

Full Length Research Paper

Cytotoxicity and antimicrobial studies of 1,6,8-trihydroxy-3-methyl-anthraquinone (emodin) isolated from the leaves of *Cassia nigricans* Vahl

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Accepted 21 May, 2007

Emodin was isolated from the ethyl acetate extract of the leaves of *Cassia nigricans* Vahl. The structure of the emodin was established by chemical spectroscopy. The LC₅₀ (lower – upper limits) of the emodin was 42.77 (11.80 – 72.94) µg/ml. Emodin was found to be highly cytotoxic. It showed significant antimicrobial activity on some common pathogens. The isolation of this active principle emodin, from the leaves of *Cassia nigricans* for the first time and the antimicrobial activity of the compound are reported in the present study. The isolation of the active principle justifies the use of the leaves of *C. nigricans* in herbal medicine for the treatment of skin diseases and gastro-intestinal disorders.

Key words: *Cassia nigricans*, leguminosae, emodin, cytotoxicity, antimicrobial activity.

INTRODUCTION

For the past two decades, there has been an increasing interest in the investigation of different extracts obtained from traditional medicinal plants as potential sources of new antimicrobial agents (Bonjar and Farrokhi, 2004). *Cassia* species have been of medical interest due to their good therapeutic value in folk medicine. Abo et al. (1999) and Eluojoba et al. (1999) showed that the leaves and pods of *Cassia fistula*, *Cassia spectabilis* and *Cassia podocarpa* possess laxative and antimicrobial activities. Other *Cassia* species studied which possess antimicrobial activities include *Cassia sieberiana*, *Cassia alata* and *Cassia occidentalis* (Abo et al., 2000). The extracts of flowers and seeds of *C. auriculata* were found to possess antidiabetic activity (Jalalpure et al., 2004).

C. nigricans Vahl (Leguminosae – Caesalpinoideae) is a woody annual herb or under shrub between 1.2 and 1.5 m high with small yellow flowers. It is widespread in India and tropical Africa including northern Nigeria, especially in cultivated or old clearings by the roadside and open grassy areas (Dalziel, 1956; Irvine, 1961). The pulverised

leaves of *C. nigricans* are used as appetizers and febrifuges. The leaves and the root powder are used for treating skin diseases such as ringworm, scabies and eczema (Benjamin, 1980). An infusion obtained from the plant is given for the treatment of sore throat. The root infusion is administered as a purgative and vermifuge in Senegal and Chad (Dalziel, 1956; Abegaz et al., 1996). Akah et al. (1998) reported that the aqueous extract of the leaves is used by traditional healers in Nigeria for the treatment of peptic ulcer. The extract is also used to treat other gastro-intestinal disorders such as stomach ache and diarrhoea (Nwafor and Okwuasaba, 2001).

The brine shrimp *Artemia salina* Leach (Artemiidae) is an invertebrate component of saline aquatic and marine ecosystems used in laboratory bioassay of toxicity and other biological actions through estimation of medium lethal concentration (LC₅₀ values). The brine shrimp lethality (BSL) bioassay has been shown to be a useful and quick *in vitro* test for predicting toxicity of plant extracts and guiding their phytochemical fractionation (Meyer et al., 1982; Fatope et al., 1993; Parra et al., 2001). It has been shown previously that the crude extracts of the leaves of the plant are very active using BSL bioassay (Ayo and Amupitan, 2004; Oyewale et al., 2004), but the active

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constituents of the leaves were not isolated. There is paucity of information in the available literature on the chemical compounds isolated from the leaves of *C. nigricans* Vahl and their biological activities.

The aims of the present study were to determine the cytotoxicity and antimicrobial activity of an active constituent emodin, isolated from the leaves of *C. nigricans* Vahl.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade.

Test organisms

Artemia salina Leach (Aquarium system, USA) was used for brine shrimp lethality bioassay. Standard strains of *Staphylococcus aureus*, *Corynebacterium pyogenes*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Neisseria gonorrhoea*, and *Klebsiella pneumoniae* were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria.

Plant material

The leaves of *C. nigricans* were collected from Jama'a village, near the Ahmadu Bello University Dam, Zaria (11° 10' N, 07° 38' E), located in the Northern Guinea Savannah zone of Nigeria. The plant was identified at the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. A voucher specimen (Voucher Specimen Number 613) has been deposited at the Herbarium of the Department. The leaves were air-dried, ground into powder and stored in polythene bags before use.

Extraction and separation procedure

The powdered *C. nigricans* leaves (250 g) were exhaustively extracted by Soxhlet extraction using each of the following solvents: petroleum ether (60 – 80°C), ethyl acetate and methanol. Each extract was concentrated and evaporated to dryness on a rotary evaporator. All the crude extracts were subjected to the BSL bioassay studies (Ayo and Amupitan, 2004), and the ethyl acetate extract was found to be very active. Therefore, the ethyl acetate extract (0.50 g) was applied on silica gel column chromatography and successfully eluted with stepwise gradient of chloroform-methanol (2:1, 1:1). The elution was monitored by thin layer chromatography, and similar fractions were pooled together, concentrated and dried under vacuum. The fractions eluted with chloroform-methanol (1:1) gave orange powder (0.080 g). The powder was further purified using silica gel preparative thin layer chromatography, which gave orange crystals of melting point 260 – 263°C. The structure of the crystals was elucidated on the basis of spectroscopic methods as emodin.

