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Evaluation of extracts of the root of *Landolphia owerrience* for antibacterial activity

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Abstract

Ethanolic and aqueous (cold and hot) extracts of *Landolphia owerrience* root parts (whole-root, root-bark and root-wood) were tested for activity against ten bacterial strains using agar-well diffusion and macro-broth dilution methods, respectively. The ethanolic extracts of the whole-root and root-wood were active against 100 and 80% of the test organisms, respectively. Ethanolic and aqueous extracts of the root-bark were moderately active while the aqueous (cold and hot) extracts of the root-wood exhibited little or no activity. Out of the nine extracts prepared, 66.7% were active against *Staphylococcus aureus* ATCC 12600, 55.6% variously against each of *Pseudomonas aeruginosa* ATCC 10145 and local clinical isolates of *P. aeruginosa*, *S. aureus*, *Escherichia coli* and *Salmonella typhi*, 44.4% against *Proteus* sp., 33.3% against *Bacillus subtilis* ATCC 6051 and 22.2% against *E. coli* ATCC 11775. The agar-well-determined MIC values for the ethanolic whole-root extract (0.78–50 mg/ml) were higher (indicating lower activity) than the corresponding macro-broth-determined values (0.39–50 mg/ml) probably because of slow diffusion rates of the active constituents of the extract in agar. On the other hand, the differences could be due to the effects of DMSO used to dissolve the ethanolic extracts in the agar-well diffusion tests. Similar discrepancies in the MIC values detectable with the two test methods were apparent in the root-wood extract and the control drug, Gentamycin, except that in the latter the agar-well-determined MIC values (0.125–8.0 μg/ml) were lower than the macro-broth-determined values (0.125–64 μg/ml). The strong activity of the ethanolic extracts against known etiologic agents of diseases traditionally treated with *L. owerrience* root of similar preparations provides scientific justification for the use of the herb in ethnomedical practice in Nigeria.

Keywords: *Landolphia owerrience*; Root parts; Antibacterial activity; Ethnomedicine; Nigeria

1. Introduction

Landolphia owerrience, family Apocynaceae, (*Igbo: Utu*) has widely varied applications in Nigerian folk medicine. Many herbalists have claimed to use the leaves and stem-bark as a colic and in treatment of venereal diseases (Watt and Breyer-Brandwijk, 1962; Ebi and Ofoefule, 1997). The whole root, root-bark and root-stem have also been reported to be effective in treatment of wound infections and various gastrointestinal disorders, including diarrhoea, food poisoning, constipation and typhoid fever. Most of the folkloric claims agree in the traditional use of the herb for treatment of diseases of known bacterial aetiology. However, except for the report of Ebi and Ofoefule (1997) that a methanolic extract of the leaves exhibited anti-microbial properties, there is apparently no scientific report on the antibacterial properties of the plant. Such lack of scientific knowledge has often constituted a major constraint to consideration of the use of traditional herbal remedies in conjunction with or as an affordable alternative to orthodox medical treatment.

This work is part of a comprehensive project to evaluate the therapeutic potentials of Nigerian herbs with a view to conserving and developing them and exploring their potential to application in orthodox medical practice. Thus, the extracts of root parts of *L. owerrience* were quantitatively evaluated for activity against ten bacterial strains, including four from the American Type Culture Collection (ATCC); one serotyped local Salmonella strain and five other local clinical isolates.

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2. Materials and methods

2.1. Collection and identification of plant materials

Fresh roots and other parts of *Landolphia owerrience* (*Igbo: Utu*) were obtained from Enugu Ezike, south-eastern Nigeria. A.O. Ozioko of the Department of Botany, University of Nigeria, Nsukka, taxonomically authenticated the plant and a voucher specimen was deposited in the departmental herbarium.

2.2. Preparation of extracts

Whole-roots (LO–WR), root-barks (LO–RB) and root-woods (LO–RW) of the plant were air-dried in the dark at room temperature before grinding to powder with a mechanical grinder. The powder was extracted by maceration in ethanol, cold water and hot water respectively. Approximately 30 g of the powder were soaked in 200 ml of either ethanol or cold water for ~18 h at room temperature (~27 °C). For hot water extraction, 30 g of powdered sample were boiled in 200 ml of water for 60 min. Each extract was first filtered through Whatman No. 1 filter paper to clarify and then through a 0.45 μm membrane filter (Sigma). The filtrate was evaporated to dryness at room temperature in a steady air current and the yield recorded as a percentage of the quantity of initial plant material (30 g) used. The dried extract was sterilised by overnight UV-irradiation and sterility checked by plating the reconstituted extract on nutrient agar. All dried crude extracts were stored at room temperature until used for phytochemical analysis or antibacterial testing.

2.3. Phytochemical screening

The dried extracts were first reconstituted in the respective solvents used for their extraction and then tested by standard phytochemical methods (Harborne, 1973; Evans, 1989) for presence of alkaloid, flavonoid, tannin, saponin, steroidal aglycon, glycosides, anthroquinone and protein.

2.4. Test bacterial strains

Typed strains of *Staphylococcus aureus* (ATCC 12600), *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 10145) were obtained from Bio-resources Development and Conservation Project (BDCP), Nsukka. Local clinical isolates (*l.c.i.*) of *S. aureus*, *E. coli*, *P. aeruginosa*, *Salmonella typhi* and *Proteus sp.* were obtained from the Clinical Laboratory, Department of Microbiology, University of Nigeria, Nsukka. The Veterinary Microbiology and Pathology Laboratory of the same University supplied the *Salmonella kintambo* (Human: 1,13,23: mt: −). All test strains were re-isolated three successive times on Mueller Hinton agar, MHA (Oxoid) to purify and identity was confirmed by standard bacteriological methods (Collins and Lyne, 1970).

2.5. Screening extracts for antibacterial activity

The inoculum size of each test strain was standardised according to the Committee for Clinical Laboratory Standards (NCCLS, 1993). The test bacterial strain was inoculated into Mueller Hinton broth, MHB (Oxoid) medium and incubated for 3–6 h at 35 °C in a shaker water bath until the culture attained a turbidity of 0.5 McFarland unit. The final inoculum was adjusted to 5×10^5 cfu/ml.

Antibacterial screening was by a modified agar-well diffusion method (Okunji et al., 1990). A 1.0-ml volume of the standard suspension (5×10^5 cfu/ml) of each test bacterial strain was spread evenly on MHA plates using sterile glass rod spreader and the plates allowed to dry at room temperature. Subsequently, 6 mm-diameter wells were bored in the agar and a 100-μl volume of each plant extract reconstituted in 50% DMSO to a concentration of 100 mg/ml was pipetted into triplicate wells. After holding the plates at room temperature for 1 h to allow diffusion of extract into the agar, they were incubated at 37 °C for 24 h and the (bacterial growth) inhibition zone diameter (IZD) was measured to the nearest mm. Gentamycin, used at concentrations of 8 and 16 μg/ml, respectively, was included as positive control while DMSO (50% concentration) served as the negative control.

2.6. Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined only for the ethanolic extracts of the LO–WR and LO–RW samples. The MIC was determined by two test methods, a modified agar-well diffusion method (Rios et al., 1988; Okunji et al., 1990) and the macrobroth dilution technique (NCCLS, 1993), respectively. In the agar-well diffusion technique, a two-fold serial dilution of each extract in 50% DMSO was prepared to obtain a 0.1–100 mg/ml concentration range. A 100-μl volume of each dilution was introduced in triplicate wells in MHA plates pre-inoculated with test bacterial cells. Similarly treated Gentamycin preparation (concentration range, 0.125–512 μg/ml) was included as positive control. All test plates were incubated at 37 °C for 24 h. The least concentration of each extract or control drug showing a clear zone of inhibition was taken as the MIC.

For the macrobroth dilution antibacterial assay, two-fold serial dilutions of the extracts and control drug were prepared in tubes with MHB as diluent. Each dilution was seeded with test organism to the standard concentration (5×10^5 cfu/ml). The MIC was taken as the last dilution showing no noticeable growth (turbidity).

MBC determination was by aspirating 0.1 ml of the culture medium from each tube (in the macrobroth MIC assay) showing no apparent growth and sub-culturing it on fresh MHA. The latter was incubated at 37 °C for 24 h. The MBC was read as the least concentration showing no visible growth on the MHA subculture. Using the values of MIC determined for each extract by the agar-well diffusion and macrobroth dilution techniques, respectively, the MIC index was calculated for each test organism.

3. Results

The phytochemical analyses showed that the ethanolic and aqueous (cold and hot) extracts of *Landolphia owerrience* root parts each contained alkaloid, tannin, saponin, steroidal aglycon, cardiac and cyanogenetic glycosides, carbohydrate and protein at the relative levels indicated in Table 1. Flavonoid, anthracene glycoside and anthroquinone were not detected.

Out of the nine extracts tested, 6 (67%) showed activity against *S. aureus* (ATCC 12600), 5 (56%) each against *S. aureus* (l.c.i), *E. coli* (l.c.i), *P. aeruginosa* (l.c.i), *S. typhi* (l.c.i) and *P. aeruginosa* (ATCC 10145), 3 (33%) against *B. subtilis* (ATCC 6051), 2 (22%) against *E. coli* (ATCC 11775) and 4 (44%) against *Proteus* sp (l.c.i) (Table 2). The proportion of test bacterial strains showing susceptibility to each extract or Gentamycin/DMSO control, with the exception of *S. kintambo* (Human 1,13,23: mt: −), which was not tested in most cases, is shown at the extreme right column of Table 2. The hot-water extract of LO–RW and the 50% DMSO (negative control) showed no activity against any of the strains tested, while the cold-water extract of LO–RW showed activity against *E. coli* (l.c.i) only. Ethanolic extract of LO–WR and the two concentrations (8 and 16 μg/ml) of Gentamycin used showed activity against all ten (100%) test bacterial strains. The IZD obtained for the positive samples ranged from 10 mm for the ethanolic extract of LO–RW against *E. coli* (l.c.i) to 18 mm for the ethanolic extract of LO–WR against *S. aureus* (l.c.i) and *Proteus* sp (l.c.i), respectively and the ethanolic extract of LO–RW against *B. subtilis* (ATCC 6051) (Table 2).

The MIC was determined for the ethanolic extracts of LO–WR and LO–RW only because these extracts showed a wider spectrum of antibacterial activity than the aqueous extracts. Secondly, alcohol maceration is the popular method for preparing root-based traditional herbal remedies, so that ethanol extracts in this study represent the traditional form in which this herbal medicine is administered. The MIC values for the LO–WR extract determined by the agar-well diffusion method ranged from 0.78 mg/ml for *P. aeruginosa* (ATCC 10145 and l.c.i., respectively) to 50 mg/ml for *E. coli* (ATCC 11775 and l.c.i.) and *S. typhi* (l.c.i.). The macrobroth-determined MIC values for LO–WR extract varied from 0.39 mg/ml for *S. aureus* (ATCC 12600 and l.c.i., respectively) to 50 mg/ml for *E. coli* (ATCC 11775 and l.c.i.). The macrobroth-determined

Table 1
Phytochemical screening of *Landolphia owerrience* whole root (LO–WR), root bark (LO–RB) and root wood (LO–RW) extracts

Plant constituent	LO–WR extracts			LO–RB extracts			LO–RW extracts		
	Cold water	Hot water	Ethanol	Cold water	Hot water	Ethanol	Cold water	Hot water	Ethanol
Alkaloid	+	+	+	+	−	++	+	+	+
Flavonoid	−	−	−	−	−	−	−	−	−
Tannin	++	++	+++	+++	++	++	++	++	++
Saponin	+++	+++	+	+++	+++	+	+++	+++	++
Steroidal Aglycon	+	+	+	+	+	+	+	+	+
Cardiac Glycoside	++	+	++	+	+	++	++	+	+
Cyanogenetic Glycoside	+	+	+	+	+	+	+	+	+
Anthracene Glycosides	−	−	−	−	−	−	−	−	−
Anthroquinone	−	−	−	−	−	−	−	−	−
Carbohydrate	++	++	+++	++	+	+	+	++	+++
Protein	+	+	+	+	+	+	+	+	+

−, not detectable; +, low concentration; ++, medium concentration; +++, high concentration.

Table 2
Inhibition zone diameter (IZD) of extracts of *Landolphia owerrience* against test bacterial strains

Plant root part (code)	Solvent of extraction	Percent Yield	Inhibition zone diameter, IZD (mm)[a] against bacterial species										Proportion susceptible (%)
			S. aureus (ATCC 12600)	*S. aureus* (l.c.i.)[b]	*B. subtilis* (ATCC 6051)	*E. coli* (ATCC 11775)	*E. coli* (l.c.i.)	*P. aeruginosa* (ATCC 10145)	*P. aeruginosa* (l.c.i.)	*S. kintambo* (Human)	*S. typhi* (l.c.i.)	*Proteus* sp. (l.c.i.)	
LO–WR	Cold water	0.3	13	14	0	0	13	0	0	ND	0	0	3/9 (33.3)
	Hot water	3.3	12	0	0	14	0	12	0	ND	12	0	4/9 (44.4)
	Ethanol	3.3	13	18	15	16	11	15	16	15	12	18	10/10 (100.0)
LO–RB	Cold water	0.3	14	0	0	0	11	14	14	ND	12	13	6/9 (66.7)
	Hot water	0.3	13	14	0	0	0	0	16	ND	11	14	5/9 (55.6)
	Ethanol	3.3	0	16	14	0	0	15	16	ND	11	0	5/9 (55.6)
LO–RW	Cold water	6.7	0	0	0	0	12	0	0	ND	0	0	1/9 (11.1)
	Hot water	0.3	0	0	0	0	0	0	0	ND	0	0	0/9 (0.0)
	Ethanol	3.3	12	15	18	0	10	13	16	12	0	14	8/10 (80.0)
Proportion showing activity per strain (%)			6/9 (66.7)	5/9 (55.6)	3/9 (33.3)	2/9 (22.2)	5/9 (55.6)	5/9 (55.6)	5/9 (55.6)	NC	5/9 (55.6)	4/9 (44.4)	–
Controls	Gentamycin	8 μg/ml	18	11	31	30	10	31	18	22	18	19	10/10 (100.0)
		16 μg/ml	24	12	33	35	13	34	21	28	22	22	10/10 (100.0)
	DMSO	50%	0	0	0	0	0	0	0	0	0	0	0/10 (0.0)

LO–WR (*L. owerrience*, LO) whole-root; LO–RB, root-bark; LO–RW, root-wood; ND, not determined; NC, not complete.

[a] Each value is a mean of three replicates; test concentration of extracts = 100 mg/ml (100 μl/well).

[b] l.c.i., local clinical isolate.

Table 3
Minimum inhibitory concentrations of ethanolic LO–WR against test bacterial strains determined by agar-well diffusion and macro-broth dilution methods, respectively

Test bacterial strain	Agar-well diffusion MIC[b]		Macro-broth dilution MIC[b]		MIC index		Effect of test methods[c]	
	LO–WR extract (mg/ml)	Gentamycin (μg/ml)	LO–WR extract (mg/ml)	Gentamycin (μg/ml)	LO–WR extract	Gentamycin	LO–WR extract	Gentamycin
Staphylococcus aureus (ATCC 12600)	3.125	0.125	0.391	1.0	8.0	0.125	S	S
Staphylococcus aureus (l.c.i.)[a]	3.125	4.5	0.391	64.0	8.0	0.07	S	S
Bacillus subtilis (ATCC 6051)	3.125	0.5	1.563	0.125	2.0	4.0	NS	NS
Escherichia coli (ATCC 11775)	50.0	0.5	50.0	4.0	1.0	0.125	NS	S
Escherichia coli (l.c.i.)	50.0	8.0	50.0	64.0	1.0	0.125	NS	S
Pseudomonas aeruginosa (ATCC 10145)	0.781	0.25	6.25	2.0	0.125	0.125	S	S
Pseudomonas aeruginosa (l.c.i.)	0.781	1.0	6.25	2.5	0.125	0.4	S	NS
Salmonella kintambo (Human: 1,13,23: mt: −)	25.0	0.25	3.125	2.0	8.0	0.125	S	S
Salmonella typhi (l.c.i.)	50.0	0.5	3.125	4.0	16.0	0.125	S	S
Proteus sp. (l.c.i.)	3.125	0.5	3.125	8.0	1.0	0.063	NS	S

[a] l.c.i., local clinical isolate.

[b] Mean of three replicates of the experiment. MIC index (MIC_i), Agar-well MIC/Macro-broth MIC; S, significant ($MIC_i = >4.0$ or ≤ 0.125); NS, non-significant ($MIC_i = >0.125$–4.0).

[c] The interpretation of MIC indices (MIC_i) for significance or non-significance is adopted from the definition of Fractional Inhibitory Concentration Index (FIC_i) as modified by Sanders et al. (1993). By this definition, MIC_i which lie outside the inherent two-fold error of MIC (i.e. $MIC_i = >4.0$ or ≤ 0.125) is considered significant, i.e. the test methods have an effect on the MIC value determined. Conversely, MIC_i which lie within the two-fold error ($MIC_i = >0.125$–4.0) imply that the test methods have no significant effect on MIC value. Two-fold error of MIC means that the true MIC may be between 2 MIC and 2 MIC (Sanders et al., 1993).

Table 4
Minimum inhibitory concentrations of ethanolic extracts of LO–RW against test bacterial strains determined by agar-well diffusion and macro-broth dilution methods, respectively

Test bacterial strain	Agar-well diffusion MIC[b]		Macro-broth dilution MIC[b]		MIC index		Effect of test methods[c]	
	LO–RW extract (mg/ml)	Gentamycin (μg/ml)	LO–RW extract (mg/ml)	Gentamycin (μg/ml)	LO–RW extract	Gentamycin	LO–RW extract	Gentamycin
Staphylococcus aureus (ATCC 12600)	3.125	0.125	3.125	1.0	1.0	0.125	NS	S
Staphylococcus aureus (l.c.i.)[a]	1.563	4.0	3.125	64.0	0.5	0.063	NS	S
Bacillus subtilis (ATCC 6051)	3.125	0.5	1.563	0.125	2.0	4.0	NS	NS
Escherichia coli (l.c.i.)	6.25	8.0	1.563	64.0	4.0	0.125	NS	S
Pseudomonas aeruginosa (ATCC 10145)	12.5	0.25	12.5	2.0	1.0	0.125	NS	S
Pseudomonas aeruginosa (l.c.i.)	12.5	1.0	6.25	2.5	2.0	0.4	NS	NS
Salmonella kintambo (Human: 1,13,23: mt:−)	50.0	0.25	3.125	2.0	16.0	0.125	S	S
Proteus sp. (l.c.i.)	12.5	0.5	6.25	8.0	2.0	0.063	NS	S

[a] l.c.i., local clinical isolate

[b] Mean of three replicates of the experiment: MIC index (MIC_i), agar-well MIC/Macro-broth MIC; S, significant ($MIC_i = >4.0$ or ≤ 0.125); NS, non-significant ($MIC_i = >0.125$–4.0).

[c] The interpretation of MIC indices (MIC_i) for significance or non-significance is adopted from the definition of Fractional Inhibitory Concentration Index (FIC_i) as modified by Sanders et al. (1993). By this definition, MIC_i which lie outside the inherent two-fold error of MIC (i.e. $MIC_i = >4.0$ or ≤ 0.125) is considered significant, i.e. the test methods have an effect on the MIC value determined. Conversely, MIC_i which lie within the two-fold error ($MIC_i = >0.125$–4.0) imply that the test methods have no significant effect on MIC value. Two-fold error of MIC means that the true MIC may be between 2 MIC and 2 MIC (Sanders et al., 1993).

Table 5
Minimum bactericidal concentrations (MBC) of ethanolic extracts of LO–WR and LO–RW against test bacterial strains

Test bacterial strain	MBC[b]			MBC/MIC Ratio[c]		
	LO–WR extract mg/ml	LO–RW extract mg/ml	Gentamycin μg/ml	LO–WR extract	LO–RW extract	Gentamycin
Staphylococcus aureus (ATCC 12600)	0.391	3.125	4.0	1.0	1.0	4.0
Staphylococcus aureus (l.c.i.)[a]	0.391	3.125	512.0	1.0	1.0	8.0
Bacillus subtilis (ATCC 6051)	>100	50.0	1.0	>64.0	32.0	8.0
Escherichia coli (ATCC 11775)	50.0	–	8.0	1.0	–	2.0
Escherichia coli (l.c.i.)	50.0	50.0	512.0	1.0	32.0	8.0
Pseudomonas aeruginosa (ATCC 10145)	25.0	50.0	16.0	4.0	4.0	8.0
Pseudomonas aeruginosa (l.c.i.)	25.0	25.0	12.0	4.0	4.0	4.8
Salmonella kintambo (Human: 1,13,23: mt:−)	50.0	>100	4.0	16.0	>32.0	2.0
Salmonella typhi (l.c.i.)	50.0	–	8.0	16.0	–	2.0
Proteus sp. (l.c.i.)	>100	50.0	8.0	>32.0	>32.0	1.0

−, no MIC and MBC/MIC values.
[a] l.c.i., local clinical isolate.
[b] Each value is a mean of three replicate experiments.
[c] Macro-broth MIC is used to calculate the MBC/MIC ratio.

MIC values for LO–WR extracts were relatively lower than the corresponding agar-well-determined values, with the exceptions of tests against *E. coli* (11775 and l.c.i.) where both showed equal values and *P. aeruginosa* (ATCC 10145 and l.c.i.) in which the agar-well-determined MIC appeared lower (Table 3). Corresponding MIC values determined by the two test methods were significantly different in all cases except for *B. subtilis* (ATCC 6051), *E. coli* (ATCC 1775 and l.c.i.) and *Proteus* sp (l.c.i.). Similar pattern of MIC variation between the agar-well diffusion and the macrobroth dilution methods was observed for the LO–RW extract but the difference between the two was not significant in all cases except *S. kintambo* (Huamn: 1,13,23: mt: −) (Table 4). The Gentamycin MIC values against the test strains also varied significantly with the test method except for *B. subtilis* (ATCC 6051) and *P. aeruginosa* (l.c.i.) (Tables 3 and 4).

The ethanolic extracts of LO-WR were bactericidal against the test organisms at MBC values of 0.39–50 mg/ml except for *B. subtilis* (ATCC 6051) and *Proteus* sp. (l.c.i.) with MBC of >100 mg/ml. The LO–RW ethanolic extracts also showed bactericidal activity against all test strains at MBC values of 3.1–50 mg/ml except for *S. kintambo* (Human: 1,13,23: mt: −) with MBC of >100 mg/ml (Table 5). The ratio of MBC to the macrobroth-determined MIC was 1.0–>64.0 for the LO–WR extracts and 1.0–>32.0 for the LO–RW extracts. The Gentamycin exhibited bactericidal activity against all test strains at MBC values of 1.0–16.0 μg/ml, except for *S. aureus* (l.c.i.) and *E. coli* (l.c.i.) at MBC value of 512 μg/ml (Table 5)

4. Discussion and conclusions

The antibacterial activity of *L. owerrience* root extracts varied with root part extracted and the solvent used for the extraction. The wider spectrum of activity exhibited by the ethanolic extract of the LO–WR sample is expected since it represents a combination of LO–RB and LO–RW. The apparent reduction in spectrum of activity in LO–RB and LO–RW may signal a possible loss in potency in the event of fractionation and further purification of the plant components. Crude preparations of whole plant parts (containing both the active and non-active components) have been suggested to have higher efficacy than semi-crude or pure plant substances (Kafaru, 1994). The relatively wider spectrum of activity of the ethanolic extracts over the water extracts is significant because traditional administration of root-based herbal medicines is in the form of the former (in *kai–kai*, a locally distilled gin).

The phytochemical metabolites detected in this study, namely, alkaloid, tannin, saponin, steroidal aglycon and cardiac and cynagenetic glycosides have been associated with the antimicrobial activities of several herbs (Leven et al., 1979). Therefore, the limited spectrum of activity of the aqueous extracts (both cold and hot) compared with the ethanolic extracts is difficult to explain since all the extracts contained the metabolites, though not in the same proportions. Perhaps, this paradox may be resolved when the active constituents have been isolated and the molar activity of the purified form determined. At that stage, a study of the interactions between the active and non-active components may throw even more light onto the differential activity of the various extracts.

It is noteworthy that the spectrum of antibacterial activity of the ethanolic extracts of LO–WR and LO–RW in this study are comparable to that of Gentamycin, the superiority of the latter in terms of lower MIC notwithstanding. Whether active components of higher purity from the plant roots would show activity approaching that of Gentamycin or lower than presently observed in this study is yet to be determined. Even though crude plant preparations have generally been reported to exhibit lower antimicrobial activity than pure antibiotic substances such as Gentamycin (Navarro et al., 1996; Ebi and Ofoefule, 1997; Ibrahim et al., 1997) the high bactericidal activity and low MBC/MIC ratio (1.0–16.0) observed for the ethanolic extracts in this study indicate strong antibacterial properties (Ibrahim et al., 1997).

Agar-well-determined MIC values of the ethanolic plant extracts were higher (indicating less activity) than the corresponding values determined by the macrobroth dilution technique. Conversely, with the control drug, Gentamycin, agar-well-determined MICs were lower (indicating higher drug activity) than the macrobroth-determined values, except for *B. subtilis* (ATCC 6051) and *P. aeruginosa* (l.c.i.). The disparity in susceptibility patterns observed with the two test methods may be explained by the differences in diffusability of the test materials in agar. The active components of the crude plant extracts presumably diffuse more slowly through the agar than the pure drug, Gentamycin. On the other hand, it is conceivable that the 50% DMSO used as diluent in agar diffusion tests would contribute significantly to the discrepancy. DMSO is an aprotic solvent in which certain reactions would proceed at a much lower temperature and at rates many times higher than in a protic solvent such as water (Morrison and Boyd, 1983). Such reactions among components of the extracts might culminate in the reduced anti-bacterial activity observed with the agar diffusion test method in this study. Hill et al. (1997) reported similar reduction of activity of cinnamon oil in DMSO against *Saccharomyces cerevisiae* and suggested that this might be due to partitioning of the oil between the aqueous phase and DMSO. In effect, when DMSO is used as solvent, the 'oil is distanced from the (yeast) cells' whereas without DMSO 'the oil may be solubilized in the lipid membrane of the organism where it could have a greater effect on cell metabolism'. Further investigation is needed to ascertain the effect of DMSO (and, indeed, any other solvent) on the antibacterial activity of different plant extracts, bearing in mind that DMSO is a highly membrane active compound with possible effects on a number of anti-microbial mechanisms of action. All the same, the influence of antimicrobial test methods on the results obtained from the susceptibility assays of different organisms to antimicrobial agents has been widely reported (Masuda and Tomoka, 1978; Jen-Chyi et al., 1997; Batch et al., 1998).

The antibacterial activity variously exhibited, particularly by the ethanolic extracts of the root parts of *L. owerrience*, is significant for two reasons with respect to their traditional medicinal use in south-eastern Nigeria. First, the more active preparations are the ethanolic extracts, the form in which medicinal root preparations are popularly administered in ethnomedical practice. Secondly, the bacterial strains used in this study which showed susceptibility to the extracts have been implicated in some of the diseases against which *L. owerrience* is the preferred herbal remedy. These diseases (and associated aetiologic agents) are diarrhoea (strains of *E. coli* and *Salmonella* sp.), typhoid (*S. typhi*), wound infections (*S. aureus*, *Proteus* sp. and *P. aeruginosa*) and food poisoning (*S. aureus* and *B. subtilis*) (Leven, 1987; Jawetz et al., 1995). Thus, these preliminary results support the folkloric claims that *L. owerrience* preparations are efficacious against these diseases. Further research, probably involving in vivo assays, would be needed to establish the relationship between the MICs obtained in this study and the effective doses at which the herbs are applied in ethnomedical practice.

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Biological activity of *Guatteria cardoniana* fractions

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Abstract

Methanolic extract of leaves and twigs of *Guatteria cardoniana* R.E. Fries (Annonaceae), a plant from the Venezuelan rain forest, was separated in alkaloid rich fractions and their biological effect on baby hamster kidney (BHK) cell line was studied. The initial plant extract (FA) induced cell proliferation, cytotoxicity as well as antiviral activity, depending on the concentration used. Further separation of this methanolic extract allowed us to separate these biological activities. The fraction with the highest antiviral activity (F7) was chromatographed and three of the nine alkaloid-rich fractions obtained, retained this activity. One of them ($F_7$11) exhibited the highest inhibitory effect against a neurotropic Sindbis virus (NSV). © 2001 Published by Elsevier Science Ireland Ltd.

Keywords: Annonaceae; *Guatteria cardoniana*; Antiviral; Sindbis virus

1. Introduction

Guatteria cardoniana R.E. Fries is a plant, which grows in the Venezuelan rain forest, and belongs to the Annonaceae family, one of the largest families of tropical plants (Leboeuf et al., 1982). Recently, phytochemical and pharmacological studies of this family have increased due to the discovery of new natural products with diverse biological activities. Indigenous people from Bolivia use *Guatteria foliosa* as insect repellent and a new antiparasitic aporphine alkaloids have been isolated from this plant (Mahiou et al., 1994). More recently an antimalarial constituent has been obtained from G*uatteria amplifolia* (Weniger et al., 2000). Ethanolic extract of *Annona muricata* (Annonaceae) had been reported to inhibit the cytopathic effect of Herpes simplex virus 1 (Padma et al., 1998).

As part of our research on plant secondary metabolites as antiviral agents, we separated different alkaloid-rich fractions from leaves and twigs of *G. cardoniana*, to study their effect on baby hamster kidney (BHK) cells, as well as their inhibitory effect on the replication of a neuro-adapted strain of Sindbis (neurotropic Sindbis virus (NSV)), which is an Alphavirus belonging to the Togaviridae family.

2. Materials and methods

2.1. Plant material

Leaves and twigs of *G. cardoniana* were collected in the rain forest along the Cataniapo riverside near Puerto Ayacucho City, Amazon State, Venezuela. The plant was identified by Professor Anibal Castillo of the Biology School, Faculty of Sciences, Universidad Central de Venezuela and a voucher specimen is preserved at the National Herbarium of Venezuela (VEN) with the collection number 3360 *AC*.

2.2. Cells and virus

BHK-21 cells were obtained from the National Health Institute of Caracas, Venezuela. Dr Guadalupe

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Guzman (Virology Department of the Pedro Kouri Institute, Havana, Cuba) kindly provided BHK-21 clone 15 cells. The neurovirulent strain (NSV) of Sindbis virus was donated by Dr Dianne Griffin (Medical School, John Hopkins University, Baltimore, USA). BHK-21 cells were grown in Eagle's minimal essential medium (MEM) supplemented with 5% inactivated fetal bovine serum (FBS), 2 mM glutamine and 40 μg/ml gentamicine (growth medium). The cells were maintained at 37 °C and 5% CO_2.

NSV was grown in BHK-21 cells with maintenance medium (MEM, 1% FBS, 2 mM glutamine and 40 μg/ml gentamicine). After infection, viral supernatants were purified. Viral titer was obtained by plaque assay (Kurokawa et al., 1995). NSV yielded a titer of 5.25×10^8 pfu/ml. The virus stocks were stored at -70 °C.

2.3. Extract and fraction

Dried leaves and twigs (800 g) of *G. cardoniana* were extracted and fractioned. Seven fractions (FA, FA_1, FA_2, FA_3 FB, FB_1, FB_2) were chromatographed on a silica gel column and eluted with polarity-increasing mixtures of CH_2Cl_2–MeOH. Fraction FA (2.5 g), was further separated on a silica gel column to yield seven fractions (F1–F7). The highest antiviral activity was found in fraction F7. Therefore, it was further chromatographed through a silica gel column, eluted with CH_2Cl_2 and nine fractions (F_70, F_72, F_74, F_77, F_711, F_714, F_717, F_723, and F_724) were obtained. Stock solutions for biological assays were prepared by dissolving each of the fractions obtained in DMSO and stored at 4 °C.

2.4. Biological tests

2.4.1. Cytotoxicity assay

Cell viability was determined by a modified MU (3-(4,5 dimethylthiazol-2-yl) 2,5 diphenyl-tetrazolium bromide) assay. Briefly, cells were adjusted to 4×10^4 cells per l00 μl in growth medium and seeded in a 96-well plate. After 24 h, cells were incubated with serial dilutions of each extract in maintenance medium, MTT 10 μl (10 mg/ml) was added to each well 24 h later, and plates were read at 570 nm after 18 h (Shearman et al., 1994).

2.4.2. Antiviral activity assay

Cells were seeded and stabilized in the same conditions as the cytotoxic assay. BHK cells were infected with NSV at a multiplicity of infection (MOI) of 0.1 pfu per cell. The infections were carried out in the presence or absence of different concentrations of extracts. After 24 h, the viability of the cells was determined by MTT assay as described before.

2.4.2.1. Plaque assay. BHK clone 15 at a final concentration of 2.5×10^5 cell was seeded in 24-wells plates. Viral suspension and the extracts were incubated for 1 h at 37 °C, then 50 μl of each solution were added to the cells to obtain a MOI of 0.02 pfu per cell and 10 μg/ml of extracts final concentration (Kurokawa et al., 1995).

2.4.2.2. Data analysis. All experiments were performed in triplicate, and each experiment was reproduced a minimum of three times. The 50% toxicity concentrations (IC50) and 50% effective concentrations (EC50) were calculated using the mean of the values of each concentration. The IC50 was estimated by linear regression analysis. The therapeutic indices (TI) were calculated as follows: IC50/EC50 (Zembower et al., 1998). Variance analysis on virus replication in the presence of the different fractions was evaluated. A $P < 0.05$ value was defined as statistically significant.

3. Results

The seven fractions obtained from the silica gel column showed a distinctive pattern of cellular responsiveness on BHK cells (Table 1). Thus, we found that FB_1 and FB_2 were able to induce cell growth in concentrations higher than 18 and 11 μg/ml, respectively, and were innocuous at lower concentrations. FA_1, FA_2, FA_3 and FB were innocuous in the wide range of concentrations tested. On the contrary, FA showed the three different biological activities (innocuous, proliferation and cytotoxicity) depending upon the concentrations used. In preliminary experiments FA, FA_1 and FA_3 showed an antiviral activity against a neurovirulent strain of the Sindbis virus (Table 2).

When FA was further chromatographed on a silica gel column, seven fractions (F1–F7) were obtained. We could separate the three biological effects (innocuous, proliferation and cytotoxicity) in different fractions at different concentrations. Thus, F1 and F2 showed the proliferating activity at low concentration (0.2–3.3 μg/ml). F3, F4, F5 and F6 were cytotoxic at concentrations higher than 42 μg/ml and the antiviral activity was rescued in fractions F2 and F7, the last one being the most effective, inhibiting NSV replication with a TI of 17 (Table 2). A high alkaloid presence was detected in F5. However, this fraction does not show antiviral activity.

