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Original article:

ISOLATION OF 3-(4-HYDROXYPHENYL) METHYLPROPENOATE AND BIOACTIVITY EVALUATION OF *GOMPHRENA CELOSIOIDES* EXTRACTS

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ABSTRACT

The efficacy of *Gomphrena celosioides* extracts in traditional medicine in the treatment of infectious diseases was evaluated by biological assays. The bioactivities of the extracts of this plant were tested against organisms. The ethyl acetate and methanol extracts of the plant displayed inhibition activities on *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli* and *Salmonella typhi*. Methanol extract was active against *Candida albican*s, *Aspergillus niger* and *Trichophyton species* with diameter zones of inhibition between 14 and 20 mm. Fractionation of the methanol extract produced 3-(4-hydroxyphenyl) methylpropenoate with mild antimicrobial activity against the test microorganisms. The anthelmintic assay showed ethyl acetate and methanol extracts to be active against *Fasciola gigantica, Taenia solium* and *Pheretima pasthuma*. Ethyl acetate was the most toxic of the extracts causing paralysis of *Taenia solium* within 15 minutes and the death of *Fasciola gigantica* within 20 minutes of application. The brine shrimp assay gave an LC_{50} of 52.15 and 77.98 µg/ml on hexane and methanol extracts respectively. The result of this work corroborated the folkloric use of *Gomphrena celosioides* in the treatment of infectious diseases.

Keywords: Phytochemical analysis, antimicrobial, cytotoxicity, anthelmintic, *Gomphrena celosioides*

INTRODUCTION

Multiple drug resistance is a common problem nowadays in the treatment of internal parasites and infectious diseases due to abuse and indiscriminate use of drugs. A number of pathogens are already developing resistance to the available drugs (Davis, 1994; Service, 1995). The continued utilization of these drugs had resulted in hypersensitivity, immune suppression and allergic reactions (Ahmad et al., 1998). Pathogens resistance to available drugs is alarming (Bhavnani & Ballow, 2000), consequently, there is a need to search for new and effective therapeutic agents for the treatment of diseases caused by these organisms. Search for cure for these diseases from natural source is growing because of degradable potential of herbal drugs apart from efficacy. Ethno-botanical information of plant traditionally used for treating these diseases is of particular importance to drug discovery, so collaborative work with traditional healers is paramount in this direction.

Gomphrena celosioides belongs to the *Amaranthacea* family and over 120 species of the family are found in America, Austra-

lia and Indo-Malaysia, while 46 species occur in Brazil. Few species occur in the East and West of Africa (Vieira et al., 1994). *Gomphrena* species in different parts of the world are used for various folkloric medicinal purposes. In Brazil, some species are employed in the treatment of bronchial infections, diarrhea, and malaria fever, while others had found application as analgesic, tonic/carminative and diuretics (Gessler et al., 1994; Vieira et al., 1994). *Gomphrena celosioides* is used in ethnomedical practice in Nigeria for treatment of various skin diseases, worms' infections and infectious diseases. In South America the plant is utilized as an abortifacient (Burkill, 1984). A decoction of the whole plant and a related species *Gomphrena globosa* is applied to gangrenous wound. *G. martiana* and *G. boliviana* are employed as antimicrobial agents by the natives (Arenac & Azorearo, 1977). Phytochemical investigation of *G. martiana* and *G. boliviana* led to the isolation of flavanones with antimicrobial properties (Pomilio et al., 1992).

Earlier research work by Botha and Gerritsma-Van der Vijver (1986) on *Gomphrena celosioides* extracts revealed the presence of saponins, steroids, amino acids, non-reducing sugars, phenols and flavonoids. Other workers had also isolated the above mentioned compounds and related compounds during the pharmacological studies of the plant (Vieira et al., 1994; Banerji et al., 1971; De Moura et al., 2004).

This present study isolated a bioactive compound from the methanol extract and carried out the biological studies, antimicrobial, anthelmintic and brine shrimp toxicity assays of the *Gomphrena celosioides* extracts.

