Ref No. UU/PHM/RAEC/VOL 1/106) and the study was carried out according to NIH Guidelines for Care and Use of Laboratory Animals [17].

Assessment of sexual behaviour

The females were brought to oestrous cycle by administering 1 mg/kg 17 β -estradiol 48 h and 0.5 mg/kg body weight of progesterone 4 h (sc) prior to the commencement of the experiment to make them receptive to the male rats. The mating behaviour studies were carried out in a separate room under red source of light between (the dark period of) 7 - 9 pm, with the females introduced to the male rats 30 min apart. Initially, the male rat was kept in a transparent plexiglass copulation cage (46 cm x 41 cm x 41 cm) for 10 min, followed by introduction of the female. The following mating behavioural parameters were observed and recorded: mount frequency (MF), intromission frequency (IF), mount latency (ML), intromission latency (IL), ejaculation latency (EL), post-ejaculatory interval (PEI), ejaculation frequency (EF) and penile erection (PE) [18]. The values of the observed parameters were compared with those of the control [19]. All the experiments were performed in triplicates.

Determination of inhibitory activity of α -amylase

This procedure was carried out as reported by Johnson *et al* [20]. The assay mixture comprising 200 µL of 0.02 M sodium phosphate buffer, 20 µL of enzyme and the crude ethanol extract, prepared in the concentrations of 20, 40, 60, 80 and 100 mg/mL was incubated for 10 min at room temperature, followed by addition of 200 µL of 1% starch (1.0 g of starch in 100 mL of 20 mM sodium phosphate buffer at pH, 6.8) to all the test tubes. The reaction was terminated by addition of 400 µL of 3, 5-dinitrosalicylic acid, placed in boiling water for 5 min, cooled and diluted with 15 mL of distilled water. The absorbance of the extract was measured at 540 nm. This procedure was repeated for the EA fraction, the isolated compound, C1 and the reference drug. Inhibition (H) was calculated as in Eq 1.

$$H (\%) = \{(Ac - Ae)/Ac\}100 \dots\dots\dots (1)$$

where Ac and Ae are the absorbance of control and extract samples, respectively.

The concentration of the test samples required to inhibit 50 % of the enzymes' action was defined by the IC₅₀ value determined from the plot of percentage inhibition against log of inhibitor concentration and was calculated by non-linear regression analysis from the mean of inhibitory values. Acarbose was used as the reference α -amylase inhibitor. All the tests were performed in triplicates.

Antimicrobial studies

Antimicrobial studies on the ethanol stem bark extract, ethyl acetate fraction and butanol fraction of the extract of *A. auriculiformis* were carried out using agar-well diffusion method. The micro-organisms, namely: *Bacillus subtilis* (NCTC 8853), *Staphylococcus aureus* (NCTC 6872), *Escherichia coli* (NCTC 10764), *Vibrio cholerae* (NCTC 5438) and *Candida albicans* (NCYC 4366) were clinical isolates from specimens of urine, abscesses, necrotizing fasciitis, osteomyelitis, diarrheal stool, wounds and vaginal swabs collected from the Medical Laboratory, University of Uyo Health Centre. The clinical isolates were collected in sterile bottles, identified and classified by convectional biochemical tests [21,22] and then refrigerated at -5 °C, prior to use. Standard procedure of the hole-in-plate agar diffusion method was strictly applied using nutrient agar for the bacteria and Sabouraud Dextrose Agar (Oxoid, England) for the fungus. Pre-labelled plates were seeded with test organisms (sub cultured overnight) and 20 mL of molten agar poured into the plates and swirled to mix. The agar was allowed to solidify. Using a 4 mm diameter cork borer, wells were bored on the agar plates in which 0.1 mL of the various extracts were placed after carrying out two-fold serial dilution to get the concentrations; 200, 100, 50 and 25 mg/mL. The standard antimicrobial agents (erythromycin, streptomycin and fluconazole, 10 μ g/mL each) were introduced accordingly. The inoculated plates were allowed to stand for 30 min for proper diffusion, and then incubated at 37 °C for 24 h [23]. The isolated compound (C1) was also evaluated for antimicrobial activity applying the same procedures and conditions used for the extracts, at concentrations of 200, 100, 50, and 25 μ g/mL. Antimicrobial property was determined by measuring the diameter of zones of inhibition produced after incubation. Each test was performed in triplicate.

Statistical analysis

All tests were carried out in triplicates and values expressed as mean \pm SEM. The data obtained were subjected to one-way analysis of variance

(ANOVA) and significant difference was determined using Turkey's multiple comparison tests with the aid of GraphPad prism software, version 5.03, at $p < 0.05$ confidence level.