A further F7 purification gave nine fractions. All of them were innocuous at the concentration tested (0.2–50 μg/ml). Three of the fractions obtained (F_711, F_714, F_717) could inhibit NSV replication using the colorimetric assay, F_714 and F_717 displayed an antiviral activity at higher concentrations; therefore, their TI is quite low (Table 2). In addition, we studied the antivi-

Table 1
Biological activity of fractions and sub-fractions of *G. cardoniana*

G. cardoniana	Innocuous[a] (μg/ml)	Cytotoxicity[b] (μg/ml)	Proliferation[c] (μg/ml)
Fractions			
FA	6.25–51	>51	0.2–6
FA_1	0.2–293	>293	
FA_2	0.2–558	>558	
FA_3	0.2–117	>117	
FB	0.2–112	>112	
FB_1	0.2–18		>18
FB_2	0.2–11		>12
FA sub-fractions			
F1	3.13–333	>333	0.2–3
F2	3.13–232	>232	0.2–3
F3	0.2–43	>43	
F4	0.2–49	>49	
F5	0.2–63	>63	
F6	0.2–49	>49	
F7	0.2–115	>115	
F7 sub-fractions			
F_70–F_724	0.2–50		

[a] Innocuous, 50–110% cellular viability.
[b] Cytotoxicity, <50% cellular viability.
[c] Proliferation, >110% cellular viability.

ral effect of these fractions, using an inhibition plaque assay. By this method, the difference in the plaque forming units between the viral control and fractions F7, F_711 and F_714 is statistically significant, the best fraction was F_711 with a frequency (F) of 31.69 and $P = 0.000493$ (Fig. 1). Also, the difference between F7 and F_711 is statistically significant, $F = 5.79$ and $P = 0.042789$, however, the difference between F7 and F_714 is not significant ($F = 1$; $P = 0.34$) when used at 10 μg/ml, these results are in agreement with the ones obtained in the colorimetric assay where F_711 showed a higher therapeutic index compared with F_714 and F_717 (Table 2).

4. Discussion

Different *Guatteria* species have shown an antiparasitic activity (Mahiou et al., 1994; Weniger et al., 2000). The present study was carried out to evaluate the biological activity of fractions obtained from *G. cardoniana* focusing in the antiviral activity against Sindbis virus. The distinct biological activity shown by a crude extract of leaves and twigs from *G. cardoniana* was separated in different fractions. F_711, F_714 and F_717 were the fractions where the antiviral activity was obtained. Due to the fact that F_714, F_717 are eluted subsequently to F_711, it is possible that the compound(s) accountable for the antiviral activity in these fractions are the same or/and with similar chemical structure to the ones responsible for this activity in F_711 fraction. F_711 showed a higher percent of inhibition in the colorimetric assay, with a TI of 20.48, by plaque assay this fraction is able to inhibit the NSV replication in approximately 45%. We are in the process of further purification, isolation and identification of the compound(s) that are responsible for the inhibition of NSV replication in these fractions. The present work is the first report of fractions from genera *Guatteria* with antiviral activity.

Table 2
Anti-NSV activity of fractions and sub-fractions of *G. cardoniana*

G. cardoniana	IC50 (μg/ml)	EC50 (μg/ml)	TI (IC50/EC50)
Fractions			
FA	50.83	2.48	20.50
FA_1	292.94	18.18	16.11
FA_3	116.51	55.91	2.08
FA sub-fractions			
F2	232.14	27.5	8.44
F7	114.53	6.73	17.08
F7 sub-fractions			
F_711	249.92	12.20	20.48
F_714	342.27	60.63	5.64
F_717	254.79	50.79	5.01

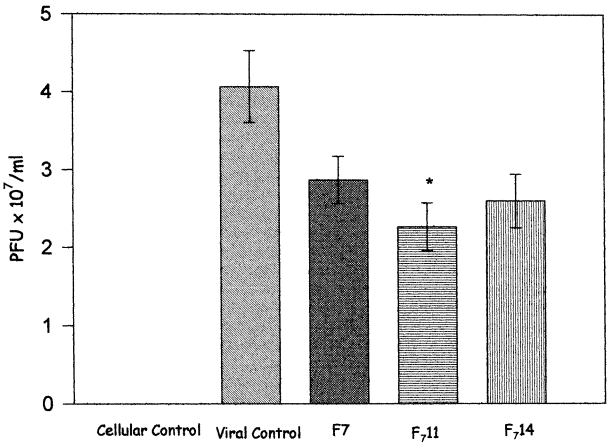

Fig. 1. Inhibition of NSV infection by *G. cardoniana* subfractions (10 μg/ml) using plaque assay. *, $P = 0.042789$.

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Immunomodulatory activity of alcoholic extract of *Mangifera indica* L. in mice

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Abstract

Mangifera indica Linn, a plant widely used in the traditional medicinal systems of India, has been reported to possess antiviral, antibacterial and anti-inflammatory activities. In the present study, the alcoholic extract of stem bark of *Mangifera indica* Linn (Extract I containing mangiferin 2.6%), has been investigated for its effect on cell mediated and humoral components of the immune system in mice. Administration of test extract I produced increase in humoral antibody (HA) titre and delayed type hypersensitivity (DTH) in mice. It is concluded that test extract I is a promising drug with immunostimulant properties. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Mangifera indica*; Cell mediated immunity; humoral immunity; immunomodulatory

1. Introduction

Immunomodulation is a procedure which can alter the immune system of an organism by interfering with its functions; if it results in an enhancement of immune reactions it is named as an immunostimulative drug which primarily implies stimulation of non specific system, i.e. granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances. Immunosuppression implies mainly to reduce resistance against infections, stress and may occur on account of environmental or chemotherapeutic factors.

Immunostimulation and immunosuppression both need to be tackled in order to regulate the normal immunological functioning. Hence both immunostimulating agents and immunosuppressing agents have their own standing and search for better agents exerting these activities is becoming the field of major interest all over the world. (Patwardhan et al., 1990).

Natural adjuvants, synthetic agents, antibody reagents are used as immunosuppressive and immunostimulative agents. But there are major limitation to the general use of these agents such as increased risk of infection and generalized effect throughout the immune system (Diasio and LoBuglio, 1996).

Traditional Indian system of medicines like Siddha and Ayurveda have suggested means to increase the body's natural resistance to disease. A number of Indian medicinal plants and various 'rasayanas' have been claimed to possess immunomodulatory activity (Atal et al., 1986; Patwardhan et al., 1990; Puri et al., 1994; Balchandran and Panchanathan, 1998; Ziauddin et al., 1996).

Mangifera indica L. (Anacardiaceae) is a medicinal plant claimed to posses number of therapeutic uses. Mangiferin (1, 3, 6, 7 tetrahydroxy xanthone 2-glucopyranoside) has been reported to be present in various parts of *Mangifera indica* viz. leaves (Desai et al., 1966), fruits (El Ansari et al., 1971), stem bark (El Ansari et al., 1967, Bhatia et al., 1967), heartwood (Ramanathan and Seshadri, 1960) and roots (Nigam and Mitra, 1964). It has been reported that *M. indica* possess immunomodulatory activity in vitro (Chattopadhyay et al., 1986). However, there is paucity of scientific data on the in vivo immunomodulatory activity of stem bark of *M. indica*. The objective of present investigation was to study the immunomodulatory activity of the alcoholic extract of the stem bark of *M. indica* Linn in animal models.

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2. Materials and methods

2.1. Animals

Swiss albino mice, (National Institute of Toxicology, Sinhagad Road, Pune) weighing between 20 and 25 g of either sex were used to evaluate the immunomodulatory activity of alcoholic extract of stem bark of *M. indica* Linn. Animals were housed under standard conditions of temperature (25 °C), 12 h/12 h light/dark cycles and fed with standard pellet diet (Chakan Oil Mill) and tap water.

2.2. Plant material and extract preparation

The fresh air dried bark (1.0 kg) of *M. indica* was collected from local gardens of Ratnagiri in Maharashtra state. It was authenticated in Agharkar Research Institute, Pune. The dried, coarsely powdered bark was successively extracted with petroleum ether (60–80) for 8 h to remove fatty matter. The defatted marc was then subjected to soxhlet extraction with 95% ethanol for 8 h. The total alcoholic extract was concentrated and air dried. During the process of concentration, a yellow material liberated from the total extract. This was filtered out and named as test extract I. The filtrate was further concentrated, dried and named as test extract II which was not used for the pharmacological study. The process of extraction was similar to that described by Iseda (1956), Bhatia et al. (1967). The test extract I was subjected to column chromatographic fractionation which afforded 2.6% of pure mangiferin (m.p. 261–264). It was further characterised by using IR and ^{1}H NMR spectral data. The test extract I containing 2.6% of mangiferin was further used for immunomodulatory activity studies.

2.3. Drugs

Weighed quantity of the test extract I having 2.6% mangiferin was suspended in 1% sodium carboxy methyl cellulose to prepare suitable dosage forms. The control animals were given an equivalent volume of the sodium carboxy methylcellulose vehicle. Cyclophosphamide (Khandelwal Laboratories, Mumbai) was used as a standard immunosuppressant agent.

Antigen: Fresh blood was collected from sheep's sacrificed in the local slaughter house. Sheep red blood cells (SRBCs) were washed three times in normal saline and adjusted to a concentration of 0.1 ml containing 1×10^8 cells for immunization and challenge.

2.4. Humoral antibody (HA) and delayed type hypersensitivity (DTH) response

2.4.1. Effect of test extract I and cyclophosphamide on HA titre and DTH response using SRBCs as an antigen in mice–7 days pre-treatment

Method described by Puri et al. (1994) was adopted. Mice were divided into seven groups, each group containing six mice. Drugs were administered in various groups, i.e. Group I–Control (Sodium carboxy methyl cellulose 1%), Group II–VI test extract I (5 dose levels 50–800 mg/kg p.o.) and Group VII-standard drug (Cyclophosphamide 50 mg/kg, p.o.).

The animals were immunized by injecting 0.1 ml of SRBCs suspension containing 1×10^8 cells intraperitoneally on day 0. Blood samples were collected in microcentrifuge tubes from individual animal by retro-orbital puncture on day 7. The blood samples were centrifuged and serum was obtained. Antibody levels were determined by the haemagglutination technique. Briefly, equal volumes of individual serum samples of each group were pooled. To serial two fold dilutions of pooled serum samples made in 25 μl volumes of normal saline in microtitration plates was added 25 μl of 1% suspension of SRBCs in saline. After mixing, the plates were incubated at 37 °C for 1 h and examined for haemagglutination under microscope. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

On day 7, the thickness of the right hind foot pad was measured using vernier calliper (Mitutoyo digimatic). The mice were then challenged by injection of 1×10^8 SRBCs in right hind foot pad. Foot thickness was measured again $+24$ h after this challenge. The difference between the pre and post challenge foot thickness expressed in mm was taken as a measure of DTH. The extract was administered orally on day 0 and continued till day 7 of challenge. Cyclophosphamide was administered on day 4 to day 6.

2.4.2. Effect of test extract I on HA titre and DTH response using SRBCs as an antigen in mice-15 day pretreatment

Mice were divided into six groups, each group containing six mice. Drugs were administered in various groups, i.e. Group I–Control (Sodium carboxy methyl cellulose 1%), Group II–VI test extract I (5 dose levels 50–800 mg/kg p.o.) The pretreatment time of 15 days was based on the method described by Sharma et al. (1996). Schedule for drug administration was 7 days prior to immunisation (days -6, -5, -4, -3, -2, -1, 0) and 7 days after immunisation (days $+1$, $+2$, $+3$, $+4$, $+5$, $+6$, $+7$). The procedure of immunisation by injecting SRBCs suspension, collection of blood sample and measure-

ment of inflammation as described in 2.4.1 was followed.

2.5. Statistical analysis

Data were expressed as the mean standard deviation of the means (S.D.) and statistical analysis was carried out employing Student's *t*-test.

3. Results

3.1. Effect of test extract I and cyclophosphamide on HA titre and DTH response using SRBCs as an antigen in mice-7 days pretreatment

The humoral antibody titre value was found to be 8.7 ± 5.9. Administration of test extract I produced a dose dependent (50–800 mg/kg per day for 7 days) increase in humoral antibody as evident by haemagglutination at that dilution (Table 1).

The result obtained in Table 1 indicated that animals treated with lower doses, i.e. 50, 100, 200 mg/kg did not show increase in paw edema. The optimum dose of the test extract I showing statistically significant increase ($P < 0.005$) was 400 mg/kg. At higher dose (800 mg/kg) increase in the paw edema was evident but the values were not statistically significant.

Administration of cyclophosphamide (50 mg/kg p.o.) on days 4, 5 and 6 resulted in significant decrease ($P < 0.05$) in humoral antibody titre compared with the animals of group I (control). After challenge on day 7 with SRBCs significant increase in the paw edema was observed on day 8.

Cyclophosphamide treatment appears to be more potent than test extract I in producing paw edema. Test extract I and cyclophosphamide actions exhibited remarkable differences in the humoral antibody titre parameter. Test extract I increased the HA titre while cyclophosphamide treatment reduced the HA titre. However, there was increase in the DTH response in case of both the drugs.

3.2. Effect of test extract I on HA titre and DTH response using SRBCs as an antigen in mice 15 days pretreatment

All the animals of groups I–VI were sensitized on day 0. The control group received only vehicle from day -7 to $+7$. The humoral antibody titre value was found to be 22.7 ± 10.6. Administration of test extract I produced a dose dependent increase in the HA titre after incubation with SRBCs (Table 2).

Administration of higher doses, i.e. 200, 400 and 800 mg/kg produced significant increase in HA titre as evident from haemagglutination after incubation of serum with SRBCs. DTH was determined 24 h after the challenge. Higher doses of test extract I (400 and 800 mg/kg) showed statistically significant increase in mean paw edema.

4. Discussion

In the present investigation two schedules of pretreatment period, i.e. 7 days pretreatment and 15 days pretreatment were selected. The results obtained in the present studies showed that test extract I displays a dose dependent immunostimulatory effects in relation to antigenic stimulation.

Injecting mice i.p. with 10^8 SRBCs suspended in saline sensitizes them for elicitation of DTH and also induces antibody formation, therefore this system has major advantages, i.e. it enables two component of immune response to be measured in the same species under ideal condition and is relatively simple and inexpensive to

Table 1
Effect of test extract I and cyclophosphamide on HA titre and DTH response using SRBCs as an antigen in mice-7 days pretreatment

Group	Treatment	Dose mg/kg p.o. for 7 days	HA titre (Mean ± S.D.)	DTH response (mm) mean paw edema ± S.D.
I	Control	–	8.7 ± 5.9	0.29 ± 0.13
II	Test Extract I	50	22.7 ± 10.6^{b}	0.31 ± 0.22
III	Test Extract I	100	21.3 ± 8.3^{d}	0.40 ± 0.10
IV	Test Extract I	200	85.3 ± 33.0^{c}	0.31 ± 0.12
V	Test Extract I	400	170.7 ± 0.1^{e}	0.57 ± 0.12^{d}
VI	Test Extract I	800	106.6 ± 33.0^{e}	0.43 ± 0.13
VII	Cyclophosphamide	50	3.00 ± 1.1^{a}	0.64 ± 0.10^{a}

Control: 1% Sodium carboxy methyl cellulose; $n = 6$ per group comparison of I with II, III, IV, V, VI, and VII.
[a] $P < 0.05$.
[b] $P < 0.025$.
[c] $P < 0.01$.
[d] $P < 0.005$.
[e] $P < 0.001$.

Table 2
Effect of test extract I on HA titre and DTH response using SRBCs as an antigen in mice–15 days pretreatment

Group	Treatment	Dose (mg/kg) p.o. for 15 days	HA titre (Mean ± S.D.)	DTH response (mm) mean paw edema ± S.D.
I	Control	–	22.7 ± 10.6	0.29 ± 0.09
II	Test Extract I	50	26.6 ± 8.3	0.33 ± 0.17
III	Test Extract I	100	46.7 ± 27.0	0.34 ± 0.16
IV	Test Extract I	200	74.7 ± 26.1[c]	0.40 ± 0.21
V	Test Extract I	400	341.3 ± 132.2[d]	0.54 ± 0.17[b]
VI	Test Extract I	800	320.0 ± 210.3[b]	0.39 ± 0.06[a]

Control: 1% Sodium carboxy methyl cellulose; $n = 6$ per group comparison of I with II, III, IV, V, and VI.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.005$.
[d] $P < 0.001$.

perform (Doherty, 1981). Test extract I produced dose dependent increase in both the parameters, i.e. antibody production and delayed type hypersensitivity.

The antibody production to T-dependent antigen SRBCs requires the co-operation of T and B lymphocytes and macrophages (Benacerraf, 1978). The data reported here for cyclophosphamide is entirely consistent with literature report on the action of drug in similar models (LaGangre et al., 1974; Turk et al., 1976; Gill and Liew, 1978; Doherty, 1981).

After administration of cyclophosphamide on day 4, 5 and 6 the humoral antibody titre is reduced significantly ($P < 0.05$) while there is elevation in DTH response. It has been established that the mechanism behind this potentiation of DTH by cyclophosphamide is the elimination of population of suppressor cells (Turk et al., 1976; Mitsuoka et al., 1976; Gill and Liew, 1978). Dosing with cyclophosphamide during period closer to elicitation of DTH is reported to have profound suppressive effect on all forms of DTH and cell mediated immunity (Bach, 1976).

Humoral and DTH responses to SRBCs administered to mice without adjuvant provide a useful system for identification of compounds with selective effect on the immune response. However, fairly precise timing of drug administration in relation to sensitization and challenge is required. In the present investigation test extract I administration appears to be in accordance with these requirements.

Guha et al. (1996) have reported that mangiferin has prominent antiviral and antitumor activities. It appears to act as a potent biological modifier with the above effects. Chattopadhyay et al. (1986) have reported a dose dependant stimulation of proliferation of thymocytes and splenic lymphocytes after 48 h exposure to mangiferin. Results obtained in the present in vivo studies seem to be in conformity with the in vitro studies of Chattopadhyay et al. (1986). It is thus apparent that the immunostimulatory effect produced by test extract I containing 2.6% of mangiferin may be due to cell mediated and humoral antibody mediated activation of T and B cell. It is thus concluded that the stem bark of *Mangifera indica*, its alcoholic extract containing mangiferin had promising immunostimulant properties.

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Effect of *Ocimum sanctum* fixed oil on blood pressure, blood clotting time and pentobarbitone-induced sleeping time

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Abstract

Ocimum sanctum fixed oil produced hypotensive effect in anaesthetised dog, which seems to be due to its peripheral vasodilatory action. The oil increased blood-clotting time and percentage increase was comparable to aspirin and could be due to inhibition of platelet aggregation. The oil also increased pentobarbitone-induced sleeping time in rats indicating probable inhibitory effect of oil towards cytochromic enzyme responsible for hepatic metabolism of pentobarbitone. © 2001 Published by Elsevier Science Ireland Ltd.

Keywords: *O. sanctum* fixed oil; Hypotensive; Antiplatelet; Metabolic inhibitor

1. Introduction

Ocimum sanctum L. (labiatae) commonly known as 'Holy basil' is a herbaceous sacred plant found throughout India. Indian materia medica describes the use of the plant in a variety of ailments (Nadkarni, 1976). In our earlier studies, *O. sanctum* fixed oil was found to possess significant anti-inflammatory (Singh et al., 1996a), antipyretic, analgesic, anti-arthritic and antiulcer activity (Singh and Majumdar, 1995a,b, 1996, 1999) without any noticeable toxicity (Singh and Majumdar, 1994). In a clinical trial, the fixed oil was also found to be effective against bovine mastitis (Singh et al., 1995). The fixed oil contains five fatty acids viz. palmitic (11.69%), stearic (3.19%), oleic (13.82%), linoleic (52.23%) and linolenic (16.63%) (Singh et al., 1996b) and linolenic acid (a dual inhibitor of arachidonate metabolism), appears to be responsible for the antiinflammatory activity of the oil (Singh and Majumdar, 1997). In the present study, the effect of fixed oil on blood pressure, blood clotting time and pentobarbitone-induced sleeping time have been evaluated.

2. Materials and methods

Dried seeds of *O. sanctum* were collected from Maidan Garhi (New Delhi, India) and were authenticated by a resident botanist of the Department of Genetics, Indian Council of Agricultural Research, New Delhi and voucher samples were deposited in the Department of Genetics. The seeds were crushed and cold macerated in petroleum ether (40–60 °C) (S.D. Fine Chemicals Ltd, India) for 3 days. The petroleum ether was evaporated from the extract and oil was filtered to clarity (weight per ml at 25 °C is 0.8750). The fixed oil thus obtained was subjected to following studies on animals. The animals were supplied by M/s Lucky Zoological House, New Delhi, India. Animals were kept under standard laboratory conditions and fed on standard diet.

2.1. Blood pressure on anaesthetised dog

Mongrel male dog weighing 10 kg was anaesthetised with pentobarbitone sodium. The femoral vein was ligated with fine venous canula connected with syringe filled with normal saline. Tracheal canula was ligated after careful incision of trachea and then carotid artery was canulated with glass canula attached with manometer and smoked kymograph. The different panel drugs,

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e.g. adrenaline, nor-adrenaline, isoprenaline, acetylcholine, histamine and *O. sanctum* fixed oil emulsion (1 ml oil emulsified in 1.9 ml distilled water containing 0.1 ml Tween 80) were administered through the venous canula to see the effect on blood pressure. Amount of emulsion used in the study was equivalent to 0.3 ml of fixed oil.

2.2. Blood clotting time

Wistar albino rats weighing between 120 and 170 g used were divided into three groups of six animals each. The groups received either distilled water (3 ml/kg Control) or *O. sanctum* fixed oil (3 ml/kg) or standard drug aspirin (100 mg/kg) (Reckitt and Colman, India) intra-peritonealy. After 1 h, under light anaesthesia, blood sample was taken with the help of a glass capillary from orbital plexus of the eye of each rat and the time was noted. Small pieces of capillary were broken from one end at every 30 s till fibrin threads of blood appeared between the broken ends of capillary (Ghai, 1990).

2.3. Pentobarbitone-induced sleeping time

Swiss albino mice weighing between 20 and 30 g were used for experimentation. The animals were divided into five groups of six animals each. Group I served as control, group II–IV received *O. sanctum* fixed oil (1.0, 2.0 and 3.0 ml/kg) and group V received chlorpromazine (4 mg/kg) (Rhone-Poulenc) intra-peritonealy. After 15 min pentobarbitone sodium (40 mg/kg) (Rhone-Poulenc) was injected intra-peritonealy to all the animals and the sleeping time was noted (Dandia and Collumbine, 1959).

3. Results

The results (Fig. 1) indicate that *O. sanctum* fixed oil produced hypotensive effect in anaesthetised dog. The fixed oil blocked the biphasic response of adrenaline and depressor response to isoproterenol. The oil partially suppressed the hypotensive response of histamine and acetylcholine. The oil increased blood-clotting time significantly and the percentage increase was comparable to that obtained with aspirin (Table 1). The oil also potentiated pentobarbitone-induced sleeping time in mice in a dose dependent manner (Table 2).

4. Discussion and conclusions

The effect of *O. sanctum* fixed oil on blood pressure was studied in anaesthetised dog. The fixed oil produced a progressive fall in blood pressure and blocked the biphasic response of adrenaline and hypotensive effect of isoproterenol. When adrenaline is injected intravenously into an anaesthetised animal, the blood pressure rises rapidly to a peak and then falls below the initial level (baseline) before returning to the baseline (biphasic response). The mechanism of the rise in blood pressure is due to (a) a direct myocardial stimulation (positive inotropic effect); (b) an increased heart rate (positive chronotropic effect); and (c) arteriolar constriction in many vascular beds specially in skin, mucous membrane and splanchic region. The blood pressure falls to initial level due to compensatory vagus reflex. The fall of blood pressure below the baseline is due to greater sensitivity to adrenaline of vasodilatary β-receptor in vascular beds than of constrictor α-receptors (Ghosh, 1984). Intravenous administration of *O. sanctum* fixed oil produced progressive fall in blood pressure that went below the baseline and did not return to baseline again. Administration of adrenaline reversed the depressant effect of oil, which appears to be due to physiological antagonism. Essential fatty acids (EFAs) are known to lower blood pressure (Jacono et al., 1982). *O. sanctum* fixed oil contains EFAs like linoleic and linolenic acids. Linoleic acid (18:2, n-6) is a precursor of gammalinolenic acid (GLA, 18:3, n-6) and arachidonic acid (A.A, 20:4, n-6). GLA is elongated to dihomogammalinolenic acid (DGLA, 20:3, n-6), which acts as a substrate for cyclooxygenase and produces PGE_1. Similarly A.A. also can produce PGE_2, PGI_2, and TXA_2 through cyclooxygenase pathway (Zurier, 1991). PGE_1, PGE_2 and PGI_2 are potent peripheral vasodilators (Horrobin, 1982; Campbel and Haluskha, 1996). Linolenic acid (an omega-3 fatty acid, 18:3, n-3) is the precursor of eicosapentaenoic acid (EPA, 20:5, n-3) which can competitively inhibit formation of cyclooxygenase products (PGE_2, TXA_2) from A.A. EPA produces TXA_3 (which has much lesser ability than TXA_2 to constrict blood vessels) and PGI_3 which has vasodilatory activity. However, EPA does not appreciably reduce formation of PGI_2 by endothelial cells (Zurier, 1991; Fischer and Weber, 1984; Lee et al., 1985). Thus, combined vasodilatory action of PGI_2 (endogenous or derived from linoleic acid) and PGI_3 derived from linolenic acid can account for the hypotensive effect of *O. sanctum* fixed oil. To know the relative contribution of linoleic acid and linolenic acid towards hypotension further studies are needed.

O. sanctum fixed oil prolonged blood clotting time and the response was comparable to that obtained with aspirin. The effect appears to be due to the antiaggregator action of oil on platelets. Linolenic acid contained in the oil can be metabolised to EPA, which can inhibit formation of TXA_2 through cyclooxygenase and produce PGI_3 and TXA_3. Like PGI_2, PGI_3 also possesses antiaggregatory property while TXA_3 has much less proaggregatory activity towards platelets compared

with TXA_2 (Zurier, 1991; Fischer and Weber, 1984; Lee et al., 1985). Combined antiaggregatory effects of PGI_2 and PGI_3 supplemented by inhibition of TXA_2 could, therefore, contribute towards anticoagulant effect of *O. sanctum* fixed oil. However, further studies are needed to confirm the same.

The fixed oil in the dose range of 2.0–3.0 ml/kg potentiated pentobarbitone-induced sleeping time significantly. The oil if given alone to animal, does not produce any sedation indicating no CNS depressant activity of its own. Lipids containing linoleic and linolenic acids absorb oxygen on exposure to air and they are called drying oil (Atherdon, 1969). *O. sanctum* fixed oil contains both linoleic (55.23%) and linolenic acid (16.63%) and hence would also have affinity for oxygen. Pentobarbitone is metabolised in the liver by oxidative pathway that involves cytochrome p450, NADPH and molecular O_2 (Benet, 1996). *O. sanctum* fixed oil may absorb the oxygen and get itself oxidised/metabolised thereby inhibiting the metabolism of pentobarbitone. In addition, the oil being a prostaglandin inhibitor, may also inhibit renal vasodilatory prostaglandins resulting in reduced blood flow to kidney and reduced clearance of pentobarbitone. It would be worthwhile to mention here that increased serum level and reduced clearance of pentobarbitone due to inhibition of hepatic metabolism by miconazole has been reported (Heinmeyer et al., 1988). Thus inhibition of hepatic

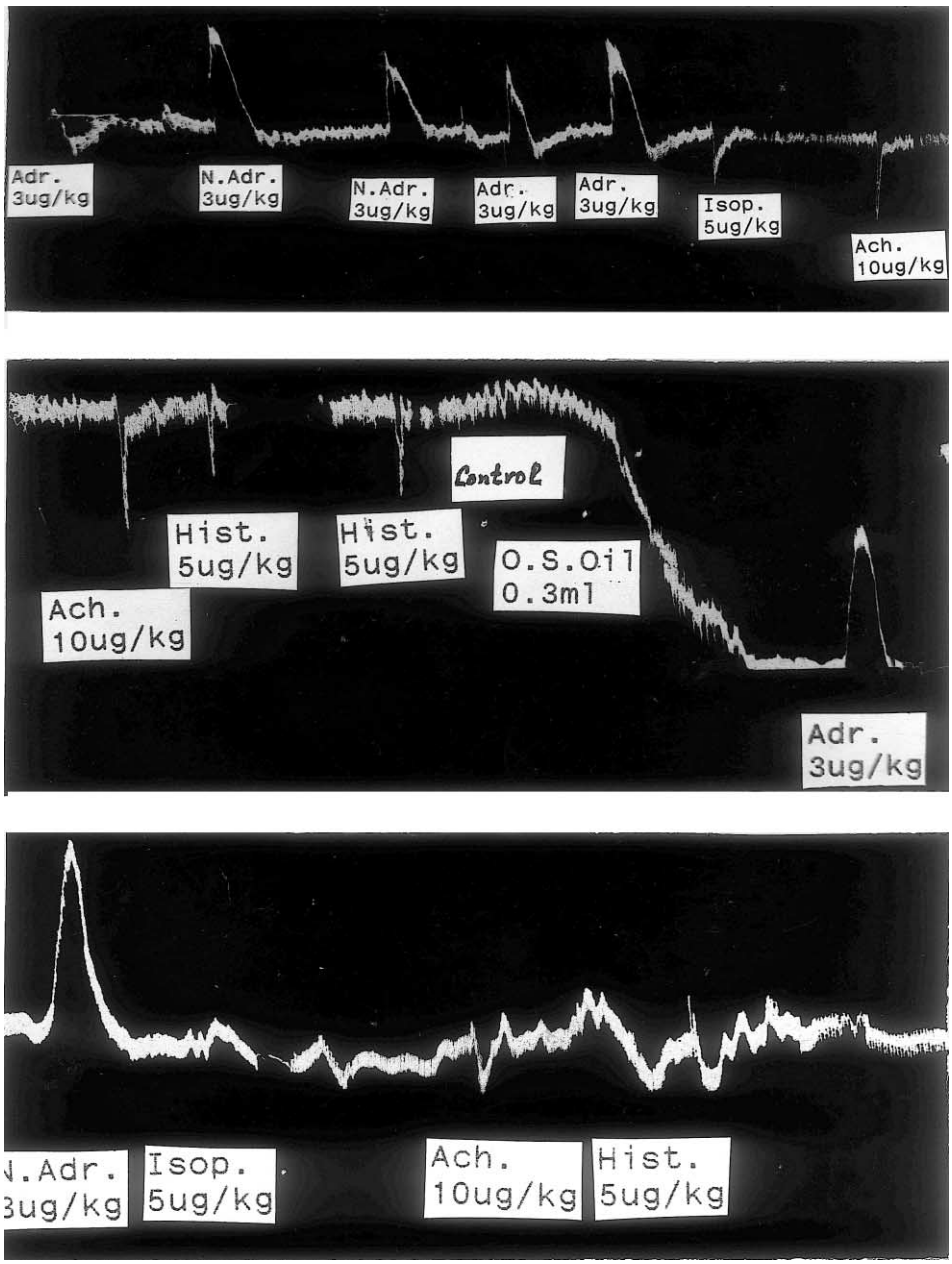

Fig. 1. Effect of *O. sanctum* fixed oil on dog blood pressure.

Table 1
Effect of *O. sanctum* fixed oil and aspirin on blood clotting time

Animal number	Blood clotting time (s)		
	Control	*O. sanctum* Fixed oil (3.0 ml/kg)	Aspirin (100 mg/kg)
1	3.5	5.0	5.5
2	4.0	6.0	6.0
3	4.5	7.0	7.0
4	4.0	6.5	6.5
5	4.0	5.5	6.5
6	3.5	5.0	5.5
Mean ± S.E.	3.91 ± 0.15	5.83 ± 3.33*	6.16 ± 0.24*
Percent increase (compared with control value)	–	32.94	36.53

Statistically significant (compared with control value) as per Student's *t*-test, $*P<0.05$.

Table 2
Effect of *O. sanctum* fixed oil and chlorpromazine on pentobartitone induced sleeping time in mice

Animal number	Sleeping time (min)				
	Control vehicle	O.S. oil (1.0 ml/kg)	O.S. oil (2.0 ml/kg)	O.S. oil (3.0 ml/kg)	Chlorpromazine (4 mg/kg)
1	46	65	78	99	137
2	37	49	69	104	147
3	29	43	82	93	129
4	28	59	63	109	134
5	41	66	76	98	151
6	40	71	80	100	119
Mean ± S.E.	36.83 ± 2.89	58.83 ± 4.42	74.66 ± 2.97*	100.5 ± 2.23*	136.16 ± 4.80*
Percent increase (compared with control value)	–	37.40	50.67	63.36	72.96

Statistically significant (compared with control value) as per Student's *t*-test, $*P<0.01$.

metabolism of pentobarbitone/renal clearance by *O. sanctum* fixed oil could be responsible for potentiation of pentobarbitone induced sleeping time. However, further studies are needed to know the exact mechanism.

On the basis of present finding, we can conclude that *O. sanctum* fixed oil possesses hypotensive and anti-coagulant activity. The oil can also inhibit hepatic metabolism of another drug due to its reducing/anti-oxidant property.

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Hypoglycemic effect of *Cecropia obtusifolia* on streptozotocin diabetic rats

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Abstract

The hypoglycemic effects of water and butanolic extracts prepared from leaves of *Cecropia obtusifolia* (Cecropiaceae) were examined in streptozotocin induced diabetic rats. A single oral administration of a water extract at doses of 90 and 150 mg/kg and of a butanol extract at doses of 9 and 15 mg/kg significantly ($P < 0.05$) lowered the plasma glucose levels in diabetic rats after 3 h administration. Glibenclamide was used as reference and showed similar hypoglycemic effect to the tested extracts at a dose of 3 mg/kg. The flavone, isoorientin and 3-caffeoylquinic acid (chlorogenic acid), were isolated as the important constituents of the plant and were identified as the main constituents in both extracts, too.

Keywords: *Cecropia obtusifolia*; Hypoglycemic effect; Streptozotocin; Diabetes; Isoorientin; Chlorogenic acid

1. Introduction

Cecropia obtusifolia Bertol. (Cecropiaceae), traditional names; 'Guarumbo' and 'Chancarro', is a monopodic tree 20 m tall, growing in form of secondary vegetation in the tropical rain forest. The main characteristics of the tree are a tall, straight, hollow trunk, stratified treetop with few large branches growing horizontally from the trunk. The leaves are in a spiral disposition located at the top of the branches and are simple, peltate or deeply palmate, with a deep green color in the upper face and gray at the under surface, the outer bark is flat. It is a fast-growing pioneer tree from tropical America, the hollow septate twigs are inhabited by ants (Pennington and Sarukhán, 1998).

