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Evaluation of anti-inflammatory and antioxidant activity of Furanocoumarins and Sterolin from the stem bark of *Ficus exasperata* Vahl (Moraceae)

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Abstract

Ficus exasperata is a popular plant among traditional healers in Africa where various parts of the plant are used for some medicinal purposes. Previous research on the stem bark revealed considerable anti-inflammatory, antioxidant and antimicrobial activities, which resided in the chloroform extract of the plant. Although, the plant enjoys a battery of pharmacological activities, it has rarely been studied for it bioactive secondary metabolites. The study was designed to fractionate the bioactive chloroform extract of the stem bark, isolate and characterize some compounds which may be responsible for these activities. Fractionation and isolation of the compounds was done using gravity column and thin layer chromatographic techniques on silica gel. The structures of the isolated constituents were elucidated using UV, mass spectrometry, 1D and 2D-NMR techniques. The carrageenan-induced foot oedema test in 7-day old chicks was used to assess the anti-inflammatory effect of the compounds. The in vitro antioxidant properties were evaluated using the DPPH radical scavenging assay. Bergapten, oxypeucedanin hydrate and the sterolin sitosterol-3-O-β-D-glucopyranoside were isolated as the active metabolites. They exhibited dose-dependent anti-inflammatory activities with respective ED50 values of 101.6±0.003, 126.4±0.011 and 225.9±0.012 mg/kg. They also showed concentration-dependent DPPH scavenging effect with IC50 of 63.38 ± 0.010 and 46.63 ± 0.011 for bergapten and oxypeucedanin hydrate respectively. Sitosterol 3-O-glucopyranoside showed the lowest activity. Bergapten, oxypeucedanin hydrate and the sterolin sitosterol-3-O-β-D-glucopyranoside display anti-inflammatory and antioxidant activities and thus contribute substantially to the bioactivities of the stem bark of F. exasperata.

Keywords: Anti-inflammatory, Antioxidant, DPPH, Bergapten, *F. exasperata*, Oxypeucedanin hydrate.

Introduction

Investigation of the efficacy of plant-based medicines used in traditional medicine is fast expanding over the globe. This is because they are readily available and accessible, and the fact that about 80% of the world population still rely on them for the treatment and management of various diseases.[1] Our on-going research is focused on the bioactive secondary metabolites of plants with alleged folkloric use as pain relievers; anti inflammatory and anti-microbial activities. New lead compounds/drugs are required because of the gastrointestinal problems, tolerance and dependence associated with the use of conventional analgesics as well as microbial resistance to most antibiotics in clinical use.

Ficus exasperata (Moraceae), is a deciduous tree with smooth gray bark, [2] commonly referred to as sand paper tree due to the scabrous surface of the leaves which makes it find use, domestically, as abrasive for polishing hard surfaces such as utensils and furniture. [3] For example the leaves are used in the treatment of inflammatory diseases, intestinal and stomach troubles, high blood pressure, ring worms, bleeding, wounds and chest pains. [2,4,5] Sap from the stem bark is used for the treatment of wounds, sores, abscesses and stomach-ache, worms, haemorrhoids, abnormal enlargement of the spleen and to relieve cough. [6-8] Previous works on extracts of the plant have demonstrated antimicrobial, [9,10] anti-inflammatory, antipyretic and antinociceptive, [11] antiarthritic and antioxidant, [3] antiulcerogenic,[12] hypotensive,[13] lipid lowering and hypoglycaemic activities.[14] The uterotonic and the potential adverse effect on kidney function have also been reported.[7,15] Oladosu *et al*.[16] reported that the essential oil of the root bark contained α-terpineol (33.7 %), α-pinene (10.8 %), sabinene (5.6 %), β-patchoulene (4.7 %), 1,8-cineole (3.1%), and α-thujopsene (2.1 %) as the major constituents. The pheophytin/pheophorbide derivatives, flavonoids, fatty acids, pyrimidine derivatives and sphingolipid and furanocoumarins have been reported from the stem bark.[15,17] Previous studies in our laboratories demonstrated the anti-inflammatory, anti-oxidant and antimicrobial activity of the pet-ether, chloroform and 70% ethanol extracts of the stem bark. [18] Therefore this study was designed to fractionate the bioactive chloroform extract of the stem bark, and isolate some compounds which may be responsible for these activities. In this publication the anti-inflammatory and antioxidant actions of these constituents are also discussed.

