Journal of Ayurveda and Integrative Medicine 7 (2016) 238–248

Contents lists available at ScienceDirect

Journal of Ayurveda and Integrative Medicine

journal homepage: http://elsevier.com/locate/jaim

Original Research Article (Clinical)

Antiparasitic and disease-modifying activity of *Nyctanthes arbor-tristis* Linn. in malaria: An exploratory clinical study

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ARTICLE INFO

Article history:
Received 25 April 2016
Received in revised form
4 August 2016
Accepted 6 August 2016
Available online 30 November 2016

Keywords:
Antimalarial
Nyctanthes arbor-tristis
Ayurveda
Traditional medicine
Reverse pharmacology
Drug resistance
Plasmodium vivax
Plasmodium falciparum
Cytokines
TNF-α

ABSTRACT

Background: An unceasing threat of drug resistance continuously poses demand for new antimalarial drugs. A scientific assessment of traditionally used antimalarial plants through reverse pharmacology is crucial for a fast track drug discovery. An Ayurvedic plant *Nyctanthes arbor-tristis* Linn. – (Parijat) is being used in clinical practice and had shown antimalarial activity, with a parasite clearance in 76.6% of 120 patients, in an earlier clinical study.
Objective: To further explore antimalarial potential of the plant through additional objective markers.
Materials and methods: An open-labelled observational study was conducted at M.A. Podar Hospital – Ayurveda (MAPH-A) after ethics committee approval. Administration of a paste of 5 fresh leaves, thrice a day for a week was a standard practice for management of malaria at MAPH-A. Clinical activity of *N. arbor-tristis* was evaluated by monitoring pyrexia, parasitemia and morbidity score (MS) in twenty patients. In addition, immune and biochemical markers and organ functions were monitored for objective markers of response. Student's paired-'t' test was applied to assess statistical significance.
Results: Ten out of 20 patients showed both fever and parasite clearance, which was confirmed by polymerase chain reaction. Remaining ten patients had persistent but decreasing parasitemia. Four of them needed chloroquine as a fail-safe procedure. Irrespective of the degree of parasitemia all the patients showed decrease in MS. There was also an increase in platelet count and normalization of plasma lactic acid. There was a good clinical tolerability and an improvement in organ function. The inflammatory cytokines showed a reduction; particularly in TNF-α within a day.
Conclusions: At the given dosage, *N. arbor-tristis* showed disease-modifying activity; early clinical recovery with a decline of TNF-α and a gradual parasite clearance. Further studies with a standardised formulation for dose-searching and optimizing the treatment schedule are needed in a larger sample size.
Clinical trial registration no: The process of trial registration had not begun when the study was conducted in 2000.

1. Introduction

In the last decade, 40% of the malaria-endemic countries reported reduction in the incidence of malaria by half. This has raised hopes to the stage of 'complete eradication' of malaria in the near future. Recently, the World Health Organization (WHO) announced an ambitious new plan to "almost" eradicate malaria by 2030; 40% by 2020 and 90% by 2030 [1]. However, alarming reports of artemisinin resistance, first from Thai-Cambodian border in 2009 and it's spread in 5 countries within 5 years have contributed to the unceasing threat of resistance development [2,3]. This has put forth again an urgent need for the development of new antimalarial drugs. In spite of persistent efforts and significant investment, availability of long lasting antimalarial drugs is still a quest.

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Peer review under responsibility of Transdisciplinary University, Bangalore.

http://dx.doi.org/10.1016/j.jaim.2016.08.003

Medicinal plants, being a source of diverse compounds, have been a major attraction for drug discovery scientists. The two major anti-malarial drugs; artemisinin and quinine, have been discovered from plants. However, efforts through extensive research on medicinal plant have been largely unproductive due to the smash and grab approach as articulated by Wells TN [4]. In the present study we have explored antimalarial potential of traditionally used Ayurvedic plant – *Nyctanthes arbor-tristis* Linn. (*N. arbor-tristis*) – family Oleaceae; through Reverse Pharmacology (RP).