The plant was first mentioned for the treatment of diabetes by Martínez (1936), since which time several ethnopharmacological reports can be found which describe its use. Traditionally the leaves, bark and root of the plant are boiled in water and the resulting infusion is drunk throughout the day (Argueta, 1994).

The hypoglycemic effect of the water extract was demonstrated on alloxan diabetic mice (Pérez et al., 1984) and on hyperglycemic rabbits (Roman-Ramos et al., 1991).

Water decoctions of 'Guarumbo' are used traditionally for the treatment of diabetes type II, particularly in the Mexican states of Oaxaca and Hidalgo. The plant is also sold in several traditional markets, either alone or mixed with other plants as an anti-diabetes complex remedy (Andrade-Cetto, 1999).

The aim of this study was to investigate the hypoglycemic effect of several extracts from *C. obtusifolia* in streptozotocin-induced diabetic rats and to identify the main chemical constituents in the plant and the tested extracts.

2. Materials and methods

2.1. Ethnobotany

Ethnobotanical studies were performed during several short visits to the communities of Tlanchinol, Hidalgo, and San Felipe Usila, Oaxaca, in Mexico

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during 1994–1999 to confirm the previously reported use of *C. obtusifolia* as a treatment for diabetic illness. In both cases we followed the same method: diabetic people were identified by the local health services and local healers; information was collected about the plant and its special usage based on structured and unstructured interviews with both the traditional healers and the diabetic people. All data were referred to plant samples (mini-herbarium) collected at its natural habitats and stored as herbarium vouchers for exact identification.

2.2. Materials

With the guidance of traditional healers, samples of *C. obtusifolia* were collected in Tlanchinol (Hidalgo) and San Felipe Usila (Oaxaca), Mexico. Their identity was confirmed by Andrade-Cetto and voucher specimens were deposited at the IMSS Herbarium in Mexico City.

2.3. Preparation of the extracts and isolation of compounds

Plant extracts were prepared from leaf samples (300 g) as already described (Andrade-Cetto et al., 2000), resulting in a yield of 45 g of aqueous extract (WE) and 4.5 g of butanolic extract (BE). The latter was used for the phytochemical identification of the main components. The BE was applied on a 100 × 2 cm Polygoprep 60–30 C_{18} (Macherey & Nagel, Düren, Germany) flash-column and eluted with H_2O/MeOH/AcCN 80:10:10, 4 ml/min (10 ml fractions; **1**: fr. 16–18, **2**: 23–26). The resulting fractions were monitored by HPLC (ET 250/8/4 Nucleosil 120–5 C_{18}, Macherey & Nagel; 0.04 m H_3PO_4/AcCN/MeOH, 0–9 min.: 85/8/7 to 70/15/15, to 20 min.: 70/15/15; 1.5 ml/min.; 220 and 255 nm UV-det. R_t of **1**: 5.1 min., **2**: 9.5 min.). Prep. HPLC (SP 250/10 Nucleosil 120–7 C_{18}, Macherey & Nagel) was used for final purification yielding compounds **1** (12 mg), **2** (8mg).

The structures (Fig. 1) were established by spectroscopy.

Here, for **1** the NMR data give evidence for a caffeoylquinic acid. The structure of **1** is especially determined by the ^{1}H coupling of H-3, H-4 and H-5. Thus, the values of 5.12 ppm, 8.5 and 9.0 Hz (C-3H), 3.53 ppm, 9.0 and 2.8 Hz (C-4H) as well as 3.94 ppm, 2.8, 2.4 and 1.5 Hz (C-5H) proof for the quinic acid part a $^1C_4$-form leading to the conclusion that **1** shows the structure of chlorogenic acid (3-caffeoylquinic acid). These date are similar to those described earlier within a range of 0.4 ppm (^{1}H) and 3 ppm (^{13}C), respectively (Corse et al., 1966; Kelley et al., 1976). The ^{1}HNMR data for **2** established the possible structure of a flavone by the aromatic protons at 7.37, 7.35, 6.82 and 6.35 ppm (C-6′H, C-2′H, C-5′H, C-8H) and the olefinic proton at C-3 at 6.55 ppm. The high-field shift of C-1″H (4.55 ppm) indicated the C-glucosidation at C-6. The ^{1}H as well as the ^{13}CNMR-data (within a range of 2 ppm) are in agreement with those already reported earlier for isoorientin (Markham et al., 1982).

2.4. Animals

Male Wistar rats, 8 weeks old (weighting 280–300 g) obtained from the Bioterium of the Science School, UNAM, and acclimatised with free access to food and water for at least 1 week in an air conditioned room (25 °C with 55% humidity) under a 12-h light:12-h dark cycle prior to the experiments.

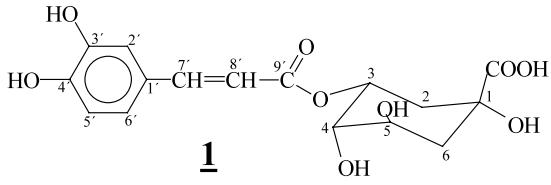
2.5. Induction of experimental diabetes

Diabetes was induced by a single intraperitoneal injection of a freshly prepared streptozotocin (STZ) solution (Sigma, No. 242-646-8) (50 mg/kg in acetate buffer 0.1 M, pH 4.5) to overnight-fasted rats. Control rats received only the buffer.

Diabetes was identified by polydipsia, polyuria and by measuring non-fasting plasma glucose levels 48 h after injection of STZ. Animals, which did not develop more than 250 mg/dl glucose levels, were rejected.

2.6. Experimental groups

The diabetic animals were classified into six groups (1–6) each of them with 11 rats and two groups with six rats (7–8). Group 1 as a control received 1.5 ml of physiological NaCl-solution (vehicle), group 2 was given a standard oral hypoglycemic agent,



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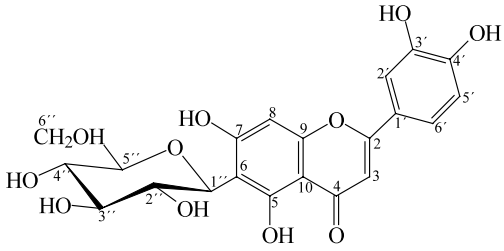

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Fig. 1. Structure of **1** and **2**.

Table 1
Traditional Uses of *Cecropia obtusifolia*

Zone	Local names	Part used	Preparation and administration
San Felipe Usila, Oaxaca, Voucher IMSS11496	Chancarro, Amac'ma	Leaves	Dry leaves are boiled in 1 l water, and the infusion is drunk during the day[a]
Tlanchinol, Hidalgo, Voucher IMSS14140	Chiflador, Hormiguillo	Leaves	Dry leaves are Boiled in 2l water, the infusion is drunk during the day[a]

[a] The amount of boiled plant material goes from three leaves approximately 36 g to five leaves approximately 60 g.

glibenclamide (3 mg/kg bodyweight (bw)), in the same vehicle, while groups 3 and 4 received WE (90 mg/kg bw) and WE (150 mg/kg bw), groups 5 and 6 received BE (9 mg/kg bw) and BE (15 mg/kg bw), respectively. Group 7 received compound **1**, Chlorogenic acid (10 mg/kg bw), and Group 8 received compound **2**, Isoorientin (10 mg/kg bw). The extracts were redissolved in 1.5 ml of physiological NaCl-solution and administered orally by a canule.

2.7. Collection of blood and determination of blood glucose

Blood samples were taken from the tail vein before oral administration of the extracts or the vehicle (time 0) and 60, 120 and 180 min thereafter. Thirty-two μl of blood were used for each assay; the glucose concentration was measured in plasma serum with a Reflotron equipment (Boehringer-Mannheim).

2.8. Statistical analysis

The data were statistically analyzed by unpaired *t*-test. The plasma glucose levels were expressed as the mean (S.E.M.).

3. Results

3.1. Ethnobotany

The results of the field study are summarized in Table 1, where the local name is listed, the voucher herbarium number the used parts of the plant and the preparation and administration. We confirmed that the main use of *C. obtusifolia* is as a hypoglycemic agent. Normally the people drink the infusion of the leaves after boiling between three (approximately 36 g) and five (approximately 60 g) leaves in 1 l water.

3.2. Identification of compounds

3.2.1. Chlorogenic acid (*1*)

LC-MS *m/z* (rel. int): 355.25 $[M]^+ + 1$ $C_{16}H_{19}O_9$ (100) (calc. 355.39), 320.21 (11.5) $C_{16}H_{16}O_7$, 248.15 (73.5) $C_{13}H_{12}O_5$, 220.08 (35) $C_8H_{12}O_7$.

3.2.2. ^1H NMR (δ = ppm) data

7.44 (1H, d, $J_{7',8'} = 15.8$ Hz, C-7′H), 7.04 (1H, d, $J_{2',6'} = 1.5$ Hz, C-2′H), 6.95 (1 H, dd, $J_{6',5'} = 8.2$ Hz, $J_{6',2'} = 1.5$ Hz, C-6′H), 6.76 (1H, d, $J_{5',6'} = 8.2$ Hz, C-5′H), 6.26 (1H, d, $J_{8',7'} = 15.8$ Hz, C-8′H), 5.12 (1 H, dd, $J_{3,2} = 8.5$ Hz (calc. 11 Hz), $J_{3,4} = 9.0$ Hz (calc. 9.2 Hz), C-3H), 3.94 (1H, ddd, $J_{5,4} = 2.8$ Hz (calc. 3 Hz), $J_{5,6\alpha} =$ 2.4 Hz (calc. 3.6 Hz), $J_{5,6\beta} = 1.5$ Hz (calc. 2.8 Hz), C-5H), 3.53 (1 H, dd, $J_{4,3} = 9.0$ Hz, $J_{4,5} = 2.8$ Hz, C-4H), 1.95 (1 H, dd, $J_{6\alpha,6\beta} = 11.9$ Hz, $J_{6\alpha,5} = 2.4$ Hz, C-6H_α), 1.81 (2H, d, $J_{2,3} = 8.5$ Hz, C-2H_2), 1.67 (1 H, dd, $J_{6\beta,6\alpha} = 11.9$ Hz, $J_{6\beta,5} = 1.5$ Hz, C-6H_β). ^{13}C NMR (δ = ppm) data: 168.0 (s, C-9′), 148.9 (s, C-4′), 146.1 (s, C-3′), 146.0 (d, C-7′), 126.7 (s, C-1′), 122.8 (d, C-6′). 116.8 (d, C-5′), 115.5 (d, C-8′), 115.1 (d, C-2′), 76.7 (s, C-1), 73.6 (d, C-4), 72.2 (d, C-3), 72.0 (d, C-5), 40.0 (t, C-2), 38.4 (t, C-6).

3.2.3. Isoorientin: (*2*)

^1H NMR: (400 MHz, DMSO-d_6, δ in *ppm*) data: 7.37 (C-6′H, dd, $J = 8.1$ Hz, 1.9 Hz, 1H), 7.35 (C-8H, s, 1H), 6.82 (C-5′H, d, $J = 8.1$ Hz, 1H), 6.55 (C-3H, s, 1H), 6.35 (C-2′H, d, $J = 1.9$ Hz, 1H), 4.55 (C-1″H, d, $J = 9.9$ Hz, 1H), 3.63 (C-5″H, d, $J = 10.7$ Hz, 1H), 3.40 (C-2″H, dd, $J = 10.5$ Hz, $J = 6.3$ Hz, 1H), 3.20 (C-3″H, d, $J = 8.5$ Hz, 1H), 3.17 (C-4″H, t, $J = 8.2$ Hz, 1H), 3.15 (C-6″H_2, t, $J = 8.0$ Hz, 2H). ^{13}C NMR: (100 MHz, DMSO-d_6, δ = *ppm*) data: 182.1 (C-4, s), 164.1 (C-2, s), 160.9 (C-7, s), 157.4 (C-9, s, C-5, s), 150.6 (C-3′, s), 146.3 (C-4′, s), 121.9 (C-1′, s), 119.8 (C-6′, d), 116.8 (C-5′, d), 113.4 (C-2′, d), 109.5 (C-6, s), 103.1 (C-3, d), 102.9 (C-10, s), 95.1 (C-8, d), 81.8 (C-5″, d), 79.4 (C-3″, d), 73.9 (C-1″, d), 70.9 (C-2″, d), 70.7 (C-4″, d) 61.9 (C-6″, t).

3.3. Activity in diabetic rats

STZ administration at a dosage of 50 mg/kg bw to normal rats significantly ($P < 0.001$) elevated the blood glucose levels compared with rats injected citrate buffer alone as reported (El-Fiky et al., 1996) for albino rats.

In our diabetic rats the extracts as well as the two isolated compounds both showed significant hypoglycemic effects. Table 2 and Fig. 2.

The water extract at doses of 90 mg/kg bw showed activity at 60 min, with a significant reduction ($P <$ 0.001). After 60 min the significance was reduced to

Table 2
Effect of oral administration of aqueous and butanolic extracts of *Cecropia obtusifolia* aerial parts on plasma glucose concentration in diabetic rats

Dose (mg/kg)	Plasma glucose (mg/ml) at			
	0 h	1 h	2 h	3 h
Control (Saline 2.5 ml)	316 ± 4	324 ± 5	314 ± 5a	312 ± 4
Glibenclamide (mg/kg)	311 ± 6	298 ± 6***	262 ± 6***	245 ± 8***
Water extract (90 mg/kg)	305 ± 8	282 ± 7***	278 ± 8**	259 ± 11***
Water extract (150 mg/kg)	308 ± 7	281 ± 7***	256 ± 7***	257 ± 8***
Butanol Extract (9 mg/kg)	310 ± 6	292 ± 8**	265 ± 8***	260 ± 11***
Butanol Extract (15 mg/kg)	303 ± 8	276 ± 7***	262 ± 7***	257 ± 6***
1 Chlorogenic acid (10 mg/kg)	304 ± 6	270 ± 10***	247 ± 12***	226 ± 11***
2 Isoorientin (10 mg/kg)	303 ± 5	265 ± 8***	229 ± 11***	221 ± 12***

The values represent the mean ± S.E.M. The number of rats for **1** and **2** was eight for the rest of the groups was = 11; $^{**}P<0.005$, $^{***}P<0.001$ as compared with control time intervals.

$P < 0.005$, and went down again at 120 min to $P < 0.001$. The water extract at doses of 150 mg/kg bw showed activity from 60 to 180 min with $P < 0.001$.

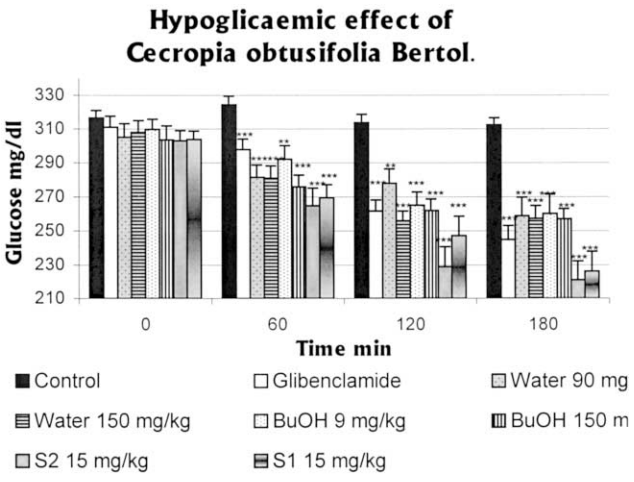

Fig. 2. Effect of oral administration of water and butanolic extract of aerial parts of *Cecropia obtusifolia* in diabetic rats. The number of rats was 11 in all cases. $^{**}P < 0.005$ and $^{***}P < 0.001$ as compared with control time intervals.

The maximum effect of the water extracts was observed after 180 min.

The butanolic extract led to a significant decrease in plasma glucose level compared with the control, at doses of 9 mg/kg bw the effect was significant after 60 min with $P < 0.005$ ongoing with $P < 0.001$ at 120 and 180 min. At doses of 15 mg/kg bw the activity was significant since 60 min until 180 min with $P < 0.001$. The maximum activity was observed after 180 min comparable to the water extract.

Chlorogenic acid **1** and the Isoorientin **2** showed a similar activity with $P < 0.001$ at 60 min ongoing to 120 and 180 min, with the same significance and the glibenclamide group (3 mg/kg) produced a significant decrease compared with the controls, with $P < 0.001$ at 60 min until 180 min.

Those results support acceptance of the null hypothesis that there is no significant difference between the tested plant mediums in comparison to glibenclamide (= standard hypoglycemic drug).

4. Discussion

Our own ethnopharmacological studies confirm the earlier reported data for the plant *C. obtusifolia* which is traditionally used as an infusion of (mainly) dried leaves by the Mexican population against type II diabetes.

There is no previous report about a hypoglycemic activity of isoorientin or chlorogenic acid. However, isorientine is reported to show antimicrobiological and antispasmodic activities (Afifi et al., 1999). Besides this, a hypoglycemic activity of some flavonoids is described and reported in literature (Lamba et al., 2000).

The STZ diabetes induction and the use of glibenclamide in this animal model were previously discussed (Andrade-Cetto et al., 2000).

Both, water and butanolic extracts of *C. obtusifolia* produce hypoglycemic effects in rats. This is in accordance with a previous report where water infusions of the plant at unspecified concentration and administered doses were used in alloxan diabetic mice (Pérez et al., 1984). Furthermore our results are similar to those, which described a hypoglycemic effect of a water extract of the same plant in hyperglycemic rabbits at doses of 528 mg/kg (Roman-Ramos et al., 1991). In contrast to the last study which described the maximum effect at 240 and 300 min. we found that the effect is significant after 60 min $P < 0.005$ and had an increased effect ($P < 0.001$) at 120 and 180 min.

Summarizing this present study we found activities from 60 min until 180 min for both extracts. These data are comparable to those that we found after glibenclamide administration.

Thus, one can speculate that the water as well as the butanolic extract of *C. obtusifolia* may possess a glibenclamide-like effect.

Besides this, we demonstrated that the main components in the plants are isoorientin and chlorogenic acid and that both substances are mainly present in both extracts, too. As these compounds showed also significant hypoglycemic activity it may be assumed that these compounds are involved in the hypoglycemic effect of *C. obtusifolia*.

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Evaluation of anti-inflammatory activity of *Pongamia pinnata* leaves in rats

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Abstract

In the present study, the anti-inflammatory activity of 70% ethanolic extract of *Pongamia pinnata* leaves (PLE) in acute, subacute and chronic models of inflammation was assessed in rats. *Per os* (p.o.) administration of PLE (300, 1000 mg/kg) exhibited significant anti-inflammatory activity in acute (carrageenin, histamine, 5-hydroxytryptamine and prostaglandin E_2-induced hind paw edema), subcute (kaolin-carrageenin and formaldehyde-induced hind paw edema) and chronic (cotton pellet granuloma) models of inflammation. PLE did not show any sign of toxicity and mortality up to a dose level of 10.125 g/kg, p.o. in mice. Both acute as well as chronic administration of PLE (100, 300 and 1000 mg/kg, p.o.) did not produce any gastric lesion in rats. These results indicate that PLE possesses significant anti-inflammatory activity without ulcerogenic activity suggesting its potential as an anti-inflammatory agent for use in the treatment of various inflammatory diseases. © 2001 Published by Elsevier Science Ireland Ltd.

Keywords: *Pongamia pinnata*; Anti-inflammatory; In vivo

1. Introduction

Inflammatory diseases including different types of rheumatic diseases are very common throughout the world. Although rheumatism is one of the oldest known diseases of mankind affecting the majority of population, no substantial progress has been made in achieving a permanent cure. The greatest disadvantage in presently available potent synthetic drugs lies in their toxicity and reappearance of symptoms after discontinuation. Therefore, the screening and development of drugs for their anti-inflammatory activity is still in progress and there is much hope for finding anti-inflammatory drugs from indigenous medicinal plants. *Pongamia pinnata* (Linn) Pierre (Leguminosae, Papilionaceae; synonym, *Pongamia glabra* Vent), popularly known as 'Karanj' or 'Karanja' in Hindi, is a medium sized glabrous tree, found throughout India and further distributed eastwards, mainly in the littoral regions of South Eastern Asia and Australia (Satyavati et al., 1987). In the ayurvedic literature of India, different parts of this plant have been recommended as a remedy for various ailments. The seed and seed oil of this plant have been used for treating various inflammatory and infectious diseases such as leucoderma, leprosy, lumbago, muscular and articular rheumatism (Nadkarni, 1954). The leaves are hot, digestive, laxative, anthelmintic and cure piles, wounds and other inflammations (Kirtikar and Basu, 1933). A hot infusion of leaves is used as a medicated bath for relieving rheumatic pains and for cleaning ulcers in gonorrhea and scroflilous enlargement (Chopra et al., 1933; Satyavati et al., 1987). While different extracts of roots and seeds (ethanol, petroleum ether, benzene extracts and others) of *P. pinnata* have been reported to have anti-inflammatory activity (Singh and Pandey, 1996; Singh et al., 1996), its leaves still remain unexplored. The present study is an attempt to address this issue. The 70% ethanolic extract of *P. pinnata* leaves (PLE) was evaluated in acute, subacute and chronic models of inflammation in rats. In addition, the effect of the extract on gastric mucosa of rats was also studied to find out whether it has any ulcerogenic activity.

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2. Materials and methods

2.1. Plant material

The mature green leaves of *P. pinnata* were collected locally from the premises of Indian Veterinary Research Institute (IVRI), Izatnagar in the month of October 1998 and were authenticated by Dr B.N. Pandey, Department of Botany, Bareilly College, Bareilly (India). The voucher specimen (ID 2000:23) is deposited in the herbarium of Indigenous Drugs Laboratory, Division of Pharmacology and Toxicology, IVRI, Izatnagar (India).

2.2. Preparation of ethanolic extract

The shade-dried and powdered leaves were subjected to extraction with 70% ethanol under reflux for 8 h and concentrated to a semi solid mass under reduced pressure. The yield was about 24% (w/w) of the starting crude material. In the preliminary phytochemical screening (Trease and Evan, 1959), this 70% ethanolic extract of PLE gave positive tests for glycosides, sterols and tannins and negative for flavones. PLE and standard reference drug were dissolved in normal saline and administered orally to rats in dose volume of 1 ml/kg body weight. The dilutions were prepared every time just prior to administration.

2.3. Chemicals

Carrageenin, histamine, 5-hydroxytryptamine (5-HT), prostaglandin E_2 (PGE_2), bradykinin, acetylsalicylic acid (ASA) and phenylbutazone (PBZ) were purchased from Sigma Chemicals, St. Louis, MO, USA. Kaolin was purchased from Hi-Media Laboratories, Mumbai, India and formaldehyde from British Drug House, Mumbai, India.

2.4. Experimental animals

Experiments were performed using Wistar rats (150–180 g) and Swiss mice (25–30 g) of either sex, procured from the Laboratory Animal Resource Section of IVRI, Izatnagar (India). All the animals were maintained under controlled room temperature (22 ± 2 °C) and relative humidity ($50 \pm 5\%$) with 12:00 h light, 12:00 h dark cycle. The animals were housed in colony cages (three animals per cage) with free access to feed (a balanced ration obtained from the Feed Technology Unit of IVRI) and water. Guidelines of Institute Animal Ethics Committee were followed while using live animals. All the animals were acclimatized to the laboratory environment for 5 days before the experiment. Six animals (rats or mice) per group comprising of three males and three females, were used in each experiment, unless otherwise specified. The animals were fasted overnight just prior to the experiment but allowed free access to drinking water.

2.5. Acute toxicity study (oral)

The PLE was administered orally in graded doses of 3.0, 4.5, 6.75 and 10.125 g/kg to four different groups of mice, while the control group received vehicle (2 ml/kg, p.o.) alone. All treated animals were closely observed for any abnormal or toxic manifestations and for mortality up to the end of 24 h in each group to calculate LD_{50} by the method described by Weil (1952). Based on the results obtained from the preliminary toxicity study, the doses for further pharmacological studies were fixed to be 100, 300 and 1000 mg/kg, p.o.

2.6. Anti-inflammatory activity

2.6.1. Study of PLE on acute inflammation

2.6.1.1. Carrageenin-induced hind paw edema in rats. The acute hind paw edema was produced by injecting 0.1 ml of carrageenin (prepared as 1% suspension in sterile normal saline) locally into the plantar aponeurosis of the right hind paw of rats (Winter et al., 1962). PLE (100, 300 and 1000 mg/kg, p.o.) was administered to three different groups while the other two groups served as negative and positive controls and received vehicle, normal saline (1 ml/kg, p.o.) and standard drug, acetylsalicylic acid (ASA, 300 mg/kg, p.o.), respectively. PLE and ASA were administered 1 h prior to the injection of carrageenin. The rat pedal volume up to the ankle joint was measured using plethysmometer (Ugo Basile, Italy) at 0 (just before) and 3 h after the injection of carrageenin. Increase in the paw edema volume was considered as the difference between 0 and 3 h. Percent inhibition of edema volume between treated and control groups was calculated as follows:

$$\text{Percent inhibition} = \frac{V_c - V_t}{V_c} \times 100$$

where V_c and V_t represent mean increase in paw volume in control and treated groups, respectively. The median inhibitory dose (ID_{50}) was calculated by the method of Reed and Muench (1938).

2.6.1.2. Autacoid-induced hind paw edema in rats. This experiment was conducted as per the methodology used by Singh and Pandey (1996). The autacoids, viz., histamine (1 mg/ml), 5-hydroxytryptamine (1 mg/ml), prostaglandin E_2 (1 μg/ml) and bradykinin (20 μg/ml) were employed as phlogistic agents. The effect of PLE (100, 300 and 1000 mg/kg, p.o.) was tested individually against each autacoid. Right hind paw edema was induced by the sub plantar injection of 0.1 ml of

respective phlogistic agent. Test compounds were administered 1 h prior to the inflammatory insult. The pedal volume was measured just before (0 h) and 3 h after the phlogistic challenge. Phenylbutazone (100 mg/kg, p.o.) was employed as reference standard in all the above experiments. The ID_{50} was calculated as mentioned above.

2.6.2. Study of PLE on subacute inflammation

2.6.2.1. Kaolin–carrageenin-induced hind paw edema in rats. According to the method of Nakamura et al. (1978), hind paw edema was produced by sub plantar injection of 0.2 ml suspension of 20% kaolin and 1% carrageenin mixture in the right hind paw of the rats, say at 16:00 h. PLE (100, 300 and 1000 mg/kg, p.o.) and ASA (300 mg/kg, p.o.) were given three times (next day at 10:00, 13:00 and 17:00 h) to four different groups, respectively, beginning at 18 h after the phlogistic challenge, while the control group received comparable volume of normal saline. The hind paw volume was measured in plethysmometer just before (0 h) and at 3, 6, 8 and 24 h after the first dosing of test compounds. The difference in the paw volume observed at 3, 6, 8 and 24 h from that of 0 h was taken as indicator of increase in paw volume at corresponding time intervals.

2.6.2.2. Formaldehyde-induced hind paw volume. The test was performed according to the technique developed by Brownlee (1950). Pedal inflammation was induced by injecting 0.1 ml of 4% formaldehyde solution below the plantar aponeurosis of the hind paw of the rats. The paw volume was recorded immediately prior to compound administration (0 h) and then at 1.5, 24 and 48 h after formaldehyde injection. Vehicle (1 ml/kg, p.o.), PLE (100, 300 and 1000 mg/kg, p.o.) and standard drug, ASA (300 mg/kg, p.o.) were administered 1 h prior to formaldehyde injection.

2.6.3. Study of PLE on chronic inflammation

2.6.3.1. Cotton pellet granuloma in rats. The effect of PLE on chronic or proliferative phase of inflammation was assessed in cotton pellet granuloma rat model as described by Winter and Porter (1957). Autoclaved cotton pellets weighing 35 ± 1 mg each were implanted subcutaneously through small incision made along the axilla or flank region of the rats anesthetized with ether. The different groups of rats were administered the PLE (100, 300 and 1000 mg/kg, p.o.) and ASA (300 mg/kg, p.o.) once daily for seven consecutive days from the day of cotton pellet insertion. The control group received normal saline alone. On the eighth day, all the rats were sacrificed and the cotton pellets covered by the granulomatous tissue were excised and dried in hot air oven at 60 °C till a constant weight was achieved. Granuloma weight was obtained by subtracting the weight of cotton pellet on 0 day (before start of experiment) from the weight of the cotton pellet on eighth day (at the end of experiment).

2.7. Ulcerogenic activity

2.7.1. Acute ulcerogenic activity

The ulcerogenic potential of PLE at three different doses (100, 300 and 1000 mg/kg, p.o.) was tested in overnight fasted male rats. The control group was administered vehicle (1 ml/kg, p.o.), while the other group received standard drug, ASA (300 mg/kg, p.o.), respectively. All the animals were killed with anesthetic ether 5 h after the administration of test compounds. The stomachs were dissected out, incised along the greater curvature, and then put in diluted formaldehyde solution (2.5%). A few minutes later, mucosa of the stomach was observed for petechial hemorrhages and ulcers, if any. The degree of ulceration was graded according to the arbitrary scale as described by Pagella et al. (1983).

2.7.2. Chronic ulcerogenic activity

The experiment was carried out using male Wistar rats with free access to feed and drinking water throughout the period of experiment. The rats were administered vehicle (1 ml/kg, p.o.), PLE (100, 300 and 1000 mg/kg, p.o.) and ASA (300 mg/kg. p.o.), once daily for 14 consecutive days. All the animals were sacrificed 24 h after the administration of the last dose of the drug and the stomachs were removed and examined as in the acute experiment (Section 2.7.1).

2.8. Statistical analysis

All data are expressed as mean $\pm$ S.E.M. The data were analyzed using Student's t-test. A value of $P < 0.05$ or less was considered statistically significant.

3. Results

3.1. Acute toxicity study (oral)

On gross examination, all animals given PLE at the doses of 3, 4.5, 6.75 and 10.125 g/kg, p.o. were devoid of toxic symptoms and mortality.

3.2. Carrageenin-induced hind paw edema

The mean increase in paw edema volume was about 0.86 ± 0.13 ml in the vehicle-treated control rats. PLE (300 and 1000 mg/kg, p.o.) significantly ($P < 0.01$) reduced the mean paw edema volume at 3 h after carrageenin injection. PLE (100, 300 and 1000 mg/kg, p.o.)

exhibited anti-inflammatory activity in a dose-dependent manner with the percent inhibition of paw edema of 27.9, 55.81 and 60.47, respectively, as compared with the control group. However, the standard drug, ASA (300 mg/kg, p.o.) showed highly significant ($P < 0.01$) anti-inflammatory activity with the percent inhibition of 74.42 (Table 1).

3.3. Autacoid-induced hind paw edema

The mean increase in paw edema volume produced at 3 h after injection of different autacoids, viz., histamine, 5-HT, PGE_2 and bradykinin was 0.25 ± 0.01, 0.44 ± 0.01, 0.26 ± 0.03 and 0.28 ± 0.04 ml respectively. PLE (300 and 1000 mg/kg, p.o.) significantly inhibited ($P < 0.05$, $P < 0.001$) hind paw edema induced by histamine, 5-HT and PGE_2 but not that of bradykinin. However, PBZ (100 mg/kg, p.o.) significantly ($P < 0.001$, $P < 0.05$) inhibited all autacoids including bradykinin-induced hind paw edema with the percent inhibition greater than that produced by PLE (1000 mg/kg, p.o.) as shown in Table 2.

3.4. Formaldehyde-induced hind paw edema

PLE (300 and 1000 mg/kg, p.o.) significantly diminished the mean paw edema volume at 1.5 ($P < 0.001$) and 24 h ($P < 0.05$, $P < 0.01$). The maximum percent inhibition of edema volume produced by PLE (1000 mg/kg, p.o.) was almost comparable to that of ASA (300 mg/kg, p.o.) (49.29 vs. 46.47 at 1.5 h). Interestingly, the effect of PLE persisted up to a period of 24 h in contrast to ASA, the effect of which was significant only at 1.5 h (Table 3).

3.5. Kaolin–carrageenin-induced hind paw edema

PLE (300 and 1000 mg/kg, p.o.) given at three different intervals produced significant ($P < 0.05$, $P < 0.01$) anti-inflammatory activity at various time intervals from 3 h onwards, which persisted up to experimental duration of 24 h. The standard drug, ASA (300 mg/kg, p.o.) showed significant as well as ($P < 0.05$, $P < 0.01$) greater percent inhibition of hind paw edema than PLE from 3 h onwards at different time intervals up to 24 h of observation period (Fig. 1).

3.6. Cotton pellet granuloma

The study of PLE on proliferative phase of inflammation indicated that PLE (300 and 1000 mg/kg, p.o.) slightly but significantly ($P < 0.05$) reduced the granuloma formation with percentage inhibition of 12.5 and 16.64 as compared with ASA (300 mg/kg, p.o.), which showed significant ($P < 0.01$) inhibition on granuloma formation with the percent inhibition of 43.04 (Table 1).

3.7. Ulcerogenic activity

Rats administered PLE as a single dose or chronically for 14 days were found to be devoid of gastric lesions in contrast to the standard anti-inflammatory agent, ASA that significantly ($P < 0.05$, $P < 0.01$) induced the gastric lesions with the mean score of severity of 2.33 ± 0.42 in acute and 2.67 ± 0.33 in chronic studies, as compared with the vehicle treated rats. The gastric lesions were associated with accumulation of fluid in the stomach, erosion or congestion of gastric mucosa, pinpoint haemorrhagic spots and few ulcers.

4. Discussion

The present study demonstrates the potent anti-inflammatory activity of the 70% ethanolic extract of PLE in different models of inflammation-acute exudative (carrageenin-induced rat paw edema), subacute (formaldehyde and kaolin carrageenin-induced rat paw edema) and chronic proliferative inflammation (cotton pellet granuloma), thereby indicating the possibility of developing PLE as the cheaper, safer and potent anti-inflammatory therapeutic agent. Although, different parts of *P. pinnata* have been used in the traditional

Table 1
Effect of PLE on carrageenin-induced hind paw edema and cotton pellet granuloma in rats

Drugs	Dose (mg/kg)	Carrageenin-induced hind paw edema volume (ml; mean ± S.E.M.)	Weight of cotton pellet granuloma (mg; mean ± S.E.M.)
Control	–	0.86 ± 0.13	107.49 ± 3.85
ASA	300	0.22 ± 0.03*** (74.42)	61.22 ± 1.57*** (43.04)
PLE	100	0.62 ± 0.11 (27.90)	100.47 ± 1.87 (6.53)
	300	0.38 ± 0.02** (55.81)	94.05 ± 4.07* (12.50)
	1000	0.34 ± 0.05** (60.47)	89.60 ± 2.98** (16.64)
		$ID_{50} = 525.21$ mg/kg	$ID_{50} = 2579.67$ mg/kg

$n = 6$ in each group; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. control group. The figures in parentheses indicate the percent inhibition of edema volume or granuloma formation in comparison to control group.

