Original Article

HPLC-DAD Fingerprinting of Ethanol Extracts from *Conyza sumatrensis* and *Spathodea campanulata* and their Additive Effect in *Plasmodium berghei* K173 Infected Mice

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ABSTRACT

The aim of the present study was to develop an appropriate HPLC method to identify different constituents from *Conyza sumatrensis* (CS) and *Spathodea campanulata* (SC) and evaluate antimalarial potential of the extract combination with reference to the traditional use. Schizontocidal activity was measured using a standard in *vivo* assay, with *Plasmodium berghei K*173, a chloroquine sensitive strain. The combination of SC and CS ethanol extracts gave statistically significant and improved suppressions which ranged from 83 to 99.01%. Remarkable suppression in parasitaemia resulted into increased mean survival time relative to vehicle control. The HPLC fingerprints of SC and CS extracts showed numerous peaks. Moreover, the same for the combination revealed 23 peaks among which gallic acid, catechol, coumarin and quercetin peaks could be detected. These compounds may be responsible for the activity of the extract towards *Plasmodium berghei* K173 infected mice. Therefore, SC and CS warrants further evaluation in the search for novel antimalarial drugs.

Keywords: *Conyza sumatrensis*, *Spathodea campanulata*; Antimalarial; *Plasmodium berghei K*173, Additive potential.

INTRODUCTION

Malaria is the world's most important tropical parasitic disease, transmitted through the bite of female *Anopheles* mosquito. Each year, 300 to 500 million new cases are detected globally. Nearly 40% of the world's population live in affected regions. Despite over a century of work to control this disease, malaria continues to take its devastating toll, largely in developing nations. The emergence of insecticide resistant mosquitoes and drug-resistant malarial parasites has made the situation much worse[1]. Consequently, new treatments for malaria are urgently needed. The long-established use of quinine and the more recent introduction of artemisinin and its derivatives as highly effective antimalarials demonstrated that plant species are an important resource for the discovery of new antimalarial agents.

The concept of combination therapy as recommended by WHO[1] is based on synergistic or additive potential of two or more drugs to improve the therapeutic efficacy and also to delay the development of resistance to individual components in the combination.

Moreover, many plant species still continue to be used in the traditional medicine for the treatment of malaria and many people depend on such remedies as they cannot afford and/or do not have access to effective antimalarial drugs.

The leaves and the stem bark of *Spathodea campanulata* individually and in combination with *Conyza sumatrensis* are widely used in Cameroon as antimalarial therapy[2]. The soft aerial part of *Conyza sumatrensis* is mixed with other plants i.e., *Rauvolfia vomitoria* stem bark, lime fruit, *Carica papaya* mature leaves and *Cymbopogom citratus* leaves prepared as decoction for the treatment of malaria in western region of Cameroon[3]. Herve et al.[4] reported the *in vitro* antiplasmodial activity of alcohol and pentane extracts of *Conyza sumatrensis* and the IC_{50} ranged from 4.3 to 10 and 39 to 49µg/ml respectively.

Our previous studies confirmed the antimalarial properties of *Spathodea campanulata* and *Conyza sumatrensis* in *Plasmodium berghei* K173 infected mice[5,6].

But no study has been reported towards qualitative analysis of ethanol extract of both plants through HPLC-DAD technique and their additive effect in *Plasmodium berghei* K173 infected mice. The present swot was undertaken to develop an appropriate method via HPLC-DAD for identification of some active components present in ethanol extract of *Conyza sumatrensis* and *Spathodea campanulata* and appraise the antimalarial potential of their combination in *Plasmodium berghei* K173 infected mice.

MATERIAL AND METHODS

Animals

Female Swiss albino mice (*Mus musculus*) in the weight range of 18-22g were used. They were bred and maintained in-house under standard environmental conditions (25 ± 2°C; 12/12 h light/dark cycle). Experiments were conducted after obtaining prior approval (AH-2012-001) for animal studies from CPCSEA, Government of India through the Institutional Animal Ethics Committee.