Material and Methods

Plant materials

The stem bark of *Ficus exasperata* voucher specimen number KNUST/HM1/2011/S004 was collected from a farmland at Effiduase in the Sekyere East District in the Ashanti Region of Ghana in October 2010. The plant was authenticated at the Department of Pharmacognosy, College of Health Sciences, Kwame Nkrumah University of Science and Technology herbarium where a specimen has been deposited.

Extraction and isolation

The coarsely powdered stem bark of *F. exasperata* (4.8 kg) was defatted with petroleum ether (40/60) and subsequently soxhlet-extracted with chloroform. The extract was concentrated under reduced pressure to a small volume by means of rotavapor (R-114, Buchi, Switzerland) at a temperature of 40ºC, and evaporated to dryness on a water bath to the give the chloroform extract FEC (Yield = 0.35%$^{W}/_{W}$). FEC (15 g) was column chromatographed over silica gel (70-230 mesh ASTM) and eluted with petroleum ether, ethyl acetate and methanol and their mixtures in increasing polarity. 153 fractions (60 ml each) were collected and these were bulked into 7 fractions, FEC_{1-7}, based on their TLC profiles. Further fractionation of FEC_4 (1.95 g) over sephadex LH_{20} and elution with $CHCl_3$ provided 70 fractions (10 ml aliquots). These were again bulked into 5 fractions, FEC_{4A-E}. Further fractionation of FEC_{4B} over silica gel yielded compound (**1)** (250 mg). FEC_5 (1.2 g) was subjected to series of fractionation over silica gel (70-230 mesh ASTM) to obtain compound (**2**) which was recrystallized in acetone as a pale yellow amorphous powder (115 mg). FEC_7 was subjected to series of column chromatography over silica gel, eluting isocratically with EtOAc:MeOH (8:2) to obtain compound (**3**) (200 mg).

Instrumentation

The UV spectra were obtained on a T90+ UV/VIS Spectrometer, ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Bruker AVANCE DPX - 400 MHz in $CDCl_3$ (compound **1),** in DMSO (Compound **2**), in a mixture of $CDCl_3$ and CD_3OD (compound **3).** EI-MS were measured on a LKB-9000S mass spectrometer at 70eV. Melting points (uncorrected) were determined on a Buchi 510 apparatus.

Chemicals

All chemicals, with the exception of the drugs, were purchased from Sigma Aldrich Co Ltd. Irvine, UK. Organic solvents were of analytical grade and purchased from BDH Laboratory Supplies (England). Diclofenac and dexamethasone were purchased from Troge, Hamburg, Germany and Pharm-Inter, Brussels, Belgium.

Animals

Cockerels (*Gallus gallus*; strain shaver 579) were obtained from Akropong Farms, Kumasi, Ghana as 1-day post-hatch and were housed in stainless steel cages (34 × 57 × 40 cm^3) at a population density of 12-13 chicks per cage. Feed (Chick Mash, GAFCO, Tema, Ghana) and water were available *ad libitum* through 1-quart gravity-fed feeders and water trough. Room temperature was maintained at 29°C, and overhead incandescent illumination was maintained on a 12 hour light-dark cycle. Daily maintenance of the cages was conducted during the first quarter of the light cycle. Chicks were tested at 7 days of

age. Group sample sizes of 5 were used throughout the study.