Table 2
Effect of PLE on autacoid-induced hind paw edema in rats

Drugs	Dose (mg/kg)	Autacoid-induced paw edema volume (ml; mean ± S.E.M.)			
		Histamine	5-HT	PGE_2	Bradykinin
Control	–	0.25 ± 0.01	0.44 ± 0.01	0.26 ± 0.03	0.28 ± 0.04
ASA	300	0.12 ± 00.02*** (52.00)	0.21 ± 0.02*** (53.53)	0.05 ± 0.01*** (80.76)	0.16 ± 0.02* (42.86)
PLE	100	0.23 ± 0.05 (8.00)	0.43 ± 0.01 (2.27)	0.24 ± 0.02 (7.69)	0.26 ± 0.03 (7.14)
	300	0.22 ± 0.02 (12.00)	0.38 ± 0.02* (13.63)	0.22 ± 0.05 (15.38)	0.28 ± 0.03 (0.00)
	1000	0.20 ± 0.02* (20.00)	0.32 ± 0.02*** (27.27)	0.19 ± 0.02* (26.92)	0.27 ± 0.02 (3.57)
		ID_{50} = 2350 mg/kg	ID_{50} = 1728.37 mg/kg	ID_{50} = 1753.58 mg/kg	–

$n = 6$ in each group; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. control group. The figures in parentheses indicate the percent inhibition of edema volume in comparison to control group.

systems of medicine for treating various ailments including febrile and inflammatory disorders and the seeds and roots of this plant have already been reported to possess significant anti-inflammatory and analgesic activities (Singh et al., 1996, 1997), there was no scientific evidence as regards to the anti-inflammatory activity of the leaves.

In acute and subacute models of inflammation, upon challenge by phlogistic stimuli, PLE showed significant anti-inflammatory activity. The edema and inflammation induced by carrageenin is shown to be mediated by histamine and 5-HT during first 1 h, after which increased vascular permeability is maintained by the release of kinins up to 2.30 h and from 2.30 to 6 h, the mediators appear to be prostaglandins, the release of which is closely associated with migration of leucocytes into the inflamed site (Di Rosa et al., 1971). In autacoid-induced inflammations, PLE produced significant inhibitory activity against histamine-, 5-HT-and PGE_2-induced hind paw edema in rats but failed to exhibit activity against bradykinin-induced hind paw edema. Inflammation induced by formaldehyde is biphasic, an early neurogenic component is mediated by substance P and bradykinin followed by a tissue mediated response where histamine, 5-HT, prostaglandins and bradykinin are known to be involved (Wheeler Aceto and Cowan, 1991). In the formaldehyde-induced inflammation, the PLE demonstrated significant anti-inflammatory activity that lasted up to 24 h in contrast to ASA, which was effective only at 1.5 h, suggesting its long duration of action. In kaolin–carrageenin-induced inflammation, PLE (1000 mg/kg, p.o.) administered three times at different intervals significantly reduced the paw edema as compared with that of ASA (300 mg/kg, p.o.). Unlike the other tests, PLE treatment was started 18 h after the injection of phlogistic agent, i.e. the activity of PLE was tested against the already 'established' inflammation that further substantiates its potential in curative purposes of various inflammatory diseases. In autacoid-induced inflammation, PLE exhibited a significant inhibitory action against histamine-, 5-HT-and PGE_2-induced hind paw edema, which indicates that the extract exhibits its anti-inflammatory action by means of either inhibiting the synthesis, release or action of inflammatory mediators, viz., histamine, 5-HT and prostaglandins involved in inflammation. However, PLE did not have any inhibitory action against bradykinin-induced hind paw edema in contrast to the seed of *P. pinnata*, which showed maximum anti-inflammatory activity against bradykinin as well as prostaglandin E_1-induced hind paw edema as reported by Singh and Pandey (1996). The difference in action between seed and leaves of *P. pinnata* on bradykinin-induced paw edema cannot be explained presently and needs further investigations. In order to assess its efficacy against proliferative phase of inflammation in which tissue degeneration and fibrosis occur, the widely used cotton pellet granuloma test was employed. During the repair process of inflammation, there is proliferation of macrophages, neutrophils, fibroblasts and multiplication of small blood vessels, which are the basic sources of forming a highly vascularised reddish mass, termed granulation tissue (Swingle, 1974; Bhattacharya et al., 1992). Though PLE (300 and 1000 mg/kg, p.o.) significantly reduced the granuloma formation the effect was of less intensity, when compared with ASA (300 mg/kg, p.o.; the maximum percent inhibition of 16.4 vs. 43.04). The mechanism of anti-inflammatory activity of PLE on proliferative phase of inflammation in a rat model of cotton pellet granuloma is not exactly known and needs further study.

The main side effect of non-steroidal anti-inflammatory drugs is their ability to produce gastric lesions (Pagella et al., 1983). During the acute and chronic ulcerogenic studies, PLE did not induce any adverse effect on gastric mucosa, indicating nonulcerogenic activity. From the acute, subacute and chronic studies, it is obvious that PLE possesses good anti-inflammatory activity, interestingly without any ulcerogenic activity.

In conclusion, the present study clearly showed that 70% ethanolic extract of PLE possessed good anti-inflammatory activity and also scientifically validated the

Table 3
Effect of PLE on formaldehyde-induced hind paw edema in rats

Drugs	Dose (mg/kg)	Formaldehyde-induced hind paw edema volume (ml)		
		1.5 h	24 h	48 h
Control	–	0.71 ± 0.04	0.95 ± 0.06	0.55 ± 0.06
ASA	300	0.38 ± 0.05*** (46.47)	0.84 ± 0.05 (11.57)	0.54 ± 0.08 (1.81)
PLE	100	0.54 ± 0.03** (23.94)	0.87 ± 0.08 (8.42)	0.54 ± 0.07 (1.81)
	300	0.46 ± 0.03*** (35.21)	0.73 ± 0.05* (23.15)	0.51 ± 0.05 (7.27)
	1000	0.36 ± 0.04*** (49.29)	0.71 ± 0.04** (25.26)	0.47 ± 0.06 (14.54)

$n = 6$ in each group; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. control group. The figures in parentheses indicate the percent inhibition of edema volume in comparison to control group.

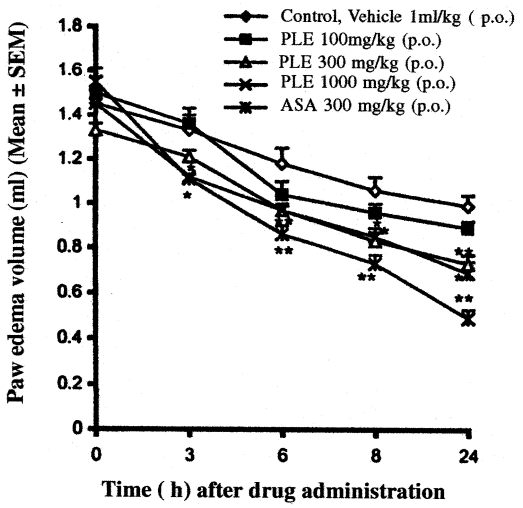

Fig. 1. Effect of PLE on kaolin–carrageenin-induced hind paw oedema in rats $n = 6$ in each group. *, $P < 0.05$; **, $P < 0.01$ vs. control group.

use of this plant for treating inflammatory disorders in the folk medicine. The advantages of PLE, viz., better and safer anti-inflammatory profile without ulcerogenic activity deserves further studies (sub fractionation of PLE and separation of active principles) to identify the possible mechanism of action as well as establishing the therapeutic value in the treatment of inflammatory diseases.

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Extracts of *Benincasa hispida* prevent development of experimental ulcers

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Abstract

Benincasa hispida (*B. hispida*) is recommended in Ayurveda for the management of peptic ulcers. Therefore, anti-ulcerogenic activity of different extracts of *B. hispida* (fresh juice, supernatant and residue fraction of centrifuged juice, alcoholic and petroleum ether extract) were studied in aspirin plus restraint, swimming stress, indomethacin plus histamine and serotonin-induced ulcers in rats and mice. The oral feeding of different doses of the extract significantly reduced the ulcer index produced by various ulcerogens. The anti-ulcerogenic effect was dose-dependent in stress induced model of ulcer and not in other models. *B. hispida* probably has a CNS component in prevention of stress induced ulceration. However, antihistaminic, anti-cholinergic effects and prevention of disturbance in gastric micro-circulation as possible modes of action cannot be ruled out. Chronic toxicity studies carried out for 3 months revealed no deleterious effect of fresh juice of *B. hispida* on various hematological and biochemical parameters studied. Thus, extracts of *B. hispida* may be considered to be a drug of natural origin possessing anti-ulcer activity.

Keywords: *Benincasa hispida*; *Benincasa cerifera*; Cucurbitaceae; Experimental ulcers; Traditional medicine; Ayurveda

1. Introduction

Although modern medicines may be available, due to socio-economical, cultural and historical reasons, herbal medicines have maintained their importance. Initially, the treatment for ulcers was primarily aimed at reducing acid secretion through blockade of H2 receptors. Later, as the role of *Helicobacter pylori* in causation of ulcers was recognized, the current therapy involves *H. pylori* eradication regimens and proton pump inhibitors (Laurence, 1996). However, recurrence rates with these therapeutic regimes are still high (Grover and Vats, 1999). Many plants have been found to possess anti-ulcer activity in experimental studies. Further, anti-ulcer activity of few plants like *Glycyrrhiza glabra* has also been demonstrated, both experimentally and clinically (Kassir, 1985).

Benicasa hispida (Thunb). Cogn (Syn. *Benincasa cerifera*) is a widely used vegetable in India and other tropical countries and belongs to the family Cucurbitaceae (Chopra et al., 1956). It is called Petha or Golkaddu in Hindi and White Gourd, Wax Gourd or Ash Gourd in English. It is a large climbing or trailing herb with stout, angular and hispid stems. Young fruit is fleshy, succulent and hairy while the mature fruit has thickly deposited hairs with easily removable waxy bloom. The flesh of the fruit is white and spongy. In Ayurveda, *B. hispida* is recommended for management of peptic ulcer, hemorrhages from internal organs, epilepsy and other nervous disorders (Warier, 1994; Sharma, 1984). Acid neutralizing and ulcer healing activities of *B. hispida* has also been described (CSIR, 2000). According to Raja Nirghantu (an ancient work on therapeutics), medicine from *B. hispida* was prepared from old ripe fruits. The pulp was scraped into thin strips and the water juice that oozes out abundantly was collected and preserved (CSIR, 2000). In our earlier studies, the fresh juice of *B. hispida* showed significant anti-inflammatory activity in cotton pellet

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granuloma and carrageenan induced edema in rats (Grover and Rathi, 1994). The fresh juice was also effective in preventing morphine withdrawal in mice (Grover et al., 2000). Phytochemical screening of *B. hispida* using bioassay-guided separation has shown the presence of 4 triterpenes and 2 sterols together with a flavonoid C-glycoside, an acylated glucose, and a benzyl glycoside (Yoshizumi et al., 1998). Previously, no scientific work has been reported on the anti-ulcerogenic activity of this plant. The present study was therefore undertaken to evaluate the anti-ulcerogenic activity of various extracts of *B. hispida*.

2. Materials and methods

2.1. Plant material

Fresh fruit of *B. hispida* was purchased from the local market during the months of October–November and was authenticated by Dr Manasi Ram, Head, Department of the Botany, Miranda House, Delhi University (Voucher Number 203/97).

2.1.1. Fresh juice supernatant and residue

For the fresh juice, the cuticle of the fruit was peeled off and juice was extracted in an electric juicer (M/S Electro Com., New Delhi). This juice was then centrifuged in a T8 electric centrifuger (Remi Udyog, Bombay) at 5000 rpm for 10 min. After aspirating the supernatant, the residue and the supernatant were collected and stored in separate containers to be used for later experimentation.

2.1.2. Alcoholic extract

For the preparation of an alcoholic extract, 1000 ml of fresh juice was mixed with 5000 ml of ethanol (Glaxo Chemicals Laboratories) and kept covered for 36 h at room temperature. The slurry was stirred intermittently for 2 h and left overnight. The mixture was then filtered and the filtrate was kept in open at room temperature (20–25 °C) for 24 h to dry. The extract was later dried completely using a lyophilizer (Christ freeze dryer, alpha 1-4, Germany). The final extract was off-white powder and the yield of the extract was 1.27 g/100 ml of the fresh juice.

2.1.3. Petroleum ether extract

For the preparation of this extract, 1000 ml of fresh juice of *B. hispida* was subjected to extraction with 90% petroleum ether (Glaxo Chemicals, India) in a soxhlet apparatus up to four cycles. After filtration through Whatman paper no. 40, the filtrate was dried using a lyophilizer (Christ freeze dryer, alpha 1-4, Germany). The yield was 1.73 g/100 ml of fresh juice.

2.2. Animals

Adult male albino rats (150–200 g) and albino male mice (30–50 g) were obtained from the experimental animal facility, All India Institute of Medical Sciences. Before the start and during the experiment, animals were fed standard chow diet (Hindustan Lever Ltd., New Delhi). All the animals were fasted for 24 h before initiation of experiment and allowed free access to drinking water. The animals were kept under standard laboratory conditions in a 12-h light and dark cycle at an ambient temperature of 25 ± 2 °C.

2.3. Animal groups

The animals were divided into groups of ten animals each for all the ulcer models. Different groups of animals received one of the extracts of *B. hispida* (i.e. fresh juice, supernatant, residue, alcohol or petroleum ether extract). Three doses of each extract were studied. The fresh juice, supernatant and the residue were administered in the dose of 1, 2 and 4 ml/animal while alcoholic and petroleum extract were administered in the doses of 12, 24, 48 and 0.75, 1.5 and 3 mg/kg, respectively. The extracts were dissolved in 1% carboxy methyl cellulose (Central Drug House, New Delhi) and given orally while the controls received the vehicle only.

2.4. Experimental procedures

2.4.1. Aspirin plus restraint-induced gastric lesions (Parmar and Hennings, 1983)

Groups of rats, previously fasted for 24 h were given different doses of the test drug or the vehicle and 30 minlater, aspirin (500 mg/kg) (M/S Reckitt and Colman of India) dissolved in 1% carboxy methyl cellulose (Central Drug House, New Delhi) was administered orally. After 30 min of administration of aspirin, the rats were immobilized by a piece of galvanized steel window screen, which was molded tightly around the rat and held with adhesive tape. After 6 h of restraint, the animals were sacrificed and their stomachs were removed and opened along the greater curvature to expose the mucosal surface. After a gentle wash with flowing water, the mucosal surface of the stomach was carefully examined under illumination using a magnifying hand lens. After identification of ulcerative areas, the length of the ulcer was measured along the greater diameter. Number of hemorrhagic spots was also calculated and every five hemorrhagic spots were considered equivalent to 1 mm of ulcer (Cho and Ogle, 1979). The mean ulcer size was calculated by dividing the total length (in mm) of ulcers for all the animals divided by the total number of animals.

2.4.2. Swimming stress-induced ulcer (Parmar and Desai, 1993)

In this model of experimental ulcer, albino mice were used. The 24-h fasted mice were treated with either test drug or vehicle. Thirty minutes later, they were placed inside a vertical cylinder filled with water up to a height of 10 cm. The temperature of the water was maintained at 20–25 °C. The mice were removed from the cylinders after 3 h and sacrificed. The mean ulcer size was calculated as described previously.

2.4.3. Serotonin-induced ulcers (Wilhelmi, 1957; Hedinger and Veraguth, 1957):

Albino rats received different doses of the test drug or vehicle orally and 30 min later, serotonin creatinine sulfate (20 mg/kg) (Sigma Chemicals, USA) dissolved in saline was injected subcutaneously. The animals were sacrificed 4 h later and mean ulcer size calculated as described previously.

2.4.4. Indomethacin + histamine induced ulcers (Takeuchi et al., 1986)

Groups of rats received either the test drug or the vehicle. After 30 min, indomethacin (5 mg/kg) (Indian Drugs and Pharmaceutical Ltd, New Delhi, India) was injected subcutaneously. Subsequently, three doses of histamine hydrochloride (Sigma Chemicals, USA) (40 mg/kg SC) were given at intervals of 2.5 h beginning at 30 min after indomethacin injection. Animals were finally killed by exsanguination 2.5 h after the last dose of histamine and mean ulcer size was determined as described previously.

2.4.5. Reference anti ulcer drug

Ranitidine (Glaxo India Ltd, Bombay, India) was used as the reference anti-ulcer drug. Ranitidine was administered orally (100 mg/kg) 30 min prior to administration of ulcerogens.

2.4.6. Toxicity studies

Chronic toxicity studies of up to 3 months duration were carried out with different doses of fresh juice with the aim of delineating any possible adverse effect of fresh juice of *B. hispida* upon long term oral administration. Albino mice weighing 30–40 g of both sexes were divided into four groups of six animals each. Group I served as control while groups II, III, IV received 1, 2 and 3 ml/animal day of fresh juice orally. After 3 months of oral feeding, blood samples were collected and analyzed for red blood cell count (RBC), white blood cell count (WBC), hemoglobin (HB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and blood sugar with the help of auto analyzer (Alpha 4, Beckman, UK) and blood urea was assessed by diacetyl monoxime method (Ellis, 1971).

2.5. Statistical evaluation

Data of independent observations are shown as means $\pm$ S.D. Statistical analysis were done using one way analysis of variance (ANOVA) and student's t-test using GraphPAD, Instat, 1990, version 1.14, INSERM 920666S, India. Probability of greater than 0.05 was considered statistically insignificant.

3. Results

The lesions in aspirin plus restraint model were deep with massive hemorrhage while the lesions in swimming stress model were superficial with acute hemorrhages in the glandular part of the stomach, respectively. The lesions in serotonin-induced model were primarily limited to the side of greater curvature of the corpus. In indomethacin plus histamine model, the lesions were round and well-demarcated and located primarily located in the duodenum but also in antral and corpus of the stomach.

The effect of fresh juice, supernatant and residue of *B. hispida* against experimental ulcerogenesis in different animal models has been compiled in Table 1. The fresh juice showed statistical significant anti-ulcerogenic activity in the dose of 4 ml/animal only in the swimming stress-induced model. In aspirin plus restraint ulcers and serotonin-induced ulcers, fresh juice of *B. hispida* in 2 ml dose significantly ($P < 0.001$ and $P < 0.05$, respectively) lowered mean ulcer size while the higher dose failed to do so in aspirin plus restraint induced ulcers. In indomethacin plus histamine-induced ulcers, all the three doses of *B. hispida* juice significantly lowered mean ulcer size but the effect was saturated at the dose of 2 ml/animal ($P < 0.05$, 0.001 and < 0.001, respectively).

Mean ulcer size was significantly lowered in all the dose groups of animals in all the four ulcer models by supernatant fraction of the centrifuged fresh juice of *B. hispida* and this effect was dose dependent in all except serotonin-induced model of ulcer. On the other hand, treatment with different doses of residue fraction of the centrifuged juice of *B. hispida* did not show any statistical significant effect on mean ulcer size.

The alcohol extract of *B. hispida* showed a dose-dependent significant reduction of mean ulcer size in aspirin plus restraint and swimming stress-induced ulcer models. However, the effect was not dose-dependent in indomethacin plus histamine-induced and serotonin-induced models of ulcer. The reduction of mean ulcer size with petroleum ether extract was statistically insignificant as compared to controls.

Ranitidine, the reference anti-ulcer agent, significantly inhibited the ulceration induced by aspirin plus restraint, indomethacin plus histamine and serotonin-

induced ulcer ($P < 0.001$ in all three models). However, it was ineffective against swimming stress-induced ulcer.

The results of the study assessing the effect of feeding fresh juice of *B. hispida* (in the dose of 1, 2 and 4 ml/animal) for 3 months on different hematological and biochemical parameters in albino mice are compiled in Table 2. The different indices (i.e. WBC, RBC counts HB, HCT, MCV, MCH, sugar and urea) were not significantly different between the control and any of the treated groups. No behavioral changes were seen in the treated groups in comparison to controls.

4. Discussion

Peptic ulcer comprises heterogeneous group of disorders with different etiologies and is a common cause of discomfort and time lost for work (Howard, 1993). The etiopathogenesis of peptic ulcer has changed from Schwartz dictum 'No acid (gastric juice) – No ulcer' to 'No mucosal damage – No ulcers' (Shankaran and Desai, 1995). It is now believed that peptic ulcers result from an imbalance between defensive (cytoprotective) and offensive factors (gastric acid), association with *H. pylori* infection and increased use of NSAIDS like aspirin and indomethacin (Grover and Vats, 1999) that causes damage by inhibiting biosynthesis of cytoprotective prostaglandins (Rainsford, 1987).

In the present study, four different models of experimental ulcers were used. The lesions in aspirin plus restraint (Nagy et al., 1983), swimming stress (Rainsford, 1975), serotonin-induced (Hashizume et al., 1978) and indomethacin plus histamine model of ulcers (Takeuchi et al., 1986) were in concurrence to the previous findings.

Oral administration of fresh juice, supernatant and alcoholic extract of *B. hispida* was effective in lowering mean ulcer size in swimming stress-induced ulcer model. Similar to Curling's or human stress ulcers (Artz and Fitts, 1966; Grosz and Wu, 1967), this model has the advantage of being simple, resemble human stress ulcers, and involves CNS factors in causing gastric pathology (Brodie, 1968). Non anti-ulcer agents such as centrally acting drugs (Brodie, 1968) and vagotomy is known to partially or completely prevent these ulcers (Brodie and Hanson, 1960). Since vagal over-activity leads to histamine release which increase the acid secretion, the protective effects of *B. hispida* in stress-induced ulcer can be attributed to its antagonistic effect on acid secretion, which in turn may be due to its

Table 1
The effect of oral feeding of three doses of fresh juice, supernatant, residue, alcoholic and petroleum ether extract of *B. hispida* on mean ulcer index in different types of experimental models of ulcers

Test treatment	Mean ulcer size (mm)			
	Aspirin (500 mg/kg + restraint)	Swimming stress	Indomethacin (5 mg/kg) + histamine (40 mg/kg)	Serotonin (20mg/kg)
Control	3.30 ± 1.03	4.20 ± 2.05	15.70 ± 5.20	9.15 ± 2.41
Ranitidine (100 mg/kg)	1.05 ± 0.64***	1.80 ± 1.15 NS	1.30 ± 1.08***	2.95 ± 2.91***
Fresh juice 1 ml	2.00 ± 0.84 NS	1.85 ± 1.24 NS	10.11 ± 2.61*	6.80 ± 1.85 NS
Fresh juice 2 ml	1.05 ± 0.79***	1.11 ± 0.54 NS	7.22 ± 2.86***	4.95 ± 1.69*
Fresh juice 4 ml	1.40 ± 0.90 NS	0.55 ± 0.28***,†	7.77 ± 2.53***	5.35 ± 2.21*
Supernatant 1 ml	1.50 ± 0.82***	2.10 ± 1.20*	10.25 ± 4.17*	7.25 ± 1.23*
Supernatant 2 ml	1.10 ± 0.52***	1.30 ± 0.95***	8.25 ± 3.32***	4.90 ± 2.57***
Supernatant 4 ml	0.70 ± 0.48***	1.00 ± 0.74***	7.05 ± 1.59***	4.95 ± 1.64***
Residue 1 ml	4.35 ± 0.88 NS	4.25 ± 1.75 NS	14.30 ± 4.27 NS	8.70 ± 2.20 NS
Residue 2 ml	3.60 ± 0.77 NS	3.90 ± 1.80 NS	12.00 ± 3.94 NS	7.50 ± 2.48 NS
Residue 4 ml	3.05 ± 1.57 NS	3.45 ± 1.93 NS	11.50 ± 4.34 NS	7.00 ± 1.96 NS
Alcohol extract 12 mg	4.16 ± 1.36*	3.00 ± 0.91 NS	15.20 ± 6.64 NS	4.88 ± 2.02*
Alcohol extract 24 mg	2.95 ± 1.46***	1.95 ± 0.68*	9.44 ± 3.74**	2.27 ± 1.03***
Alcohol extract 48 mg	2.15 ± 1.05***	1.00 ± 0.70***	9.60 ± 4.69**,†	3.00 ± 1.20***
PE extract 0.75 mg	4.00 ± 1.45 NS	3.27 ± 0.90 NS	14.20 ± 5.05 NS	7.35 ± 1.79 NS
PE extract 1.5 mg	3.40 ± 1.85 NS	2.44 ± 1.33 NS	12.00 ± 4.50 NS	7.50 ± 3.37 NS
PE extract 3 mg	3.45 ± 1.25 NS	2.18 ± 1.36 NS	12.33 ± 6.63 NS	6.65 ± 2.18 NS

Each value represents the mean ulcer index ± S.E.M. from ten animals in each group. NS—not significant.
* $P < 0.05$ as compared to control.
** $P < 0.01$ as compared to control.
*** $P < 0.001$ as compared to control.
† $P < 0.05$ as compared to ranitidine.

Table 2
The effect of feeding fresh juice of *B. hispida* for 3 months on different hematological and biochemical parameters in albino mice

Parameter	Control (normal saline)	Group I (1 ml fresh juice of *B. hispida*)	Group II (2 ml fresh juice of *B. hispida*)	Group III (3 ml fresh juice of *B. hispida*)
WBC ($\times 10^3$/μl)	7.46 ± 1.50	7.56 ± 2.53	8.46 ± 1.61	7.38 ± 2.97
RBC ($\times 10^6$/μl)	8.32 ± 1.55	9.01 ± 0.55	8.84 ± 0.81	8.52 ± 0.95
HB% (g/dl)	12.31 ± 1.97	13.00 ± 0.75	13.06 ± 1.72	13.18 ± 1.50
HCT (%)	34.58 ± 5.71	41.00 ± 3.98	40.60 ± 5.29	39.73 ± 6.14
MCV (fL)	42.19 ± 6.51	45.58 ± 2.79	45.88 ± 3.77	46.25 ± 2.29
MCH	14.89 ± 0.99	14.55 ± 0.79	14.75 ± 0.98	15.55 ± 2.39
Sugar (mg/dl)	109.00 ± 16.00	96.16 ± 18.11	110.00 ± 23.78	109.16 ± 9.80
Urea (mg/dl)	16.98 ± 4.02	14.96 ± 4.88	14.77 ± 4.61	16.37 ± 2.40

Each value represents the mean $\pm$ S.D. from six animals in each group. No statistical significant difference was seen between the control and I, II and III groups.

anti-histaminic, anti-cholinergic or CNS activity. However, the standard anti-ulcer drug, ranitidine was ineffective in preventing ulcers in this model. Since ranitidine blocks H_2 receptors, ultimately leading to blockade of histamine release and blunting of acetylcholine and gastrin action, factors other than vagal over activity may be involved in pathophysiology of stress-induced ulcers. Therefore, it is reasonable to assume that *B. hispida* probably inhibited these mechanisms also. Moreover, extracts of *B. hispida* also reduced ulceration in another stress model i.e. restraint plus aspirin, supporting the hypothesis that anti-ulcerogenic activity of *B. hispida* has a prominent central component in prevention of ulceration. The presence of sigma receptors in the GIT has also been demonstrated and KB 5492 (Ekbald et al., 1985), a novel anti-ulcer agent with a selective sigma receptor antagonistic activity has been shown to act through mechanisms other than inhibition of gastric acid secretion (Morimoto et al., 1991, 1994). Apart from GIT, the gastroprotective actions of specific sigma receptor ligands may also be mediated by central action (Scoto, and Parenti, 1996). Since the fresh juice of *B. hispida* is also effective in morphine withdrawal (Grover et al., 2000), sigma receptor mediated CNS action is a possibility.

Mechanisms involved in serotonin-induced ulcers are not clear yet but it is believed to result from a disturbance in gastric mucosal microcirculation (Pal and Nagchaudhary, 1991) and the extracts seem to prevent such a disturbance. In this model, ranitidine showed a slightly better protection than the fresh juice and the supernatant, but alcohol extract was as effective as ranitidine in bringing down the mean ulcer size.

Development of duodenal ulcers induced by indomethacin plus histamine involves both an increase in gastric acid secretion and an impairment of acid induced duodenal bicarbonate secretion (Takeuchi et al., 1986). While histamine increases acid secretion, indomethacin inhibits the mucosal defense. As expected, ranitidine exhibited better response in this model. The fresh juice, supernatant and alcohol extract significantly lowered the mean ulcer size as compared to controls. The anti-ulcerogenic effect was dose-dependent with supernatant while the fresh juice and alcohol extract failed to exhibit a dose-dependent effect. In this model, *B. hispida* might have acted through inhibition of histamine-induced acid secretion or by preventing inhibition of mucosal defense or by stimulating the synthesis of cytoprotective prostaglandins. The possibility of inhibition of histamine induced acid secretion is more likely as two triterpenes, alnusenol and multiflorenol, present in the methanolic extract of *B. hispida* have been shown to inhibit histamine release from the rat exudate cells induced by antigen-antibody reaction (Yoshizumi et al., 1998).

Overall, the different fractions of fresh juice and alcoholic based extracts of *B. hispida* showed varied response. The supernatant portion of the whole fresh juice was more effective than the whole fresh juice while residue and PE was not all effective. This clearly indicates that the active anti-ulcerogenic component of B. hispida is water-soluble.

No adverse effect or any behavioral changes were seen with oral feeding of fresh juice of *B. hispida* for 3 months on the hematological and biochemical parameters (i.e. RBC, WBC, HB, HCT, MCV, MCH, blood sugar and urea levels). Since, the fresh juice of *B. hispida* along with supernatant fraction of the centrifuged fresh juice and alcohol extract were effective in reducing ulceration caused by different mechanisms (in different models), in both stomach and the duodenum, it is likely that these two extracts act at multiple points.

Ulcer being a multifactorial disease, therapeutic options with a broad spectrum of anti-ulcer mechanisms are needed and may be preferred over the synthetic compounds, which most often are unidirectional in their approach. The fresh juice of *B. hispida* besides having these advantages also possesses a unique combination of both anti-ulcer and anti-inflammatory activities. Further being a non-toxic plant (it is a commonly

consumed vegetable in India), it is advantageous over the presently available anti-ulcer agents whose utilities are limited by adverse effects. As *B. hispida* is a widely and easily available natural product, it can be a scientifically justified home remedy for treatment of ulcers.

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Anti-anxiety studies on extracts of *Passiflora incarnata* Linneaus

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Abstract

Passiflora incarnata Linn. has been used to cure anxiety and insomnia since time immemorial. Despite the worldwide use of *P. incarnata*, the pharmacological work on this plant had been inadequate, inconclusive and wage as the earlier reports were unable to infer the mode of action of the plant as well as the phytoconstituents responsible for the much acclaimed anxiolytic and sedative effects of *P. incarnata*. An attempt has been made to isolate and identify the bioactive phytomoiety of *P. incarnata* by resorting to bioactivity directed fractionation and chromatographic procedures. A fraction derived from the methanol extract of *P. incarnata* has been observed to exhibit significant anxiolytic activity at a dose of 10 mg/kg in mice using elevated plus-maze model of anxiety. This fraction comprises mainly two components which are visible as blue and turquoise colored fluorescent spots at 366 nm of the UV light. The possibility of a phytoconstituent having benzoflavone nucleus as the basic moiety being responsible for the bioactivity of *P. incarnata* is highly anticipated. © 2001 Published by Elsevier Science Ireland Ltd.

Keywords: *Passiflora incarnata* Linn.; Anxiolytic; Elevated plus-maze model; Column chromatography; Flavonoids

1. Introduction

Anxiety has affected 1/8th of the total population of the world. Researchers even in the advanced world are exploring their traditional remedies to find a suitable cure for these 'mind affecting diseases' which have been the outcome of man's zest to win the nature. *Passiflora incarnata* Linn. (Passifloraceae), also known as May-pop, Maracuja or Passion flower, is a plant which has been used as an anxiolytic and sedative throughout the world since time immemorial (Bergner, 1995; Foster, 1998; Gremillion, 1989; Handler, 1962; Heci, 1999; Raintree Nutrition Incorporation, 1999; Rawat, 1987 Vasudev, 1955). The worldwide clinical applications of the plant are evident from the fact that *P. incarnata* is an official plant drug in the British Herbal Pharmacopoeia 1983, Homoeopathic Pharmacopoeia of India 1974, United States Homoeopathic Pharmacopoeia 1981, Pharmacopoeia Helvetica, 1987 and the pharmacopoeias of Egypt, France, Germany and Switzerland. The plant has also been described in the British Herbal Compendium 1992, the European Scientific Cooperative on Phytotherapy (ESCOP) monographs 1997, Deutsches Arzneibuch 1997 (DAB), Deutsches Homeopathisches Arzneibuch 1978 (DHAB), Bundesanzeiger (Banz.) Monographien der Kommission E 1998 and American Materia Medica 1983. Flavonoids are reported to be the major phytoconstituents of *P. incarnata*. These include apigenin, luteolin, quercetin, kaempferol (Gavasheli et al., 1974a,b), vitexin, iso-vitexin (Lutomski et al., 1981), 6-β-D-allopyranosyl-8-β-xylo pyranosylapigenin (Grandolini et al., 1997); C-glycosyl flavonoids schaftoside, isoschaftoside, isovitexin-2″-*O*- glucopyranoside, orientin, iso-orientin, iso-orientin-2″-*O*-glucopyranoside, 2″-*O*-glucosyl-6-C-glucosylapigenin, 6-β-D-glucopyranosyl-8-β-D-ribopyranosyl apigenin and swertisin (Congora et al., 1986; Geiger and Markham, 1986; Li et al., 1991; Proliac and Raynaud, 1988; Rahman et al., 1997). Besides flavonoids, various indole alkaloids based on β-carboline ring system viz., harman, harmine, harmalol and harmaline are reported to be present in *P. incarnata* (Poethke et al., 1970). Various other phytoconstituents reported to be present in *P. incarnata* include: carbohydrates (Gavasheli et al., 1975), essential oil (Buchbauer and Jirovetz, 1992), amino acids (Gavasheli et al., 1974a,b) and a cyanogenic glycoside gyanocardin (Spencer and Seigler, 1984).