RESULTS

Spectral characteristics of C1

C1 was obtained as light brown amorphous powder, mp 147 °C. The molecular formula C₁₀H₁₂O₃ was determined on the basis of EI-MS. (calcd. 180.00 for C₁₀H₁₂O₃). On the FTIR spectrum the following peaks were identified: 3295. (OH); 2922.2 (C-H); 1169.97 (C=O) 1606.0 (C=C stretch) 1196.5 (C-O) and 1110.7 (phenolic C-O), 838.7. For the proton NMR peaks were identified as follows: δ_H 7.049 (aromatic), 6.806 (aromatic) 4.072 (-OCH₂-); 1.794 (CH₂) 0.900 (CH₃) (see Figure 1 a); For C-13 NMR spectrum the following diagnostic peaks were identified: δ_C 172.976 (-COOH), 162.765(Ar - C-O-), 134.224(Ar=CH), 129.033 (Ar=CH), 115.648 (Ar=CH), 62.936 (CH₂), 26.000 (CH₂); 16.036 (CH₃) (see Figure 1 b).

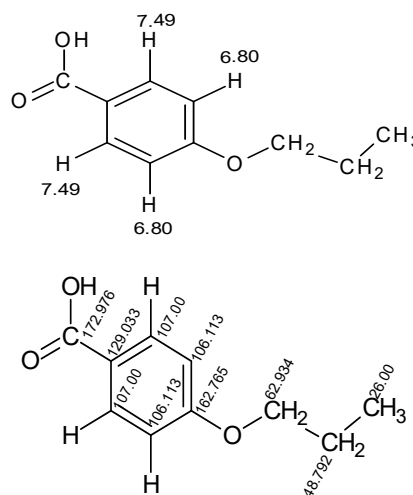


Figure 1: (a) Structure of C1 showing proton NMR peaks; (b) structure of C1 showing C-13 NMR peaks

Table 1 shows the results of the effect of test samples, the isolated compound C1 and the control drugs on sexual behaviour of the male rats

Antidiabetic properties of the plant samples

Table 2 shows the result of inhibitory actions of the extract, fractions, the isolated compound C1 and the standard drug (acarbose) on α -Amylase inhibitory activity of *A. auriculiformis*.

Table 1: Summary of effect of test samples on sexual behaviour in male rats

Dose (mg/kg)	ML	MF	IL	IF	EL	PEI	EF	PE
Normal Saline (10 ml/kg)	0.64 ± 0.28 ^a	6.50 ± 1.28 ^b	1.17 ± 0.17 ^c	5.00 ± 0.82 ^d	4.73 ± 0.41 ^e	7.81 ± 1.27 ^f	3.50 ± 1.29 ^h	6.73 ± 1.26 ⁱ
Testo (1 mg/kg)	0.22 ± 0.09 ^j	7.50 ± 2.65 ^k	0.46 ± 0.06 ^l	6.85 ± 2.22 ^m	1.64 ± 0.46 ⁿ	2.77 ± 0.61 ^l	6.25 ± 2.63 ^o	2.21 ± 0.46 ^p
C1 (50 µg/mL)	0.61±0.003 ^a	0.65±0.002 ^b	1.11±0.005 ^c	5.01±0.005 ^d	4.61±0.002 ^e	7.68±0.004 ^f	3.60±0.001 ^h	6.40±0.005 ⁱ
EA. (LD; 136.90 mg/kg)	0.64 ± 0.28 ^a	6.50 ± 1.29 ^b	1.17 ± 0.17 ^c	5.00 ± 0.82 ^d	4.73 ± 0.41 ^e	7.81 ± 1.27 ^f	3.50 ± 1.29 ^h	6.73 ± 1.26 ⁱ
EA (MD; 273.86 mg/kg)	0.69 ± 0.29 ^a	6.50 ± 1.29 ^b	1.98 ± 0.30 ^c	5.50 ± 1.73 ^d	4.58 ± 0.60 ^e	7.67 ± 1.17 ^f	3.50 ± 1.00 ^h	6.48 ± 0.96 ⁱ
EA. (HD;410.79 mg/kg)	0.64 ± 0.39 ^a	6.75 ± 1.71 ^b	1.15 ± 0.14 ^c	5.05 ± 2.22 ^d	4.60 ± 0.89 ^e	7.80 ± 0.97 ^f	3.25 ± 2.22 ^h	6.71 ± 0.92 ⁱ

Values are expressed as mean ± SEM(n=3); data with different letters are significantly different at *p < 0.05. Key: Testo = Testosterone; EA. = Ethyl acetate fraction; LD = low dose; MD = middle dose; HD = high dose; MF = mount frequency, IF= intromission frequency, ML = mount latency, IL = intromission latency, EL = ejaculation latency, PEI = post-ejaculatory interval, EF = ejaculation frequency, PE = penile erection

Table 2: Inhibition of α-amylase by the test samples

Conc.	ETOH		EA		C1		Acarbose	
	%	IC ₅₀	%	IC ₅₀	%	IC ₅₀	%	IC ₅₀
20	13	>100	25	65±0.50 ^b	35	50±0.45 ^a	45	30±0.18 ^a
40	16		33		45		58	
60	21		47		57		72	
80	23		58		65		81	
100	37		67		73		89	

Conc. in mg/mL for ETOH and EA; µg/mL for C1 and Acarbose; Data are given as mean ±SEM (n=3); data with different letters are significantly different at p < 0.05

Antimicrobial activity of C1

Figure 2 shows the results of the antimicrobial studies carried out on the isolated compound (C1) and the standard drugs, with methanol as negative control.

