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Full Length Research Paper

Antimicrobial activity of *Crataeva religiosa* Forst against bacteria isolated from *Thryonomys swinderianus* Temminck

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An attempt has been made to carry out a screening on the antibacterial activity of leaves of *Crateva religiosa* Forst used in Benin traditional veterinary medicine against bacterial infection of *Thryonomys swinderianus* (class of *Mammalia*, family of Thryonomyidae) commonly called *agouti or kholan*. The aim of this study was to select the most active extracts and fractions which may be useful to combat these bacterial infections. Seven extracts from *C. religiosa* were screened for their antibacterial. The antibacterial activity was evaluated by both microtest method using p-iodonitrotetrazolium and bioautography against five microorganisms obtained from *T. swinderianus* (*Escherichia coli, Shigella sonei*, *Staphylococcus aureus, Pasteurela pestis* and *Yersinia enterocolitica*). The minimum inhibitory concentration (MIC) and the total activity (TA) were determined. All extracts were effective against tested microorganisms at different levels (0.31 ≤ MIC ≤ 10 mg/ml). The screening experiment revealed that ethyl acetate extract was more potent than other extracts with the MIC values of 0.62 mg/ml against *E. coli* and 0.31 mg/ml against *S. aureus*, *S. sonei, P. pestis* and *Y. enterocolitica*. The results provide an evidence for the traditional use of *C. religiosa* for the treatment of infective diseases of *T. swinderianus* Temminck.

Key words: *Crataeva religiosa*, *Thryonomys swinderianus* Temminck, ethnomedicine, Republic of Benin.

INTRODUCTION

The breeding of ruminants is an important activity in developing countries. This activity, which is often traditional, has some problems. The most recurring problems, concerning infection and diarrhea, cause enormous losses at the breeders. To protect animals, the veterinarian services recommend the use of pharmaceutical products such as antibiotics, but due to the cost and increase in microbial resistance against antibiotics, many farmers have resorted to the treatment of their animals with medicinal plants. It is known that many plants especially those used by traditional healers produce pharmaceutically active compounds that have antimicrobial, antihelminthic, antifungal, antiviral, anti-inflammatory and antioxidant activity (McGaw et al., 2000). Medicinal plants are usually easier to obtain than pharmaceutical products by breeders. Furthermore, the emergence of antibiotic resistance has heightened interest in medicinal plants as a source of antibacterial compounds. This led to an increase in the research work on the *in vitro* efficacy of plant extracts (Carraminana et al., 2008; Cava et al., 2007; Gutierrez et al., 2008; Mytle et al., 2006; Furiga et al., 2009).

Crateva religiosa Forst (Capparaceae; syn Crataeva

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religiosa) is found in the forest galleries of African Sudanese area, in India and Burma (Adjanohoun et al., 1989). It is found in many parts of Africa where it has different uses. In Bénin, the plant has different vernacular names: goriguiberou (Bariba, northern region), wonton-zonzwen (Fon and Goun, southern region), tanilabia and tcharouwenwe (Yoruba, middle and southern region). The leaves were used in combination with other plants by traditional healers and local populations as analgesic, antispasmodic, antimalarial and antidiarrheic. The decoction of fresh leaves and branches is taken orally to treat hypertension (Adjanohoun et al., 1989). In the rural areas in India, it is reported that *C. religiosa* is currently used for the treatment of different diseases (Khan et al., 2003; Dilip and Tamuli, 2004). In Bénin, the decoction of this species was used by traditional breeders to treat the digestive disorders of the bred animals such as ruminants and *Thryonomys swinderianus.*

This study looks into the investigation of the *in vitro* antimicrobial activity of extracts and fractions obtained from *C. religiosa* against five pathogenic micro-organisms that cause the most common cases of digestive infectious diseases in *T. swinderianus* Temminck breeding in the Republic of Benin.

MATERIALS AND METHODS

Plant material

The aerial part of *C. religiosa* was collected in a rural zone close to Cotonou, Department of Atlantic (Southern Benin), in August 2008. The botanical identification of the collected material was performed by botanists from the botanic garden at the University of Abomey-Calavi in Benin. A voucher specimen (AA 6366/HNB) had been deposited in the National Herbarium at the same university.

