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Anti-inflammatory, antioxidant and antimicrobial activity of the stem bark extract and fractions of *Ficus exasperata* Vahl. (Moraceae)

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ABSTRACT

Ficus exasperata is used in various ethnomedicines for the treatment of pain, inflammatory diseases, wounds and abscesses. Different solvent extracts of the stem bark of *F. exasperata*, which is rarely studied, were tested for anti-inflammatory, antioxidant and antimicrobial activities. Anti-inflammatory activity of the extract was evaluated using the carrageenan induced foot pad edema model in seven day old chicks. In the test for antioxidant activity, a DPPH radical scavenging assay was performed. Antimicrobial evaluation of the extract was done using the agar well diffusion and micro-dilution assays. The hydro-alcoholic stem bark extract (30-300 mg/kg, *p.o*) dose dependently inhibited carrageenan induced foot edema with maximum inhibition of 68.57±3.342% (300 mg/kg) which was comparable to that of diclofenac (71.56±3.43% at 100mg/kg) and dexamethasone (74.53±5.21% at 3 mg/kg). The extract also showed antibacterial activity against both gram positive and negative organisms. The most sensitive pathogenic strains inhibited by the extract were *Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumoniae* and *Staphylococcus aureus* with respective zones of inhibition of 14.33±0.33, 12.33±0.33, 17.33±0.67 and 15.0±0.58mm. The MIC's ranged between 250-1000µg/ml. The lowest MIC of 250µg/ml was recorded for *Klebsiella pneumoniae*. The anti-inflammatory activities and antimicrobial activities of the stem bark were highest in it chloroform fraction. The activities shown by the extract in this study may be due to the presence of phytosterols, triterpenes, tannins, flavonoids and glycosides which were found in the phytochemical screening of the powdered stem bark. The present study validates the traditional uses of the plant in the treatment of inflammation and infectious diseases.

Keywords: Anti-inflammatory, Antimicrobial, Antioxidant, *Ficus exasperata*, Stem bark.

1. Introduction

Documented evidence demonstrates that inflammation is associated with virtually all diseases. Typical inflammatory diseases such as rheumatoid arthritis, asthma, colitis and hepatitis are the leading cause of disability and death [1]. The steroidal and non-steroidal anti-inflammatory agents, which are the main agents used in conventional anti-inflammatory therapy, have been associated with side effects such as gastric ulceration, hemorrhage, bronchospasm, kidney dysfunction [2], dyslipidaemia, Cushing's syndrome, hypertension and immunosuppression [3]. This has limited their use in some cases. Great attention and extensive studies have been devoted to evaluate the therapeutic benefits of plants used in traditional medicine as alternatives to these drugs.

The excessive production of reactive oxygen metabolites by phagocytic leucocytes during the inflammatory process, as part of host defence, deregulates cellular function causing tissue injury which in turn augments the state of inflammation leading to chronic inflammatory diseases [4]. Antioxidants, which scavenge these reactive oxygen metabolites, have been found to complement the anti-inflammatory process, promote tissue repair and wound healing. A number of plant secondary metabolites such as apigenin, quercetin, luteolin and silymarin have been found to exhibit anti-inflammatory activities due to their antioxidant properties [5, 6].

Plants have also been a source of various anti-infective agents. The bacteriostatic and fungicidal properties of lichens and the antimicrobial action of allicin in garlic (*Allium sativum*) are a few examples of age-old antibacterial therapy [7].