Inoculation of the mice

Plasmodium berghei K173 (sensitive to chloroquine at 10mg/kg) was used to assess the antimalarial activity *in-vivo*. The strain was procured from Central Drug Research Institute, Lucknow and maintained through serial passage in mice as earlier reported from our laboratory[7]. Briefly, each mouse was inoculated with 0.2ml of infected

blood containing about 1×10^6 parasitized red blood cells in acid citrate- dextrose (ACD) solution.

Plant material

Fresh leaves of *Spathodea campanulata* (A) and *Conyza sumatrensis* (B) collected in February 2011 from Dschang, located in West Region of Cameroon were shade dried and reduced to powder. The specimens were identified by Dr. S.C. Singh (Taxonomist) and were deposited at the herbarium of Central Institute of Medicinal and Aromatic Plants of India under voucher number 12542 and 13780 respectively. See fig. A and b.

Chloroquine, the standard drug for this experiment was obtained commercially from Sigma Aldrich, USA.

Extraction

The powders, 80 and 100g of *Conyza sumatrensis* and *Spathodea campanulata* respectively were extracted in 95% ethanol (750ml) by soxhlet apparatus till the solvent became transparent. The liquid filtrate was concentrated in rotary vacuum evaporator at 45°C and lyophilized at -45°C / 10.00 mbar. The yields were 7.9 and 11.52% (W/W) respectively. The extracts were stored in the fridge until use.

Qualitative analysis of the extracts by HPLC

The HPLC fingerprinting was performed using a Waters modular HPLC system (Waters, Milford, USA) consisting of 2996 photo diode array detector, 600 E pump, 717 auto sampler and C18 column (150 mm × 4.6 mm i.d.; 4 µm). Samples were diluted adequately with eluent prior to the injection. The gradient elution was carried out by using solvent system comprising water: trifluoro-acetic acid (99.99:0.01, v/v), and solvent-B encompassing acetonitrile: trifluoroacetic acid (99.99:0.01, v/v). A linear gradient programming was accomplished at 27°C with initial composition of 95% of A and 5% of B with a flow rate of 1.2ml/min changing to 60% of A and 40% of B at 30min with flow rate of 0.6ml/min while the same remained unchanged at 35 min. The initial conditions were restored beyond 37min with a flow rate of 0.8 ml/min. The extracts were defatted with equal amount of hexane. 10µl of each sample was injected for each analysis and detection was done at 240nm.

Combination of Conyza sumatrensis with Spathodea campanulata and bio evaluation for possible antimalarial activity

HPLC fingerprints of ethanol extracts of *Conyza sumatrensis*, *Spathodea campanulata* as well as the extract combination guided for the type of extract chosen during antimalarial study. Antimalarial activity was carried upon *Plasmodium berghei* infected mice based on percent parasitaemia, haemoglobin, survival kinetics and mean survival time. The schizontocidal activity of the extract was evaluated using the method described by Knight and Peters[8] with modifications in the increase of doses from 4 to 7. To derive a logical conclusion from the antimalarial testing, the extract combination was administered at 1000, 1500 and 1600 mg/kg. Briefly, the animals were infected intraperitoneally with standard inoculum of 1×10^6 *Plasmodium berghei* K173 infected erythrocytes (day0). Following the infection, the animals were orally administered with the extract for a period of 7days after suspending them in 0.7% CMC (Carboxymethyl Cellulose) along with 5% DMSO (Dimethyl sulfoxide) and 2.5% Tween 80. However, chloroquine was orally administered till day3 (4 doses). The smears were drawn every alternate day till day 28 from snipped tail of each mouse, stained with Giemsa (Sigma) and observed under 100X objective (Leica DMLB2) to determine the parasitaemia through the formula:

% parasitaemia = (No of parasitized red blood cells× 100)/ Total number of red blood cells).

Besides parasitaemia, the haemoglobin was also quantified at peak parasitaemia (Day8) through cyanmethe-moglobin (CMG) method. Briefly, 4µl of blood were diluted into 1ml of Drabkin's reagent[9] and the absorbance was read at 540nm against blank using CMG as standard through spectrophotometer (Spectramax, Mol Devices, USA).