MATERIALS AND METHODS

General methods

Infrared spectra were recorded on Bruker FT vector 22 spectrophotometer in KBr discs, in cm^{-1}. EIMS (ionization voltage 70 ev) was measured on a Varian MAT 311/A mass spectrometer and HR EIMS were taken by MS JEOL-MS route (JMS-600H). ^{1}H and ^{13}C-NMR spectra were run on Bruker AMX400 and AMX500 MHz NMR spectrometers at 200(^{1}H) and 75(^{13}C). The chemical shifts are given in ppm (δ) relative to TMS as internal standard and coupling constant, J in H_z.

In the biological assay, the inoculums size of the microorganisms was adjusted to 2.6×10^7 cfu/ml using Unicam gamma 1000 spectrophotometer at 540 nm for the molten nutrient agar.

Plant materials

Fresh *Gomphrena celosioides* were collected in November 2005 from Abdulsalami Abubakar Graduate Hall, University of Ibadan, Nigeria. The identity was authenticated by Mr. Felix Nsong of Forest Research Institute of Nigeria (FRIN), Ibadan. A voucher specimen with herbarium number FHI 106429 was deposited in the FRIN herbarium.

Extraction and isolation

Air dried and ground *G. celosioides* (3.2 kg, whole plant) was exhaustively extracted with hexane, ethyl acetate and methanol successively. The extracts were concentrated under pressure to yield hexane (24.4 g), ethyl acetate (19.50 g) and methanol (93.80 g) extracts.

Crude methanol residue (10 g) was fractionated by vacuum liquid chromatography on silica gel (Merck). Partitioning was done in hexane followed by gradient mixtures of diethyl ether. Fractions were separated further on small column and 20 % ether in hexane afforded compound **1**. Repeated crystallization in methanol afforded white crystal and structural elucidation resolved compound **1** as 3-(4-hydroxyphenyl) methylpropenoate.

Spectral data

- IR (KBr) ν_{max} (cm^{-1}): 3420, 2940, 2850, 1745, 1670 1458, 1375.
- ^{1}H-NMR (200 MHz $CDCl_3$): δ7.63 (H, d, J = 15.95), δ7.41 (H, d, J = 15.9Hz),

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δ6.85 and δ6.30 (2H each, dd, J = 9Hz), δ3.8 (3H, s, CH_3), δ7.24 (1H, s, -OH)

- ^{13}C-NMR (75MHz, $CDCl_3$): δ167.78 (C-1^1), δ51.57 (-CH_3O), δ157.57 (C-4), δ144.46 (C-3^1), δ127.36 (C-2^1), δ115.8 (C-6), δ115.3 (C-5), δ115.3 (C-3), δ115.6(C-2), δ129.93(C-1)
- HR EIMS ^{m}/z 178.1 (calculated for $C_{10}H_{10}O_3$, 178.06)
- EIMS ^{m}/z (rel. int): 147.0 (100), 119.0 (40.0), 91.0 (13.2), 64.9 (32.6), 59.4 (49.2)

Antimicrobial assay

Test organisms

Laboratory strains of bacteria and fungi were obtained from Pharmaceutical Microbiology Department of Faculty of Pharmacy, University of Ibadan. Five bacteria, consisting of gram positive and gram negative were used in this study: *Staphylococcus aureus* NCTC6571, *Escherichia coli* NCTC9001, *Pseudomonas aeruginosa* NCTC6750, *Bacillus subtilis (* Lab-stock), *Salmonella typhi* ATCC14028 *and three fungi, Candida albicans* (NCTC 7534), *Aspergillus niger* and *Tricophyton species* (clinical isolates). Nutrient agar, sabouraud dextrose agar (SDA), tryptone soy agar (Oxoid Ltd, UK) were the media used in the assay. Dimethylsulphoxide (DMSO) (Merck) was used in dissolving the extracts and the same was used as negative control in the assay.

Standard drugs

Ampicillin (Beecham) 25 µg/ml, Tioconazole (Pfizer) 1 % w/v (0.5 mg/ml) and piperazine citrate (Pfizer) were used as reference drugs in the assay.