Ficus exasperata, is a deciduous tree with smooth gray bark which exudes a gummy sap. It grows in the drier types of forest, secondary vegetation, in rocky places and sometimes persisting in cleared land [9]. It is widely distributed in tropical Africa, from Senegal to Cameroon and extending north-east to Ethiopia and Djibouti and southward to Angola and Mozambique. It also occurs in Yemen, India and Sri Lanka [10]. It is commonly referred to as sand paper plant 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. The rough leaves are also used for scratching patches of ringworm before further treatment [11]. Natives of Bosomtwe-Atwima-Kwanwoma district of Ghana use poultices of the leaves and stem bark for the treatment of new, old and deep wounds as well as boils and burns [12]. Sap from the stem bark is used for the treatment of wounds, sores, abscesses and stomach-ache [13]. Decoctions of the bark are taken against worms, haemorrhoids, abnormal enlargement of the spleen and also to relieve cough [14]. Chest pains are treated in the Gambia by steam inhalation of the leaves boiled in water [15]. The leaves are also reported to exhibit anti-hypertensive [16], anti-ulcerogenic [17], anti-inflammatory [18] and hypolipidemic activities. Research on *F. exasperata* have been focused on the leaves whereas the stem bark has rarely been studied for it biological activities and phytochemistry. This study was therefore designed to assess the stem bark of *Ficus exasperata* for anti-inflammatory, antioxidant and antimicrobial activities and fractionate it into polar, medium polar and non-polar fractions in an attempt to track down the compounds which may be responsible for these activities.

2. Material and methods

2.1 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 herbarium specimen has been deposited.

2.2 Extraction

The coarsely powdered stem bark of *F. exasperata* (0.25 kg) was soxhlet-extracted with 70% ethanol. The extract was concentrated under reduced pressure to a small volume by means of rotavapor (R-114, Buchi, Switzerland) at a temperature of 50ºC, and evaporated to dryness on a water bath to give the total crude ethanol extract (SFE, yield = 3.64% $^{W}/_{W}$). 4.8 kg of the powdered bark was further successively soxhlet-extracted with petroleum ether (40/60), followed by chloroform and 70% ethanol to give pet-ether fraction FEP (Yield = 0.29 $\%^{W}/_{W}$), chloroform fraction FEC (Yield = 0.35%$^{W}/_{W}$) and 70% ethanol fraction FEE (Yield = 2.08 $\%^{W}/_{W}$), after evaporation under reduced pressure and drying on a water bath.

2.3 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.

2.4 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.

2.5 Anti-inflammatory Assay

The carrageenan-induced foot oedema model of inflammation in the chick by Roach and Sufka [19] with modifications by Woode *et al*. [18] was used to evaluate the anti-inflammatory properties of the extracts. Dexamethasone and diclofenac served as reference drugs. Chicks were randomly divided into groups of 5 and had access to food and water ad *libitum*. Carrageenan (10 µl of a 2% suspension in saline) was injected sub-plantar into the right footpads of the chicks. The foot volume was measured before injection and at hourly intervals for 5 hours after injection by water displacement plethysmography as described by Fereidooni *et al*.[20] using an electronic Von Frey plethysmometer (Model 2888, IITC life science inc. Ca 91367 Canada). The oedema component of inflammation was quantified by measuring the difference in foot volume before carrageenan injection and at the various time intervals. The extracts were orally administered at 10, 30 and 100 mg/kg whereas the standard drugs dexamethasone (0.3, 1 and 3 mg/kg) and diclofenac (10, 30 and 100 mg/kg) were given intraperitoneally. The control animals received only saline which served as the vehicle. 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.

2.6Anti-oxidant assay

2.6.1 Scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical assay

The free radical scavenging activity was determined as described by Govindarajan *et al*.[21] with few modifications. 1 ml of the extract (0.25-2 $mgml^{-1}$ in methanol) was added to 3 ml methanol solution of DPPH (20 mg l^{-1}) 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 (1 ml) methanol (50%) was added to 3 ml DPPH solution incubated at 25 °C for 30 minutes and used as control. N-propyll gallate (3.75-30 μgml^{-1}) was used as a standard free radical scavenger. 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 (EC_{50}). Each test was carried out using three replicates.