Anti-inflammatory assay

The anti-inflammatory properties of the compounds were evaluated using the carrageenan-induced foot oedema model of inflammation in the chick[19] with modifications by Woode *et al.* [11] The chicks were divided into fifteen groups, each group consisting of five animals and had access to food and water *ad libitum*. Oedema was induced by sub-plantar injection of freshly prepared carrageenan (10 µl of a 2% suspension in saline) into the right footpads of each chick. The foot volume was measured before injection and at hourly intervals for 5 hours after injection by water displacement plethysmography as described by Fereidoni *et al*.[20] using an electronic Von Frey plethysmometer (Model 2888, IITC life science inc. Ca 91367 Canada). The compounds at 3, 10 and 30 mg/kg were administered orally to nine groups of chicks. The tenth to fifteenth groups of chicks received the standard drugs dexamethasone (0.3, 1 and 3 mg/kg) and diclofenac (3, 10 and 30 mg/kg) intraperitoneally. The control animals received only saline which served as the vehicle. The oedema component of inflammation was quantified by measuring the difference in foot volume before carrageenan injection and at the various time intervals. All experimental protocols were in compliance with the National Institute of Health guidelines for the care and use of laboratory animals and were approved by the Department of Pharmacology Ethics Committee.

Anti-oxidant assay

Scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl (DPPH)

The hydrogen atoms or electrons donation ability of the compounds were measured from the bleaching of purple colored methanol solution of DPPH. The effect of methanol solutions of the compounds on DPPH radical was estimated according to Govindarajan *et al*.[21] with few modifications. One milliliter of various concentrations (3.75-30 µg/ml) of the compounds and *n*-propyll gallate (standard antioxidant) was added to 3ml methanol solution of DPPH (20mg /l) in a test tube. The reaction mixture was kept at 25°C for 30 minutes. The absorbance of the residual DPPH was determined at 517 nm in a spectrophotometer (Cecil CE 7200 spectrophotometer, Cecil instrument limited, Milton Technical Centre, England). One milliliter (1ml) methanol (50%) was added to 3 ml DPPH solution incubated at 25°C for 30 minutes and used as control. The absorbance decreases with increasing free radical scavenging ability. Results were expressed as percentages of blank (100%). The concentration required to cause a 50% decrease in the absorbance was calculated (IC_{50}). Each test was carried out using three replicates.

The % DPPH scavenging effect (% of control) of the antioxidant was calculated as follows

$$\% \text{ DPPH scavenging effects} = (Ac - At)/Ac \times 100$$

Where: Ac = Absorbance of the control, At = Absorbance of the test drug/ extract

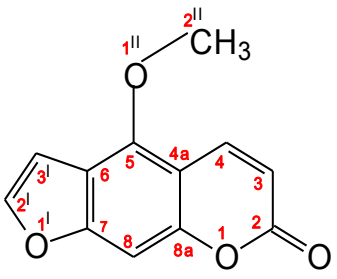
Results and Discussion

Chromatographic analysis of the active chloroform fraction of the stem bark yielded bergapten **(1)**, oxypeucedanin hydrate **(2)** and β sitosterol- 3-O-β-D-glucopyranoside **(3)**. They were characterized using ^{1}H-NMR, ^{13}C-NMR, HSQC and HMBC spectroscopy and comparing with the literature data. Bergapten **(1),** was obtained as white needle-like crystals with a characteristic odour and melted at 189-191°C. The UV spectrum in methanol exhibited four absorption maxima at 223, 249, 267 and 311 nm, characteristic of a furanocoumarin nucleus.[22] The ^{13}C-NMR spectrum exhibited 12 carbon resonances including five methines, one methoxy, one carbonyl and five quaternary carbons. The ^{1}H-NMR spectrum showed two proton doublets at δ_H 6.28 (J = 9.6 Hz) and δ_H 8.16 (J = 9.6 Hz) characteristic of α-pyrone protons assignable to H-3 and H-4 respectively, and a pair of doublets occurring at δ_H 7.03 (J = 2.2 Hz) and δ_H 7.61 (J = 2.2 Hz) typical of furanic protons assignable to H-3' and H-2' respectively.[23] The spectrum further showed a proton singlet at δ_H 7.37 and a methoxy signal at δ_H 4.42, assignable to C-5 of the furanocoumarin structure. This NMR data compared favourably with that published for bergapten **(1)** [24] with molecular formular of $C_{12}H_8O_4$.