Statistical analysis

The data, expressed as Mean±SE, were subjected to Kruskal–Wallis one way analysis of variance (ANOVA) through Graphpad PRISM Software. Inter group comparisons were made by Duns-test (non parametric) for only those responses which yielded significant treatment effects in the ANOVA test. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

HPLC fingerprinting of Spathodea campanulata, Conyza sumatrensis and their combination

An HLPC chromatographic method was successfully developed using a reversed phase column with UV detection at 240nm having acetonitrile: trifluoroacetate and water: trifluoroacetate as mobile phase that could resolve 18, 19 and 23 peaks from *Conyza sumatrensis*, *Spathodea campanulata* and their combination respectively. A standard solution including gallic acid, catechol, coumarin and quercetin injected in the same chromatographic conditions, produced 4 peaks at the retention time of 2.17, 5.6, 21.6 and 29.33 respectively. *Conyza sumatrensis* fingerprint allowed for the detection of two marker compounds (coumarin and quercetin) while fingerprints of *Spathodea campanulata* and mixture of both plants sensed the four marker compounds (gallic acid, catechol, coumarin and quercetin) at 240 nm with overall satisfying peak resolution (Figure 1). The elevated number of peaks appearing on chromatogram of combined extract suggested that the combination might contain more secondary metabolites and might be the better choice for antimalarial study. The fingerprint profile of the extracts can also be used for quantification of the chemical constituents present in *Conyza sumatrensis* and *Spathodea campanulata*.

Response of Plasmodium berghei K173 infected mice towards treatment with Conyza sumatrensis and Spathodea campanulata in combination

The ethanol extracts of *Conyza sumatrensis* and *Spathodea campanulata* when considered individually were effective in suppressing the early infection of malaria in our previous studies [5,6].

Upon seven days administration post infection, the parasitaemia was monitored at regular intervals and the mortality was noted. The peak parasitaemia was observed on day8 when the animals in the infected, untreated group started succumbing. *Conyza sumatrensis* in combination with *Spathodea campanulata* (1000, 1500 and 1600mg/kg) exhibited extremely significant ($P<0.001$) suppression of parasitaemia in mice infected with *Plasmodium berghei* K173 (Table 1). The optimum mean survival time (12.75 days) and percent suppression (99.08±1.64%) were exhibited by the group administered with 1500mg/kg of the ethanol extract. The mean parasitaemia got correlated with the mean survival time. Interestingly, one of the six animals (16.66%) in this group survived till the end of the experiment (Day 28) with no parasitaemia. This observation might be made clear through the presence of more secondary metabolites in the extract combination as put forward by more number of peaks on the HPLC chromatogram. Flavonoids like quercetin and gallic acid and

phenolic compounds such as catechol which are known to possess anti-oxidant activity might aid in boosting the innate immunity of the infected subjects thus lowering the mortality and morbidity rate.

This confirms the additive potential of different components of *Conyza sumatrensis* and *Spathodea campanulata* and the use of these species in the treatment of malaria. The haemoglobin quantified at peak parasitaemia also concludes that the treated groups at all the doses had significantly restored the haemoglobin levels ($p<0.05$) towards normalcy[10] (Figure 2).

CONCLUSION

In conclusion, the present study has demonstrated the potential of combining *Conyza sumatrensis* and *Spathodea campanulata* in the treatment of malaria. HPLC fingerprint of these plants confirmed the presence of gallic acid, quercetin, coumarin and catechol which might be responsible for the antimalarial activity. Further, development of both species for this purpose would no doubt provide cost effective strategy of malaria control especially in developing countries. Therefore, it would be interesting if the active principles of *Conyza sumatrensis* and *Spathodea campanulata* are isolated and characterised. Additionally, toxicity profile of the combination is envisaged.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Table 1. Antimalarial activity of *Conyza sumatrensis* in combination with *Spathodea campanulata* in terms of parasitaemia, meansurvival time and percent suppression frommice infected with *Plasmodium berghei*K173. Figures in paren thes is indicate the number of animalssurviving.