Quantitative antimicrobial evaluation

The agar cup diffusion method (Perez et al., 1990; Kavanagh 1972) was used. Two-fold dilutions of overnight broth cultures (0.1 ml) of organisms at inoculums size adjusted to 2.6 x 10^7 cfu/ml using Unicam gamma 1000 spectrophotometer at 540 nm were inoculated into cooled but molten nutrient agar (for fungal cultures, surface spread method was used for inoculation on SDA). The plates were allowed to solidify and wells were made using a sterile 7 mm diameter cork borer. The extracts (12.5 mg/ml) in DMSO were introduced into the wells with the aid of a dispenser. Controls and standards were set up containing the solvent (DMSO) and ampicilin (25 µg/ml) for bacteria and tioconazole (0.5 mg/ml of 1 % w/v) for fungi.

Diameters of zones of inhibition were determined after incubating plates at 37^o C for 24 h for bacteria and at 25^o C for 72 h for fungi. Antimicrobial studies were done in triplicates and diameters of zones of inhibition (mm) were expressed as the mean and standard deviations of the means. Zones of inhibition ≥ 10 mm were considered active (Zwadyk, 1972). Student 'T' tests was used to test for probability at $P < 0.05$.

Anthelmintic assay

Fasciola gigantica (liver fluke mean weight of 0.05 - 0.07 g) and *Taenia solium* (Tapeworm, 2.4 - 2.8 g) were obtained from freshly slaughtered cows at the Bodija abattoir, in Ibadan metropolis. *Pheretima pasthuma* (Earthworm, 0.06 – 0.6 g) were collected from the Awba dam and the water logged areas of Staff school, both within the campus of University of Ibadan. All the three types of worm were authenticated at the Parasitology Research Unit of Zoology Department in University of Ibadan.

Five worms of the same type were placed in 9 cm Petri dishes containing solutions of different concentration of the extracts made in DMSO (10, 20, 30, 40, 50, 70, 80, 100 mg/ml) respectively. This was done in duplicate for all worm types. Times of paralysis (P), in minutes were taken when no movement of any sort could be observed, except when the worms are shaken vigorously. Time of death of worms (D), in minutes were recorded after ascertaining that worms neither moved when shaken vigorously nor when dipped in warm water (50^o C). Mean time was calculated for P and D. Piperazine citrate

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(10 mg/ml) was included as a reference drug while DMSO in distilled water served as control in accordance with modified Ajaiyeoba and Okogun methods (1996).

Brine shrimp toxicity assay

The brine shrimp lethality test was used to determine extracts and isolated compound toxicity (McLaughlin et al., 1993). 10 brine shrimp *nauplii* were introduced into vials containing 1000, 100 and 10 ppm concentration of the extracts and isolated compound. The effect of this extract on the brine shrimp *nauplii* were examined after 24 h. The ratio of dead *nauplii* was compared to the total number of *nauplii*. The result was analysed by Finney program and the LC_{50} calculated. Podophylotoxin was used as a positive control in the bioassay.

RESULTS AND DISCUSSIONS

Isolation

Fractionation of the crude methanol residue afforded compound **1**, a white crystal with melting point of 87^{o}C. The IR spectrum of this compound showed absorption peaks at 3420, 2940, 2850, 1745, 1670, 1450 and 1375 cm^{-1}. The absorptions at 3420 and 1670 cm^{-1} are characteristic of OH and C=O stretching vibrations respectively. Earlier workers have reported similar absorption frequencies for OH and C=O in vanillic acid (de Moura *et al.*, 2004). The ^{1}H-NMR showed two pairs of characteristic doublets at δ7.63 and δ7.41 with large coupling constant (J = 15.95Hz) allotted to the olefinic α- and β-protons of the (E)-hydroxycinnamoyl moiety (Terahara et al., 2001). The ^{1}H-NMR exhibited two *ortho* and *meta* coupled protons with signals at δ6.85 and δ6.25 (2H each, J= 9Hz) characteristic of a *p*-disubstituted benzene ring. The downfield shift of the former signal indicated the presence of an electron-withdrawing group on the benzene ring. A singlet at δ3.80 was due to methyl protons of the ester.