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

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

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

2.7 Antimicrobial assay

2.7.1 Microbial strains

The extracts and standard drugs (gentamicin and fluconazole) were tested against a set of 7 microorganisms. Two Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* NCTC 10073) and four Gram-negative bacteria including *Salmonella typhi* NCTC 6017, *Escherichia coli* NCTC 9002, *Klebsiella pneumonia* ATCC 10031 and *Pseudomonas aeruginosa* ATCC 27853 and the fungus *Candida albicans* (ATCC 10231) were provided by the Pharmaceutical Microbiology Section of the Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Science, KNUST, Kumasi. Bacterial strains were cultured overnight at 37 °C in nutrient broth and Fungi were cultured overnight at 37 °C in sabouraud dextrose agar.

2.7.2 Screening for Antimicrobial Activity of Extracts

The antimicrobial activities of the different extract/drugs were determined using the agar well diffusion method outlined by Dickson *et al*.,[12] The inocula was prepared by inoculating the test organisms in nutrient broth and incubating them for 24 hours at 37˚C for bacteria, while for *Candida albicans,* Sabouraud's dextrose broth was incubated for 48 hours. 1ml of the diluted cultures was inoculated into sterile molten agar at 45 °C and poured into a sterile petri dish. These were swirled gently and allowed to solidify. Wells were bored into the solidified inoculated nutrient agar plates using cork borer number 6. The extract was reconstituted in 2% DMSO. The agar-wells were filled respectively with equal volume of 0.1ml of each extract (10 mg/ml), gentamicin (500µg/ml), fluconazole (500 µg/ml). One hour was allowed for the extract/drug to diffuse into the agar after which the plates were incubated overnight at 37 °C for bacteria and at 37 °C for 48 hours for fungi. At the end of the incubation period, the diameter of the inhibition zone(s) were measured and recorded. The extract and antibiotics were tested in triplicates and mean zones of inhibition were calculated for each extract and the standard antibiotics.

2.7.3 Micro dilution assay

Minimal inhibitory concentration (MIC) values of the extracts were determined based on a micro-well dilution method [23]. The inocula of microorganisms were prepared from 12-hour broth cultures and serial dilutions were made to achieve a suspension of approximately 10^5 CFU/mL. The plant extract was screened at concentrations between 1000 µg/mL and 7.8 µg/mL. The 96-well sterile plates were prepared by dispensing into each well 100µL of double strength nutrient broth and 100µL of test samples together with 20µL of the inoculum. The microplates were incubated at 37 °C for 24 hours. Growth of the microorganisms was determined by adding 20 µL of a 5% solution of tetrazolium salt (MTT) and incubating for further 30 minutes. Dark wells indicated the presence of microorganisms as the dehydrogenase enzymes in the live bacteria reacts to form a dark complex with the tetrazolium salt. All experiments were carried out in triplicates.

2.8 Statistical analysis

The raw scores for right foot volumes were individually normalized as percentage of change from their values at time zero then averaged for each treatment group. Total foot volume for each treatment was calculated in arbitrary unit as the area under the curve (AUC). To determine the percentage inhibition for each treatment, the following equation was used.

$$\%\ inhibition\ of\ edema = \left(\frac{AUC_{control} - AUC_{treatment}}{AUC_{control}} \right) \times 100$$

Differences in AUCs were analyzed by one way analysis of variance (ANOVA) followed by Student-Newman-Keuls' *post hoc t* test. Doses and concentrations responsible for 50 % of the maximal effect (EC_{50}, $ED_{50}$and IC_{50}) for each drug/extract were determined using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation.

$$Y = \frac{a + (b - a)}{(1 + 10^{(LogEC50 - X)})}$$

Where, *X* is the logarithm of dose and *Y* is the response. *Y* starts at *a* (the bottom) and goes to *b* (the top) with a sigmoid shape. Graph Pad Prism for Windows version 5.0 (Graph Pad Software, San Diego, CA, USA) was used for all statistical analyses. $P<0.05$ was considered statistically significant.

2.9 Phytochemical screening

The total crude extract SFE was screened for the presence of secondary metabolites such as tannins, flavonoids, alkaloids, phytosterols, reducing sugars, terpenoids by using simple qualitative test described by Harbone [31].