Preparation of extracts

The collected aerial part of *C. religiosa* was left at room temperature (20°C) in the laboratory for two weeks to dry. Samples were chopped into smaller pieces and then ground into powder using NAKIA Blender MX-738 (Comptoir Scientific, Benin). The dry powdered aerial parts obtained (100 g) were exhaustively extracted three times with 500 ml of ethanol (96°C), distilled water (aqueous) and water/ethanol (hydroethanol, v/v), by maceration at room temperature for 24 h. On the other hand, 20 g of dried powder was extracted successively with diethyl ether, methylene chloride, ethyl acetate and acetone. The suspension was further filtered through Whatman filter paper (Whatman International Ltd, Maidstone, England). The filtrate was concentrated in vacuum using a rotary evaporator (STUART, RE300, Bd Scientific, England via Comptoir Scientifique, Benin) to obtain each extract. The quantity of the extracts was determined, after which the yield was calculated and the extracts were then stored at 4°C before they were used for assay.

Tested microorganisms

The microorganisms were obtained from *T. swinderianus*, bred in the experimental "Programme Elevage des Espèces Animales Non Conventionnelles" (PEEANC), (Laboratory of Zootechnic, Veterinarians and Halieutic Researches) in the "National Institute of Agricultural Researches in Benin" (INRAB). The microorganisms were taken from the intestinal flora of *T. swinderianus* suffering from diarrhoea. All tested bacteria were isolated three times on Mueller Hinton agar (MHA) oxoid, and the identification of the microorganisms was confirmed by standard bacteriological methods (Collins and Lyne, 1970; Gregerson, 1978).

Inhibitory percentage determination at high concentration

To determine the inhibitory percentage, the extracts were reconstituted to a concentration of 200 mg/ml. The aim of this method was to eliminate the extracts, which in high concentration (100 mg/ml) do not inhibit the growth of the microorganisms. A 150 µl of each extract (200 mg/ml) was introduced in triplicate tubes already seeded with 150 µl of the Muller Hinton broth culture inoculum (10^6 CFU/ml) of the tested bacteria. All test tubes were incubated at 37°C. After 18 h of incubation, four successive 1/100 serial dilutions of the test bacteria were prepared in sterile distilled water to achieve a decreasing concentration range from 100 to 10^{-6} mg/ml. 10 µl of each dilution was spread on sterile Muller Hinton agar plates. Simultaneously, a positive control was realized in the same conditions with 150 µl of bacterial broth and 150 µl of sterile distilled water. The plates were incubated at 37°C for 18 h, and the inhibitory percentage of extracts and fractions was calculated.

Minimum inhibitory concentrations (MICs)

Minimum inhibitory concentration values of crude extracts and fractions against isolated microorganisms were determined by slightly modified serial dilution microplate bioassay using specific dye p-iodonitrotetrazolium violet as an indicator of growth (Eloff, 1998). MIC was determined by two fold serial dilutions of extracts beyond the level where no inhibition of growth of microorganisms was observed. Plant extracts and fractions were reconstituted to 20 mg/ml with a mixture of acetone/Muller Hinton (v/v), while about 100 µl of Muller Hinton broth culture of bacteria (10^6 CFU/ml) was inoculated to each well. Sulfadiazine was used as a reference antibiotic and eight wells were used for positive growth control containing both MH and the tested bacteria. The microplates were incubated at 37°C for 18 h, after which 40 µl of 0.2 mg/ml solution and *p*-iodonitrotetrazolium solution were added to each well and then the microplates were incubated at 37°C. After 1 h of incubation, the MIC values which were the lowest extracts concentration at which bacteria growth was inhibited were recorded. The total activity (TA) values of each extract were determined by dividing the MICs with the quantity extracted from 1 g of the plant material (Eloff, 2004).

Qualitative antibacterial activity assay by bioautography

The bioautography procedure described by Begue and Kline (1972) was used. Five TLC plates (Macherey-Nagel, Alugram SIL G) were prepared and developed in ethyl acetate/methanol/water (100:17:13) solvent system and dried overnight under a chemical hood to remove residual solvent. One of the plates was sprayed with the vanillin sulfuric spray reagent and the others with one of the concentrated suspension of actively growing bacteria cultures (*Escherichia coli, Shigella sonei*, *Staphylococcus aureus, Pasteurela pestis* and *Yersinia enterocolitica*). The plates were then incubated at 37°C in a chamber at 100% relative humidity. After 18 h, plates were sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma-Aldrich Chemicals). Inhibition of

Table 1. Yield of extracts from *C. religiosa* using seven different extractants.