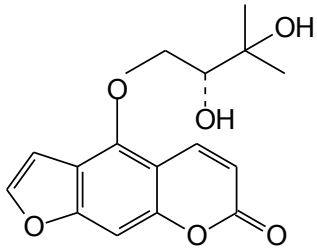
Oxypeucedanin hydrate **(2),** was isolated as a pale yellow amorphous compound and melted at 136-138°C (uncorrected). The UV spectrum showed characteristic absorption maxima similar to that of bergapten **(3)** at 223, 250, 259, 267, and 309 nm, suggesting a furanocoumarin structure. The ^{1}H-NMR spectrum was also similar to that of **(1)**, except that it lacked the methoxy signal at δ_H 4.42 but also had additional signals consistent with a prenyl group made up of two methyl singlets (δ_H 1.11 and δ_H 1.18), an oxymethylene multiplets (δ_H 4.27 and δ_H 4.77), one oxymethine multiplet (δ_H 3.64) and two hydroxyl protons (δ_H 4.48 and δ_H 5.43). These were confirmed in the DEPT 135 ^{13}C-NMR spectrum which displayed a total of

16 carbon resonances. It showed the eleven (11) carbon resonances for the furanocoumarin nucleus, as described for bergapten, and five additional signals arising from carbons at the side-chain that accounted for: 2 methyl groups (δ 24.7 and 28.2)**,** one oxymethylene (δ 75.5), one oxymethine (δ 76.9) and a quaternary oxygenated carbon (71.2 ppm). In the HMBC spectrum, the oxymethylene proton signals H_g (δ_H 4.27) and H_f (δ_H 4.77) correlated with the carbon signal at δ_C 149.8 (C-5), suggesting that the oxyprenyl unit was connected to C-5. These data confirmed the structure of **(2)** as oxypeucedanin hydrate with a molecular formular of $C_{16}H_{16}O_6$.

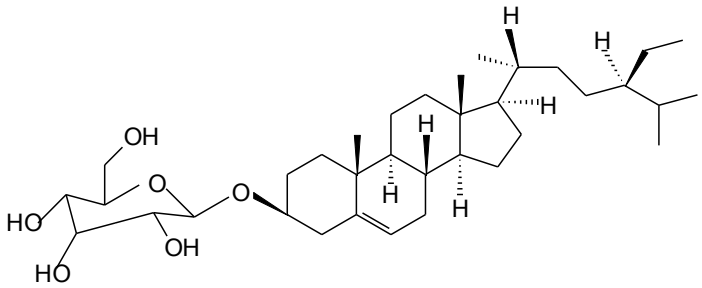
β sitosterol- 3-O-*β*-D-glucopyranoside **(3),** was obtained as a white amorphous powder with a melting point of 280-282°C. The ^{1}H-NMR spectrum were similar to that published for *β*-sitosterol [25] except for additional signals at δ_H 3.88 [1H (d, d), *J* = 2.3, 2.1 Hz)] assignable to H-5', and multiplets at δ_H 3.38, 3.29 and 3.19, each integrating for one proton, assignable to H-2', H-3' and H-4' of a sugar moiety. The anomeric proton **(1)** H-1') occurred as a doublet at δ_H 4.41 with a *J* value of 7.8 Hz. This confirmed the orientation of the glucose as a *β* linkage due to the coupling constant of the anomeric proton. [26] The ^{13}C-NMR spectrum confirmed the presence of 35 carbon atoms. The anomeric (C-1') and oxygenated methylene (C-6') carbons of the sugar moiety, appeared at δ_C 101.1 and δ_C 61.5. The remaining sugar carbons resonated at δ_C 75.6 (C-2'), δ_C 76.4 (C-3'), δ_C 73.7 (C-4') and δ_C 78.6 (C-5'). The MS-ES exhibited a maximum peak at *m/z* 599 corresponding to the sodium adduct ion $(M+Na)^+$, which is 23 Da higher than the expected molecular mass. This agreed with the molecular formula $C_{35}H_{60}O_6$. The spectrum also showed a weak signal at *m/z* 414 ($C_{29}H_{50}O$; *β*-sitosterol) and a characteristic fragmentation pattern similar to *β* -sitosterol. On the basis of the above evidence and comparison with published data,[25, 27] the structure of **(3)** was established as *β*-sitosterol-3-O-*β*-D-glucopyranoside.