3. Results and Discussion

Induction of acute inflammation in control chicks resulted in a prominent increase in paw thickness, which began 1 h after intraplantar injection of carrageenan and reached a peak of inflammation after 3 hours (Figure 1a) and slowly declining for the duration of the experiment. Oral administration of the hydro-alcoholic stem bark extract, SFE (30-300 mg/kg), dose dependently and significantly ($P<0.001$) reduced the increase in foot volume induced by injection of carrageenan (Figure 1a) with ED_{50} of 50.65±0.012 mg/kg (Table 1). The extract inhibited the increase in foot volume significantly ($P<0.001$) from the second hour (Figure 1a) and thus, presumably, inhibited the synthesis and release of prostaglandins responsible for the first phase of the inflammation [24, 25]. Similarly the NSAID diclofenac and the steroidal anti-inflammatory agent dexamethasone, significantly ($P<0.001$) and dose-dependently reduced the foot oedema (Figure 1c-f) over the period of the experiment. The extract was three times less potent than diclofenac and 17 times less than dexamethasone (Table 1). This is the first report of the anti-inflammatory activity of the stem bark of *F. exasperata*. In an attempt to track down the anti-inflammatory constituents, successive petroleum ether, chloroform and ethanolic fractions of the stem bark were also tested for anti-inflammatory activity. The chloroform extract (FEC) dose dependently exhibited the highest anti-inflammatory activity with maximum percentage inhibition of 70.80±2.951 at 100 mg/kg body weight, followed by the 70% ethanolic extract (FEE) and the petroleum ether extract (FEP) respectively. Their respective ED_{50} values are presented in Table 1. Thus the study has shown that extracts of the stem bark of *Ficus exasperata* possessed significant anti-oedematogenic effect on foot pad oedema induced by carrageenan. The anti-inflammatory activity of the chloroform

fraction (FEC) was twice lower than the total hydro-alcoholic extract of the stem bark (SFE), suggesting a possible synergism with the other fractions. Thus fractionation may reduce the anti-inflammatory potency of the stem bark of *Ficus exasperata*. The anti-inflammatory activity of the stem bark justifies it use traditionally for the treatment of abscesses and stomach-ache, haemorrhoids, abnormal enlargement of the spleen and also to relieve cough [13].

Table 1: Effect of extracts, standard drugs on carrageenan-induced oedema in 7 - day old chicks

Extract/drug	ED_{50} (mg/kg) ± SEM
SFE	50.65 ± 0.012
Diclofenac	16.97 ± 0.011
Dexamethasone	2.95 ± 0.013
FEP	215.0±0.011
FEC	97.76±0.013
FEE	109.70±0.013

SFE (total ethanol extract), FEP (Pet-ether extract), FEC (Chloroform extract), FEE (ethanol extract)

The EC_{50} values for the DPPH free radical scavenging assays are presented in Table 2. The stem bark extract (SFE) was twice a weaker scavenger of the DPPH radical than *n*-propyl gallate, used as standard antioxidant (Table 2). The antioxidant compounds of the stem bark were distributed in the chloroform and ethanol fractions (FEC and FEE) as shown by their EC_{50} values. The antioxidant activity of the extracts may, partly, contribute to their anti-inflammatory activities. This is because inflammatory tissue injuries are mediated by reactive oxygen metabolites from phagocytic leukocytes (e.g neutrophils, monocytes, macrophages and eosinophils) that invade the tissues. These radicals may injure cells and tissues directly via oxidative degradation of essential cellular components [26]. Thus the ability of the extracts to act as antioxidant agent may play a significant role in the amelioration of acute inflammatory response as shown in this study. The anti-inflammatory activities of some NSAIDS have been linked to their ability to inhibit lipoxygenase and decelerate the rate of glutathione depletion [26].