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Despite a long history of use, supported by the well-documented phytochemical reports on *P. incarnata*, the exact mode of its anti-anxiety effects and the phytoconstituents responsible for the much acclaimed CNS effects have never been described clearly. Earlier researchers have postulated different theories on the bioactive phyto-moieties of *P. incarnata* and there has been no consensus of opinion regarding the exact mode of the pharmacological activity of *P. incarnata*. The harman alkaloids, due to their MAO enzyme inhibiting properties have been suggested to be the main bioactive phytoconstituents (Natural Health Encyclopedia, 2000; The Saw Palmetto Trading Company, 1999). Other groups of researchers have highlighted the role of the flavonoid chrysin (Wolfman et al., 1994; Zanoli et al., 2000) and even the β-pyrone derivative maltol to be responsible for the CNS effects of the plant (Aoyagi et al., 1974). In the latest reports, the sedative and anxiolytic activities in *P. incarnata* have been attributed to the benzodiazepine and GABA receptors mediated biochemical processes in the body (Simmen et al., 1999; Viola et al., 1998). Contrary to all these reports, the exhaustive pharmacological studies on *P. incarnata* by Soulimani et al. (1997) have ruled out the role of any of the known phytoconstituents being responsible for the well-established anxiolytic and sedative activity of *P. incarnata*.

The present studies were undertaken with an objective to lay hands on the phytoconstituents responsible for the anxiolytic and sedative effects of *P. incarnata*. The bioactivity directed fractionation and chromatographic methods were employed to reach at the final bioactive fraction containing the major chunk of the phytoconstituents liable for the CNS effects of this potential plant.

2. Materials and methods

2.1. Plant material

P. incarnata aerial parts were procured from a commercial supplier and cultivator Rati Ram who had cultivated *P. incarnata* in his nursery at village Khurrampur, district Saharanpur (Uttar Pradesh, India) in January 1999. The identity of the plant material was confirmed from the Department of Systematic Botany, Botany Division, Forest Research Institute, Dehra Dun, India (FRI). A voucher specimen (code no. 1325/2000) was deposited in the Herbarium-cum-Museum of the FRI.

2.2. Animals

Swiss albino mice (either sex) procured from the Disease Free Small Animals House, College of Veterinary Sciences, Haryana Agriculture University, Hisar, India, were bred at the Central Animal House of the Panjab University, Chandigarh. The mice were allowed standard laboratory feed and water ad libitum. Groups of five mice (20–24 g) were used in all sets of experiments.

2.3. Chemicals and instruments

Solvents viz., petroleum ether (60–80 °C, MERCK), methanol (s.d. Fine-Chem Ltd, Mumbai), chloroform (MERCK) and 1-butanol (s.d. Fine-Chem Ltd., Mumbai), all of LR grade were employed for the extraction of the plant material. Silica gel (# 60–120, s.d. Fine-Chem Ltd., Mumbai) was used for column chromatography. Pre-coated TLC sheets (Macherey-Nagel D-5160 DUREN, 0.25 mm, Polygram® SiLG) and 2 μL capillary tubes (CAMAG) were utilized for developing thin layer chromatograms. The chromatograms were visualized under 254/366 nm UV light (DESAGA, Heildberg, Min. UVIS) and also by spraying with 60% v/v aqueous sulfuric acid (BDH). Diazepam I.P. was procured from Triko Pharmaceuticals, Rohtak, Haryana (India). Reference samples of chrysin, apigenin, hesperidin, flavone, benzoflavone and β-carboline alkaloid harman were procured from Sigma, USA and Alkem International Ltd., Ballabhgarh, Haryana (India).

2.4. Elevated plus-maze model of anxiety

The plus-maze apparatus consisting of two open arms (16×5 cm.) and two closed arms ($16 \times 5 \times 12$ cm.) having an open roof, with the plus-maze elevated (25 cm.) from the floor was used to observe anxiolytic behavior in animals (Kulkarni and Reddy, 1996). The animals were fasted 18 h prior to the experiment. Extracts of *P. incarnata* were administered orally using a tuberculin syringe fitted with oral canula. The dose administration schedule was so adjusted that each mouse was having its turn on the elevated plus-maze apparatus 45 min after the administration of the dose. Each mouse was placed at the center of the elevated plus-maze with its head facing the open arms. During this 5 min experiment, the behavior of the mouse was recorded as (a) preference of the mouse for its first entry into the open or closed arms, (b) the number of entries into the open or closed arms, (c) average time spent by the mouse in each of the arms (average time = total duration in the arms/number of entries). During the entire experiment, the animals were allowed to socialize. Every precaution was taken to ensure that no external stimuli could invoke anxiety in the animals. Similar observations were recorded for the standard group (Diazepam 2 mg/kg) as well as the control group (vehicle, 0.25 ml).

2.5. Statistics

The anxiolytic activities of test substances, diazepam (standard) and control were analyzed by analysis of variance (ANOVA) and the test groups were compared with standard/control by Fischer-LSD test. Differences were considered significant at $P < 0.05$.

2.5.1. Preparation of extracts and evaluation of anxiolytic activity

Aerial parts of *P. incarnata* were dried in shade and powdered (# 60). Two hundred and fifty grams of the dried and powdered *P. incarnata* were Soxhlet extracted successively with petroleum ether, chloroform, methanol and water. Exhaustive extraction with each of the solvents was ensured. The four extracts were dried using Buchi 461 Rotary Vacuum Evaporator and the dried extracts were preserved in vacuum desicator containing anhydrous silica gel blue (Sarabhai M Chemicals, Baroda, India). The weight of various extracts obtained after exhaustive extraction was: petroleum ether extract (6.8875 g), chloroform extract (8.2314 g), methanol extract (11.8787 g) and water extract (4.8876 g). The extracts of P. incarnata were separately suspended in a vehicle comprising 1% w/w carboxymethylcellulose (CMC) in Simple Syrup I.P. Five sets of doses viz., 300, 200, 125, 100 and 75 mg/kg of each extract of *P. incarnata* were prepared by suspending the dried extracts in the vehicle. Weights of extracts were so adjusted as to administer 0.25 ml of the suspension of the extracts (test substance). Diazepam 2 mg/kg suspended in the vehicle was used as a standard anxiolytic. The suspending vehicle (0.25 ml) was used as control. The relative anxiolytic profile of the four extracts of *P. incarnata* is depicted in Fig. 1.

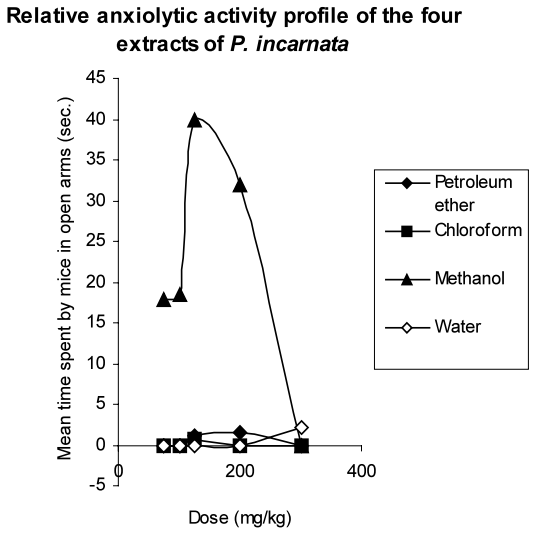

Fig. 1. Dose–response curve showing the anxiolytic profile as the mean time (seconds) spent by mice in the open arms of the elevated plus-maze.

Table 1
Column chromatography of F-4

Fraction (50 ml)	Obtained by pooling fractions	Eluants
F-4.1	1–38	chloroform
F-4.2	39–50	chloroform: methanol (95:5)
F-4.3	51–79	chloroform: methanol (90:10)
F-4.4	80–91	chloroform: methanol (85:15)
F-4.5	92–110	chloroform: methanol (85: 15)

2.5.2. Fractionation and bioactivity determination of methanol extract

The methanol extract (11.8787 g) was shaken successively with 5×10 ml volume of each of 1-butanol, petroleum ether and chloroform. The four different fractions were dried and weighed as: butanol soluble fraction (F-1; 1.4338 g), petroleum ether soluble fraction (F-2; 1.0034 g), chloroform soluble fraction (F-3; 2.1437 g) and the methanol soluble fraction (F-4; 7.2978 g). The four different fractions (F-1 to F-4) were again evaluated for the anxiolytic activity using elevated plus-maze apparatus.

2.5.3. Column chromatography of F-4

The bioactive fraction F-4 (7.2978 g) was subjected to column chromatography using silica gel (# 60-120, MERCK). Elution was done with chloroform and chloroform-methanol in the increasing order of polarity (Table 1). All the five fractions were subjected to screening for anxiolytic activity at various doses (Fig. 2). The fraction F-4.2 (2.7443 g) exhibited anxiolytic activity at a dose of 50 mg/kg.

2.5.4. Fractionation of F-4.2

The fraction F-4.2 (2.7443 g) was shaken successively with 5×10 ml volume each of petroleum ether and petroleum ether-chloroform (Table 2). The relative anxiolytic profile of the sub-fractions of F-4.2 has been depicted in Table 3.

2.5.5. Phytochemical screening of F-4.2.3

Thin layer chromatographic profile of F-4.2.3. showed two distinct spots under UV light (366 nm) at R_f 0.69 (bright turquoise) and 0.65 (blue) using the mobile phase comprising petroleum ether: toluene: ethyl acetate: acetone in the proportion 13:4:2:1. TLC of this fraction along with the reference standards viz., harman alkaloid, maltol, flavonoids including vitexin, isovitexin, chrysin, orientin, isoorientin and apigenin, confirmed the absence of these reported phyto-constituents of *P. incarnata*. The fraction also tested nega-

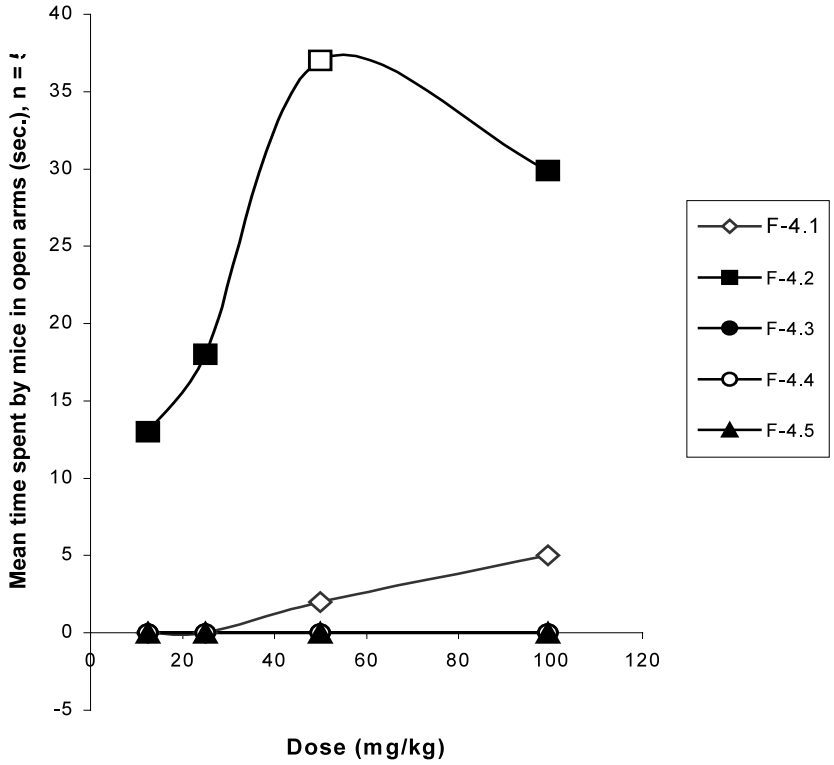

Fig. 2. Dose–response curves of the five sub-fractions of F-4.

tive for C and *O*-glycosides reported in *P. incarnata*. However, the fraction gave positive phyto-chemical tests for flavones (cyanidin test) and sitosterol. When separated by preparative TLC, 332 mg of the flavone moiety could be generated.

3. Results and discussion

In light of the findings and observations of the earlier studies, this is strongly affirmed that certain important factors have to be taken into account prior to starting any pharmacological work on *P. incarnata*. The plant bears morphologic and microscopic features that are also present in another species of the same genus, i.e. *P. edulis* Sims. The similarity between *P. incarnata* and *P. edulis* which was first reported in 1843 (Hooker, 1843) had baffled the taxonomists as well as researchers to an extent that at many places the two species were reported as synonyms (Chakravarty, 1949; Finzelberg, 1999; Jackson, 1895; Raintree Nutrition Incorporation, 1999; Scheper, 1998; Sengbusch, 1999; Vecchia, 1998; Wooten, 1999). Interestingly, *P. edulis* is mainly an edible species (McGuire, 1999) and is not having significant CNS depressant effects. Complete or even partial substitution of the bioactive *P. incarnata* with biologically inert *P. edulis* can not be ruled out and can lead to contradictory pharmacological results. Till date, the proper identification of these two closely related species had been a subject of controversy. Authors have reported the key differential parameters to establish the proper identity of *P. incarnata* and *P. edulis* (Dhawan et al., 2000). Resorting to any of these parameters can ensure proper selection of the bioactive plant. In the earlier work by Soulimani et al. (1997), *P. incarnata* was reported to possess anxiolytic activity at a dose of 400 mg/kg and sedative activity at a very high dose of 400 g/kg. According to their findings, the two forms of tranquillizer activities depended upon the solvent used to prepare the extract. In their work the hydro-alcoholic extract (70:30) was reported to possess anti-anxiety activity at a dose of 400 mg/kg in mice and accordingly the dose of 10 mg/kg of the fraction derived from methanol extract by successive partitioning and chromatographic separations in our work is highly justified. Recently, a group of Italian researchers (Zanoli et al., 2000) working on *P. incarnata* reported

Table 2
Fractionation of F-4.2

Fraction	Shaken with	weight (g)
F-4.2.1	petroleum ether	0.3572
F-4.2.2	petroleum ether–chloroform (85:15)	0.4004
F-4.2.3	Petroleum ether–chloroform (70:30)	0.4356
F-4.2.4	Petroleum ether–chloroform (55:45)	0.5346
F-4.2.5	Petroleum ether–chloroform (50:50)	0.6200
F-4.2.6	fraction leftover un-dissolved	0.3965

Table 3

Fraction	Doses (mg/kg)	Mean* ± S.D.	$P<0.05$	
			D**	C***
	50	6.0 ± 1.2	S	S
F-4.2.1	25	3.2 ± 1.1	S	S
	10	4.8 ± 0.8	S	S
	5		S	NS
F-4.2.2	50	6.0 ± 1.6	S	S
	25	1.4 ± 0.8	S	NS
	10	1.0 ± 0.7	S	NS
	5	–	S	NS
F-4.2.3	50	12.0 ± 3.3	S	NS
	25	18.8 ± 2.4	S	S
	10	4 04 ± 5.6	NS	S
	5	16.2 ± 3.3	S	S
F-4.2.4	50	-	S	NS
	25	-	S	NS
	10	-	S	NS
	5	-	S	NS
F-4.2.5	50	-	S	NS
	25	-	S	NS
	10	-	S	NS
	5	-	S	NS
F-4.2.6	50	-	S	NS
	25	-	S	NS
	10	-	S	NS
	5	-	S	NS
Diazepam (D)	2.0	37.4 ± 3.5		
Control (C)	0.25 ml	-	S	

Anxiolytic activity determined as mean time spent by the mice in open arms after the oral administration of the various doses of six sub-fractions of F-4.2, diazepam and control. S = significant, NS = non significant; $n = 5$, * = mean time spent in open arms (s), ** = S or NS with respect to standard, *** = S or NS with respect to control.

the possible role of the flavonoid chrysin to be partly responsible for the anxiolytic activity of the plant. However, chrysin was not present in the bioactive fraction, as per the TLC profile of the fraction showing significant anti-anxiety activity at 10 mg/kg in mice. Similarly, the other reported flavonoids of *P. incarnata* viz., apigenin, quercetin, hesperidin and orientin were also absent. In our studies, we strictly confined to our objective to lay hands on the bioactive phyto-moiety. Since initially, the maximum anxiolytic activity of the plant was confirmed at a dose of 125 mg/kg in the methanol extract, therefore, only methanol extract of *P. incarnata* was processed further. Upon shaking the methanol extract with 1-butanol, petroleum ether and chloroform, the anxiolytic activity was observed only in the methanol fraction, that too, at the same dose of 125 mg/kg in mice. The methanol fraction, thus obtained, was subjected to column chromatography using eluents like chloroform and mixtures of chloroform and methanol. The fraction F-4.2. (Table 1) showed maximum anxiolytic activity at a dose of 50 mg/kg in mice using the same experimental model. Upon shaking F-4.2 with petroleum ether and petroleum ether-chloroform mixtures, the sub-fraction F-4.2.3 (Table 2) was isolated in an almost pure form which exhibited anxiolytic activity at 10 mg/kg dose in mice, using diazepam as the standard anxiolytic. Throughout the entire process, the approach was to remain confined to the most active fraction only and it was not deemed appropriate to evaluate all the fractions from the phyto-chemical point of view. The last fraction (F-4.2.3) was subjected to phytochemical screening, and was found to be devoid of all the known phytoconstituents. This fraction, upon further separation, yielded β-sitosterol and a compound that tested positive for flavone moiety. Identity of this compound is being ascertained using standard physico-chemical and spectroscopic techniques. That this compound is a benzoflavone derivative has been confirmed (Dhawan et al., 2001).

Finally, in view of our observations and findings, it is concluded that a fraction derived from the methanol extract of *P. incarnata* shows significant anti-anxiety activity at a dose of 10 mg/kg p.o. in mice using elevated plus-maze model of anxiety. The studies are still under process for characterizing the benzoflavone moiety and to evaluate its mode of CNS depressant action.

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Curing animals with plants: traditional usage in Tuscany (Italy)

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Abstract

Tuscany is an area rich in traditions, many of an ethnobotanical nature, and those of veterinary practice are of special interest. Almost a 100 different plant species are used to treat animals; sometimes old remedies are used to cure similar human ailments, other times the cure is used exclusively for veterinary treatment.

Keywords: Ethnobotany; Tuscany; Italy

1. Introduction

Tuscany is located in central Italy; on the west it faces the Ligurean and Tyrrhenian Seas while to the north, east and south it lies between the Ligurean, Emilia-Romagna, Marche, Umbria and Lazio regions, respectively.

Tuscany has derived its name from Etruria whose ancient inhabitants were the Etruscans or Tusci.

Tuscany has a surface area of 22,992 km^2 and from an orographical point of view it is rather complex with plains on only about 1\10 of its territory, and mountains on 1\5 while the most part consist of hills and foothills (Landini, 1979).

Tuscan history, its geographical position, and geological structure (Giusti, 1993) have characterised the development of a large and varied collection of traditions, very different from area-to-area, mostly of an ethnobotanical nature. In such an environment, there are numerous reports concerning plant usage in local folk medicine (Caffaro Corti and Gastaldo, 1980; Uncini Manganelli and Tomei, 1999), some of which are also used in veterinary practice, whilst others are used exclusively to treat animals (Viegi et al., 1999) which also results from extended research (Pieroni, 1999).

Until a few years ago, agriculture was more widespread and animals were widely employed on the land (De Simonis, 1982) so curing them with plants was common in the region; moreover, cheese, meat and by-products were also obtained from these animals.

Even today Tuscany supplies rich agricultural produce, the most part represented by sowable areas (Fig. 1); this surface area corresponds to approximately 567,383.71 ha. The most important crops are cereals, in particular wheat, corn and barley. Crop rotation follows with a surface area of 130754 ha, that is plants for industry-seed oils from sunflower, tobacco and soya (53,328 ha), vegetables (13,298.74 ha), sugarbeet (8058.43 ha), dried pod vegetables (5803.30 ha) mostly for forage; potatoes (1504.64 ha), and flower and house plants (1487.96 ha; ISTAT, 1990).

Also orchards and vinyards are present (75,000 wine farms and 70,500 olive farms). The use of farming land for livestock even though the pasture area is only about 9%, is well above this percentage because, as mentioned before, among the sowable areas specific crops are planted for this purpose. Although animals are no larger used for farm work, livestock breeding for meat and dairy products is present in this region. A general estimation based on recent surveys regarding the main kinds of livestock present can be made, there has been a considerable increase in sheep and goats (717.534 head) with a reduction in pigs (292.785 head) and cattle (149.725 head) which, however, still indicate a large number (ISTAT, 1990). Concerning secondary stock breeding, horse breeding is increasing due to the growing number of tourists in Tuscany.

In conclusion, although there is a reduction in the number of rabbit and wild bird farms, the number of

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stock is increasing due to fewer and larger farms which are linked to chain distributors. However, small family-owned farms are still very common.

2. Methodology

This work has been carried out during a 4-year period, from February 1997 to August 2000. The authors interviewed 772 people, mostly female (Fig. 2) and in the 50–60 age range (Fig. 3).

The majority of the people interviewed have been or are still farmers, either full time or using the farm as an extra source of income; others are shepherds or cattlemen or employed on small family-owned farms.

Also people living in small rural villages were contacted because they too raise small animals such as chickens, pigeons, rabbits and turkeys.

The areas selected for our data collecting were Grosseto, Livorno, Massa Carrara, Pisa and Siena (Fig. 4) mostly small communities living in the mountains or rural areas with a population ranging from 40 to 1000.

In order to facilitate our data collecting we drew up a questionnaire (Fig. 5) in which we listed a number of pre-defined or open questions, to stimulate, especially in the elderly, a recollection of past local traditions regarding the most frequent animal illnesses (dysentery, distemper, mastitis, etc.).

The data collected was assessed eliminating any information which could have been linked to the media (books, newspapers, TV, radio, etc.) and therefore not from common folk notions.

Various plants, with their given local name, were found and identified by the authors following the botanical reference nomenclature for Italian flora (Pignatti, 1982).

Two specimens were picked of each plant species, one was used for the preparation of a voucher herbarium specimen, kept in the Herbarium Facultatis Agrariae of Pisa University (PI. AGR); the other was planted in the Tuscan Ethnobotanical Garden at La Rottaia (PI). The Garden was founded very recently. Two hundred of the over 400 plants that are used in various branches of Tuscan ethnobotany, are cultivated there. The plants that are cultivated have various uses, magical; dyeing; nourishing; medicinal; cosmetic; domestic; veterinary. This Garden has the double purpose of popularising the rich heritage that lies in Tuscan ethnobotanical culture, and maintaining the local genetic patrimony which risks extinction, because of the rapid decline of the countryside.

3. Results and discussion

Results obtained showed that over 90 plant species are in use in ethnoveterinary practice in Tuscany. Results of this work are shown in Table 1; plant names are arranged with their family names in alphabetical order.

Following the on-the-spot investigations you can see that the majority of the ethnobotanical veterinary practices which were collected have been substituted by current veterinary treatments. The wide use of vaccines together with modern hygienic care have much reduced the onset of diseases.

The shepherd or farmer who looks after his own animals has been replaced by the veterinarian who is now part of the Public Health Authorities.

In fact numerous animal diseases, presently under control can became dangerous when contagious for man. Among the most widespread and feared here in Tuscany but today under control thanks to current prevention methods, is tuberculosis whose most typical form especially in cattle is *Mycobacterium bovis*. Because it is so common constant inspection and prevention are required (regulated in Italy by a Government law dated 20 June, 1977); approximately 10% of human tuberculosis is caused by *M. bovis*, even if the most serious pathogen for man is *Mycobacterium tuberculosis*, which is also hosted in animals. In this case because of its high risk suppression of the stock is carried out.

Another form of animal tuberculosis is *Mycobacterium avium-intracellulare* which strikes mainly wild and domestic birds, especially if in captivity but also

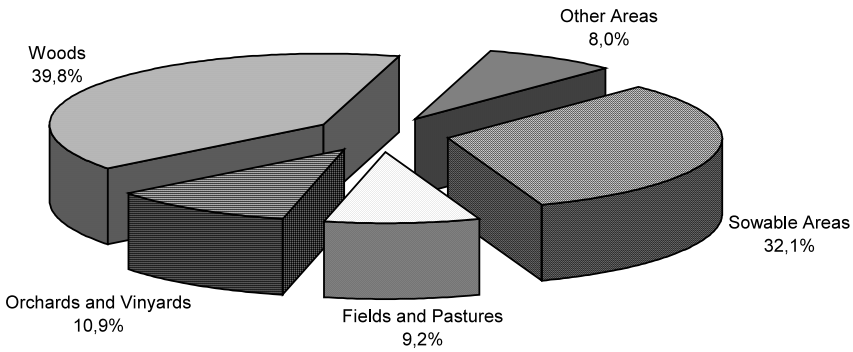

Fig. 1. Cultivations and areas now present in Tuscany.