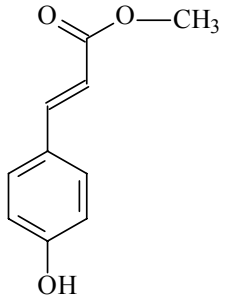
The ^{13}C-NMR and DEPT analysis of the compound indicated that there are 10 carbon atoms in the molecule, three of which are quaternary (including a carbonyl), six are methine (CH) and one is methyl (CH_3). Signal at δ167.78 was due to carbonyl carbon, δ51.57 signal was that of the methyl carbon next to oxygen, while the carbinol carbon showed signal at δ157.57. The olefinic carbons had absorptions at δ144.46 and δ129.93.

The HR-EIMS gave molecular ion peak at 178.1 which translated to a compound with molecular formula of $C_{10}H_{10}O_3$. The EIMS measurement showed the base peak at $^{m}/_{z}$ 147 (100 %) and this peak was resulted from the fragmentation of $–OCH_3$ ion from the molecular ion, while the peak at 119 (40.42 %) resulted from cleavage of $–COOCH_3$ ion from the parent molecule.

The IR, NMR and MS spectra data with a molecular mass of 178 coupled with the fragmentation pattern recorded elucidated compound **1** to be 3-(4-hydroxyphenyl) methylpropenoate with molecular formula of $C_{10}H_{10}O_3$. 3-(4-hydroxyphenyl) methylpropenoate is a derivative of vanillic acid which had earlier been isolated from other species of the family.

Table 1: NMR spectra data of 3-(4-hydroxyphenyl) methylpropenoate

C/H	δC	δH
1	167.78	-
2	129.93	7.41
3	144.46	7.63
1’	127.36	-
4’	157.57	-
2’/6’	115.85	6.85
3’/5’	115.40	6.25
OCH_3	51.57	3.85



3-(4-hydroxyphenyl) methylpropenoate

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Bioactivities

The presence of OH group in phenolic compounds had shown responsibility for inhibitory activities on microorganisms (Aziz et al., 1998; Friedman et al., 2003). This explained the antimicrobial activities recorded for this plant extracts and the isolated compound with an OH group. Gourma et al. (1989) explained that the OH group is very reactive and can easily form hydrogen bonds with active sites of enzymes. This was buttress with 0.4 mg/disc and 2.1 mg/ml of vanillic acid which completely inhibited the growth of *Escherichia coli* and its related strains (Aziz et al., 1998; Chamkha et al., 2002). Table 2 shows the antibacterial activity of crude ethyl acetate extract, methanol extract and 3-(4-hydroxyphenyl) methylpropenoate. The isolate, 3-(4-hydroxyphenyl) methylpropenoate, an ester of the vanillic acid inhibition activity is therefore not surprising, hence *S. typhi, E.coli, P. aeruginosa, B. subtilis* and *S. aureus* growth were curtail with diameter zone of inhibition of 9±0.2, 9±0.3, 10±0.5, 11±0.7 and 10±0.4 mm respectively at 25 µg/ml. The phenolic compounds inhibition activity has also been found to be proportional to the number of OH group present in the compound (Friedman et al., 2003). The crude extract which contain many compounds is likely to have more than one OH groups as obtained in the isolated compound and the high concentration of application of the extract are likely possibilities for the higher inhibitory activities recorded for the extract than the isolated compound.

Apart from higher number of OH groups that are present in the extracts, synergism may account for higher inhibitory activities of the extracts also and lack of it may be responsible for lower activity recorded for the isolated compound (Odebode et al., 2004). Ethyl acetate and methanol extracts displayed higher inhibitory activities on the microorganisms than the isolated compound (Table 2) for reasons stated above. The relatively lower inhibitory activities of the extracts recorded compared with the standard antibacterial drug, ampicilin in this study might probably be due to metabolism of the extracts by the bacteria which reduced their activities. A selective sensitivity was observed for *S. aureus* and *S. typhi* against *P. aeruginosa*, *P. mirabilis* and *E. coli* which were resistant to ethanol extract of *G. celosioides* and vanillic acid in a study conducted by De Moura et al., (2004). Metabolism of the extract by the bacteria was adduced to be responsible for this observation. Methanol extract (12.5 mg/ml) had pronounced effect on the activities of the fungi, *C. albicans*, *A. niger* and *Trichophyton species*, with higher activity on *C. albican* (20 ± 0.3 mm) (Table 3). The inhibition activities of the extracts on the fungi were comparable to that of reference drug, tioconazole.