Also the antioxidant activity of the extracts, shown in this study, may also support it traditional use in Ghana, where poultices of the leaves and stem bark are used for the treatment of new, old and deep wounds as well as boils and burns [12]. This is because in local and chronic wounds, oxidants cause cell damage and function and serve as inhibitory factors to wound healing [27]. The administration of antioxidants or free radical scavengers is reportedly helpful, notably to limit the delayed sequelae of thermal trauma and to enhance the healing process [27].

Table 2: DPPH scavenging activity of extracts and compounds isolated from the stem bark

Extract/Drug	DPPH Assay EC_{50} (µg/ ml)
SFE	20.0±0.01
FEP	1174.0±0.010
FEC	134.0±0.031
FEE	125.3±0.011
N-propyl gallate	10.80±0.002

SFE (total ethanol extract), FEP (Pet-ether extract), FEC (Chloroform extract), FEE (ethanol extract)

In the present study, the ethanol extract of the stem bark of *F. exasperata* exhibited a wide range of antimicrobial activities, covering both gram positive and negative organisms as well as the fungus *Candida albicans* (Figure 2). *S. aureus* was the most susceptible organism. The pet-ether fraction (FEP) was inactive against all test organisms whereas the chloroform and ethanol fractions showed considerable activity against both gram negative and positive organisms (Figure 2). The chloroform fraction was most active against *P. aeruginosa* and *S. aureus* with MIC 1000 µg/ml (Table 3) for both organisms. The susceptibility of both gram positive and negative organisms to the extract supports the folkloric use of the plant for wound healing [12], treatment of veneral diseases and nasopharyngeal infections [13]. Also the high susceptibility of *S. aureus* to the extract and fractions further supports the use of the stem bark for the treatment of wounds since *S. aureus* is a common pathogen in most infected wounds [28].

The significant anti-inflammatory, antioxidant and antimicrobial shown by the stem bark extract of *F. exasperata* may be linked to the tannin, flavonoids, triterpenoids, glycosides and sterols which were found in the phytochemical screening of the plant extract. Flavonoids are well known to show anti-inflammatory properties due to their inhibitory effects on enzymes involved in the production of the chemical mediators of inflammation [29]. Tannins, steroids and triterpenoids are documented to interfere with several components of the inflammatory cascade [30]. These secondary metabolites are also reported to show considerable antioxidant and antimicrobial activities and therefore their identification in the plant supports the bioactivities shown in the present studies.