Extractant	Plant powder (g)	Extract quantity (g)	Yield (%)
Ethanol	100	17.23	17.23
Water	100	9.36	9.36
Ethanol-water (1:1)	100	23.78	23.78
Diethyl ether	20	1.62	8.10
Methylene chloride	20	0.08	0.40
Ethyl acetate	20	0.2	1.00
Acetone	20	2.68	13.40

growth was indicated by clear zones on the chromatogram after incubating for about 1 h.

RESULTS AND DISCUSSION

Preparation of extracts

The yield of the extracts is shown in Table 1. The hydroethanol extract gave the highest yield (23.78%), while the lowest yield (9.36%) was recorded with the aqueous extract. There was significant difference in the yield of the hydroethanol extract as compared to the aqueous and ethanol extracts. As far as the fractions obtained by successive extractions are concerned, the diethyl ether gave the highest yield (8.10%), while the lowest yield was recorded with the methylene chloride fraction (0.4%). Significant difference was also noticed in the yield of diethyl ether and acetone fractions as compared to methylene chloride and ethyl acetate.

Tested microorganism isolation

The analysis of the intestinal flora of 3 specimens of *T. swinderianus* led to the isolation and identification of five bacteria known as *E. coli*, *S. sonei*, *S. aureus, P. pestis* and *Y. enterocolitica.* Amongst various aetiological factors in rabbit 'diarrhoea', *E. coli* or its toxins have been found to be commonly incriminated. *E. coli and Yersinia*, isolated in this study, confirmed the previous results (Smith et al., 2004; Peter et al., 1984; Newcomer et al., 1984).

Inhibitory percentage determination at high concentration

The inhibitory percentages of the extracts and fractions were determined against *E. coli*, *S. sonei, S. aureus, P. pestis* and *Y. enterocolitica*. The results obtained showed that all tested extracts and fractions (100 mg/ml) were inhibited at 100% growth of the microorganisms. These extracts and fractions were thus used to determine the MIC.

Minimum inhibitory concentrations (MIC)

The MIC determination was performed to compare the antimicrobial effect of crude extracts and fractions from *C. religiosa* against the bacteria which caused many problems in *T. swinderianus* breeding. The results of the antimicrobial screening of all extracts are shown in Table 2. All extracts showed antimicrobial activity by inhibiting one or more microorganisms ($0.31 \leq MIC \leq 2.5$ mg/ml). These results indicated that the ethyl acetate fraction was the most effective against the tested bacteria with MIC values of 0.62 mg/ml against *E. coli* (0.31mg/ml) and against *S. sonei*, *S. aureus, P. pestis* and *Y. enterocolitica*. It is followed by an aqueous extract on *E. coli* with a MIC value of 0.62 mg/ml. The acetone extract and the diethyl ether fraction are the least active with a MIC value of 5 mg/ml. These results were consistent with those obtained by Eloff (2004), in that the ethyl acetate would be the best solvent of extraction during the evaluation of the MIC of extracts. There is no report on the antimicrobial information available for this species. Some reports on the chemical study of *C. reliogisa* reveal the presence of lupeol which possess antiarthritic activity through possible suppression of the immune system. It was found to suppress various immune factors such as the phagocytic (cell-killing) activity of macrophages and the T-lymphocyte activity that included CD4+T cell mediated cytokine generation (Sarang et al., 2006). Also, the methanolic and aqueous extracts of this species have been evaluated (Parekh et al., 2006).

Qualitative analysis of antibacterial activity

The results obtained with bioautography were shown in Figures 1 to 3. The bioautography method worked well with Gram-positive *S. aureus* and Gram-negative *P. pestis*, but did not with gram-negative *E. coli* and *S. sonei*. The diethyl ether showed one minor antibacterial compound against *S. aureus*, whereas ethyl acetate, methylene chloride and acetone extracts, showed two antibacterial compounds against the same bacteria (Figure 2). These two antibacterial compounds are the major compounds found in non-polar extractant (methylene chloride) than in the intermediate polar

Table 2. MIC values in mg/ml and the total activity in ml (Eloff, 2000) of *C. religiosa* per gram leaves extracted with ethanol (ET), water (W), ethanol/water (ETW), diethyl ether (DEE), methylene chloride (MC), ethyl acetate (EA) and acetone (ACN). Positive controls: Sulfadiazine (µg/ml) (S).