(1)



(2)



(3)

The isolates were also evaluated for anti-inflammatory and antioxidant activities. They exhibited dose-dependent anti-inflammatory activities (Figure 1) with ED_{50} values of 101.6±0.003, 126.4±0.011 and 225.9±0.012 mg/kg for bergapten (1), oxypeucedanin hydrate (2) and sitosterol 3-O-glucopyranoside (3) respectively (Table 1). The isolates were however less potent than dexamethasone and diclofenac used as positive control (Table 1). Thus the study has shown that bergapten, oxypeucedanin hydrate and sitosterol 3-O-glucopyranoside possessed significant anti-oedematogenic effect on foot pad oedema induced by carrageenan and thus contributed to the anti-inflammatory activity of the stem bark. Bergapten, isolated from *Angelica pubescens*, has been shown to demonstrate anti-inflammatory and analgesic activities in mice. [28] However, to the best of our knowledge, this is the first report of the *in vivo* anti-inflammatory activity of oxypeucedanin hydrate. In the antioxidant assay, the compounds showed concentration-dependent DPPH scavenging effect with IC_{50} of 63.38 ± 0.010 and 46.63 ± 0.011 for bergapten and oxypeucedanin hydrate respectively (Table 2). Sitosterol 3-O-glucopyranoside showed the lowest activity (Table 2). It has been proposed that addition of the oxyprenyl group to the furanocoumarin skeleton (as is the case of

oxypeucedanin hydrate), results in an increase in antioxidant activity. Hydroxyl groups on the 5-oxyprenyl side chain may account for the relatively higher antioxidant activity of oxypeucedanin hydrate than bergapten, as hydroxyl and imino groups are known to increase free radical scavenging or antioxidant activity. [29]

The sterolin sitosterol-3-O-*β*-D-glucopyranoside **(3)** is one of the most ubiquitous sterols found in plants.[30] It has been shown to reduce secretion of pro-inflammatory cytokines by macrophages thereby decreasing inflammation. [31] It is also reported to reduce DNA damage, reduce the level of free radicals in cells and cause an increase in the levels of glutathione and other antioxidant enzymes. [32]

Thus the results obtained in this study, and that reported in literature, clearly indicate that bergapten , oxypeucedanin hydrate and sitosterol-3-O-*β*-D-glucopyranoside display considerable anti-inflammatory and antioxidant properties and contributes to the anti-inflammatory and antioxidant activity of the stem bark of *F. exasperata*. This gives scientific credence to the use of the stem bark of *F. exasperata* in various ethno-medicines for the treatment of inflammatory and infectious conditions.

Table 1: Effect of isolates on carrageenan-induced oedema in 7 - day old chicks

Isolate/drug	ED_{50} (mg/kg) ± SEM
Diclofenac	16.97 ± 0.011
Dexamethasone	2.95 ± 0.013
Bergapten	101.6±0.003
Oxypeucedanin hydrate	126.4±0.011
Sitosterol-3-O-glucopyranoside	275.9±0.012

Table 2: DPPH scavenging activity of extracts and isolated compounds

Isolate/Drug	IC_{50} (µg/ ml) ± SEM
Bergapten	63.38 ± 0.010
Oxypeucedanin hydrate	46.64 ± 0.011
Sitosterol-3-O-glucopyranoside	220.3 ± 0.03
N-propyl gallate	10.80 ± 0.002