RP has been recently evolved, in India, as a fast-track trans-disciplinary research and development path to discover and develop a standardized formulation, extract or active principle – from Ayurvedic medicinal plants of therapeutic importance [5]. It is defined as *'The science of integrating documented clinical experiences and experiential observations (hits) into leads by trans-disciplinary exploratory studies to further develop these into drug candidates or formulations through robust preclinical and clinical research'*. RP is one of the paths of drug development from traditional medicine, which essentially relates to reversing the standard "laboratory to clinic" progress of the discovery pipeline to "clinic to laboratory" or "bedside-to bench" approach [5,6].

N. arbor-tristis a sacred plant from India, has been described for its use in *vishamjwara* (malaria) in the classical text [7], and for malaria in notes of Aryavaidya Mayaram Sundarji [8], Waman Ganesh Desai [9] and several others (Fig. 1). Vaidya Antarkar and Vaidya Tathed at MAPH-A had introduced the use of *N. arbor-tristis* in the management of malaria, as an Ayurvedic line of treatment. Chloroquine (CQ) was used when there was failure of response to this treatment or in severe cases. The early clinical observations of the antimalarial activity and safety of the plant were recorded during MD (1995) and PhD (1997) thesis work by Karnik [10]. In that collaborative experiential study (published in 2008), we have reported antimalarial activity – clinical cure and parasite clearance, in 92 of 120 patients (76.7%), when treated for 7–10 days with the paste of the fresh leaves of the plant (as a traditional dosage form) [11]. Clinically, cure was associated with not only the fever clearance but also an early decrease in the severity of all malaria-related symptoms. That experiential study was followed by systematic exploratory studies in clinic and laboratory. The present study is being published after a delay as there was an ongoing experiment to generate intellectual property rights (IPR) vis-à-vis disease-modifying novel action demonstrated by objective markers of response. (In view the uncertainty of a precise phytoactive, despite substantial isolation efforts, the attempt for IPR is dropped.) The parasite clearance was confirmed by the use of polymerase chain reaction (PCR) for the absence of Plasmodium species. Markers for organ functions, and inflammatory severity were assessed for tolerability, and disease-modifying activity respectively.

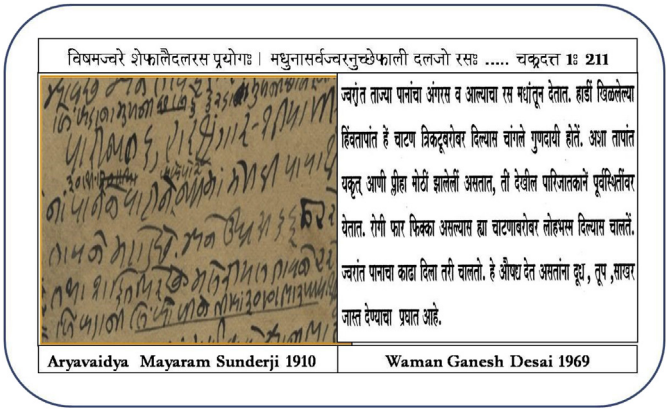

Fig. 1. Shows description in classical texts for the use of *N. arbor-tristis*. Sanskrit shloka [7] (Top): *Dalarasa*, Juice of leaves of *N. arbor-tristis* (*Shefali*) in malaria (*Vishamjwara*), Notes in Gujarathi [8] (Left): *Parijat*, *Harshringara pan … N. arbor-tristis* leaves, *Malarainu Tavane …* in malarial fever, '*Aushadhisamgrah*' [9] in Marathi (Right): Dosage form: *Tajya panacha amgarasa*: juice of fresh leaves. *Lohabhasma* may be added for anaemia. Excess milk, ghee and sugar intake recommended.

2. Material and methods

2.1. Patient material

The present observational study of *N. arbor-tristis* was carried out during rainy season (June 2000 to Aug 2000), at the peak of malaria incidence within the endemic area – Worli, Mumbai, at the same hospital i.e. MAPH-A a postgraduate teaching institution. Patients were strongly suspected to have malaria based on their clinical presentation. In view of the antimalarial activity shown in 76.7% of 120 patients in an earlier experiential study, the number of patients for screening was decided to be thirty. A sample size of completed twenty patients was considered to be adequate with a more frequent and detailed clinical and parasite monitoring by rigorous methods of assessment.