Table 1
Plants used in popular Tuscan veterinary treatment

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
Adiantaceae						
A. capillus-veneris L. (PI. AGR 1 42)	W	Felcina	Frond	Bovines	Post partum	The decoction is given after calving to facilitate expulsion of the placenta (GR).
Apocynaceae						
Nerium oleander L. (PI. AGR 57 2841)	C	Oleandro	Flower tip	Rabbits, cats, dogs	Against dermatosis	The powder obtained by grinding dried flower tips is applied locally on the skin of animals affected by tinea (fungal disease) (GR).
Araliaceae						
Hedera helix L. (PI. AGR 48 2400)	W	Edera	Leaf	Ovines, caprines, bovines	Abortive-anti-inflammatory	Fresh leaves eaten by ovines and caprines cause spontaneous abortion (LU). The decoction was used to bathe the genital area of cows when inflamed, this could even cause sterility (SI).
Aspleniaceae						
Asplenium trichomanes L. (PI. AGR 2 54)	W	Felcini	Frond	Ovines	Ruminative	Fresh fronds are added to other herbs for animals to eat (LU).
C. officinarum DC. (PI. AGR 2 69)	W	Erba ruggine, felcini	Frond	Bovines, ovines	Post partum	The decoction is drunk by sheep and cows after delivery as depurative (LU).
Asteraceae						
Achillea millefolium L. (PI. AGR 80 3976)	W, C	Erba giogaia	Leaf	Bovines	Wound healing	Leaves are used to make an ointment cooking them over a low heat with olive oil, tallon, bee's wax and egg yolk; this is applied locally to cure sores on bovines' withers caused by yoke rubbing; sometimes *Cynoglossum creticum* Miller is added (GR, SI).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
Artemisia abisinthium L. (PI. AGR 81 4028)	W	Erbo bon, assensio, ascenzio	Whole plant without roots	Bovines	Favours digestion-against parasite	Added to calves' feed or an infusion is given to favour digestion (LU). As a vermifuge it is mixed with garlic and lard (PI).
Cirsium arvense (L.) Scop. (PI. AGR 84 4156)	W	Stroppioni, stoppioni	Inflore scence	Rabbits	Favour digestion	Given as food (PI)
H. italicum (Roth) Don (PI. AGR 78 3872)	W	Canutello, tombolo, muschio di S. Giovanni, cannugiori/o	Whole plant without roots, leaf	Ovines, mules, asses, rabbits	Against asthma, reconstituent, improves quality of milk	The quality of sheep's milk improves when they are fed this plant; the leaf decoction is used in fumigations for asthmatic asses and mules (LU). A mixture of 'elicriso', *Ruta chalepensis* L. and *Satureja montana* L. leaves is given to rabbits as a reconstituent (MS).
Inula viscosa (L.) Aiton (PI. AGR 78 3890)	W	Céppita	Leaf	Bovines, ovines	Anti-inflammatory	The cold infusion is applied by compresses to the bruises (PI).
L. tuberosus L. (PI. AGR 88 4356)	W	Radicchio, puppoline	Root	Rabbits	Galactogogue	Added to rabbit food (LU).
Betulaceae						
A. glutinosa (L.) Gaertner (PI. AGR 4 184)	W	Ontano	Leaf	Chickens, geese	Insecticidal	Leaves are put in the chicken run to keep away lice (LU).
Boraginaceae						
Cynoglossum creticum Miller (PI. AGR 62 3065)	W	Lingua di cane	Leaf	Bovines	Wound healing	The leaves combined with those of 'sambuco' (*Sambucus nigra* L.) and 'ginestra puzzola' (*Cytisus scoparius* L.) are cooked in olive oil and bee's wax to obtain an ointment; this is applied locally to cure sores on the withers where the yoke has rubbed; some also add *Achillea millefolium* L. for the same purpose (GR, SI).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
Echium vulgare L. (PI. AGR 61 3004)	W	Lingua di bue	Leaf	Bovines	Against dermatosis	The fresh juice is applied on the skin several times a day (PI).
Buxaceae						
Buxus sempervirens L. (PI. AGR 44 2174)	C	Bussolo	Leaf	Bovines	Anti-mastitic	After calving cows are put on a bed rich in *Buxus* leaves (LU).
Caprifoliaceae						
Sambucus nigra L. (PI. AGR 73 3611)	W	Sambuco	Leaf	Bovines, ovines, equines, chickens	Anti-mastitic-for gastro intestinal disorders-wound healing	In cases of mastitis, cows' udders are exposed to fumigations of 'sambuco'; the bark together with 'frassino' (*F. ornus* L.) bark is macerated for twelve hours and drunk by animals having gastro intestinal inflammation (LU). The leaves, mixed with those of *Cytisus scoparius* L. and of *Cynoglossum creticum* Miller are cooked in olive oil and bee's wax to make an ointment, which is applied locally to the inflamed withers of bovines where the yoke has rubbed (GR, SI, PI).
S. ebulus L. (PI. AGR 73 3610)	W	Sambuco	Leaf	Chickens	Insecticidal	Dried leaves are hung in the chicken house to keep away lice and other parasites (LU).
Caryophyllaceae						
S. media (L.) Vill. (PI. AGR 10 463)	W	Centocchio	Leaf	Chickens	To increase egg production	The leaves are added to chicken feed to increase egg production (GR).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
Crassulaceae						
Sempervivum tectorum L. (PI. AGR 25 1226)	W,	Sopravvivo	Whole plant	Bovines, ovines, chickenss	Ruminative-against pestilence	Ground together with rancid lard, until a paste is formed, then small balls are made and eaten by cows and sheep as cud; to cure chicken pestilence a mash is prepared also adding *Allium cepa* L. (SI).
Cruciferae						
Capsella bursa-pastoris (L.) Medicus (PI. AGR 22 1093)	W	Borsa del pastore	Whole plant without roots	Ovines	Post partum	The decoction is drunk by sheep after lambing to prevent haemorrhage (LI).
Cupressaceae						
C. sempervirens L. (PI. AGR 3 123)	C	Cipresso	Bark, juniper berries, small branches	Equines, bovines, swine, rabbits	Anti-rheumatic-for gastro intestinal disorders-against dermatosis	Bark softened in water is applied to the legs of horses suffering from rheumatics; the immature juniper berries are eaten by animals for gastro intestinal inflammation (LI). Small branches and juniper berries are eaten to cure the so called 'ventrina'; in cases of dermatosis due to excessive perspiration, a decoction of juniper berries in vinegar is applied locally to the skin (PI).
Juniperus communis L. (PI. AGR 3 126)	W, C	Ginepro	Juniper berries, small branch	Bovines, rabbits	Favour digestion-strengthens joints	Juniper springs are added to the forage of rabbits (PI). A decoction, made in white wine, is applied by compresses on the joints of 'working animals' (SI).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
Equisetaceae						
E. telmateja Ehrh. (PI. AGR 1 26)	W	Coda cavallina, cucito, cucitoli	Sterile shoot	Bovines, equines, ovines, dogs	Wound healing	The dried and finely chopped cauli are applied to small wounds and abrasions (LI).
Euphorbiaceae						
E. lathyris L. (PI. AGR 42 2086)	W	Cacaprussia, catapuzia	Fruit	Bovines	Purgative	The oil—obtained by cooking the fruit in olive oil—is given in small doses to bovines affected by intestinal block; some, for the same purpose, prepare the decoction (LU). Ingestion of this herb can cause dysentery in young calves (SI).
M. annua L. (PI. AGR 41 2043)	W	Marcorella, marcuella, mercorella, mercuriella	Whole plant	Bovines, ovines	Post partum	The decoction is drunk by cows and sheep post partum (LU). According to some, ingestion of this plant can kill rabbits (PI).
Gramineae						
A. sativa L. (PI. AGR 102 5072)	C	Avena, biada	Fruit	Bovines, ovines	Fertility enhancer-anti-mastitic	The caryopsises are left to germinate and then eaten by cows before they mate, in order to favour fertilisation (LU). A poultice is applied to the udders (PI).
Echinochloa crus-galli Beauv. (PI. AGR 61 3004)	W	Saginella	Fruit	Birds	Reconstituent	Given mixed with birdfeed (LI, PI).
Oryza sativa L. (PI. AGR 105 5247)	C	Riso	Fruit	Bovines, equines, ovines,	Anti-diarrheal	The decoction is drunk by cattle (LU).
Phragmites australis (Cav.) Trin. (PI. AGR 104 5165)	W	Cannella	Whole plant without roots	Bovines	Anti-diarrheal	Given as forage (LU, PI).
Secale cereale L. (PI. AGR 102 5056)	C	Segale	Fruit	Bovines	Wound healing, rectal prolapsus	The decoction is used in enemas in cases of rectal prolapse; a poultice of maize flour and rye is applied to umbilicus of calves (LU).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
Triticum aestivum L. (PI. AGR 102 5058)	C	Grano	Fruit	Bovines, equines, ovines	Ruminative, laxative, against dermatosis	Bran, salt and vinegar are used to make a paste eaten by animals to reactivate end chewing; for the same purpose sometimes *Rosmarinus officinalis* L. is added; a decoction of *Parietaria diffusa* M. et K. together with wheat flour is drunk by animals as a laxative (LU). After washing inflammed skin of horses and cows with a decoction obtained by boiling juniper berries of *C. sempervirens* in vinegar, flour is applied topically (PI).
**Zea mays* L. (PI. AGR 106 5294)	C	Mais	Fruit	Bovines	Wound healing	A poultice made with corn and rye flour is applied to the umbilicus of calves (LU).
Guttiferae						
Hypericum perforatum L. (PI. AGR 18 872)	W	Iperico, erba di San Giovanni	Whole plant without roots	Dogs, bovines	Analgesic-wound healing	The macerate in olive oil is used to massage painful joints or applied on wounds (PI).
Labiatae						
M. vulgare L. (PI. AGR 63 3117)	W	Marrubio	Leaf	Bovines, equines	Favor digestion	A few leaves are given to working animals to eat when their stomachs are bloated from eating forage which is too fresh and damp (SI).
Mentha aquatica L. (PI. AGR 65 3249)	W	Menta d'acqua, mentastro	Whole plant	Rabbits, cats, dogs	Insecticidal	A decoction is made in wine or vinegar and is rubbed and washed inside or around the anomal's ears to eliminate parasites and mites (LU, PI).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
Mentha pulegium L. (PI. AGR 65 3247)	W	Menta	Leaf	Dogs, cats, bovines	Against cold	A decoction is prepared and used for fumigation to treat colds (PI).
Rosmarinus officinalis L. (PI. AGR 66 3254)	W, C	Rosmarino	Small branch	Ovines	Ruminative	The decoction is added to wheat flour and given as feed (LU).
Satureja montana L. (PI. AGR 64 3195)	W	Trombo	Whole plant without roots	Rabbits	Reconstituent	The whole plant without roots, mixed with that of *Ruta chalepensis* L. and of *H. italicum* (Roth) Don is an excellent reconstituent (MS).
Stachys officinalis (L.) Trevisan (PI. AGR 64 3156)	W	Bertonica	Leaf	Bovines, equines, dogs, cats	Wound healing	The fresh leaf is chewed and applied locally to wounds even if infected and purulent (SI).
Teucrium chamaedrys L. (PI. AGR 62 3099)	W	Querciola	Whole plant	Bovines, caprines, equines, ovines	Anti-inflammatory	The decoction is used for cleaning affected hooves (SI).
Lauraceae						
L. nobilis L. (PI. AGR 18 875)	W, C	Orbaco	Leaf	Bovines, ovines	Post partum	The decoction is drunk by cattle and sheep post partum (LU).
Leguminosae						
Cytisus scoparius (L.) Link (PI. AGR 32 1588)	W	Ginestra puzzola	Leaf	Bovines	Wound healing	In cases of sores of the withers due to irritation from the yoke, an ointment, obtained by cooking 'ginestra', *Sambucus nigra* L. and *Cynoglossum creticum* Miller leaves in olive oil and bee's wax, is applied (GR, SI).
G. officinalis L. (PI. AGR 33 1624)	W, C	Avanese	Whole plant without roots	Caprines	Galactogogue	Given as food (SI).
Lupinus albus L. (PI. AGR 33 1621)	W, C	Lupino	Seed	Bovines, swine	Insecticidal	The decoction is used for dips and local applications against lice (LU).
M. sativa L. (PI. AGR 37 1808)	W, C	Erba medica	Whole plant without roots	Bovines	Galactogogue	Given as feed (GR).
**Robinia pseudacacia* L. (PI. AGR 33 1623)	W	Acacia	Leaf	Rabbits	Reconstituent	Added to forage (PI).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
Spartium junceum L. (PI. AGR 33 1614)	W	Ginestra	Caulis	Ovines	Ruminative	To reactivat rumination a few sprigs are put in sheep's mouths held by a muzzle, a pratice called ''romico'', so the juice can be swallowed (SI).
Liliaceae						
Allium cepa L. (PI. AGR 94 4685)	C	Cipolla	Bulb	Chickens	Against pestilence	Bulbs mixed with *Sempervivum tectorum* L. and ground are added to feed to prevent pestilence (SI).
Allium sativum L. (PI. AGR 93 4650)	C	Aglio	Bulb	All kinds	Against parasite	Eaten raw by both large and small animals (GR, PI).
Linaceae						
L. usitatissimum L. (PI. AGR 41 2030)	W, C	Lino	Seed	Bovines, ovines, equines	Post partum-anti-diarrheal-rumi native-laxative	The decoction is drunk in cases of diarrhea and as a post partum depurative; to reactivate rumination animals are given the seeds together with *M. sylvestris* L. leaves to eat (LU). The decoction together with boiled seeds is given to cure constipation (PI).
Malvaceae						
Althaea officinalis L. (PI. AGR 45 2216)	W	Altea	Root	Bovines	Anti-inflammatory	The decoction is applied by compresses to inflamed withers where the yoke rubs (LU, PI).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
M. sylvestris L. (PI. AGR 44 2199)	W	Malva	Leaf	Bovines, swine	Evacuant-ruminative-anti-mastitic-laxative	The decoction is administered to swine by enema; malva leaves and lino (*L. usitatissimum* L.) seeds, are eaten by cows to reactivate rumination (LU). The decoction of leaves is used by compresses to treat mastitis in bovines (GR). Some people give pigs the decoction to drink for constipation (PI).
Moraceae						
Ficus carica L. (PI. AGR 5 218)	W, C	Fico	Leaf	Bovines	Galactogogue	Leaves are added to forage (GR)
**Morus nigra* L. (PI. AGR 5 215)	C	Gelso	Leaf	Bovines, rabbits	Anti-inflammatory	Leaves are added to forage (PI).
Oleaceae						
F. excelsior L. (PI. AGR 56 2776)	W	Frasso, frassine	Bark, small branch, sap	Ovines, chickens, geese	For gastro intestinal disorders-ruminative-antiseptic	Bark macerated in water for 12 hrs is drunk by poultry for gastrointestinal problems; to reactivate rumination a few branches are put in sheep's mouths, a pratice called ''romico'' (LU). Tappings are made in the bark at the end of June, the sap is collected, heated and added to chicken feed as an intestinal disinfectant (GR). Bark from branches is put into chicken drinking troughs to prevent various infective diseases (SI).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
F. ornus L. (PI. AGR 56 2775)	W	Frassino, ornello	Bark, leaf	Bovines, caprines, equines, chickens, ovines, swine	For gastro intestinal disorders, anti-diarrheal, anti-bacterial, egg production intensifier-ruminative-against cold-against parasites	'frassino' bark, and sometimes *Sambucus nigra* L. bark, is macerated for a whole night and the solution obtained is drunk by animals having gastrointestinal inflammation, and diarrhea; a watery macerate -made by soaking the bark several days in water—is drunk by poultry to cure the so called 'calcinaccio'; by chicks to prevent bacteriosis and by hens in particular to increase egg production (LU, LI). Fresh leaves are eaten by cows to reactivate rumination (LU). A liquid macerate made from leaves and bark is drunk by chickens to cure colds (PI). For anthelmintic purposes, leaves are added to equine forage (GR)
Olea europaea L. (PI. AGR 7 325)	C	Olivo	Leaf	Bovines, ovines, swine, equines	Detoxicating	The decoction is given orally against food intoxication (PI).
Papaveraceae						
Papaver rhoeas L. (PI. AGR 18 878)	W	Papavero, pappavero tenero	Leaf	Rabbits, geese, chickens	Laxative	The leaves, added to other herbs, are eaten by poultry as a bland laxative; other species of the genus *Papaver* are used in the same way (SI).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
Phytolaccaceae						
**Phytolacca americana* L. (PI. AGR 9 404)	W	Lùvino, lòvino	Fruit	Birds	To improve plumage	The fruit is added to birdseed (LU)
Pinaceae						
Pinus halepensis Miller (PI. AGR 3 119)	W	Pino	Resin	Bovines, dogs, caprines, equines, ovines	Antiseptic, wound healing	The resin—added to olive oil or lard—is heated in double boiler and applied locally on infected sores,to aid healing (LI). The buds macerated in vinegar are used for bathing affected hooves (SI).
Plantaginaceae						
Plantago major L. (PI. AGR 72 3584)	W	Tirafila	Leaf, fruit	Bovines, dogs, caprines, equines, ovines, birds	Wound healing	The leaves are crushed and applied on infected wounds, alone or mixed with olive oil (LU, PI). The fruit is used as birdfeed (MS, PI).
Polygonaceae						
Rumex acetosa L. (PI. AGR 6 299)	W	Romice	Fruit	Chickens, ducks, geese	Reconstituent	Added to feed (PI).
Rumex crispus L. (PI. AGR 6 310)	W	Rombicia, erba maligna	Leaf	All kinds	Anti-diarrheal	The decoction is given orally (PI)
Ranunculaceae						
C. vitalba L. (PI. AGR 15 725)	W	Vitalba	Whole plant	Bovines	Anti-inflammatory	To treat ocular inflammation or conjunctivitis, it is tied round the animal's neck (PI).
H. foetidus L. (PI. AGR 14 671)	W	Erba nocca, nocca, ombrelli, gafarelli, farfarelli, maschio	Root, whole plant without roots	Swine	Anti-bacterial, antiseptic, antipyretic	To treat 'mal rossino' (*E. rhusiopathiae*) in pigs (LU, PT) e and fevers of unknown nature (PI) the root is grafted underneath the tail; for abscesses the insertion is behind an ear (GR); for dog distemper the root is grafted near the trachea (PI). The decoction is used as an antiseptic lavage for new-born animals (LU).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
Helleborus odorus W. et K. (PI. AGR 14 674)	W	Erba nocca, radice (*sic*)	Root, leaf	Bovines, ovines, swine	Anti-bacterial, anti-pyretic	The root of this species, like that of *H. foetidus*, is grafted underneath the skin of pig ears when the animal has 'mal rossino'; the same procedure is used in cow and sheep tails as an anti-pyretic and as anti-bacterial in pneumonia; fresh leaves are crushed and applied locally to treat the so called 'incollatura', a purulent abscess of bovine's withers where the yoke has rubbed; it is advisable to lance the skin to free from pus before treatment (LU).
H. viridis L. (PI. AGR 14 673)	W	Radicchia, radichiella, spazzaforni	Root	Bovines, ovines	Antipyretic, anti-convulsive, fertility enhancer -cough sedative	An incision is made in ovine and bovine tails and the root is inserted to act as an antipyretic (LU, PI). It is also inserted behind lambs' ears to cure 'capo matto', that is epileptic crisis due to cranial oedema; leaves are tied under sheep tails to aid pregnancy (PI). The decoction obtained from the leaves is drunk by animals with colds and coughs (LU).
Rosaceae						
Potentilla reptans L. (PI. AGR 30 1457)	W		Leaf	Bovines, ovines	For urinary infections	The infusion is drunk by animals suffering from urinary infections (PI).
P. avium L. (PI. AGR 32 1568)	C	Ciliegio	Leaf	Bovines	Fertility enhancer	Leaves are eaten by cows before being covered, to favour pregnancy (LU).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
Rubiaceae						
Galium verum L. (PI. AGR 58 2887)	W	–	Whole plant	Bovines, swine	Antidiarrheal	The decoction is drunk to treat dysentery (GR, SI).
R. peregrina L. (PI. AGR 59 2937)	W	Sgrubbia, erba dei conigli, strozza l'oci	Whole plant without roots	Bovines, rabbits	Post partum	Eaten by cows post partum to facilitate expulsion of the placenta (GR). Likewise it is put in rabbit feed (LI, LU).
Rutaceae						
Ruta chalepensis L. (PI. AGR 43 2115)	W	Ruta	Leaf, small branch	Swine, rabbits	Anti-bacterial, reconstituent	The decoction used to be drunk by pigs affected by 'mal rossino' (*E. rhusiopathiae*) (PI). 'Ruta' and *Satureja montana* L. leaves and *H. italicum* (Roth.) Don small branches, are eaten as an excellent reconstituent for rabbits (MS).
Salicaceae						
Populus nigra L. (PI. AGR 4 176)	C	Pioppo	Leaf	Rabbits	Reconstituent	Added to forage (PI).
S. alba L. (PI. AGR 3 139)	W	Torchio, salcio	Small branches, leaf	Bovines, ovines	Ruminative	The decoction is drunk or fresh leaves are eaten to restore rumination. Some people prepare the decoction in wine by boiling 'salcio' branches and fennel flowers for one hour (LU).The practice of 'romico' is used by some, which involves forcing the animal to chew fresh branches (LU, PI).
Salix viminalis L. (PI. AGR 4 167)	W	Vinco	Branch	Ovines	Ruminative	To reactivate rumination a few small branches are tied in their mouths so they swallow the juice (SI).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
Scrophulariaceae						
Verbascum thapsus L. (PI. AGR 67 3323)	W	Erbo tasso	Leaf	Swine	Rectal prolapsus	To cure rectal prolapse an ointment made by cooking leaves in olive oil is applied locally (LU).
Solanaceae						
**Nicotiana tabacum* L. (PI. AGR 67 3310)	C	Tabacco	Foglie	Rabbits	Insecticidal	The liquid obtained by soaking cigarette butts in water for a few days is used externally to cure scabies (MS).
S. nigrum L. (PI. AGR 66 3293)	W	Cacabuzzi	Fruit	Bovines, equines, ovines, swine	Purgative	The decoction is given orally (LU).
Ulmaceae						
Ulmus minor Miller (PI. AGR 5 209)	W	Olmo	Leaf	Rabbits, ovines	Favors digestion	Leaves are added to forage (PI).
Umbelliferae						
A. sylvestris L. (PI. AGR 52 2564)	W	–	Whole plant	Bovines, ovines, rabbits	Depurative	Added to animals' hay (LU, PI).
Apium nodiflorum (L.) Lag. (PI. AGR 51 2535)	W	Sberna, sedano selvatico	Whole plant	Bovines	For gastro intestinal disorders-post partum-insecticidal-laxative	The infusion is given orally for abdominal inflammation and pain; the decoction is drunk by bovines post partum as a laxative; it is applied locally on the skin of animals affected by scabies (LU). Some add it to hay exploiting its laxative properties (SI).
Foeniculum vulgare Miller (PI. AGR 50 2498)	W	Finocchio, finocchio selvatico	Fruit, flowers	Bovines	Galactogogue-ruminative	To increase the milk supply the fruits are added to hay (LI, PI); to reactivate rumination a decoction of fennel flowers with *S. alba* L. branches cooked in wine is given by animals (SI).

Table 1 (*Continued*)

Scientific name	Cultivated (C) or wild (W)	Local name	Part used	Animal	Plant uses	Mode of preparation
Petroselinum sativum Hoffm. (PI. AGR 51 2540)	C	Prezzemolo	Whole plant without roots	Bovines, ovines, swine	Diuretic-abortive	Eaten fresh by cattle; high doses can cause abortion (LU).
Urticaceae						
Parietaria diffusa M. et K. (PI. AGR 5 229)	W	Vetriola	Small branches	Bovines	Pre partum	The decoction mixed with wheat flour is given orally before calving (LU).
U. dioica L. (PI. AGR 5 223)	W	Ortica	Seed, whole plant without roots	Caprines, chickens, ovines, turkeys	Galactogogue-egg production intensifier-reconstituent	Stinging sheep and goat udders daily with the whole plant increases milk supply; the seeds are added to chicken feed to increase egg production (LU, GR). Nettles cooked and mixed with bran, wheat flour and a few drops of oil make a highly nourishing food, suitable to be given as feed to turkeys; *Urtica urens* L. (PI. AGR 5 225) is also used (SI).
Urtica membranaceae Poiret (PI. AGR 5 226)	W	Ortica	Root	All kinds	Depurative	Instead of simply drinking water an infusion is given (PI).
Vitaceae						
V. vinifera L. (PI. AGR 44 2189)	C	Vite	Fruit, shoot	Bovines, ovines	Anti-mastitic, ruminative, against dermatosis	A mixture of vinegar and clay is applied locally to sheep or cow udders to treat mastitis; a mixture of bran, salt and vinegar, called 'biasciotto', is eaten to reactivate rumination (LU). Juniper berries of *C. sempervirens* L. are boiled in vinegar and with this liquid skin spoiled by excessive sweating is washed (PI).

*, Introduced species.

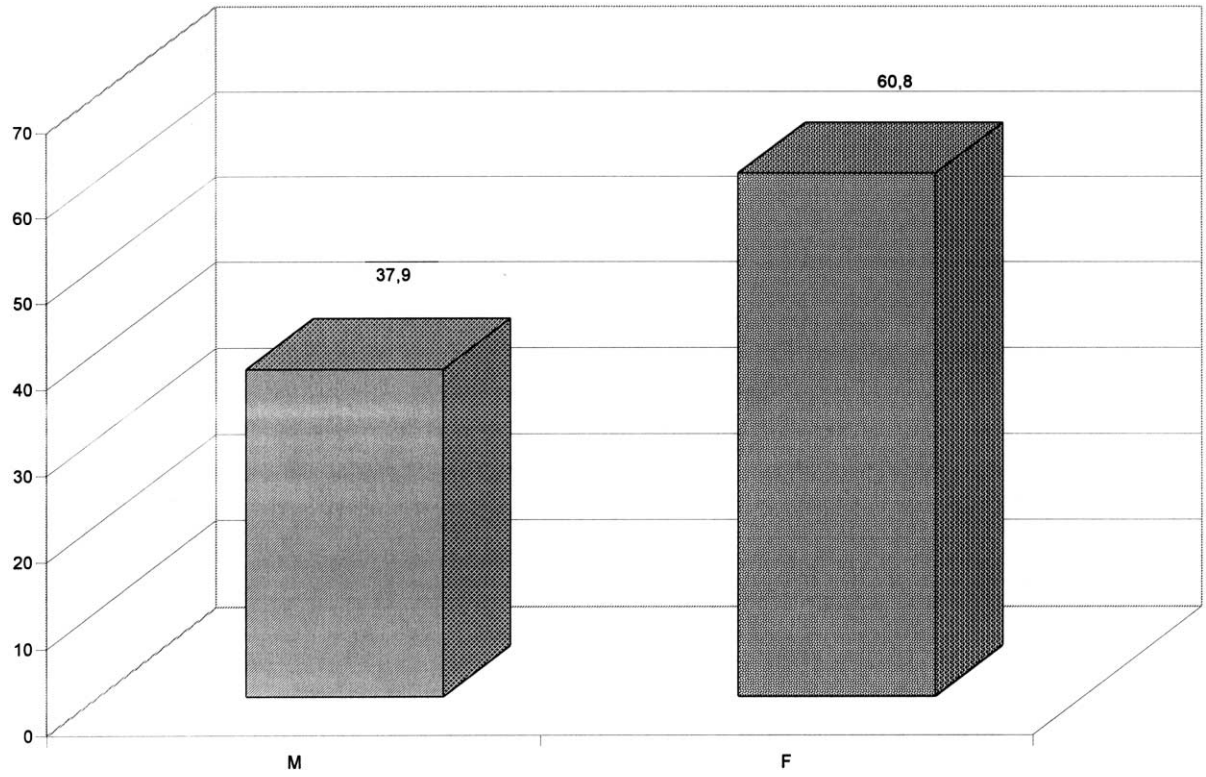

Fig. 2. Percentage of males (M) and females (F) which supplied information about Tuscan ethnoveterinary medicine.

involves other species, above all pigs. Not many contagious cases are noted here except in those individuals affected by acquired immuno-deficiency syndrome (AIDS; Redaelli, 1980).

Another feared pathogen, very widespread up until a few years ago now under control thanks to prevention methods, is *Listeria monocytes*, which causes listeriosis. It strikes mostly ruminants but also pigs and birds; it can be transmitted to man through ingestion of infected animal products like milk and dairy products; it cause serious fetal damage and in the adult forms of meningitis and septicaemia especially in the liver, spleen and lungs (Gualandi, 1980). For these diseases, no ethnoveterinarian treatments exist in this region.

For other animal diseases, more or less serious still present in this region, occasionally natural remedies are used as in the case of *Salmonella pullorum* e *Salmonella gallinarum*; the most common forms of salmonellosis which strike farm poultry, like chickens and turkeys. The first has a main symptom, white diarrhoea (called 'calcinaccio' which derives from 'calce'—lime referring to its colour and consistency) while the second is referred to as 'tifosi'—typhous (Andreani, 1980). In this second case a watery solution containing macerated *Fraxinus ornus* L. is used. The bark is left to steep in water for 3–4 days, after which the liquid obtained replaces normal drinking water to force the animal to drink this solution. The animals were kept without food for a whole day after which they were given dry feed. This treatment lasted for 5–7 days.

Another disease which was very common up until a few years ago which strikes mainly pigs but can infect ovines and other farm animals is 'mal rossino', caused by *Erysipelothrix rhusiopathiae*; its symptoms are high fever, skin rash and septicaemia and is contagious for man (Jones, 1986).

The therapy in practice today in the rare cases that appear utilises the roots of *Helleborus foetidus* L. and *Helleborus odorus* W. et K., a cut is made behind the animal's ear or tail and a piece of *Helleborus* root is inserted; a few days later the area swells with body fluid, the root is removed enabling the fluid to discharge and thus the recovery of the animal. This treatment is also used in sheep and pigs to cure fever states of unknown origin or pneumonia, while the curing of 'taroni', abscesses on necks and ears is carried out by inserting the stem of *Helleborus* instead of the root.

Even dogs with distemper, until a few decades ago, were cured with the insertion of *Helleborus* root; in this case the insertion took place near the trachea.

Still another common illness present in Tuscan breeding farms and often cured with plant derivatives is the so-called 'ventrina'. It consists of a large and noticeable swelling of the animal's belly caused by gastric fermentation due to ingestion of fresh grass or hay, which mostly affects rabbits and ovines. *Cupressus sempervirens* L. is often used to treat this by feeding the animal the branches and fruit. Another plant species, which can cure this illness is *Marrubium vulgare* L. whose leaves are eaten.

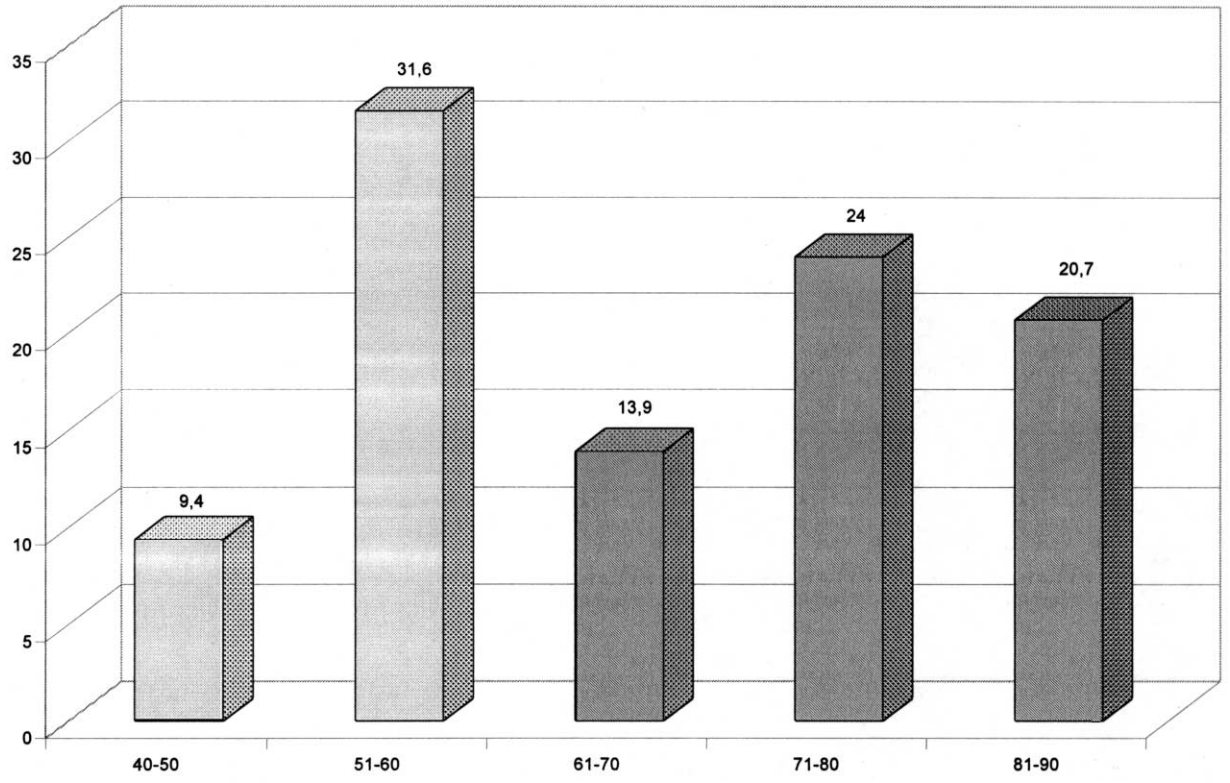

Fig. 3. Age ranges referring to those who were familiar with Tuscan ethnoveterinary medicine.

Other plants used in much of the region are those necessary to reactivate rumination, some by means of 'romico'. This practice consists of placing a branch of the chosen plant between the animal's jaw. This can be done in two ways. In the first, a hole is made at both ends of the branch and a string is passed through the holes. Once the branch is placed in the animal's mouth the ends of the string are tied behind the animal's head to block the stick between its teeth and force the animal to chew. In the second, a kind of muzzle is used which is placed around the animal's head after having fixed the ends of the branch inside the muzzle. The species used mostly for 'romico' are *Fraxinus excelsior* L. and *Salix alba* L. It is to be noted that while plants which are considered 'ruminative' are specifically used to reactivate end chewing in ruminants, others which favour digestion are given to animals mixed with normal forage or during particular ailments (for example after indigestion or an illness).

A particular custom used to treat indigestion in sheep in the province of Siena is the following, a cut is made with a knife behind the animal's ear and a branch of *Vitis vinifera* L. is inserted so treat the sides of the cut are separated to enhance bleeding which is considered extremely efficacious.

Regarding plants which are generically defined as 'depurative', they are believed to bring about a sort of general detoxication. This is the case of *Laurus nobilis* L., *Linum usitatissimum* L., *Mercurialis annua* L., *Ceterach officinarum* DC., used especially for post partum, or *Angelica sylvestris* L., which is mixed with hay after an illness, after physical stress or fright or simply in certain periods during the year, in particular spring and fall.

An original use of plants is that of increasing production of specific products of animal origin. For example,

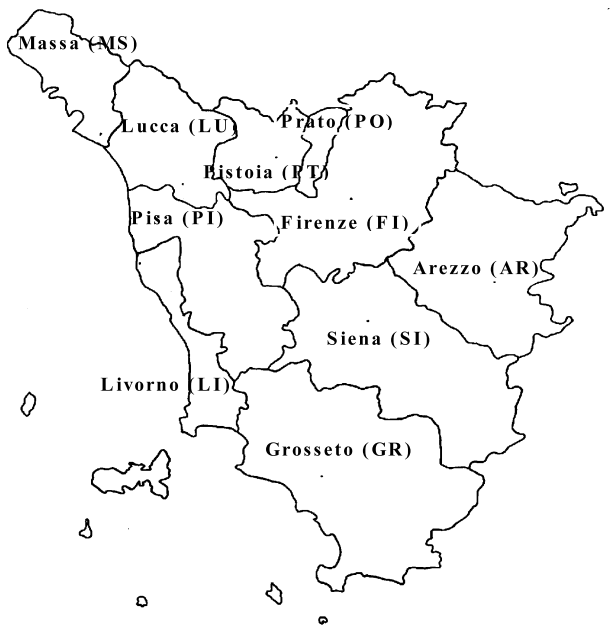

Fig. 4. The region of Tuscany divided into provinces.

Form for collecting ethnobotanical/veterinary data

1. Area of data collecting

Place:
Town:
Province:

2. Personal information of interviewed person

First name and Last name:
Age:
Sex:
Education:
Job:

3. Data referring to the plant

Scientific name:
Local name (s):
Habitat:
Parts used:
Season picked:
Type of extract (decoction, infusion, steeped in water, oil, etc.)
Local name:
Kind of animal treated:
Illness treated:
Used with other plants (indicate which):
Kind of preparation:
How used and how often:
If product still used in the area: YES □ alot □ not much□ rarely NO secify how long and why:

Fig. 5. Data questionnaire.

to increase the quantity and improve the quality of sheeps' milk, a few branches of *Helichrysum italicum* (Roth) Don are added to their daily diet, while *Stellaria media* (L.) Vill. leaves, *Urtica dioica* L. seeds and an infusion prepared with the bark of *F. ornus* L. are added to chicken feed to increase egg production.

Among other plants found in the regional ethnoveterinarian traditions, toxic plants are present, some of which are orally administered. An example is *Euphorbia lathyris* L. whose fruit is used to prepare an oily solution and a watery decotion which cattle, affected by constipation, are forced to swallow with the aid of needle less syringes and injected into the animal's mouth.

The *M. annua* L. is another toxic plant having laxative properties which can be used only in large animals due to its high toxicity. In small amounts, it is also used in the regional cuisine in preparing the typical Lucca soup dishes (Tomei et al., 1996).

Another toxic plant taken orally is *Solanum nigrum* L.; in low doses its fruit has a laxative effect on large animals having long and troubled intestinal constipation.

The use of *Clematis vitalba* L., even though it is toxic, is applied externally. However, the plants shoots are considered edible in the popular Tuscan cuisine and picked for the preparation of omelettes (Camangi and Uncini Manganelli, 2000).

Artemisia absinthium L. is given orally to cattle both to favour digestion and as vermifuge. The dosages are low and used with large animals.

The species pertaining to the *Helleborus* genus are highly toxic and subject to a particular use as indicated in the table, concerning an under the skin root insertion. This tradition has existed in Italy for centuries (Mattioli, 1568) and can be found in other European countries (Bogdan et al., 1990).

By analysing the obtained data, the following information indicates the various plant uses and their per-

centages, the most exploited is ruminative (9%) followed by wound healing (8%), anti-inflammatory and post partum (6%), digestive (5.5%), anti-mastitis, anti-bacterial, gastro-intestinal and anti-diarrhoeal (4%), followed by others of minor use.

Statistically speaking, the animals treated mostly with popular remedies are, bovines (32%); ovines (20%); rabbits (10%); equines (8%); pigs (7%); chickens (6%); goats (5%); others (12%).

In particular bovines and horses are more prone to wounds, abrasions and swellings due to their employment on the farm.

Dysentery is quite widespread among large and small animals due to unbalanced nutrition, hygienic norms or bacteria.

Skin wounds are very common during the summer months caused by haematophage insects like mosquitoes (*Anopheles* sp. pl.) and horse-flies (*Tabanus* sp. pl.). Treatments are prepared to apply on the animal's hide for this purpose (*Equisetum telmateja* Ehrh.). Furthermore, to keep lice and other parasites away from chicken coops and stables, *Alnus glutinosa* (L.) Gaertner and *Sambucus ebulus* L. leaves are put in every corner.

Finally, great attention is placed on the reproductive cycle of the various animals, in particular bovines, due to the economic impact they have with rapid and prolific reproduction. In this area, plants like *Avena sativa* L., *Helleborus viridis* L. and *Prunus avium* L. are used as fertility enhancers. Others in the post partum group like *Adiantum capillus-veneris* L., *Rubia peregrina* L., favour the expulsion of the placenta; the anti-haemorrhaging plants (*Capsella bursa-pastoris* (L.) Medicus), the depurative plants (*M. annua* L., *L. nobilis* L., ecc.), the galactagoguing plants (*U. dioica* L., *Medicago sativa* L., *Galega officinalis* L., *Leontodon tuberosus* L.) and the anti-mastitis plants (*Malva sylvestris* L., *A. sativa* L., *Sambucus nigra* L., ecc.).

The richness of data collected induces us to make further considerations regarding the ethnoveterinarian customs in Tuscany. As we have already indicated in our report, many practices are unusual and original both in the choice of plants, many not recognised by official veterinary medicine, some being even toxic, and in the extravagant preparation methods. Some uses, originating from folk empiricism to cure specific ailments connected to the use of work animals have disappeared caused by technological changes in the agricultural environment. For example 'mal rossino', a bacterial disease in pigs, was once treated with *Helleborus* sp. pl. root, today vaccines, hygiene and prevention under the supervision of the National Health Service deal with these illnesses.

What remains, in our opinion, are all those practices and customs used for curing minor ailments (dysentery, mycosis, gastro-intestinal disorders, etc.) which afflict small farm animals (chickens, rabbits, geese, turkeys, etc.) raised for family use.

In a future publication, it would be interesting to examine in detail the properties given to *U. dioica* L., *F. ornus* L., *S. media* (L.) Vill. e *H. italicum* (Roth) Don which are used in the popular Tuscan tradition to increase production and improve the organo-electric qualities of some animal products (milk and eggs) together with those species (*A. glutinosa* (L.) Gaertner e *Sambusus ebulus* L.) used to keep away insects which could be employed to manufacture natural insecticides.

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Antiprotozoal activities of Colombian plants

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Abstract

In our search for therapeutical alternatives for antiprotozoal chemotherapy, we collected a selection of 44 plants from western Colombia upon ethnopharmacological and chemotaxonomic considerations. Polar and apolar extracts of these species were examined for antimalarial activity using in vitro tests with two clones of *Plasmodium falciparum*. Leishmanicidal and trypanocidal activity were determined in vitro using promastigote and amastigote forms of several strains of *Leishmania* sp. and epimastigotes of *Trypanosoma cruzi*. Among the selected plants, the 15 following species showed good or very good antiprotozoal activity in vitro: *Aspidosperma megalocarpon*, *Campnosperma panamense*, *Conobea scoparioides*, *Guarea polymera*, *Guarea guidonia*, *Guatteria amplifolia*, *Huberodendron patinoi*, *Hygrophila guianensis*, *Jacaranda caucana*, *Marila laxiflora*, *Otoba novogranatensis*, *Otoba parviflora*, *Protium amplium*, *Swinglea glutinosa* and *Tabernaemontana obliqua*. Cytotoxicity was assessed in U-937 cells and the ratio of cytotoxicity to antiprotozoal activity was determined for the active extracts. Ten extracts from eight species showed selectivity indexes ≥ 10. Among the extracts that showed leishmanicidal activity, the methylene chloride extract of leaves from *C. scoparioides* showed a selectivity index in the same range that the one of the Glucantime control. Several of the active leishmanicidal plants are traditionally used against leishmaniasis by the population of the concerned area.

Keywords: Antimalarial activity; Leishmanicidal activity; Trypanocidal activity; Colombian plants

1. Introduction

The crisis of reemerging infectious diseases and the resistance of many pathogens to currently used drugs is widely recognized as being of serious and immediate concern. Morbidity and mortality due to malaria remains an important health problem in many countries and chloroquine-resistant *Plasmodium falciparum* is widespread in tropical zones. The different forms of leishmaniasis require expensive treatments, and the currently used medicines, pentavalent antimonials and/or pentamidine salts, show toxicity together with numerous side effects. Nifurtimox and benznidazole, which are used to treat the acute stages of Chagas' disease, are poorly tolerated. Higher plants are clearly a potential source of the new antiprotozoal drugs, which are needed (Kondrachine and Trigg, 1997; Phillipson and Wright, 1991).

The pacific coast of Colombia is part of the biogeographical Chocó region, which goes from Panama to the Ecuadorian coasts. The region is predominantly populated by black ethnic population and is an endemic area for malaria and cutaneous and mucocutaneous leishmaniasis. Traditional therapies against protozoal infections still play an important role among these communities. However, this knowledge is progressively disappearing, due to a lack of systematization of this heritage and to severe deforestation in some parts of this area in recent years. In this paper, we present the results of our ethnopharmacological and botanical in-

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vestigations, together with the results of a biological study using in vitro bioassays to confirm the parasitological activity. The results have been tabulated and we have added the bibliographic data about the use of the described material together with biological and chemical references.