Treatment groups	% Parasitaemia on days post infection														MeanSurvival Time (days)	% Suppression
	9	4	6	8	10	12	14	16	18	20	22	24	26	28		
P.berghei	00 (6/6)	0.97±0.43 (6/6)	17.48±3.1 (6/6)	17.5±3.0 (6/6)	Died (0/6)	-	-	-	-	-	-	-	-	-	6.25	00±00
1000mg/kg	00 (6/6)	0.0±0.0 (6/6)	0.0±0.0 (6/6)	2.90±0.54 (6/6)	9.08±3.22 (3/6)	26.04±0.0 (1/6)	20.00±0.0 (1/6)	Died (0/6)	-	-	-	-	-	-	9.1	83.00±2.63***
1500mg/kg	00 (6/6)	0.0±0.0 (6/6)	0.0±0.0 (6/6)	0.16±7.61 (6/6)	4.92±1.99 (6/6)	5.38±3.9 (4/6)	11.22±4.4 (3/6)	16.5±00 (3/6)	0.0±0.0 (2/6)	0.0±0.0 (1/6)	0.0±0.0 (1/6)	0.0±0 (1/6)	0.0±0.0 (1/6)	0.0±0.0 (1/6)	12.75	99.08±1.64***
1600mg/kg	00 (6/6)	0.0±0.0 (6/6)	0.0±0.0 (6/6)	0.2±0.1 (5/6)	6.5±1.07 (3/6)	15.01±8.6 (1/6)	22.5±8.83 (1/6)	27.04±7.9 (1/6)	35±0.0 (1/6)	Died	-	-	-	-	10.705	98.85±0.96***
Chloro-quine (10mg/kg)	00 (6/6)	0.0±0.0 (6/6)	0.0±0.0 (6/6)	0.0±0.0 (6/6)	0.0±0.0 (6/6)	0.0±0.0 (6/6)	0.0±0.0 (6/6)	0.0±0.0 (6/6)	0.0±0.0 (6/6)	0.0±0.0 (6/6)	0.0±0.0 (6/6)	0.0±0 (6/6)	0.0±0.0 (6/6)	0.0±0.0 (6/6)	28	100±0.0***

*p<0.05, **p<0.01, ***p<0.001 treatment vs *P.berghei* on day8.

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(A) *Spathodea campanulata*

(B) *Conyza sumatrensis*

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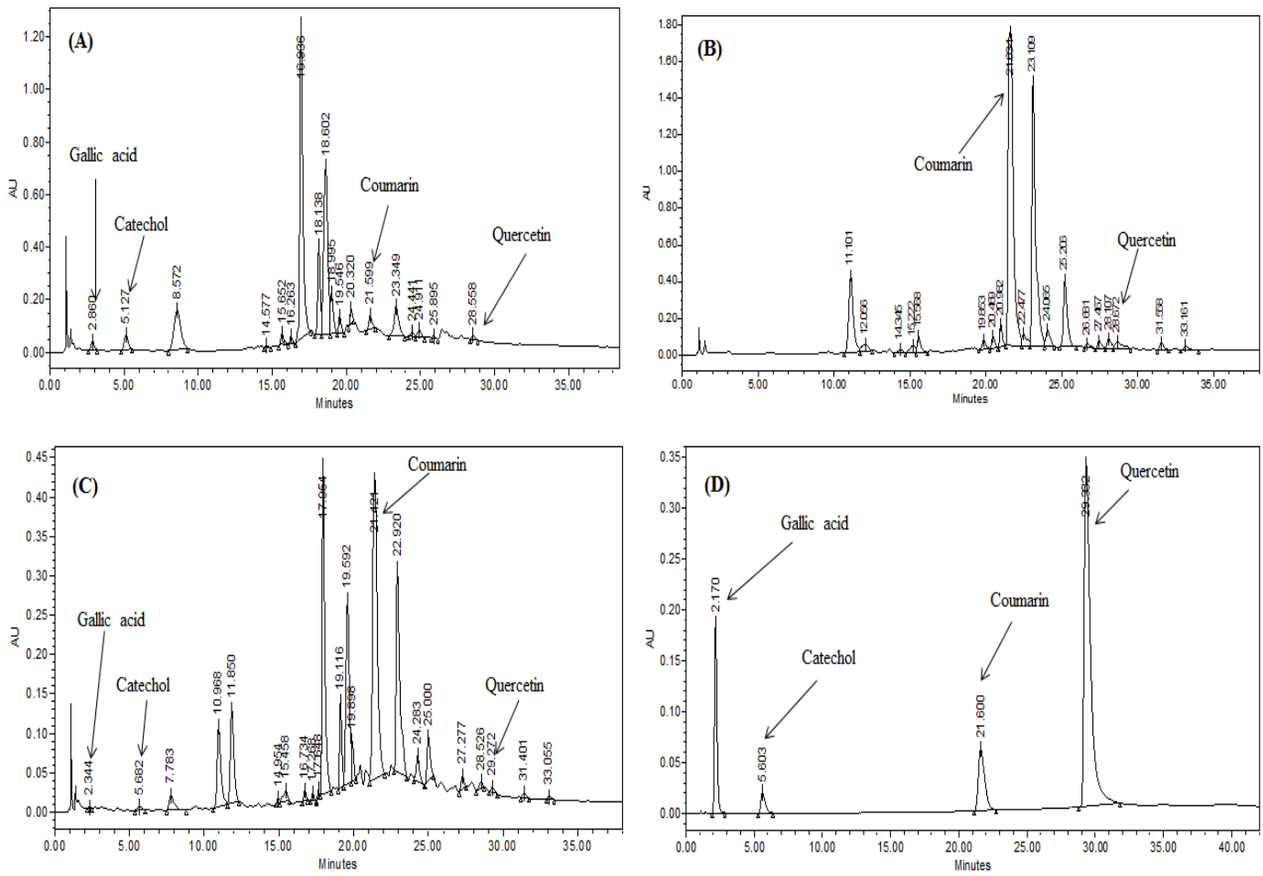