2.2. Preparation of paste of N. arbor-tristis

Fresh leaves of *N. arbor-tristis* were obtained from a single tree in the garden of MAPH-A as described earlier [10,11]. The plant was identified by the botanist. Medium-sized leaves were plucked by a trained attendant every day, washed with distilled water and crushed in mixer for fresh preparation of the paste. The paste was then distributed in small containers equivalent to a single dose (5 leaves approx. wt. 6.8 to 7 gm) per container and used fresh for the day.

2.3. Study design

An open-labelled observational study of exploratory stage of Reverse Pharmacology; with baseline as control was chosen for the individualized in-depth evaluation. Protocol, case record form (CRF) and informed consent form were approved by the independent ethics committee – Inter-Systems Biomedical Ethics Committee (ISBEC) of Mumbai. The informed consent form in regional language comprised of the information of the study, risk and benefits to the patient, and patients' responsibility and rights during the study. The Ayurvedic experts of the ISBEC approved the drug administration at the Ayurvedic hospital under the guidance of an expert Ayurvedic physician (PST – one of the co-authors), as per the Good Clinical Practice Guidelines by Indian Council of Medical Research.

2.4. Selection criteria

Patients visiting the outpatient clinic of MAPH-A, with age between 15 and 55 years, presenting fever and chills, were screened and selected if confirmed to have malaria (*Plasmodium falciparum and/or Plasmodium vivax*) by microscopy and by a rapid diagnostic test (RDT), OptiMal™. The severity of malaria in selected patients was mild to moderate with the haemoglobin value > 8 gm % and absence of cerebral or renal complications. Patients having other systemic diseases or on drugs were excluded.

2.5. Enrolment of patients

The study details were explained to patients in their language as to the drug information, study procedure, the degree of inconvenience due to frequent venipunctures, temperature recording and

modes of assessment for clinical response and safety. Patients were enrolled, only if they agreed to participate in the study, after taking their written informed consent and were admitted to the *Kaya-chikitsa* (Internal Medicine) ward. In case of patients below 18 years old (n = 2), consent was given by the parents.

2.6. N. arbor-tristis administration

Each patient was provided three containers with a fixed dosage of the paste of leaves for the day and was explained to ingest the dose under supervision at 9 am, 1.30 pm, and at 9 pm i.e. after breakfast, lunch and dinner respectively. The duration of the treatment was for a minimum period of 7 days. The treatment was continued beyond 7 days till the complete cure, only in those patients, who were showing improvement. This was decided based on the clinical judgement of the Ayurvedic expert (PST – one of the co-authors). Compliance was monitored by the nursing staff and the resident physicians. No other antimalarial or anti-pyretic drugs were administered during the study. In case of high fever, non-drug modalities like tepid sponging, cold water enema were used.

2.7. Criteria of clinical response

Clinical response to *N. arbor-tristis* was monitored daily by assessing the reduction in the severity of the twenty target features of malaria (Table 1 in result section). These target features were chosen based on the high frequency of the dominant signs and symptoms noted in the earlier larger experiential study of 120 patients. These features were graded individually for their severity on every day as: absent = 0, mild = 1, moderate = 2 and severe = 3. The MS was calculated for each patient as the sum of the severity scores of all the twenty symptoms and signs daily. The mean morbidity score (MMS) at the baseline and on each day till 7th day of the treatment for all patients was calculated for the analysis of the clinical response. Apyrexia as one of the criteria of the clinical response was determined by a careful record of temperature by ear thermometer at every 2 h excluding sleep hours. Consistent temperature of 98° F for consecutive three days was considered a complete fever clearance.

2.8. Assessment of antiparasitic activity

The change from the basal parasite count was monitored on the days 1st, 3rd and 7th, and the absence of parasites was considered as a parasitic cure. The parasite clearance was judged by blood smear examination, RDT (OptiMal™) and confirmed by PCR on the preserved blood dry blots in all the twenty patients (*vide infra*). PCR was done by one of the authors (CG) at the Centre of Molecular Parasitology, Drexel University, Philadelphia, USA.