2. Materials and methods

2.1. Ethnopharmacological and botanical studies

Ethnopharmacological and botanical researches were carried out in the Department of Valle, in the Occidental part of Colombia, particularly on the Pacific coast near Buenaventura, and around Cali. The coastal area near Buenaventura, inhabited by afro-Colombian communities and characterized by a very hot and humid climate, is occupied by primary and secondary forest and is an endemic region for malaria and cutaneous and mucocutaneous leishmaniasis. The area near Cali (1000–1500 m), where the forest has been largely replaced with sugar cane, coffee and fruit cultures, has a subtropical climate. Ethnopharmacological and botanical information was collected from the natives, particularly from the afro-Colombian communities of the pacific coast. Besides selecting plants on the basis of ethnopharmacological criteria, we collected the other species on the basis of bibliographic or chemotaxonomic criteria. Herbarium samples were determined by Lic. Nestor Paz and Lic. Robert Tulio Gonzalez. Voucher specimens were deposited at the Herbarium of the Universidad del Valle, Cali (CUVC).

2.2. Preparation of extracts

For each part of plant, the methylene chloride extract was prepared by macerating 5 g of powdered dry plant material in stoppered flasks containing 50 ml of methylene chloride for 3 days. After extraction, the same plant material was dried and used again for the preparation of the methanolic extract, using 50 ml of methanol in a stoppered flask for 3 days. After filtration, the solvent was evaporated under reduced pressure.

2.3. Biological assays

2.3.1. Antimalarial activity in vitro

For the in vitro tests, cultures of the D2 (chloroquine resistant) and F32-Tanzania (chloroquine sensitive) strains of *P. falciparum* kindly provided by Dr T. Fandeur (Pasteur Institute Cayenne, France) were maintained according to Trager and Jensen (1976), in human red blood cells on glucose-enriched RPMI 1640 medium supplemented with 10% human serum at 37 °C.

Fifty microlitres of dimethyl sulfoxide (DMSO) were added to the plant extracts, which were dissolved in RPMI 1640 medium with the aid of mild sonication in a sonicleaner bath (Branson Ltd.), and then diluted as required in culture medium. The final DMSO concentration was never greater than 0.1%. One hundred and fifty microlitres of total culture medium with the diluted extract and the suspension of infected human red blood cells (0+ group, 5% haematocrit, 1% parasitaemia), were distributed into a 96-well microtitre plates. All tests were performed in triplicate. After 24 h of incubation at 37 °C in a candle jar incubator, the medium was replaced by fresh medium with the diluted extract, and incubation was continued for a further 48 h. On the 3rd day of the test, a blood smear was taken from each well and parasitaemia counted. The parasitaemia for each well was obtained and the percentage inhibition of parasitaemia for each concentration of extract was calculated in relation to the control. IC_{50} values were determined graphically by plotting concentration versus percentage inhibition. Each test also included an untreated control with solvent and a positive control with a *Cinchona calisaya* Wedd. (Rubiaceae) bark extract. Chloroquine diphosphate (Sigma, USA) was also used as control. It was considered that if the extracts displayed an IC_{50} less than 5 μg/ml, the antimalarial activity was very good; from 5 to 25 μg/ml the antimalarial activity was good; over 25 μg/ml the extract was considered inactive. The IC_{50} of *C. calisaya* stem bark extract used as the standard drug in these assays was 0.2 μg/ml against both strains. The IC_{50} of Chloroquine diphosphate was 0.006 μg/ml for the F32 strain and 0.100 μg/ml for the D2 strain.

2.3.2. Leishmanicidal and trypanocidal activity in vitro

Leishmanicidal assays on the promastigote forms of *Leishmania* spp. and trypanocidal activity on the epimastigote forms of *Trypanosoma cruzi* were performed in vitro. Three strains of *Leishmania* and one strain of *T. cruzi* were used during these investigations: *Leishmania mexicana amazonensis* (IFLA/BR/67/PH8) responsible for the cutaneous form, *Leishmania braziliensis* (MHOM/BR/75/M 2903) responsible for the mucocutaneous form of the disease, *L. donovani infantum* (MHOM/IN/PP75) responsible for the visceral form and *T. cruzi* (Tulahuen) responsible for Chagas disease. All strains were obtained from IBBA (La Paz, Bolivia). *Leishmania* promastigote were cultivated at 28 °C in Schneider's Drosophila medium (Sigma) supplemented with heat inactivated (56 °C for 30 min) fetal calf serum (10%). Epimastigotes of *T. cruzi* were maintained in liver infusion tryptose medium (LIT, Bacto) supplemented with 10% fœtal calf serum at 28 °C. Plant extracts passed through 0.22 μm Millipore filters, were previously dissolved in saline or DMSO (with a final concentration not exceeding 0.1%) and then dissolved

in the culture medium. Parasites in logarithmic growth phase were dispatched in 96 flat bottom well plates at a concentration of 10^6/ml. Each well contained increasing concentration of the extract, from 10 up to 100 μg/ml during 72 h. The activity was determinated by evaluating the movements of the parasites with an inverted microscope and compared to control wells (without extract and with reference drugs). The movements were estimated as follow: 0 cross means that the parasite are in good conditions and the drug inactive; 1 cross, the drug is poorly active; 2 crosses, the drug is active; 3 crosses, no movement is detected, the drug is very active. Pentamidine (Aldrich chemical) and ketoconazole (Janssen Pharmaceutica) for *Leishmania* spp. and Nifurtimox (Bayer) for *T. cruzi* were used as reference drugs. All assays were carried out in triplicate (Moretti et al., 1998).

The evaluation of leishmanicidal activity on intracellular amastigotes of *Leishmania* (*Viannia*) *panamensis* (M/HOM//87/UA140) strain obtained from infected human promonocytic U-937 cells (Sundstrom and Nilsson, 1976) was performed only on extracts that showed activity (2 or 3 crosses) against *Leishmania* spp. promastigotes. To maintain the virulence of the parasites and obtain a good in vitro infection, parasites were maintained by passage in golden hamsters (*Messocrycetus aureatus*) (Rey-Ladino et al., 1990). Periodically, lesions were aspirated and the material obtained was cultivated in NNN medium until stationary phase growth of promastigotes. Briefly, after 48 h of growth, the U-937 cells were washed twice with Dulbecco's phosphate buffer saline (DPBS) (Gibco BRL). 100.000 cells/ml were exposed to stationary phase growth promastigotes at a ratio of 25 parasites/cell. Infected cell cultures were incubated for 2 h with 5% CO_2 at 34 °C. Free parasites were removed by washing twice with warm DPBS. After 24 h of incubation with 5% CO_2 at 34 °C in RPMI 1640 medium containing 10% FCS, the medium was replaced with complete RPMI 1640 medium containing the corresponding concentration of extract. The range of concentration varied between 0.1 and 10 μg/ml, depending of the LD_{50} for each extract. Thereafter, the medium was renewed every 2 days. After 96 h of incubation in the presence of extract or compound, cells were washed, fixed with methanol (JT Baker) for 20 min and stained with Giemsa (Merck). Similarly, infected cells cultured in absence of extract or compound served as control of infection. For each extract, three independent experiments in triplicate were performed for the determination of the leishmanicidal activity. All assays were evaluated blindly. For each test 200 cells/well were examined at random; the number of infected and uninfected cells was recorded. Percentage of infection was calculated by dividing the number of infected cells obtained in presence of each extract or compound by the number of infected cells obtained in absence of treatment (Robledo et al., 1999). Glucantime (Rhône-Poulenc) was used as reference drug. Results were expressed as ED_{50}, which was calculated by Probit analysis (Finney, 1964).

It was considered that if the extracts displayed an ED_{50} lower than 25 μg/ml, the leishmanicidal activity against intracellular amastigotes was very good; from 25 to 100 μg/ml the leishmanicidal activity was good; over 100 μg/ml the extract was considered inactive. The ED_{50} of Glucantime used, as the standard drug in these assays was 6.7 μg/ml.

2.3.3. Cytotoxicity

Cytotoxic activity of active extracts and test compounds was evaluated on human promonocytic U-937 cells. To estimate the 50% lethal doses (LD_{50}), the 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) enzymatic micromethod was used (Sereno and Lemesre, 1997). U-937 cells were cultured in suspension in complete RPMI 1640 medium (Gibco BRL, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (FCS) in the presence of 5% CO_2 at 37 °C. Medium was renewed at 2-days intervals. Cells were harvested and washed by centrifuging for 10 min at $400 \times g$, then counted and adjusted to a final concentration of 1×10^6 cell/ml. Hundred microlitre were seeded in 96-well-flat-bottom microplates (Nunc). Plant extracts passed through 0.22 μm Millipore filters, were previously dissolved in saline or DMSO (with a final concentration not exceeding 0.1%) and then dissolved in the culture medium. Then, 100 μl of each extract was added at concentrations ranging from 3 to 100 μg/ml. Cells were incubated at 37 °C with 5% CO_2; the medium was changed after 48 h of incubation and cells were reincubated in presence of the same concentrations of extract. After 96 h of incubation, 10 μl of MTT (10 mg/ml) was added to each well. Plates were further incubated for 3 h. The enzymatic reaction was then stopped by addition of 100 μl of 50% isopropanol–10% sodium dodecyl sulfate solution. The plates were incubated for an additional 30 min under agitation at room temperature. The optical density at 570 nm was measured using an ELISA plate reader (Bio Rad). Cells cultivated in absence of treatment but maintained under the same conditions were used as control. Three independent experiments in triplicate were performed for the determination of cytotoxicity of each extract or test compound. Results were expressed as LD_{50} and calculated by Probit analysis.

3. Results

In the present study, we report the use of one plant against malaria and five plants used topically to treat cutaneous leishmaniasis on the Pacific coast of Colom-

bia near Buenaventura (see Table 1). Polar and apolar extracts from these six species, together with polar and apolar extracts from a selection of 38 species collected only on the basis of bibliographic or chemotaxonomic criteria, were tested in vitro for antiprotozoal activity.

Table 2 summarizes the positive results obtained with the extracts that showed antiprotozoal activity against two clones of *P. falciparum*, *Leishmania* spp. promastigotes and/or *T. cruzi* epimastigotes, as well as the results against *L.* (*V.*) *panamensis* amastigotes, for the extracts that showed previous activity against *Leishmania* spp. promastigotes.

Table 3 summarizes the cytotoxicity against U-937 cells in culture as well as the ratio of cytotoxicity to biological activity (selectivity index) of the plant extracts and reference drugs.

4. Discussion

Hygrophila guianensis Nees (Acanthaceae), Chupador. The leaves of this herbaceous plant are used as a topical application against leishmaniasis by black and indigenous groups of Southwest Colombia (Caballero, 1995). This is the only clear case of convergence of use of a species with antiprotozoal reputation between our study and other similar investigations carried out on the Ecuadorian and Colombian Pacific coasts (Vasquez et al., 1991; Kroeger et al., 1993; Weigel et al., 1995; Blair et al., 1991). Neither biological nor chemical data about this species could be found in the literature.

Campnosperma panamense Standl. (Anacardiaceae), Sajo. This tree is used as a commercial source of timber on the Pacific coast of Colombia (Poyry, 1982). Neither biological nor chemical data about this species could be found in the literature.

Guatteria amplifolia Triana & Planch. (Annonaceae), Cargadero. The bark of this medium-sized tree, distributed through the Occidental coast of Central and Northern South America, is used in Nicaragua for diarrhea and venereal diseases (Coee and Anderson, 1996). The presence of aporphine alkaloids in the genus, and the trypanocidal activity for some of them, were described (Hsu et al., 1977; Mahiou et al., 1994). As leishmanicidal and trypanocidal activities were reported in the literature (Fournet et al., 1994), this is the first time that antimalarial activity is demonstrated in the genus.

Aspidosperma megalocarpon Müll. Arg. (Apocynaceae), Costillo acanalado. Various trees of the genus *Aspidosperma* are used in the Northwest Amazonia against fever and rheumatism, and as a source of timber (Perez-Arbelaez, 1994; Schultes and Raffauf, 1990). Previous studies report that the bark of *A. megalocarpon* exhibits antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*, and the aerial parts show antiviral activity (Verpoorte et al., 1983; Roming et al., 1992). Recently, three alkaloids identified as fendlerine, aspidoalbine and aspidolimidine, isolated from the stem bark of the same plant, demonstrated strong antimalarial activity in vitro (Mitaine et al., 1998).

Tabernaemontana obliqua (Miers) Leeuwenb. (Apocynaceae), syn. *Bonafousia obliqua* Miers, Mierda de guagua. Various species from this genus are used in Colombia and in all the Amazonian area as antirheumatic (García Barriga, 1992; Duke and Vasquez, 1994). The genus is well known for the presence of indole alkaloids. Neither biological nor chemical data about this species could be found in the literature.

Jacaranda caucana Pittier (Bignoniaceae), Gualanday. This tree, and other species from the same genus, is commonly used in Colombia against rheumatism, colds and skin diseases (Bartholomäus et al., 1995; Schultes and Raffauf, 1990). A work of Colombian investigators mentions the use of this species for the treatment of leishmaniasis by a predominantly black ethnic population living in an endemic area of Southwestern Colombia (Vasquez et al., 1991). This use, however, was not reported in our survey. Aerial parts of the plant contain quinoid derivatives and triterpenes, and show cytotoxic activity (Ogura et al., 1976, 1977). As leishmanicidal activity has been reported in the literature (Sauvain et al., 1993), this is the first time that antimalarial activity is demonstrated in the genus.

Huberodendron patinoi Cuatrec. (Bombacaceae), Carrá. This large tree is used as a commercial source of timber on the Pacific coast of Colombia (Poyry, 1982).

Table 1
Plant species used for protozoal infections in Western Colombia near Buenaventura

Family	Scientific name	Local name	Part used[a]	Use	Voucher number
Acanthaceae	*H. guianensis* Nees	Chupador	AP	Leishmaniasis	BW147
Loganiaceae	*P. amara* Aubl.	Amargo andré	L and R	Malaria	BW140
Moraceae	*Castilla elastica* Sessé	Caucho negro	L	Leishmaniasis	BW120
Myristicaceae	*O. novogranatensis* Moldenke	Otobo	RE	Leishmaniasis	BW099
Myristicaceae	*O. parviflora* (Markgr.) A.H. Gentry	Otobo	RE	Leishmaniasis	BW070
Scrophulariaceae	*C. scoparioides*(Cham. & Schltdl.) Benth.	Hierba de sapo	AP	Leishmaniasis	BW109

[a] AP, aerial part; L, leaves; R, root; RE, resin-like bark exudate.

Table 2
In vitro antiprotozoal activity of some Colombian plant extracts

Family	Scientific name	P[a]	E[b]	Antiprotozoal activity							V[e]
				P. falciparum[c]		*Tc*	Promastigotes			Amastigotes[d]	
				F32	D2		*La*	*Lb*	*Li*	*Lp*	
Acanthaceae	*H. guianenensis* Nees	AP	D	>50	>50	0	++	0	++	24.0	BW147
Anacardiaceae	*C. panamense* Standl.	L	D	3	35	0	0	0	0	NT[f]	BW067
Anacardiaceae	*C. panamense* Standl.	L	M	15	5	0	0	0	0	NT	BW067
Annonaceae	*G. amplifolia* Triana & Planch	AP	M	1.9	1.5	0	0	0	0	NT	BW030
Apocynaceae	*A. megalocarpon* Mull. Arg	B	M	25	8	0	0	0	0	NT	BW031
Apocynaceae	*T. obliqua* (Miers) Leeuwenb.	L	M	25	40	0	++	+	++	47.4	BW119
Bignoniaceae	*J. caucana* Pittier	L	M	14	4.6	0	0	0	0	NT	BW017
Bombacaceae	*H. patinoi* Cuatrec.	B	M	3	18	0	0	0	0	NT	BW094
Burseraceae	*P. amplum* Cuatrec.	FR	D	32	50	+	++	++	++	8.2	BW092
Clusiaceae	*M. laxiflora* Rusby	L	D	20	28	+++	+++	+++	+++	11.0	BW137
Meliaceae	*G. guidonia* (L.) Sleumer	S	D	10	10	0	0	0	0	NT	BW104
Meliaceae	*G. polymera* Little	L	D	>50	>50	+++	+++	+++	+++	4.0	BW066
Meliaceae	*G. polymera* Little	L	M	>50	>50	+	+++	+++	+++	10.0	BW066
Meliaceae	*G. polymera* Little	B	D	>50	>50	+++	+++	+++	+++	4.0	BW066
Myristicaceae	*O. novogranatensis* Moldenke	L	D	>50	>50	+++	+++	+++	+++	177.0	BW099
Myristicaceae	*O. novogranatensis* Moldenke	L	M	20	26	+++	+++	+++	+++	>40	BW099
Myristicaceae	*O. novogranatensis* Moldenke	FR	D	>50	>50	+++	+++	+++	+++	6.5	BW099
Myristicaceae	*O. novogranatensis* Moldenke	FR	M	>50	>50	+++	+++	+++	+++	10.6	BW099
Myristicaceae	*O. parviflora* (Markgr.) AB.H. Gentry	B	D	>50	>50	+++	+++	+++	+++	98.0	BW070
Rutaceae	*S. glutinosa* Merr.	B	D	2.6	5	0	+	+	+	NT	BW103
Rutaceae	*S. glutinosa* Merr.	B	M	10	10	0	+	+	+	NT	BW103
Scrophulariaceae	*C. scoparioides* (Cham. & Schltdl.) Benth	L	D	>50	>50	+++	+++	+++	+++	1.3	BW109
	Chloroquine diphosphate			0.006	0.1	NT	NT	NT	NT	NT	
	Glucantime			NT	NT	NT	NT	NT	NT	6.7	

[a] AP, aereal parts; B, bark; FR, fruits; L, leaves; S, seeds.
[b] D, methylene chloride extract; M, methanol extract.
[c] IC_{50} (μg/ml) at 72 h; F32, Tanzania (chloroquine sensitive) strain of *P. falciparum*; D2, chloroquine resistant strain of *P. falciparum*; *Tc*, epimastigotes of *T. cruzi*; *La*, promastigotes of *Leishmania* (*L.*) *amazonensis* (IFLA/BR/67/PH8), *Lb*: *L.* (*V.*) *braziliensis* (MHOM/BR/75/M2903), *Li*, *L. (L.) infantum* (MHOM/IN/PP75), at 100 μg/ml of extract.
[d] ED_{50} (μg/ml) at 96 h, amastigotes of *Lp*, *L.* (*V.*) *panamensis* (MHOM/CO/87/UA140).
[e] Voucher number.
[f] NT, no tested.

Table 3
Cytotoxicity and selectivity index of some Colombian plant extracts

Family	Scientific name	P[a]	E[b]	Cytotoxicity[c] U-937 strain	Selectivity Index[d]		
					P. falciparum		*L. panamensis* Amastigotes
					F32	D2	
Acanthaceae	*H. guianensis* Nees	AP	D	101.8 ± 7.2	NC	NC	4.2
Anacardiaceae	*C. panamense* Standl.	L	D	172.5 ± 7.3	5.6	0.5	NC
Anacardiaceae	*C. panamense* Standl.	L	M	653.5 ± 19.5	43.6	130.7	NC
Annonaceae	*G. amplifolia* Triana & Planch	AP	M	75.7 ± 15.7	39.8	50.5	NC
Apocynaceae	*A. megalocarpon* Mull. Arg	B	M	0.4 ± 0.0	0.02	0.05	NC
Apocynaceae	*T. obliqua* (Miers) Leeuwenb.	L	M	231.6 ± 2.7	9.3	5.8	4.9
Bignoniaceae	*J. caucana* Pittier	L	M	281.7 ± 23.4	20.1	61.2	4.9
Bombacaceae	*H. patinoi* Cuatrec	B	M	501.0 ± 45.8	167.0	27.8	NC
Burseraceae	*P. amplum* Cuatrec.	FR	D	56.8 ± 11.8	1.8	1.1	5.5
Clusiaceae	*M. laxiflora* Rusby	L	D	203.1 ± 0.0	10.2	7.3	18.5
Meliaceae	*G. guidonea* (L.) Sleumer	S	D	28.8 ± 8.2	2.9	2.9	NC
Meliaceae	*G. polymera* Little	L	D	6.1 ± 1.2	NC	NC	1.5
Meliaceae	*G. polymera* Little	B	D	6.1 ± 0.5	NC[e]	NC	1.5
Myristicaceae	*O. novogranatensis* Moldenke	L	D	27.6 ± 3.6	NC	NC	0.2
Myristicaceae	*O. novogranatensis* Moldenke	L	M	76.6 ± 18.4	28.4	21.9	NC
Myristicaceae	*O. novogranatensis* Moldenke	FR	D	28.5 ± 2.8	NC	NC	4.4
Myristicaceae	*O. novogranatensis* Moldenke	FR	M	38.1 ± 4.1	NC	NC	3.6
Myristicaceae	*O. parviflora* (Markgr.) AB.H. Gentry	B	D	233.7 ± 16.8	NC	NC	2.4
Rutaceae	*S. glutinosa* Merr.	B	D	>400	>153.8	>80.0	NC
Rutaceae	*S. glutinosa* Merr.	B	D	>400	>40.0	>40.0	>4.8
Scrophulariaceae	*C. scoparioides* (Cham. & Schltdl.) Benth	L	D	63.4 ± 0.1	NC	NC	48.8
	Chloroquine diphosphate			57.9 ± 0.2	9650	579	NC
	Glucantime			416.4 ± 3.4	NC	NC	62.1

[a] AP, aereal parts; B, bark; FR, fruits; L, leaves; S, seeds.
[b] D, methylene chloride extract; M, methanol extract.
[c] LD_{50} (μg/ml) at 96 h.
[d] Selectivity index, LD_{50}/IC_{50} (*Plasmodium* sp.) or LD_{50}/ED_{50} (*Leishmania Viannia panamensis*).
[e] NC, not calculated.

The species is endemic of the Chocó region. Neither biological nor chemical data about this species could be found in the literature.

Protium amplum Cuatrec. (Burseraceae), Anime. Several species from this genus are sources of balsamic resinous latex used in Latin America against tumors and heavy colds (Pernet, 1972; Schultes and Raffauf, 1990). The resin essential oil of several *Protium* species, mainly constituted of monoterpenes and phenylpropanoids, show anti-inflammatory-related activity (Siani et al., 1999). Neither biological nor chemical data about *P. amplum* could be found in the literature.

Marila laxiflora Rusby (Clusiaceae), Aceitillo. The genus *Marila* is distributed in the tropics of Central and South America and the West Indies. The roots of various species of this genus are used against dysentery by the Siona Indians of South Colombia (Schultes and Raffauf, 1990). Recently, antifungal xanthones were isolated from the roots of this species (Ioset et al., 1998).

Guarea guidonia (L.) Sleumer (Meliaceae), syn. *G. trichiloides* L., Cedro macho. The seeds of this species are used in Brazil for rheumatism, and show anti-inflammatory activity in rats (Oga et al., 1981). The aerial parts of the same plant show weak antimalarial activity in vitro (Antoun et al., 1993). Limonoids and other triterpenes have been found in various parts of the plant (Zelnik and Rosito, 1971; Furlan et al., 1993; Lukacova et al., 1982).

Guarea polymera Little (Meliaceae), syn. *Guarea chalde* Cuatrec., Chalde. This medium-large tree is used as a commercial source of timber on the Pacific coast of Colombia (Poyry, 1982). Neither biological nor chemical data about this species could be found in the literature.

Otoba novogranatensis Moldenke (Myristicaceae), syn. *Dialyanthera otoba* (Humb. & Bonpl.) Warb., Otobo. The genus *Otoba* comprises about 10 species of shrubs to tall trees native to upland areas from Costa Rica to the western Amazon and Venezuela (Schultes

and Raffauf, 1990; Gentry, 1993). Neither biological nor chemical data about this species could be found in the literature.

Otoba parviflora (Markgr.) A.H. Gentry (Myristicaceae), syn. *Dialyanthera parvifolia* Markgr., Otobo. The Waorani Indians from the Ecuadorian Amazon crush the bark and the red resin and rub it on the skin for treating infections caused by mites and fungi (Schultes and Raffauf, 1990). Farnesyl-homogentisic acid derivatives have been isolated from the seeds of the species (Ferreira et al., 1989, 1995).

Swinglea glutinosa Merr. (Rutaceae), Swinglea. This species, native to Asia, is cultivated as ornamental and hedge. From the bark of this species, we recently isolated and determined four acridone alkaloids with antimalarial activities (Weniger et al., 2001).

Conobea scoparioides (Cham. & Schltdl.) Benth. (Scrophulariaceae), Hierba de sapo. This aromatic herb or low shrub is also used in the Chocó region as anticonceptive (García Barriga, 1992). The aerial parts of the plant show cell adhesion inhibition in vitro, and contain cucurbitacin E and monoterpenes (Musza et al., 1994; Alpande De Morais et al., 1972).

5. Conclusions

Considering the six plants selected for their traditional antiprotozoal use in the mentioned area, the only species used for malaria, *Potalia amara*, was inactive in vitro against the two clones of *P. falciparum*. Extracts from four out of the five species used traditionally against leishmaniasis (80%) were active in vitro at 100 μg/ml against *Leishmania* spp. promastigotes: *C. scoparioides*, *H. guianensis*, *O. novogranatensis* and *O. parviflora*. Furthermore, extracts from three of the five species (*C. scoparioides*, *H. guianensis* and *O. novogranatensis*) showed good activity against *L.* (*V.*) *panamensis* amastigotes. In addition, extracts from *C. scoparioides*, *O. novogranatensis* and *O. parvifolia* were active in vitro against epimastigotes of *T. cruzi*. Considering the 38 species selected only on the basis of bibliographic or chemotaxonomic criteria, extracts from five of them showed very good antimalarial activity in vitro, but only four showed leishmanicidal activity against *Leishmania* spp. promastigotes in vitro (see Table 2).

Out of a total of 13 active extracts against promastigotes, 10 showed very good leishmanicidal activity against *L.* (*V.*) *panamensis* amastigotes, two showed mild activity, and one was considered as inactive, showing, in our case, a good correlation between the activities against the two forms of the parasite. Furthermore, the methylene chloride extracts of leaves and bark from *G. polymera*, fruit from *O. novogranatensis* and leaves from *C. scoparioides* were more active than the Glucantime control in this assay. On the other hand, only extracts from two species (*M. laxiflora* and *O. novogranatensis*) showed a simultaneous antimalarial, leishmanicidal and trypanocidal activities (see Table 2).

The assessment of cytotoxicity on U-937 cells in culture was performed in order to determine the ratio of cytotoxicity to biological activity (selectivity indexes). It is generally considered that biological efficacy is not due to in vitro cytotoxicity when this index is ≥ 10. In relation with antimalarial activity, eight extracts from seven species showed selectivity indexes ≥ 10, with some indexes higher than 100 (*C. panamense*, *H. patinoi* and *S. glutinosa*). In the case of leishmanicidal activity, only two extracts showed selectivity indexes ≥ 10, but it is worthwhile to notice that the selectivity index of the methylene chloride extract of leaves from *C. scoparioides* is in the same range that the one of the Glucantime control (see Table 3).

The most promising extracts were prioritized for the determination of the active constituents, a work that is already under way.

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Antioxidant and antimicrobial activities of *Tamarix ramosissima*

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Abstract

The ethylacetate and water–acetone extracts of *Tamarix ramosissima* were screened for their antioxidant, antibacterial, antifungal and DNA damaging activities through in vitro experiments. All fractions as well as precipitates showed significant antioxidant activity. A known compound tamarixetin (**1**) was isolated which showed significant DNA damaging activity in mutant yeast bioassay. Results revealed that antioxidant and antibacterial activities were associated with the presence of polyphenolic substances.

Keywords: *Tamarix ramosissima*; Tamaricaceae; Antioxidant; Antimicrobial; DNA damage; Tamarixetin

1. Introduction

Tamarix ramosissima commonly known as tamarisk or salt cedar belongs to the family Tamaricaceae. The *Tamarix* species prefer alluvial soil but grow well on saline and alkaline soil. They are cultivated as ornamental plants in gardens for their pleasing pendulous panicles or racemes of small pink or whitish flowers and graceful scaly leaves. Various species of *Tamarix* have been used as medicament and tonic. They are useful in leucoderma, spleen troubles, and eye diseases (Sharma and Parmar, 1998). About 125 species have been reported from different regions of Europe, USA, Asia and Africa. Only 11 species and two hybrid species are found in Kazakhstan, while 16 species are reported from Pakistan. Leaves of *T. ramosissima* are one of the oldest herbal medicines, which have been used for the treatment of rheumatism and jaundice. The bark of this plant is used as astringent and galls possess medicinally important tannins (Komaroz, 1949; Pavlov, 1963). This plant is found to be rich in polyphenolic compounds such as flavonoids, sulphur-containing flavonoids, phenolic acids, hydrolyzable tannins and coumarins (Sokolov, 1986). The pure compound tamarixetin (**1**) (3′,3,5,7-tetrahydroxy-4′-methoxyflavone) was isolated from the butanolic fraction of the plant, showed significant DNA damaging activity. However, this compound had not shown any antioxidant and antimicrobial activities. The plant, *T. ramosissima* has not been investigated earlier, although there are many reports of phytochemical work on other *Tamarix* species.

2. Materials and methods

2.1. Plant material

Leaves of *T. ramosissima* Ledeb. (1 kg) were collected from the southern Kazakhstan in September 1999. Voucher specimen was deposited at the Department of Biological Sciences, Kazakh State National University (Almaty). The leaves were air-dried and powdered before extraction.

2.2. Extraction of plant material

The powdered air-dried leaves (1 kg) of *T. ramosissima* were soaked in water:acetone (1:1) (5 l) at room temperature for 24 h. The resulting extract was concentrated at 40 °C, and precipitates thus obtained were filtered off (30 g, **I**). The remaining layer was further

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extracted with benzene (10 g), chloroform (15 g), ethylacetate (20 g, **II**) and butanol (30 g, **III**), respectively. Butanolic extract contained precipitates, which were filtered off (2.7 g). The remaining water extract was found to be 25 g (**IV**). The ethylacetate fraction (20 g) was loaded on the Sephadex LH 20 column using water as eluent. The polarity of column was gradiently increased with ethanol and acetone. The mixture of tannins and other plant phenols (4 g, **V**) were obtained at 70% ethanol and acetone. The presence of tannins in fraction **V** was inferred by spraying its TLC with a saturated solution of potassium iodinate (galloyl esters as red to pink spots and gallic acid as orange–red) and freshly prepared 10% solution of sodium nitrate and acetic acid (hexahydrodiphenoyl esters as rose–red spots turning rapidly green, brown, purple and finally, indigoblue; Porter, 1989). The butanolic extract (20 g) of the plant was subjected to column chromatography over silica gel, using eluents CH_2Cl_2:MeOH (100–0%) gradiently to afford compound **1** which was identified as 3′,3,5,7-tetrahydroxy-4′-methoxyflavone (tamarixetin). The yield of the compound was 8 mg (percent yield was 8×10^{-4}%).

2.3. Chemicals

All the chemicals and nutrients were of analytical grades and were obtained from various sources. 1, 1-Diphenyl-2-picrylhydrazyl radical (DPPH, Sigma), Dimethylsulfoxide (Sigma), Methanol (Sigma), Propyl gallate (Sigma), Nutrient Agar Media (Merck), PYD Agar Media (Merck).

2.4. Microorganisms

Bacterial strains (clinical isolates) including *Bacillus cereus*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Pseudomonas aeroginosa*, *Staphylococcus aureus*, *Shigella boydii*, *Streptococcus pyogenes* and *Proteus mirabilis* were used in the antibacterial assay. Tetracycline was used as standard antibiotic in these assays. Eight different fungal pathogens, including human and plant pathogens, were used in the study. The human fungi studied were *Trichophyton schoenleinii*, *Trichophyton simii*, *Trichophyton mentagrophytes*, *Pseuallescheria boydii*, *Candida albicans*, *Aspergillus niger* and *Microsporum canis*. Plant fungi, *Macrophomina phaseolina*. Miconazole and ketoconazole were used as standard drugs in antifungal assay. The microorganisms were identified by a trained microbiologist. They are mainly of wild types. Wild type RAD + and mutant RAD52 *Saccharomyces cerevisiae* strains (Sigma) were used in DNA damaging yeast bioassay.

2.5. Antioxidant assay for DPPH free radical scavenging activity

The reaction mixture contains 5 μl of test samples (various extracts and compound **1** dissolved in DMSO) and 95 μl of DPPH in ethanol. Different concentrations of test samples were prepared while the concentration of DPPH was 300 μM in the reaction mixture. These reaction mixtures were taken in 96-well plate microtitre plates (Molecular Devices, USA) and incubated at 37 °C for 30 min, the absorbance was measured at 515 nm. Percent radical scavenging activity by sample treatment was determined by comparison with a DMSO treated control group. IC_{50} values denote the concentration of sample which is required to scavenge 50% DPPH free radicals (Smith et al., 1987; Fujita et al., 1998). *Emblica officinalis* (methanolic extract) and propyl gallate were used as positive controls.

2.6. Bactericidal bioassay

Bactericidal activity was determined by Broth dilution method (Finegold and Martin, 1982). In this method, decreasing concentrations of samples (extracts **I**–**V** and compound **1**) were prepared in serial two-fold dilutions in DMSO and placed in screw-capped tubes of a broth medium. One drop of the test organism culture (each drop contained 10^5–10^6 colony-forming units 'CFU' of overnight broth culture) was inoculated in each tube. Tubes containing DMSO and reference antibacterial drug (Tetracycline) served as negative and positive controls, respectively. All tubes (sample and controls) were incubated at 37 °C for 24 h, and growth of bacteria was visually monitored. Concentration at which no growth of bacteria was observed was taken as the minimum inhibitory concentrations (MIC) expressed in μg/ml.

2.7. Antifungal bioassay

Antifungal activity was determined by agar tube dilution method. Test samples (400 μg/ml, DMSO) were diluted in Sabouraud dextrose agar, and allowed to solidify in slanting positions. Test fungal cultures were inoculated on the slanting position of the media in the test tubes and tubes were incubated at 29 °C for 7–10 days. After completion of the incubation period, the test tubes were observed for linear growth inhibition of fungi in mm. Percentage inhibition was calculated with reference to negative and positive controls (Paxton, 1991).

2.8. Mechanism-based yeast bioassay for DNA damaging activity

The assay was carried out by introducing the test

sample dissolved in a MeOH:DMSO (1:1) to 100 μl well in agar plates, separately impregnated with normal wild type RAD+ yeast cells and with mutant yeast cells RAD52 and incubated for 48 h at 30 °C. A clear zone of inhibition was observed around the active sample with significant activity. The result is expressed as IC_{12} values, which represents the concentration of the test sample in mg/ml required to produce a zone of 12 mm diameter (Gunatilaka and Kingston, 1998).