Extract	Minimum inhibitory concentration (mg/ml)							
Microorganism	**ET**	**W**	**ETW**	**DEE**	**MC**	**EA**	**ACN**	**S**
E. coli	1.25	0.62	2.5	5.00	5.00	0.62	5.00	18.8
S. aureus	2.5	10	10	2.5	0.625	0.31	-	<1.21
S. sonei	2.50	1.25	1.25	5.00	2.50	0.31	5.00	18.8
P. pestis	1.25	1.25	0.62	5.00	1.25	0.31	1.25	18.8
Y. enterocolitica	1.25	2.50	1.25	5.00	2.50	0.31	5.00	4.69
Total quantity in mg extracted from 1 g								
	173	936	237.8	81	4	10	134	
Total activity in ml/g								
E. coli	138	151	95	16	0.8	16	27	
S. aureus	69	93.6	23.7	32.4	6.4	32	-	
S. sonei	69	749	190	16	1.6	32	27	
P. pestis	138	749	384	16	3.2	32	107	
Y. enterocolitica	138	374	190	16	1.6	32	27	

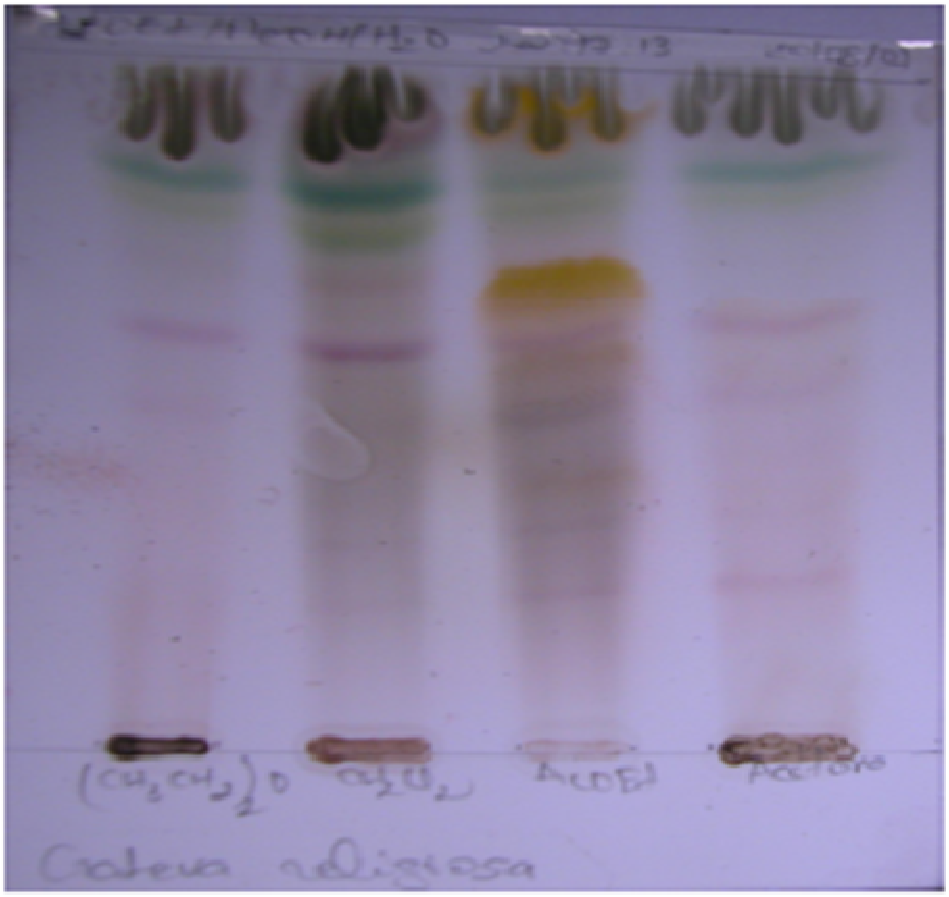

Figure 1. Separation of the components present in 50 µg of 4 different extracts with EMW as eluent and vanillin-sulphuric acid spray reagent. From left to right: diethyl ether, methylene dichloride, ethyl acetate and acetone.