2.9. Criteria of tolerability assessment

The clinical tolerability of the paste of *N. arbor-tristis* was assessed by a careful daily monitoring of the adverse events and physical examination. Adverse events were evaluated and the causality was ascribed as per the criteria of Karch and Lasagna [12]. Any new symptom, sign or a marked change in organ function test, after intervention and not expected to a natural history of malaria was considered as adverse drug event (ADE). ADEs were recorded as to their onset, severity and duration. Measures taken for the assessment and treatment of ADE were recorded. Laboratory investigations (day 0, 1st, 3rd, 7th) viz. complete blood count and markers of liver and kidney functions for the organ safety were carried out.

2.10. Fail-safe procedures

In the situation where patient failed to respond, had severe aggravation of pyrexia or severe side effects the patient was to be discontinued from the study and prescribed standard CQ therapy. In addition, if patient chooses to withdraw from the trial CQ therapy was to be prescribed.

2.11. Parasite identification and count: microscopy, rapid test and PCR

Venous blood was collected from the antecubital vein, in vacutainers with EDTA, on day 0, 1st, 3rd and 7th of the treatment for parasite counts. Thin blood smears were stained with Giemsa by using standard method and a number of infected RBCs were counted in total 2000 RBCs/slide. The percent parasitemia was estimated as described previously [11]. RDT was also used to confirm presence of parasite and its type. Parasite clearance during the treatment period was monitored by both microscopy and RDT. Blood samples were preserved for PCR, in the form of dry-blots, on the Whatman Paper No. 1. Deoxyribonucleic acid (DNA) was extracted from the dry blots by the method described by Newbold et al. [13]. In brief, the dry blot was washed with phosphate buffered saline followed by incubation with saponin. Then the blot was boiled with alkaline chelex-100, then was smashed with pipette tip to release DNA. The extracted DNA in supernatant was obtained by centrifuging the mixture. The DNA was subjected to nested PCR for the amplification of *P. vivax*-specific Merozoit Surface Protein–3α (MSP-3α) gene [14] and *P. falciparum*-specific Glutamate Rich Protein (GLURP) antigen gene [15].

2.12. Reagents, chemicals and instruments

The RDT kit – OptiMal™ from Flow INC, Philadelphia, was supplied by Global Diagnostic, Mumbai. Giemsa stain was obtained

Table 1
Target features of malaria: Baseline frequency and change by 7th day.

Symptoms and signs	Frequency (n)		Symptoms and signs	Frequency (n)	
	Baseline	7th day		Baseline	7th day
Fever	20	6	Excessive sweating	11	1
Nausea	20	3	Thirst	10	3
Chills	18	0	Body heaviness	10	2
Headache	18	7	Abdominal pain	8	3
Anorexia	17	1	Giddiness	7	0
Bodyache	17	4	Splenomegaly	7	7
Exhaustion	16	1	Lethargy	6	0
Vomiting	16	2	Cough	6	0
Bitterness of mouth	14	5	Hepatomegaly	6	6
Loss of taste	14	6	Anaemia	4	4

n: Number of patients; out of 20, showing the symptoms at the baseline and on 7th day.

from Sigma. Various kits were obtained from the local dealers viz. lactic acid Spineract SA, Espana; human tumour necrosis factor (TNF)-α, Interleukin (IL)-10 and IL-6, Interferon-γ (IFN-γ) R & D system – Quantikine®; C-reactive protein (CRP) Nycocard Single test, Axis-shield, Norway; biochemical test kits viz. ALT, creatinine, ERBA Test, Transasia Bio-Medicals, India.

The following instruments and accessories were used for various studies viz. Sysmex K 1000 Haematology analyser (TOA Medical electronics Co. Ltd. Japan) for haemogram and platelet count; Semi-auto analyser (ERBAChem Pro) for biochemical markers; Labomed Vision 2000 Microscope for parasite identification and count; Nycocard Reader II for CRP, Stat Fax 303 Plus, Awareness Technology INC an enzyme linked immunosorbent assay (ELISA) reader for cytokines and interleukin assays. Vacutainers for blood collection were obtained from Becton Dickinson. An ear thermometer (ThermoScan PLUS, Germany) was used for monitoring internal body temperature.

2.13. Statistical analysis

The values of all the investigations were calculated for means and standard error (mean ± SE). The levels of statistical significance were determined by applying the student's paired 't' test by using GraphPad Prism 5 software.