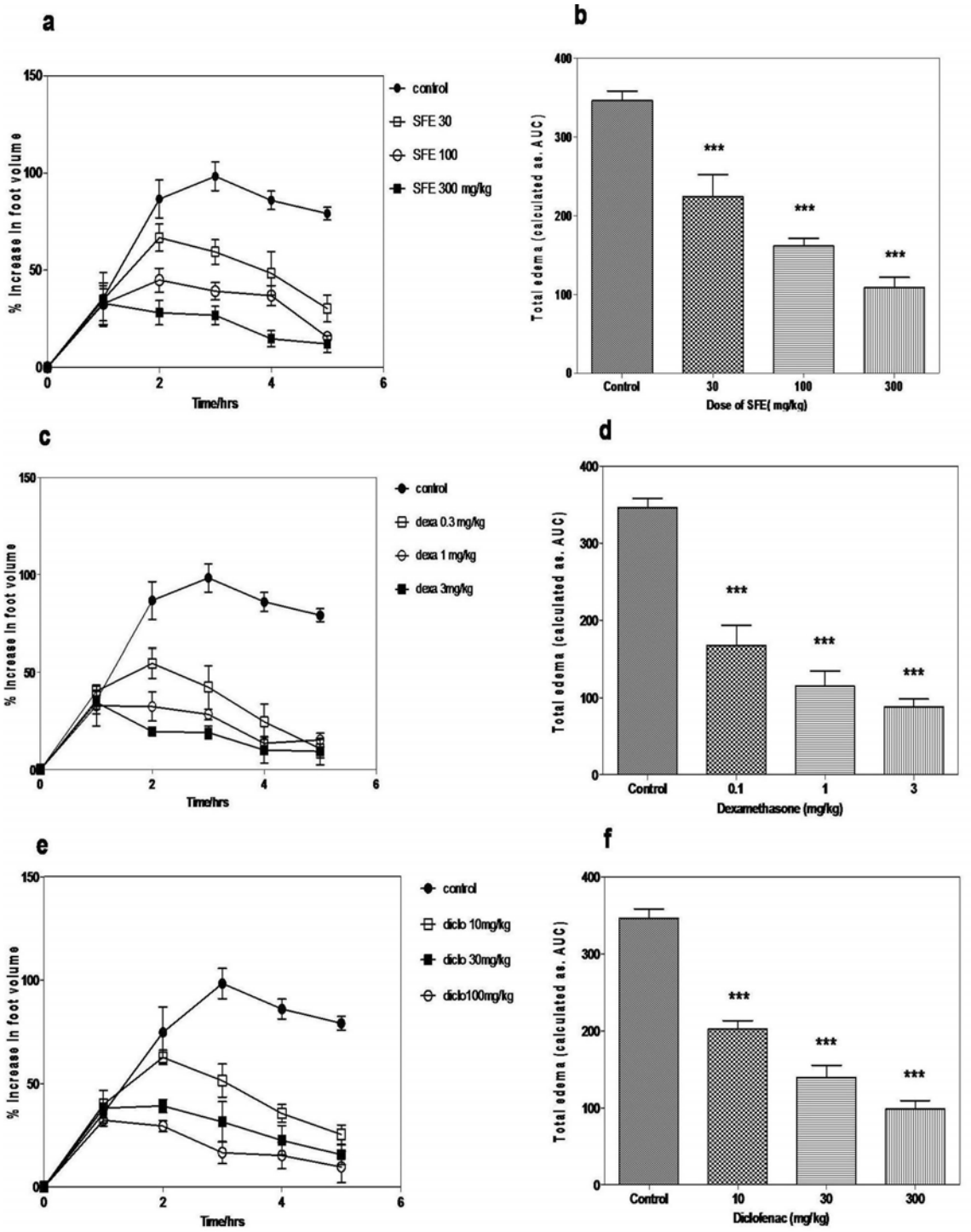
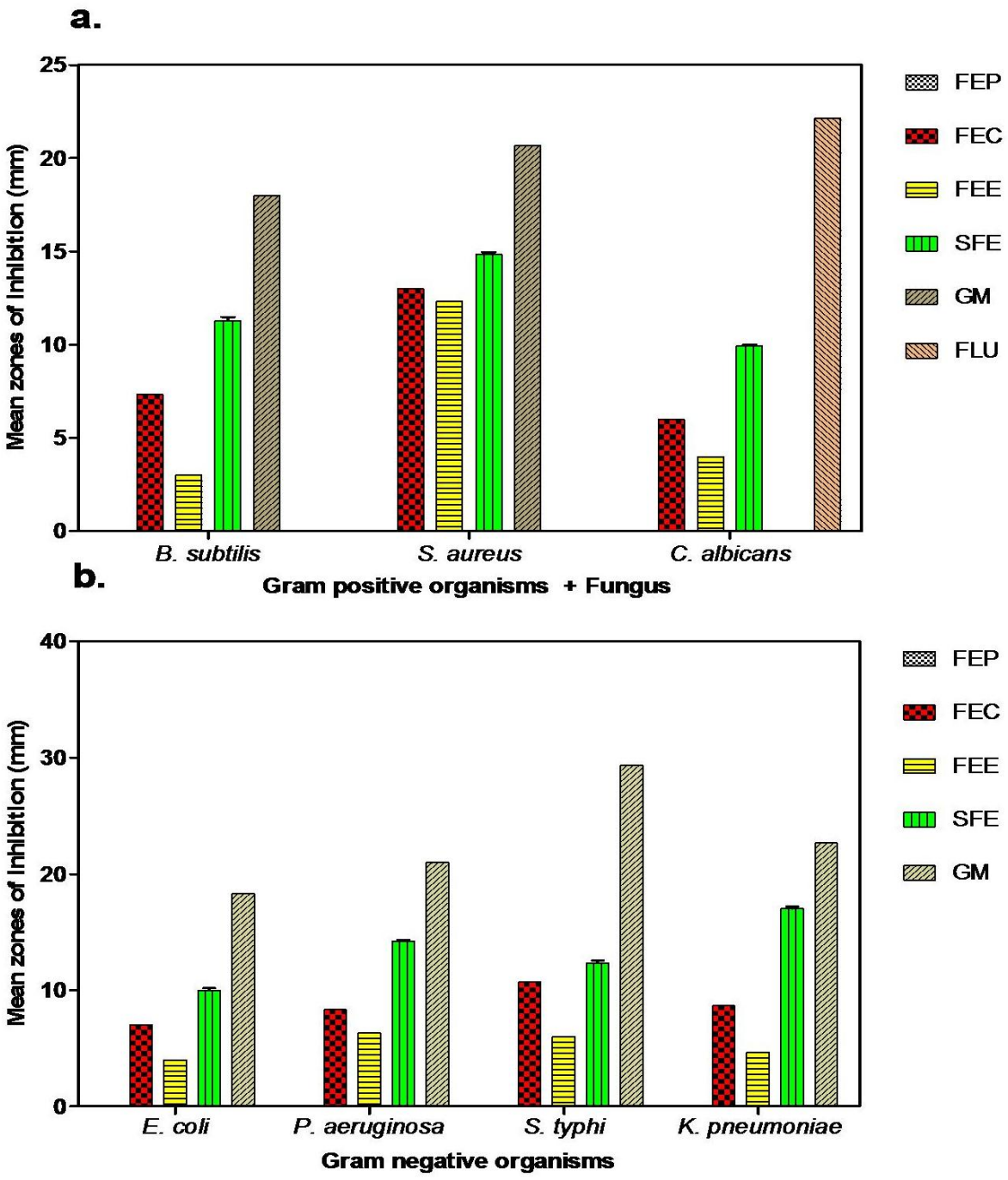