3. Results and discussions

The extracts showed positive colour test with 1% aqueous $FeCl_3$, diazotised *p*-nitroaniline with 20% Na_2CO_3 and ammonia vapour which indicated the presence of polyphenolic substances. The present study on different extracts of *T. ramosissima* unravelled their ability to scavenge stable DPPH free radicals. These extracts were also screened against a number of pathogens to identify their antibacterial and antifungal activities. DNA damaging activities were also screened by employing mutant yeast bioassay. The structure of known compound, tamarixetin (**1**) was identified with the help of spectroscopic studies (EI, ^{1}H-NMR).

Table 1
Free radical scavenging activities of extracts by DPPH reduction

Extracts	IC_{50} (μg/ml)[a]
I	9.9 ± 0.07
II	8.6 ± 0.07
III	14.2 ± 0.19
IV	12.0 ± 0.09
V	7.4 ± 0.07
E. officinalis (seeds). Standard MeOH extract having antioxidant activity	6.8 ± 0.06
Propyl gallate. Standard antioxidant	6.4 ± 0.0003

[a] IC_{50} is the concentration of sample which is required to scavenge 50% DPPH free radicals. IC_{50} are the S.E.M. of three assays.

Table 2
In vitro antibacterial activities of extracts of *T. ramosissima*

Name of bacteria	MIC (μg/ml)[a]					
	Extracts of *T. ramosissima*					
	I	**II**	**III**	**IV**	**V**	Tetra
C. diphtheriae	–	25	100		25	12.5
B. cereus	–	–	–	–	–	–
E. coli	–	–	–	–	–	–
K. pneumoniae	–	–	–	–	–	–
P. aeroginosa	–	–	–	–	–	–
S. typhi	–	–	100	–	–	12.5
S. boydii	–	–	–	–	–	–
S. aureus	–	–	–	–	–	–
S. pyogenes	–	–	–	–	–	–
P. mirabilis	–	100	–	–	100	25

[a] Tetra, standard antibiotic tetracycline; MIC, minimum inhibitory concentration; –, inactive.

3.1. Free radicals scavenging activity of T. ramosissima

DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Due to its odd electron, the ethanolic solution of DPPH shows a strong absorption band at 515 nm. DPPH radicals react with suitable reducing agents and then electrons become paired off and the solution loses colour stiochiometrically with the number of electrons taken up (Blois, 1958). Such reactivity has been widely used to test either the ability of compounds to act as free radical scavengers or the antioxidant activity of plant extracts (Dinis et al., 1994; Ursini et al., 1994; Lamaison et al., 1990; Navarro et al., 1993).

Reduction of DPPH radicals can be observed by the decrease in absorbance at 515 nm. Different extracts of *T. ramosissima* reduced DPPH radicals significantly. IC_{50} (μg/ml) are shown in Table 1. The tannins (**V**) and ethylacetate (**II**) fractions of the plant showed low IC_{50} (7.4 μg/ml and 8.6 μg/ml, respectively), suggesting a high free radical scavenging activity, whereas the other fractions also showed moderate activities. The antioxidant potentials of test samples were also compared with the propyl gallate and methanolic extract of *E. officinalis*.

3.2. Antibacterial activity of T. ramosissima

Table 2 shows MIC of different extracts of *T. ramossisima* against human pathogens. Ethylacetate (**II**) and tannins fraction (**V**) exhibited antibacterial activity against *C. diphtheriae* (25 μg/ml) and *P. mirabilis* (100 μg/ml). Butanolic fraction (**III**) also showed activity against *S. typhi* (100 μg/ml) and *C. diphtheriae* (100 μg/ml). Tetracycline was used as a reference drug to compare the extent of activity.

3.3. Antifungal activity of T. ramosissima

Table 3 shows that the plant extracts have some antifungal activity against a number of pathogens. Precipitates (**I**) and butanol (**III**) and ethylacetate (**II**) fractions showed antifungal activity at a concentration of 400 μg/ml against human pathogen, *A. niger*. The extent of activity was compared with standard drugs, miconazole and ketoconazole.

Table 3
In vitro antifungal activities of extracts of *T. ramosissima*

Fungal pathogens	Linear growth (mm) inhibition% (at 400 μg/ml)						
	Extracts of *T. ramosissima*					Standard drugs	
	I	**II**	**III**	**IV**	**V**	Ketoconazole	Micronazole
T. schoenleinii	50	50	46	25	28.5	100	100
T. simii	25	66	47	32	50	100	100
A. niger	62.5	62.5	75	33	25	100	100
P. boydii	57	60	60	17	14	100	100
M. phaseolina	–	–	50	–	–	100	100

Ketoconazole, standard drug; Miconozole, standard drug; –, inactive.

Table 4
DNA damaging activities of extracts of *T. ramosissima*

Test samples	Concentration (μg/ml)	Zone of inhibition (mm)		IC_{12} (μg/ml)
		S. cerevisiae RAD52	*S. cerevisiae* RAD+	
Tamarixetin (**1**)	100	14 ± 1	–	50
I	100	–	–	–
II	100	–	–	–
III	100	8 ± 1	–	–
IV	100	–	–	–
V	100	10 ± 1	7	–
Streptonigrin	100	28 ± 1	10	1.0

Streptonigin, standard anticancer drug; IC_{12}, the concentration (μg/ml) is required to produce a zone of inhibition of 12 mm; –, inactive.

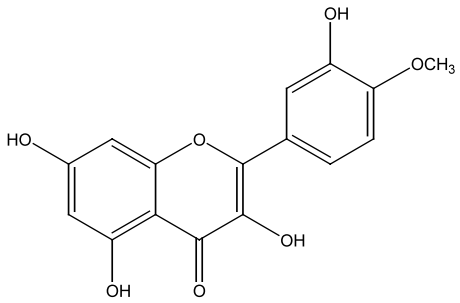

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Fig. 1.

3.4. Yeast bioassay

This assay utilises DNA repair or recombination-deficient mutants of the yeast *S. cerevisiae* for the screening of compounds, which induce DNA damage. This mechanism-based yeast assay depends on the different responses of DNA repair-deficient and repair-proficient yeast (*S. cerevisiae*) strains to the sample. The major DNA repair pathway is RAD52 pathway associated with the repair of double strand break and meiotic recombination. The results are shown in Table 4. Tamarixetin (**1**) (Fig. 1) exhibited some toxicity towards DNA-repair deficient mutants of *S. cerevisiae*. Its IC_{12} value was found to be 50 μg/ml while no significant zone of inhibition was observed for wild type yeast strain at the same concentration. This indicates specific cytotoxic activity through DNA damage. Streptonigrin, one of the potent DNA damaging anticancer drug, was used as a reference drug to compare the extent of activity (Patil et al., 1997).

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Short communication

Analgesic and anti-inflammatory activity of *Crinum glaucum* aqueous extract

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Abstract

The anti-inflammatory and analgesic effects of the aqueous extract of *Crinum glaucum* were evaluated in mice and rats using the carrageenan- and dextran-induced paw oedema, acetic acid-induced writhing, cold water tail flick and formalin pain tests. The extract (100–400 mg/kg) and acetylsalicylic acid (100 mg/kg) produced a significant ($P < 0.05$) inhibition of the second phase response in the formalin pain model, while only the high dose (400 mg/kg) of the extract showed an antinociceptive effect in the first phase. The extract also showed a dose-dependent inhibition of acetic acid-induced abdominal writhes. The tail flick latency was dose dependently enhanced by the extract but this was significantly ($P < 0.05$) lower than that produced by morphine (2 mg/kg). The extract (125–500 mg/kg) administered 1 h before or after carrageenan-induced paw swelling produced a dose dependent inhibition of the oedema. No effect was observed with the dextran-induced oedema model. The data obtained suggest that the anti-inflammatory and analgesic effects of the extract may be mediated via both peripheral and central mechanisms.

Keywords: *Crinum glaucum*; Analgesic; Anti-inflammatory; Formalin test; Tail flick

1. Introduction

Crinum glaucum (Amaryllidaceae) is a bulbous plant widely used in folk medicine in West Africa as an antispasmodic. It is known as 'ISU MERI' in South West, Nigeria, where traditional medicine practitioners claim that it is effective in the treatment of cough, asthma and convulsions.

Bulbs of various crinum species are used to treat ailments such as cold, renal and hepatic conditions (Watt and Breyer-Brandwijk, 1962). They are also used as anthelmintics and emetics (Oliver, 1959), and in the treatment of sores (Kokwaro, 1976), sexually transmitted diseases and backache (Duri et al., 1994).

The aqueous extract of *C. glaucum* bulbs was found in an earlier study to produce a non-specific relaxant effect on the gastrointestinal smooth muscles (Okpo and Adeyemi, 1998). In the present study, we have evaluated the anti-inflammatory effects of the aqueous extract using the carrageenan-and dextran-induced rat paw oedema test and the analgesic activity using the formalin test, rat tail flick and mouse writhing assays.

2. Materials and methods

2.1. Preparation of plant extract

Fresh bulbs of *C. glaucum* were collected in Ogun State, Nigeria and botanical authentication was confirmed by Professor J.D. Olowokudejo of the Department of Botany, Faculty of Science, University of Lagos, Lagos, Nigeria. Samples of this plant were deposited in the herbarium of the Pharmacognosy Department of the College of Medicine, University of Lagos.

Fresh bulbs of *C. glaucum* were cut into pieces and macerated in distilled water. The liquid was decanted on day 4 and filtered. The filtrate was evaporated to

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dryness in an oven set at 40 °C. The dried extract was weighed and dissolved in distilled water to a concentration of 400 mg/ml.

2.2. Animals

Wistar rats (150–200 g) and Swiss mice (18–22 g) of either sex kept at the Laboratory Animal Centre of the College of Medicine, University of Lagos, Lagos, Nigeria were used. The animals maintained under standard environmental conditions had free access to standard diet (Pfizer Feeds, PLC, Lagos, Nigeria) and water ad libitum.

2.3. Analgesic activity

2.3.1. Mouse writhing assay

This was carried out according to the method described previously (Koster et al., 1959). The extract (100–400 mg/kg, orally) or distilled water (10 ml/kg) were administered to mice before intraperitoneal injection of acetic acid (0.6% v/v in normal saline, 10 ml/kg). Acetylsalicylic acid (100 mg/kg, sc) was used as the reference drug. The number of writhes was counted for 15 min.

2.3.2. Formalin test

The method used was similar to that described previously (Shibata et al., 1989; Vianna et al., 1998). Twenty microlitres of 1% formalin was injected subcutaneously into the right hind paw of mice. The time (in seconds) spent in licking and biting responses of the injected paw was taken as an indicator of pain response.

Responses were measured for 5 min after formalin injection (first phase) and 15–30 min after formalin injection (second phase).

Extract (100–400 mg/kg, orally) and acetylsalicylic acid (100 mg/kg, sc) were administered 30 min before formalin injection. Control animals received the same volume of distilled water orally.

2.3.3. Tail flick assay

The rat cold water tail flick assay was based on a modification (Clark et al., 1988) of the method originally described by Pizziketti et al. (1985). Rats were closely restrained in a wire mesh cage and the lower half of their tails dipped in a beaker of cold water (0–1 °C). The time (in seconds) for tail withdrawal from the water was taken as the reaction time. Measurement of threshold was made 30 min before and after administration of extract (100–400 mg/kg, orally) or morphine (2 mg/kg, sc). Distilled water (10 ml/kg) served as the control.

2.4. Anti-inflammatory activity

Rats (5–9 per group) were alloted to different treatment groups. Oedema was induced in the rats by injection of carrageenan (0.1 ml, 1% w/v in normal saline) or dextran (0.1 ml, 1% w/v in normal saline) into the sub-plantar tissue of the right hind paw (Winter et al., 1962). The linear paw circumference was measured using the cotton thread method (Bamgbose and Noamesi, 1981). Measurements were made immediately before injection of the phlogistic agent and at hourly intervals for 3 and 6 h in the animals injected with dextran and carrageenan, respectively.

The paw swelling at each time was calculated as the difference between the linear circumference at time t (C_t) and that at 0 h (C_0).

Extract (125–500 mg/kg), distilled water (0.1 ml/100 g rat), indomethacin (10 mg/kg) and methysergide (1 mg/kg) were administered orally 1 h before injection of phlogistic agents.

The effect of the extract (125–500 mg/kg) administered orally 1 h after the induction of carrangeenan-induced oedema was also investigated.

2.4.1. Preliminary phytochemical screening

Preliminary phytochemical analysis of the extract was carried out as described previously (Odebiyi and Sofowora, 1978). The presence of alkaloids was tested with Dragendorff's and Mayer's reagents, flavonoids with dilute NaOH and HCl, phenols with ferric chloride and potassium ferricyanide, and reducing sugars using Fehling's reagent.

2.4.2. Acute toxicity

Mice (10 per group) were administered intraperitoneally and orally with different doses of the extract. Mortality in each group within 24 h was recorded. LD_{50} was estimated by log dose–probit analysis (Miller and Tainter, 1944).

2.4.3. Statistical analysis

Results are expressed as mean $\pm$ SEM. Statistical analysis of the data was done using Student's t-test and the results were considered significant when $P < 0.05$.

2.4.4. Drugs

The drugs used were indomethacin, carrageenan, acetylsalicylic acid (Sigma Chemical Company, St. Louis, USA), methysergide, (Sandoz, Basle, Switzerland) dextran (UNIC Pharmaceuticals, Lagos, Nigeria) morphine (Evans Medical Ltd., England). Other chemicals used were of analytical grade and purchased locally.

3. Results

3.1. Mouse writhing

In control mice, the number of writhes during the 15

min test period was 68.4 ± 4.7 ($n = 6$). The treatment of animals with *C. glaucum* aqueous extract (100–400 mg/kg) produced a significant and dose dependent inhibition of the control writhes (Table 1). The inhibition by 400 mg/kg extract was similar to that produced by 100 mg/kg acetylsalicylic acid (78.8 and 74.2%, respectively).

3.2. Formalin test

The extract demonstrated a dose-dependent relationship in both phases of formalin induced pain. A significant inhibition (13.6%) was produced only with the dose of 400 mg/kg extract compared to control, in the first phase (Table 2). However, all the doses significantly ($P < 0.05$) inhibited the second phase, similar to acetylsalicylic acid (100 mg/kg).

3.3. Tail flick

Table 3 shows the effect of the extract on tail flick response in rats. All doses of the extract used significantly ($P < 0.05$) increased the reaction time compared

Table 1
Effect of *C. glaucum* aqueous extract on acetic acid-induced writhing in mice

Group	Dose (mg/kg)	No. of writhings (per 15 min)	% Inhibition
Control	–	68.4 ± 4.7	–
C. glaucum	100	31.5 ± 1.7*	53.2 ± 2.5
	200	30.7 ± 2.4*	55.2 ± 3.6
	400	14.5 ± 2.6*	78.8 ± 3.8
Acetylsalicylic acid	100	17.7 ± 3.2*	74.2 ± 4.7

Values are mean ± SEM.
* $P < 0.05$ significantly different from control (Student's *t*-test).

Table 2
Effect of *C. glaucum* on aqueous extract on formalin-induced pain

Group	Dose (mg/kg)	0–5 min	% Inhibition	15–30 min	% Inhibition
Control	–	114.8 ± 3.9	–	94.6 ± 7.1	–
C. glaucum	100	109.0 ± 7.8	5.1	41.4 ± 9.1**	56.2
	200	99.8 ± 6.4	13.1	23.8 ± 7.9**	74.8
	400	99.2 ± 3.0*	13.6	19.6 ± 5.5***	79.3
Acetylsalicylic acid	100	135.8 ± 8.1	–	29.4 ± 9.9**	68.9

Values are mean ± SEM.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ significantly different from control (Student's *t*-test).

Table 3
Effect of *C. glaucum* aqueous extract on tail flick test

Group	Dose (mg/kg)	Pre-treatment (s)	Post-treatment (s)	% Inhibition
Control	–	6.4 ± 1.1	8.4 ± 1.0	3.7 ± 1.1
C. glaucum	100	12.1 ± 2.5	20.3 ± 2.7	17.1 ± 3.4*
	200	13.1 ± 1.9	32.5 ± 3.6	41.6 ± 6.6**
	400	12.4 ± 2.4	32.6 ± 4.2	44.2 ± 6.7**
Morphine	2	11.6 ± 2.2	53.9 ± 3.1	88.6 ± 5.8**

Values are mean ± SEM.
* $P < 0.05$.
** $P < 0.001$, significantly different from control (paired *t*-test).

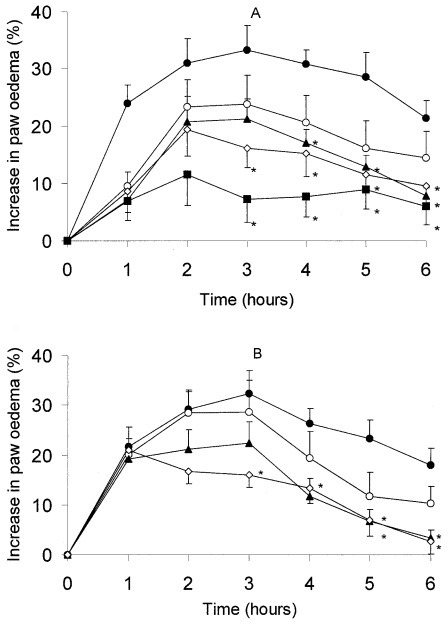

Fig. 1. Effect of *C. glaucum* aqueous extract on rat paw swelling induced: (A) 1 h before; and (B) 1 h after injection of carrageenan. Control (●); aqueous extract of *C. glaucum* (○, 125; ▲, 250 and ◇, 500 mg/kg); indomethacin (□, 10 mg/kg). Vertical bars are mean ± SEM ($n = 5$–9 animals). *$P < 0.05$ compared to control.

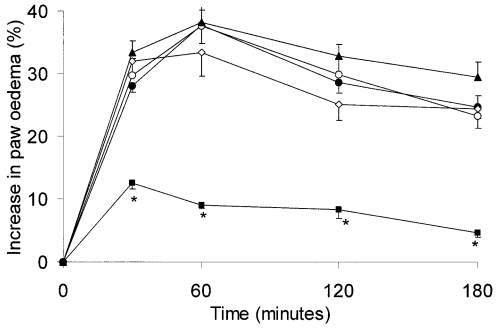

Fig. 2. Effect of *C. glaucum* aqueous extract on rat paw swelling induced by dextran. Control (●); aqueous extract of *C. glaucum* (○, 125; ▲, 250 and ◇, 500 mg/kg); methysergide (□, 1 mg/kg). Vertical bars are mean ± SEM ($n = 5$ or 6 animals). *$P < 0.05$ compared to control.

to control. The effect of morphine (2 mg/kg) was significantly higher ($P < 0.01$) than that produced by the highest dose of the extract.

3.4. Anti-inflammatory activity

The extract administered 1 h before carrageenan showed a dose dependent inhibition of the induced oedema. Carrageenan produced a swelling of the rat paw which reached a peak (33.3 ± 4.2%) in 3 h and gradually declined over the next 3 h (Fig. 1). The extract showed the highest inhibition of oedema at 500 mg/kg compared to the lower doses. Indomethacin (10 mg/kg) produced a greater oedema inhibition (69%), which was not significantly different ($P > 0.05$) from that of the extract (500 mg/kg, 52%) within the same period.

Administration of extract (125–500 mg/kg) 1 h after the onset of carrageenan induced oedema showed a significant ($P < 0.05$) reduction in paw swelling (Fig. 1). Dextran produced a rapid swelling, which reached a peak in 60 min and gradually declined over the period of experiment.

Pre-treatment of rats with extract (125–500 mg/kg) did not suppress the dextran oedema but methysergide inhibited the same paw oedema by 76% (Fig. 2).

3.5. Phytochemical tests

Preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, reducing sugars and phenols.

3.6. Acute toxicity

The LD_{50} of the extract when administered intraperitoneally and orally were 119 mg/kg and 1420 mg/kg, respectively.

4. Discussion

The data obtained from the present study indicate that *C. glaucum* aqueous extract produced a dose dependent anti-inflammatory effect on carrageenan-induced paw oedema but this effect was less than that produced by the standard anti-inflammatory drug-indomethacin. The effect of the extract was most pronounced at the later stages of the inflammatory response, which corresponds to the phase of prostaglandin release. Carrageenan oedema consists of three distinct phases; an initial release of histamine and 5HT, a second phase mediated by kinins and finally a third phase, the mediator of which is suspected to be prostaglandin (Di Rosa et al., 1971). The extract was however, ineffective in the dextran model showing that it does not inhibit inflammation by blocking the release of histamine and 5HT, two mediators which are released by dextran (Nishida et al., 1979; Pearce, 1986).

In this study, it was shown that the extract inhibited both phases of formalin-induced pain, a model which is very useful for elucidating the mechanism of pain and analgesia (Tjolsen et al., 1992). Drugs which act mainly centrally, such as narcotics, inhibit both phases of formalin-induced pain while peripherally acting drugs, such as aspirin, only inhibit the late phase (Santos et al., 1994).

The effect of the extract on tail flick response provides a confirmation of its central effect since the assay

is specific for opioid induced antinociceptive effect (Clark et al., 1988).

The extract also inhibited acetic acid-induced writhing in mice hence it can be suggested that the analgesic effect of the extract is also peripherally mediated.

The ability of the extract, in this study, to suppress abdominal writhes, increase tail flick latency, inhibit both phases of formalin induced pain as well as suppress the carrageenan- induced inflammation confirm the analgesic and antiinflammatory activities of the extract.

It is concluded that the aqueous extract of *C. glaucum* possesses analgesic and anti-inflammatory properties, which are probably mediated via inhibition of prostaglandin synthesis as well as central inhibitory mechanisms. The extract will, therefore, be of potential benefit in the management of pain and inflammatory disorders.

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Short communication

Inhibition of drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis* by diospyrin, isolated from *Euclea natalensis*

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Abstract

The binaphthoquinoid, diospyrin, was isolated from *Euclea natalensis* A.DC., and evaluated for its activity against drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis*. The minimal inhibitory concentration (MIC) of diospyrin was found to be 100 μg/ml for all the *M. tuberculosis* strains.

Keywords: Diospyrin; *Euclea natalensis*; *Mycobacterium tuberculosis*

1. Introduction

The resurgence of tuberculosis (TB) as a major disease in many parts of the world, is prompting the search for novel compounds, active against the causative organism, *Mycobacterium tuberculosis*. It is estimated that TB claims between 2 and 3 million lives a year and shows every sign of spiraling out of control (*New Scientist*, July 2001). According to estimates by the World Health Organization (WHO), there were 8.4 million new TB cases in 1999 up from 8.0 million in 1997. If present trends continue, 10.2 million new cases are expected in 2005, and Africa will have more cases than any other WHO Region (WHO Report 2001). The increase in prevalence of multidrug-resistant (MDR) strains of this organism and the emergence of AIDS-related TB are, probably the reasons why TB is the disease with the fifth highest fatality rate in the world (World Health Organization, 1997; Kochi, 1997).

The use of some medicinal plants, such as *Acacia nilotica*, *Cassine papillosa*, *Chenopodium ambrosioides*, *Combretum molle*, *Euclea natalensis* etc. by South Africans in curing TB related symptoms has been reported (Watt and Breyer-Brandwijk, 1962; Bryant, 1966; Pujol, 1990). Out of 20 medicinal plants investigated in our laboratory, *E. natalensis* exhibited the best activity against drug-sensitive and drug-resistant strains of *M. tuberculosis* (Lall and Meyer, 1999).

E. natalensis A.DC., a tree of the Ebenaceae family is used extensively in oral health care, for chest complains, bronchitis, pleurisy, chronic asthma, urinary tract infections, venereal diseases etc. by the indigenous people of South Africa (Watt and Breyer-Brandwijk, 1962; Bryant, 1966; Van Wyk and Van Wyk, 1997). The large subtropical genus *Euclea* is well known as a source of naphthoquinones. Monomers, complex dimers and trimers such as 7-methyljuglone, diospyrin and mamegakinone have been isolated from it (Van der Vijver and Gerritsma, 1974; Khan et al., 1978; Hazra et al., 1984) but, there have been no reports concerning their antimycobacterial activity. In a previous paper, we have reported the inhibitory effect of the crude extract of *E. natalensis* against a drug-sensitive and two drug-resistant strains of *M. tuberculosis* (Lall and Meyer, 1999). We now report on the isolation and identification of the active principle from *E. natalensis* and its inhibitory effect against the drug-sensitive and drug-resistant strains of *M. tuberculosis*.

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2. Materials and methods

2.1. Plant material

Roots of *E. natalensis* were collected from Mputaland, a region in the KwaZulu-Natal province of South Africa. A voucher specimen (N.L. 22) was deposited and identified at the H.G.W.J. Schweickerdt Herbarium, at the University of Pretoria.

2.2. Extraction, isolation and purification of the compound

The dried powdered root (100 g) of *E. natalensis* was left to macerate in acetone (1 l) at room temperature for 2 days. After filtration, the solvent was concentrated under reduced pressure to give a crude extract of 4.2 g.

A direct bioassay of the crude acetone extract against *Staphylococcus aureus* was done by applying 20 μl extract dissolved in acetone (20 mg/ml) to silica gel 60 plates (Merck) according to the procedures described previously by Lund and Lyon (1975). These TLC plates were then developed with chloroform–hexane (1:1) and a fine spray was used to spray a bacterial suspension of *S. aureus* onto the TLC plates. The plates were then incubated for 48 h in humid conditions. After incubation, the plates were sprayed with an aqueous solution of 2 mg/ml *p*-iodonitrotetrazolium violet.

It was found that a compound of R_f 0.30, had antibacterial activity, and in order to purify this antibacterial compound the crude extract of the plant (4.2 g) was dissolved in $CHCl_3$ and fractionated on silica gel 60 by column chromatography, using $CHCl_3$ as eluent. This achieved a preliminary separation from more polar materials, which are unable to pass through the silica column. The bioactive antibacterial fraction also showed activity against *M. tuberculosis* and was then subjected to Sephadex LH-20 column chromatography, using ethanol as eluent. The compound was finally purified by HPLC utilizing an analytical phenomenex reverse phase 250 × 4.60 mm column, at a flow rate of 1.0 ml/min, temperature 40 °C and monitored at a wavelength of 206 nm. An ethanol:water (50:50) solution was employed as mobile phase.

2.3. NMR spectroscopy

^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{13}C short and long range correlation HETCOR and COSY spectra were obtained at 300 MHz on a Bruker ARX 300 NMR spectrometer using $CDCl_3$, as solvent with TMS as internal standard.

2.4. Bioassay on M. tuberculosis

The radiometric respiratory technique with the BACTEC apparatus was used for susceptibility testing of *M. tuberculosis* as described earlier (Middlebrook et al., 1977; Siddiqi et al., 1981). Bacterial cultures utilized in this study were grown from specimens received from the Medical Research Council (MRC) in Pretoria. A sensitive strain of *M. tuberculosis*, H37Rv reference strain, and six other multidrug resistant strains were used in the screening procedure (Table 1).

Diospyrin was dissolved at 10 mg/ml in 1% DMSO and stored at −4 °C until used. Subsequent dilutions were done in DMSO and added to 4 ml of BACTEC 12B broth to achieve the desired final concentrations of 100, 50 and 10 μg/ml, together with PANTA (Becton Dickinson & Company), an antimicrobial supplement. BACTEC drug susceptibility testing was also done for the two primary TB-drugs, streptomycin and ethambutol at concentrations of 6.0 and 7.5 μg/ml, respectively, against H37Rv strain.

A homogenized culture (0.1 ml) of all the strains of *M. tuberculosis*, yielding 1×10^4–1×10^5 colony forming U/ml (CFU per ml), were inoculated in the vials containing the compound, as well as in the control vials (Heifets et al., 1985). Two compound-free vials were used as controls: one vial was inoculated in the same way as the vials containing the compound, and the

Table 1
Effect of diospyrin on the growth of the drug-sensitive strain (H37Rv) and drug-resistant strains of *M. tuberculosis* by the radiometric method

M. tuberculosis strains; resistance profile to antibiotic drugs[a]	Lab ref. No. of strains	MIC (μg/ml)	ΔGI[b] values of diospyrin	ΔGI values of the control vials
Drug-sensitive strain (H37Rv)	ATCC27294	100	-1 ± 1.41	20 ± 4.24
Resistant to I and R	CCK028469V	100	3.5 ± 0.70	25 ± 7.07
Resistant to S, I and E1	C9	100	4 ± 2.12	29 ± 1.41
Resistant to S, I, R and E1	C84	100	5 ± 2.82	25 ± 2.82
Resistant to I, S, R, T1 and C	CGT1296429	100	10 ± 1.41	22.5 ± 3.53
Resistant to I, R, E2, T1, T2 and O	CCK070370H	100	9 ± 2.82	30 ± 1.0
Resistant to I, S, E1, E2, K, R, and T1	CGT1330497	100	13.5 ± 3.2	28 ± 3.1

[a] I, isoniazid; R, rifampin; S, streptomycin; E1, ethambutol; E2, ethionamide; T1, thiacetazone; T2, terizidone; C, cycloserine; O, ofloxacin; K, kanamycin.

[b] ΔGI values are mean ± S.D.

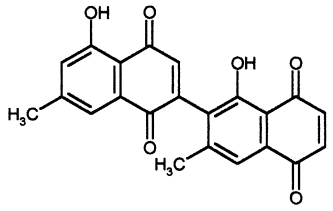

Fig. 1. Diospyrin isolated from *E. natalensis*.

other was inoculated with a 1:100 dilution of the inoculum (1:100 control), to produce an initial concentration representing 1% of the bacterial population (1×10^2–1×10^3 CFU per ml) found in the vials containing diospyrin. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the compound that inhibited more than 99% of the bacterial population.

Inoculated bottles were incubated at 38 °C and each bottle was assayed everyday at about the same hour until cumulative results were interpretable. The difference in the GI values of the last 2 days is designated as ΔGI. The GI reading of the vials, containing the compound was compared with the control vial, containing a 1:100 dilution of the inoculum. Readings were taken until the control vials containing a 100 times lower dilution of the inoculum, than the vials with the compound, reached a GI of 30 or more. If the ΔGI value of the vial containing the plant extract was less than the control, the population was reported to be susceptible to the compound.

3. Results and discussion

3.1. Identification of the isolated compound

The complex UV spectrum of the compound with maxima at 206, 254 and 430 nm and the bathochromic shift on the addition of alkali was typical of a naphthoquinone. The compound was identified as the binaphthoquinone, diospyrin, by comparison of its ^{1}H-NMR and ^{13}C-NMR spectral data with published spectra (Hazra et al., 1984) (Fig. 1). The identification was also confirmed by direct comparison with authentic samples on TLC. Diospyrin has also been isolated from other species of *Euclea* such as *E. pseudebenus* (Ferreiera et al., 1974), *E. crispa*, *E. divinorum*, and *E. schimperi* (Van der Vijver and Gerritsma, 1974), and from the *Diospyros* species, *D. mannii* (Jeffreys et al., 1983), *D. montana* (Hazra et al., 1984), *D. chamaethamus* (Costa et al., 1998) and *D. piscatoria* (Adeniyi, 2000).

3.2. Activity of diospyrin against drug-sensitive and drug-resistant strains of M. tuberculosis

Results were interpreted on day 6 or 7, when the control vials containing the 1:100 dilution of the inoculum reached a GI value of 30 or more (Table 1). The MIC of diospyrin was found to be 100 μg/ml against the H37Rv strain as well as against the two to seven drug-resistant strains. However, the ΔGI values of the vials containing diospyrin at 0.05 mg/ml concentration level, for the two and three drug-resistant strains were found to be 20 ± 3.60 and 13 ± 6.55, respectively. This indicated that, these strains are partially susceptible to the compound. The ΔGI values of the vials containing streptomycin and ethambutol was found to be 2 ± 1.41 and 5 ± 2.12, respectively.

Over the past decade, there has been a proliferation in literature on the antimycobacterial properties of plant extracts. The quinones exhibiting inhibitory activity against *M. tuberculosis* were found to be multiorthoquinone and 12-demethylmultiorthoquinone isolated from *Salvia multicaulis* (Ulubelen et al., 1997). The MIC values of a few compounds such as allicin isolated from garlic oil (Delaha and Garagusi, 1985), Hypargenin F, from the roots of *Salvia hypargeia* (Ulubelen et al., 1988), triterpenes from *Borrichia frutescens* (Cantrell et al., 1996) were found to be generally higher than that of diospyrin for *M. tuberculosis*. In contrast, compounds, such as ambroxol, a semi-synthetic derivative of vasicine from the Indian shrub *Adhatoda vasica* (Grange and Snell, 1996), alkaloids isolated from *Galipea officinalis* (Houghton et al., 1999) etc. exhibiting better activity than diospyrin has also been previously reported.

The antifungal, antibacterial and termite resistant properties of *Diospyros* and *Euclea* species have all been attributed to the occurrence of naphthoquinones (Khan et al., 1978; Hazra et al., 1984). There is ample evidence from various studies done earlier, that the strong antibacterial action of the root of *E. natalensis* is due to naphthoquinones (Khan et al., 1978; Stander and Van Wyk, 1991; Lall and Meyer 2000). These compounds act bactericidally by denying access of essential metabolites through the membrane into the interior of the cell (Olenick et al., 1971). According to a previous report, a naphthoquinone, kigelinone, isolated from *Kigelia pinnata* synonymous with *K. africana* have been shown to be active against Gram-positive bacteria at concentrations ranging from 0.05 to 0.2 mg/ml. They have attributed this activity to the position of a hydroxyl group on the aromatic ring of the naphthoquinone nucleus. The position of the hydroxyl group relative to the quinone carbonyl as in kigelinone makes the formation of chelation with an active site possible (Bintu et al., 1996). Our findings, reported here, would seem to support such a hypothesis, based on the fact that diospyrin also has a hydroxyl group adjacent to the quinone carbonyl.

The traditional use of *E. natalensis* extract against sores, purulent lesions and skin infections, cough could possibly be attributed to the activity of diospyrin against *S. aureus* and *M. tuberculosis*. This might explain the plant's use in traditional remedies against TB and adds credibility to earlier reports of the beneficial antimicro-

bial (Khan et al., 1978), antitrypanosomal, antiprotozoal (Yardley et al., 1996) and antischistosomiasic (Sparg et al., 2000) effect of this binaphthoquinone. The antimycobacterial properties of the root extract, probably, provides the reason for its use in folk medicine also, to treat leprosy, which is caused by another mycobacterial species (Bryant, 1966; Heifets et al., 1985).

Isolation of the active principle from the root extract of *E. natalensis* was initiated by a preliminary bioassay using *S. aureus* and therefore, it is possible that there could be other antimycobacterial compounds present in the plant. Moreover, in a previous study, it was found that MIC of crude extract of *E. natalensis* against *M. tuberculosis* was 100 μg/ml (Lall and Meyer, 1999). Research is underway to isolate and identify other compounds with antimycobacterial activity from the roots of *E. natalensis*.

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