3. Results

3.1. Baseline profile of patients and symptomatic response

Total 30 patients of fever and chills were screened and diagnosis of malaria was confirmed in 27 patients by microscopy and OptiMal™. Twenty of them agreed to follow the study protocol and hence were enrolled for the study.

The baseline syndromic profile of the patients was done for twenty target features of malaria. Table 1 shows the frequency of these features. While fever and nausea were present in all patients, other most frequent symptoms were chills and headache (n = 18), body ache, anorexia (n = 17), exhaustion and vomiting (n = 16), bitterness of mouth and loss of taste (n = 14), and excessive sweating (n = 11). Ten patients had thirst and body heaviness. Remaining symptoms were less frequent, as shown in Table 1. None of the patients had drowsiness or mental changes. *P. vivax* infection was detected in 8 patients and *P. falciparum* in 9 patients. The remaining three patients having mixed infection were positive for both the parasites.

All the twenty patients; irrespective of the type of plasmodial infection and the degree of parasite count, showed distinct symptomatic improvement, began from day 1 (24 h) of the treatment. Table 1 also shows change in frequency of all the symptoms with treatment of 7 days.

3.2. Antimalarial activity of N. arbor-tristis: fever and parasite clearance

Antimalarial activity of the plant paste was assessed by clinical response that comprised of apyrexia and parasite clearance. The results are summarized in Fig. 2. Based on these data, patients were divided in to two subsets: subset A and subset B as cured and partially responded respectively.

3.2.1. Subset A

This subset comprised of 10 patients (4 *P. falciparum*, 4 *P. vivax* and 2 mixed infection) out of 20 who showed complete cure by

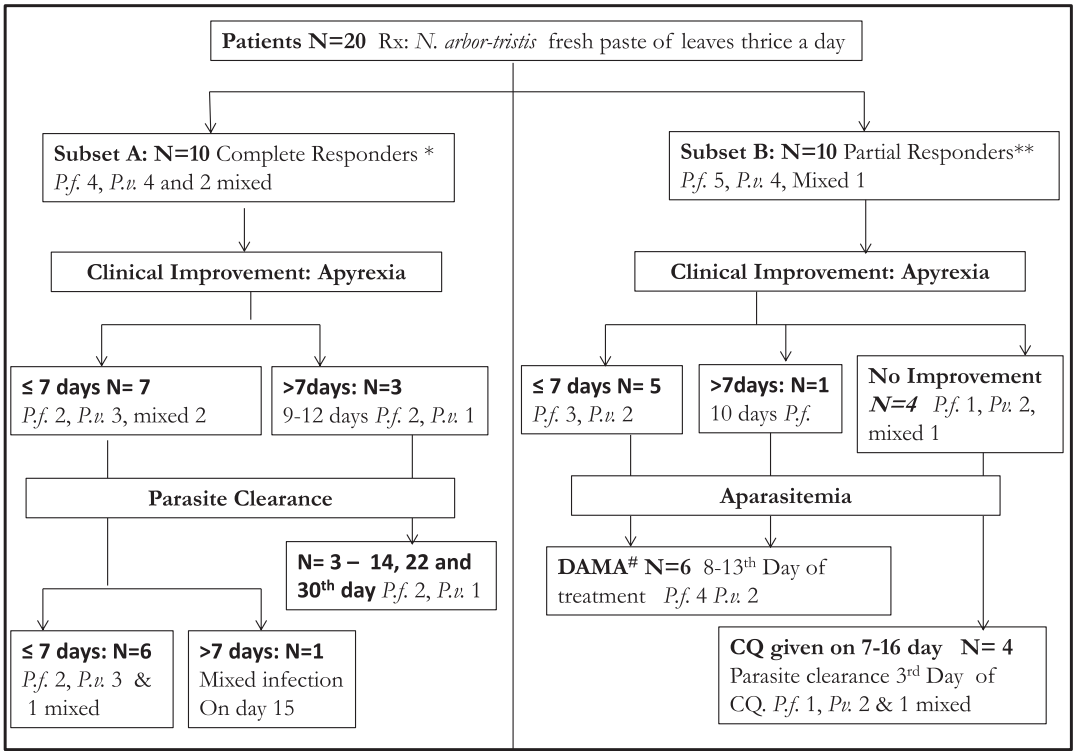

Fig. 2. Shows clinical response (apyrexia) and parasite clearance in subset A and B. All the patients were treated with the paste of fresh leaves of *N. arbor-tristis*. (* Both clinical improvement and parasite clearance ** Clinical improvement but parasite present # Discharge against medical advice as patients were not willing to continue.