Brine shrimp lethality bioassay (cytotoxicity)

Cytotoxicity test was carried out using the standard procedure as described by Meyer et al. (1982), McLaughlin (1991), and Parra et al. (2001). Briefly, samples were prepared by dissolving emodin (30

mg) in DMSO (3 ml). From this solution, the concentrations 1000, 500, 250, 125, and 62.5 µg/ml were obtained, respectively by serial dilution. Each concentration was tested in triplicate, 15 test-tubes per test fraction and one control were prepared using DMSO. Brine shrimp eggs (*A. salina* Leach) were hatched in a hatching chamber, filled with fresh sea water. Ten larvae of brine shrimps were transferred to each sample test-tube using disposable pipettes. The test-tubes were maintained under illumination. Survivors were counted after 24 h, and the percentage death at each concentration was determined (Meyer et al., 1982; McLaughlin, 1991). The LC₅₀ value at 95% confidence interval was determined from the count using the statistical method of Probit analysis (Finney, 1971; Saunders and Fleming, 1971).

Antimicrobial screening test

The paper disc diffusion method was used to determine the antimicrobial activity of the emodin isolated from *C. nigricans* leaves using standard procedure (Erickson et al., 1960; Bauer et al., 1966). Solutions of emodin of varying concentrations, ranging from 1.0×10^3 to 5.0×10^3 µg/ml were prepared. Nutrient agar was prepared, sterilised and used as the growth medium for the microorganisms. 20 ml of the sterilized medium was poured into each sterilized Petri dish, covered and allowed to solidify. The Mueller-Hinton sensitivity agar (oxid) plate was then seeded with the test microorganisms by the spread plate technique, and was left for about 30 min to dry. The sterilized paper discs were soaked in the prepared solutions of the extracts with varying concentrations and were dried at 50°C. The dried paper discs were then planted on the nutrient agar seeded with the test microorganisms. The plates were incubated at 37°C for 24 h, after which they were inspected for the zones of inhibition of growth. The zones of inhibition of growth produced by the minimum inhibitory concentrations (MICs) were measured in millimetres and the values obtained were recorded. A control experiment was also set up using pure DMSO for each of the test organisms.

RESULTS

The extraction of the leaves of *C. nigricans* gave 9.4 g (3.76%) of dry crude petroleum extract, 10.5 g (4.20%) ethyl acetate extract and 17.1 g (6.84 %) methanol extract. The spectral analyses of the active constituent, emodin (Figure 1), from the leaves of *C. nigricans* are shown below:

Orange crystals; melting point 260 – 263°C; ESI-MS molecular peak m/z : 269 [M-H]⁺ molecular weight 270.

Molecular formula C₁₅H₁₀O₅

IR: ν_{\max} 3245 (–OH), 1677, 1627 (C=O) ¹H – NMR (400 MHz, DMSO –d₆) showed peaks at δ 12.06, 11.9 (each s, each 1H, OH–1, OH–8), 7.44 (1H, d, J = 1.1 Hz, H–5), 7.13 (1H, s, H–7), 6.97 (1H, d, J = 2.3 Hz, H–4), 6.50 (1H, d, J = 2.5 Hz, H–2), 2.39 (3H, s, CH₃). ¹³C – NMR (400 MHz, DMSO –d₆)

δ_c : 164.4 (C–1), 107.9 (C–2), 165.6 (C–3), 108.8 (C–4), 120.4 (C–5), 148.1 (C–6), 124.0 (C–7), 161.4 (C–8), 189.5 (C–9), 181.1 (C–10), 134.9 (C–4a), 113.2 (C–8a), 108.9 (C–9a), 132.6 (C–10a), 21.5 (–CH₃). The results of cytotoxicity of emodin were summarised in Table 1. The LC₅₀ value was found to be 42.77 µg/ml. Table 2 shows the results of MICs. The MIC value was found to be 2×10^3 µg/ml against *S. aureus* and *C. pyogenes*, while for *S. pyogenes*, *B. subtilis*, *S. typhi* and *E. coli*, the value

Table 1. Cytotoxicity of emodin isolated from *Cassia nigricans* leaves.

Concentration ($\mu\text{g/ml}$)	1000	500	250	125	62.5
Number of shrimps per test sample	30	30	30	30	30
Number of survivors	0	2	4	7	12
Number of deaths	30	28	26	23	18
Percentage mortality	100	93.3	86.7	76.7	60

$\text{LC}_{50} = 42.77$ (11.80 – 72.94) $\mu\text{g/ml}$.

Table 2. Minimum inhibitory concentration of emodin isolated from *Cassia nigricans* leaves.