Table 2: Antibacterial activity of crude ethyl acetate extract, methanol extract and 3-(4-hydroxyphenyl) methylpropenoate

	Diameter of zone of inhibition (mm)[a)]			
Microorganism	EtOAc extract[b)]	MeOH extract[b)]	Isolate[c)]	Ampicilin[c)]
S. typhi	12 ± 0.3	12 ± 0.3	9 ± 0.2	19 ± 0.2
E. coli	12 ± 0.4	12 ± 0.3	9 ± 0.2	15 ± 0.5
P. aeruginosa	12 ± 0.7	12 ± 0.1	10 ± 0.5	18 ± 0.1
B. subtilis	14 ± 0.4	13 ± 0.2	11 ± 0.7	22 ± 0.3
S. aureus	13 ± 0.3	13 ± 0.2	10 ± 0.4	18 ± 0.4

[a)] Values are mean ± SD
[b)] 12.5 mg/ml
[c)] 25 µg/ml

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Table 3: Antifungal activity of crude methanol extract

Diameter of zone of inhibition (mm)[a)]

Microorganism	EtOAc extract[b)]	Tioconazole[c)]
C. albican	20 ± 0.3	28 ± 0.4
A. niger	17 ±0.5	22 ± 0.3
Trichophyton species	14 ± 0.1	19 ± 0.1

[a)] Values are mean ± SD
[b)] 12.5 mg/ml
[c)] 0.5 mg/ml of 1% w/v

Both ethyl acetate and methanol extracts (10 mg/ml) were very active in invoking paralysis and death of *F. gigantica*, *T. solium* and *P. pasthuma*. The methanol extract was more potent in causing paralysis of *P. pasthuma* than the ethyl acetate extract and reference drug, piperazine citrate. The time of death of *T. solium* and *P. pasthuma* by piperazine citrate, ethyl acetate and methanol extracts was similar but piperazine citrate used far less time to paralysis and caused death of *F.gigantica* than the extracts (Table 4).

Compounds with low LC_{50} (i.e. < 100 µg/ml) show indication for presence of cytotoxic and insecticidal compounds (Krishnaraju et al., 2005). Therefore, LC_{50} of 52.148 and 77.978 µg/ml recorded for hexane and methanol extracts respectively (Table 5) may suggest the presence of cytotoxic and/or insecticidal compounds in the extracts while 3-(4-hydroxyphenyl) methylpropenoate with LC_{50} of 110.654 indicates weak cytotoxicity. The low LC_{50} values of the extracts support the use of the aqueous extract of this plant in folk medicine for the treatment of gangrenous wounds.

Table 5: Brine shrimp assay of the extracts and 3-(4-hydroxyphenyl) methylpropenoate

Extract/isolate	LC_{50}, µg/ml, 24h
Hexane	52.146
Methanol	77.978
3-(4-hydroxyphenyl) methylpropenoate	110.654

Table 4: Anthelminthic activity of ethyl acetate and methanol extracts (min)[a)]

Helminths	EtOAc extract[b)]	MeOH extract[b)]	Piperazine citrate[b)]
F. gigantica	P 20 ± 0.8	40 ± 0.5	3 ± 0.2
	D 35 ± 0.5	45 ± 0.1	5 ± 0.5
T. Solium	P 15 ± 0.4	37 ± 0.3	5 ± 0.5
	D 40 ± 0.3	42 ± 0.3	40 ± 0.5
P. Pasthuma	P 40 ± 0.2	8 ± 0.9	20 ± 0.3
	D > 60	60 ± 0.5	60 ± 0.5

[a)] mean time in minutes ± SD
[b)] 10 mg/ml
P = Mean time of helminth paralysis
D = Mean time of helminth death

CONCLUSION

The activities shown by the extracts of the plant corroborate the use of the plant by the traditional healer for antimicrobial and cytotoxic purposes as in the treatment of wounds, skin diseases, bronchial infections, diarrhea and other microbes' related diseases, and the good anthelmintic activity displayed by the plant extracts suggests the plant as a possible source of potent anthelmintic drug isolation of 3-(4-hydroxyphenyl) methylpropenoate is been reported for the first time in *G. celosioides* to the best of our knowledge.

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