Figure I: A representative chromatographic fingerprinting of ethanol extracts from *Spathodea campanulata* (A), *Conyza sumatrensis* (B), their mixture (C) and assortment of standards (D) at 240 nm.

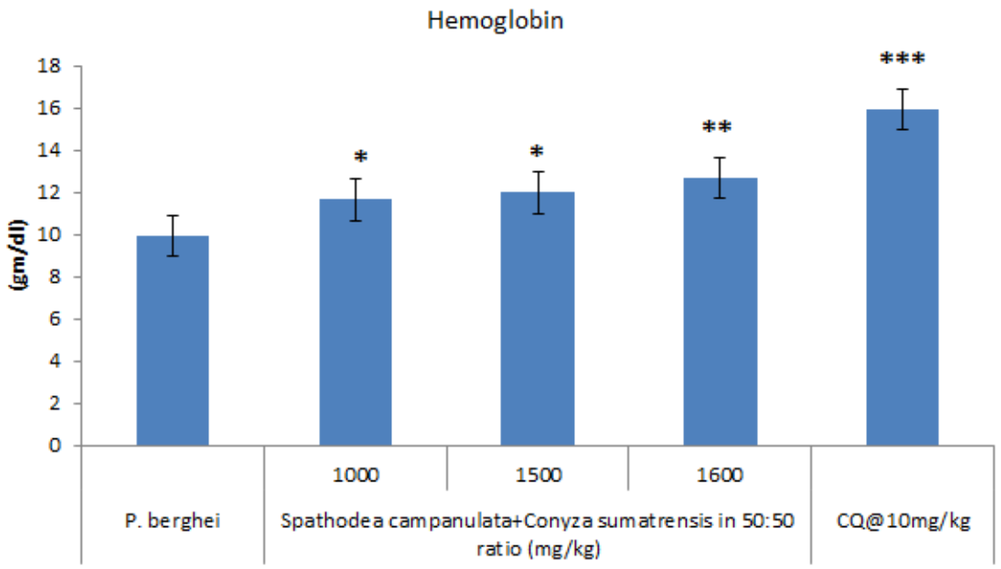

Fig. II. Haemoglobin level of mice infected with *plasmodium berghei* K173 and treated with varying concentration of ethanol extract from the combination of *Conyza sumatrensis* and *Spathodea campanulata.* n=6, *p<0.5, **p<0.01 and ***p0.001 infected vs treated.