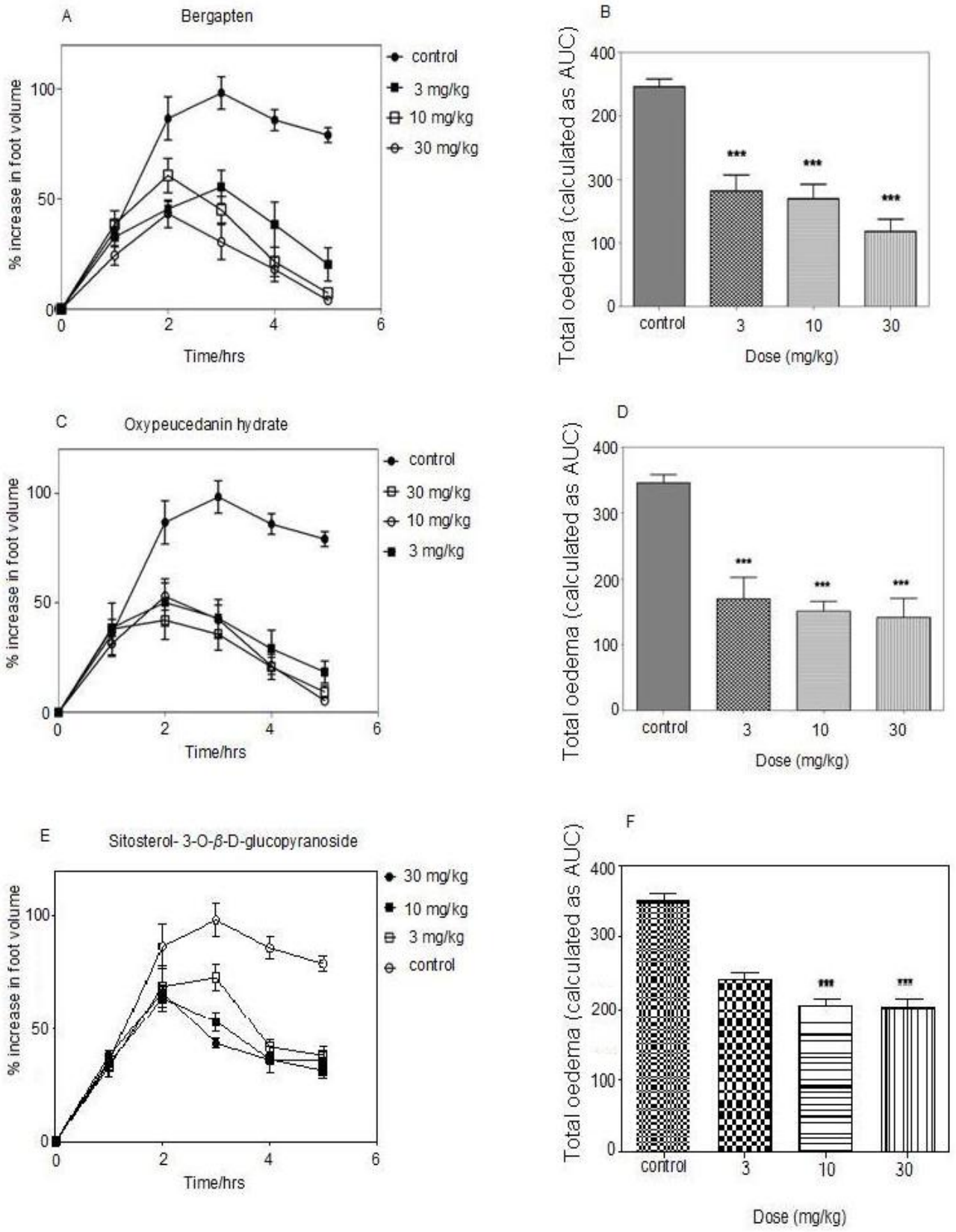


Figure 1: Effect of bergapten [(3-30 mgkg^{-1}oral)(A-B)] , oxypeucedanin hydrate [(3-30 mg/kg; *i.p*)(C-D)] and sitosterol-3-O-*β*-D-glucopyranoside [(3-30 mg/kg oral); (E-F)] on time course curve and the total oedema response, calculated as AUC's, for 5 hours, in carrageenan induced paw oedema in chicks. Values are means ± S.E.M (n=5) *** $p < 0.001$, ** $p < 0.01$. *P<0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

Conclusion

This work has demonstrated that extracts of the stem bark of F. exasperata exhibits considerable anti-inflammatory and antioxidant activities. The activities resided in the chloroform extract of the stem bark. The furanocoumarins bergapten and oxypeucedanin hydrate as well as the sterolin sitosterol-3-O-β-D-glucopyranoside, were isolated from the chloroform extract of the stem bark. The compounds showed anti-inflammatory and antioxidant and antimicrobial activities and thus contributed substantially to the bioactivities of F. exasperata observed in this study.

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