Test organism	Concentrations of emodin ($\mu\text{g/ml}$)				
	1×10^3	2×10^3	3×10^3	4×10^3	5×10^3
<i>S. aureus</i>	–	0+	+	+	+
<i>S. pyogenes</i>	–	–	0+	+	+
<i>C. pyogenes</i>	–	0+	+	+	+
<i>B. subtilis</i>	–	–	0+	+	+
<i>S. typhi</i>	–	–	0+	+	+
<i>E. coli</i>	–	–	0+	+	+
<i>P. aeruginosa</i>	–	–	–	0+	+
<i>C. albicans</i>	–	–	–	0+	+
<i>N. gonorrhoea</i>	–	–	–	0+	+
<i>K. pneumonia</i>	–	–	–	–	0+

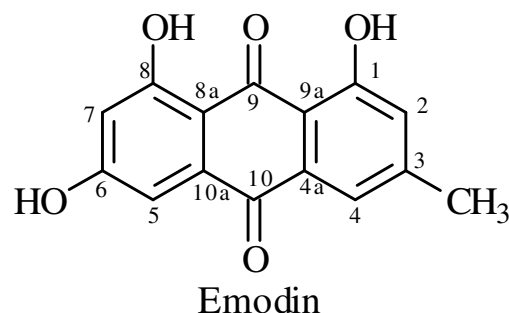
+ = Inhibition; 0+ = minimum inhibition; – = no inhibition.

was 3×10^3 $\mu\text{g/ml}$. For *P. aeruginosa*, *C. albicans*, and *N. gonorrhoea*, the MIC value was 4×10^3 $\mu\text{g/ml}$, while that of *K. pneumonia* was 5×10^3 $\mu\text{g/ml}$. The results of the diameters of zones of inhibition showed that *S. aureus* had the highest zone of inhibition (Table 3).

DISCUSSION

The structure of the orange crystals, emodin, isolated from the ethyl acetate extract was established by spectral data shown above. It was confirmed by comparison with authentic samples and spectra data, previously reported by Cohen and Towers (1995) and Demirezer et al. (2001).

The LC_{50} values have been reported for many toxins and plant extracts (Parra et al., 2001; Oyewale et al., 2004). The results of brine shrimp lethality (BSL) obtained in the present study agreed with the findings of Parra et al. (2001) that the *in vitro* test is highly correlated with *in vivo* tests, and that it is a useful alternative model for predicting toxicity in plant extracts. According to Meyer et al. (1982) and Parra et al. (2001), LC_{50} value lower than 1000 $\mu\text{g/ml}$ is considered bioactive in toxicity evaluation of plant extracts by BSL bioassay. Therefore, emodin is a highly cytotoxic compound. This fraction was then subjected to more elaborate bioassay for specific antimicrobial test. The results of the zone of inhibition demonstrat-

**Figure 1:** 1,6,8-trihydroxy-3-methyl-anthraquinone (Emodin).

ed that emodin had very high growth inhibitory effects on all the microorganisms. The findings were consistent with those of Singh (1982), who observed that *Cassia* species containing anthraquinone, flavonoids and polysaccharides showed considerable activity against Gram-positive microorganisms. They also agreed with the findings of Abo et al. (1999) that extracts from the leaves and pods of *C. fistula*, *C. podocarpa* and *C. spectabilis* showed significant antimicrobial activity. Abo et al. (2000) also found out that the methanol extracts of the leaves and pods of *C. alata* and *C. sieberiana* exhibited significant antimicrobial activity against *P. aeruginosa*, *S. aureus*, *Proteus mirabilis*, *C. albicans*, *A. niger* and *A. flavus*. The isolation of emodin for the first time from the leaves of *C.*

Table 3. Antimicrobial activity of emodin isolated from *Cassia nigricans* leaves.

Microorganisms	Strain	Diameter of zone of inhibition (mm)
<i>Staphylococcus aureus</i>	ATCC 13709	31
<i>Streptococcus pyogenes</i>	Local	25
<i>Corynebacterium pyogenes</i>	Local	21
<i>Bacillus subtilis</i>	NCTC 8236	19
<i>Salmonella typhi</i>	ATCC 9184	20
<i>Escherichia coli</i>	NCTC 10418	17
<i>Pseudomonas aeruginosa</i>	NCTC 6750	20
<i>Candida albicans</i>	ATCC 10231	17
<i>Neisseria gonorrhoea</i>	Local	19
<i>Klebsiella pneumonia</i>	ATCC 10031	18

nigricans and the antimicrobial activity of the active constituent are reported in the present study. The results of the study justified the use of the extract of the leaves of *C. nigricans* in the treatment of diseases of microbial origin in herbal medicine.

Conclusions

The action of the isolated emodin from the leaves of *C. nigricans* against some common pathogenic microorganisms has demonstrated the great potential of the plant as a source of an antimicrobial agent. The isolation of this active principle, emodin, justifies the use of the leaves of *C. nigricans* in herbal medicine for the treatment of some skin diseases and gastro-intestinal disorders.

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