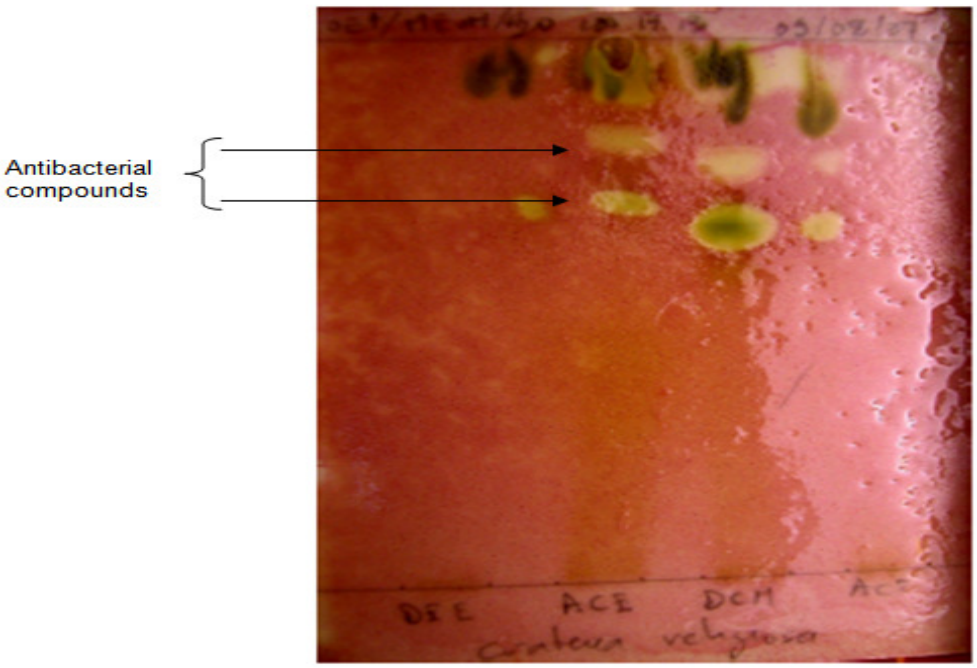

Figure 2. Bioautogram of *C. religiosa* aerial parts. TLC developed in EMW and sprayed with *S. aureus* culture incubated overnight then sprayed with INT. Growth inhibition indicated by lighter zones on TLC plates. DEE: Diethyl ether; ACE: ethyl acetate; DCM: methylene chloride; ACT: acetone.

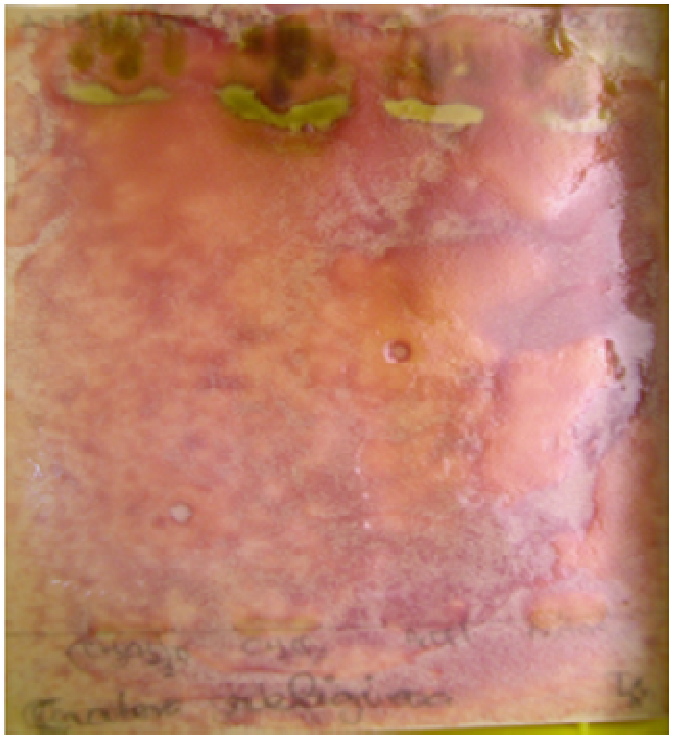

Figure 3. Bioautogram of *C. religiosa* aerial parts. TLC developed in EMW and sprayed with *P. pestis* culture incubated overnight then sprayed with INT. Growth inhibition indicated by lighter zones on TLC plates. DEE: Diethyl ether; ACE: ethyl acetate; DCM: methylene chloride; ACT: acetone.

extractant (ethyl acetate). According to the Rf, the antibacterial compounds have an intermediate polarity.

Conclusion

All the extracts showed varying degrees of antimicrobial activity on the tested microorganisms. The chance to find antimicrobial activity compounds was more apparent in ethyl acetate extract. The results confirm the use of this plant by traditional breeders to treat microbial infections in traditional *T. swinderianus* culture.

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