Fig 1: Effect of SFE [(30-300 mgkg^{-1}oral) (a-b)], diclofenac [(10-100 mg/kg; *i.p*) (e-f)] and dexamethasone [(0.1-3 mg/kg; *i.p*) (c-d)] on time course curve and the total oedema response, calculated as AUC's, for 5 hours, in carrageenan induced paw oedema in chicks. Values are means ± S.E.M (n=5) *** *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).

Table 3: Minimum inhibitory concentration (MIC) of extracts against susceptible microorganisms

Extracts	MIC (µg/ml)			
	PA	SA	ST	KP
SFE	500	500	250	250
FEC	1000	1000	>1000	>1000
FEE	>1000	>1000	>1000	>1000
FEP	NT	NT	NT	NT

PA (*P. aeruginosa*), **SA** (*S. aureus*), **ST** (*S. typhi*), **KP** (*K. pneumoniae*), NT= Not tested. **SFE** (total ethanol extract), **FEP** (pet-ether extract), **FEC** (chloroform extract), **FEE** (ethanol extract)



GM= Gentamicin, Flu= Fluconazole

Fig 2: Mean zones of inhibition of extracts and standard drugs against organisms

4. Conclusions

This work has demonstrated that extracts and fractions of the stem bark of *F. exasperata* exhibits considerable anti-inflammatory, antimicrobial and antioxidant activities. The activities resided in the chloroform extract of the stem bark. The study has provided some justification for the folkloric use of stem bark of *F. exasperata* for the treatment of inflammatory conditions, wounds and infections. Isolation of the compounds responsible for these activities is in progress in our laboratories.

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