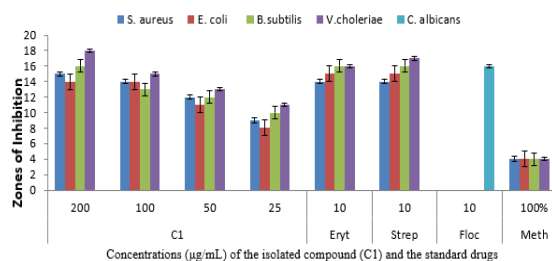


Figure 2: Antimicrobial profile of the isolated compound (C1), standard and control drugs. Key: C1 = Isolated compound, Eryt = Erythromycin, Floc = Fluconazole, Meth = Methanol, B. subtilis = *Bacillus subtilis*, S. aureus = *Staphylococcus aureus*, E. coli = *Escherichia coli*, V. cholerae = *Vibrio cholerae*, C. albicans = *Candida albicans*

DISCUSSION

Phytochemical screening of the ethanol stem bark extract of *Acacia auriculiformis* indicated the presence of typical plant chemical constituents namely: saponins, terpenoids, alkaloids and flavonoids. **C1** was isolated from the EA fraction of the ethanol extract as a light brown amorphous powder, with mp, 147 °C. The compound was elucidated by a combination of physical properties (melting point) and spectroscopic analyses. FTIR absorption at 3295 cm^{-1} indicated the presence of OH from carboxylic acid; 11699.7 cm^{-1} indicated C=O from carboxylic acid and the absorption at 1606.0, indicated aromatic structure; while 838.7 cm^{-1} showed a para-substituted aromatic system [24], [25]. Thus, FTIR analysis suggested a structure of a para-substituted aromatic acid. The proton NMR showed the presence of two aromatic protons each at: δ_{H} 7.049 ppm (deshielded), adjacent to COOH group and at 6.806 ppm (upfield), adjacent to CO group on the benzene ring. The proton signals at 4.072 ppm 1.794 ppm and 0.900 ppm are attributed to the propoxy side chain (see figure 1a). For the ^{13}C NMR: δ_{C} 172.976 ppm indicated signal for acids while the peaks at 162.765 ppm, 134.224 ppm and 129.033 ppm are attributable to carbon atoms on the benzene. The peaks at 115.648 ppm and 62.936 ppm 26.000 ppm and 16.036 ppm came from the side chain [24,25]. The FTIR, ^1H and ^{13}C NMR showed that the compound has 10 carbon atoms, a benzene ring with para substitution, one of which is a carboxylic acid group. These data and the melting collaborate

aply with the literature values for 4-propoxybenzoic acid [26] hence C1 is identified as 4-propoxybenzoic acid or para-propoxybenzoic acid. From the result obtained, the extract, fractions and the isolated compound showed aphrodisiac activity that was not significantly different from that of the normal saline (the negative control drug) but lower than that of the standard drug (testosterone – positive control) indicating a weak aphrodisiac activity of the plant extract and the isolated compound. This result is not surprising because *A. auriculiformis* and its isolate - acasiaside-B, have been associated with spermicidal activity [14]. The isolated C1 dose-dependently inhibited the actions of α -amylase implicated in the metabolism of carbohydrate in human body. Alpha amylase catalyses the breaking down of starch to disaccharides and oligosaccharides [20]. This inhibition of digestion of the carbohydrate in turn decreases the rise in postprandial hyperglycemia. It was observed that the antimicrobial inhibition of the test sample was concentration-dependent. The inhibition exhibited by the isolated compound compared favourably with that of the standard drugs with highest inhibition of 18.00 ± 0.23 mm at 200 $\mu\text{g}/\text{mL}$ against *Vibrio cholerae* (NCTC 5438)

CONCLUSION

The findings of this research reveal that the ethanol stem bark extract of *Acacia auriculiformis* contains a compound identified as 4-propoxybenzoic acid that can inhibit bacterial growth and α -amylase action indicating potentials for antibacterial and anti-diabetic activity respectively. Interestingly, all the samples tested showed no antifungal activity and very low aphrodisiac potentials.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the

authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Ekarika C. Johnson conceived, designed the study, analyzed, interpreted the spectra, and wrote the manuscript; Richard A. Ukpe and Emmanuel I. Etim performed the physicochemical analyses; Olorunfemi A. Eseyin designed and supervised the anti-diabetic experiment; Tina Mboho carried out the extraction and antimicrobial study; Paschal C. Anthony performed the anti-diabetic experiment, Uwemedimoh F. Umoh collected and prepared the plant materials; Anwanabasi E. Udoh performed the aphrodisiac test. All authors read and approved the manuscript for publication.

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