both apyrexia and parasite clearance. Seven out of these 10 patients had fever clearance within 7 days and six of these 7 also had parasite clearance by 7 days. One of these 7 patients showed parasite clearance on 15th day of treatment. Remaining 3 of 10 patients, on continuous treatment beyond 7 days showed fever clearance by 9–12 days. Parasite clearance in these 3 patients was between 14 and 30 days of treatment.

3.2.2. Subset B

Patients in this subset (n = 10) were showing directionality in the clinical response. Six of these 10 cases showed apyrexia – 5 within 7 days and 1 took 10 days. These 6 patients however, had persistent but decreasing parasitemia. These patients were not willing to stay in hospital and were discharged against medical advice (DAMA) between 8th to 13th day of treatment. Though these patients were advised to take standard CQ regimen, their follow up after CQ was not available. The four remaining patients; due to repeated fever episodes were shifted to standard CQ regimen during 7–16 days as per the fail-safe procedure.

In-depth comparative analysis was carried out, for the selected objective markers in subset A and subset B. The objective markers analysed during the 7 days of treatment were MS, parasite count, platelet count and lactic acid, TNF-α, CRP and organ functions. Parasitemia and MS were monitored until discharge (beyond 7 days).

3.3. Antimalarial activity of N. arbor-tristis: MS and parasite count

The symptomatic response was semi-quantitatively judged by change in the MS and fever clearance. The decrease in MS was associated with the decrease in severity of all the major symptoms shown in Table 1. However in the short period of 7 days observation, there was no remarkable change in hepatosplenomegaly. Parasite count monitored in these patients on day 1st, 3rd and 7th showed gradual decrease from the baseline count. The scatter gram for the MS and parasite count in these patients are shown in Figs. 3 and 4 respectively.

Both the subsets had similar MS at the baseline (subset A 19.8 ± 1.9 and 19.4 ± 1.5). A significant reduction; > 50% in MS ($p < 0.0005$ and $p < 0.0001$ in subset A and B respectively) was seen within 24 h of treatment. A further drop in MMS was significant on 7th Day in both the subsets as 4.1 ± 0.7 in subset A and 4.0 ± 1.2 in subset B ($p < 0.0001$).

Mean parasite count was monitored in both the subsets. Mean baseline parasite count in subset A was 0.62 ± 0.24% and of subset B was 0.39 ± 0.1%. Six patients from Subset A cleared parasites and remaining four patients showed a decrease by 7th day. Remaining four patients continued treatment beyond 7 days and showed complete clearance between 14 and 30 days (*vide infra*). Subset B showed marginal decrease in parasite count on 7th day (0.31 ± 0.12%), which was not significant from the baseline mean count 0.39 ± 0.03%.

Parasite clearance in these patients was confirmed by PCR. Fig. 5 shows representative data – Amplified DNA on agarose gel electrophoresis.

A significant correlation was found in MS and parasite count at baseline ($p < 0.05$ and Spearman r = 0.44) in 20 patients. This correlation did not remain significant (Spearman r = −0.033) on 7th day. This indicates discordance between MS and parasite clearance, due to early clinical response despite presence of parasites during treatment.

In addition there was no association of the baseline MS or parasite count with the type of species or with the therapeutic response. Individual analysis of 4 delayed responders from subset A distinctly showed this feature. Two of the 4 delayed responders from subset A had very high parasitemia at base line. The patient having highest baseline parasitemia as 2.35% also had the highest MS as 31 with *P. vivax* infection. Another patient had baseline parasite count 1.58%, with MS 23 and was positive for *P. falciparum*. They showed a decrease in parasite count as 0.13% and 0.18% respectively on the 7th day and complete parasite clearance by 14th and 30th day. Other two delayed responders from subset A had mixed and *P. falciparum* infection with MS 19 and 22 and baseline parasitemia 0.3% and 0.5% respectively. These patients showed parasite clearance by 15th and 22nd day respectively.

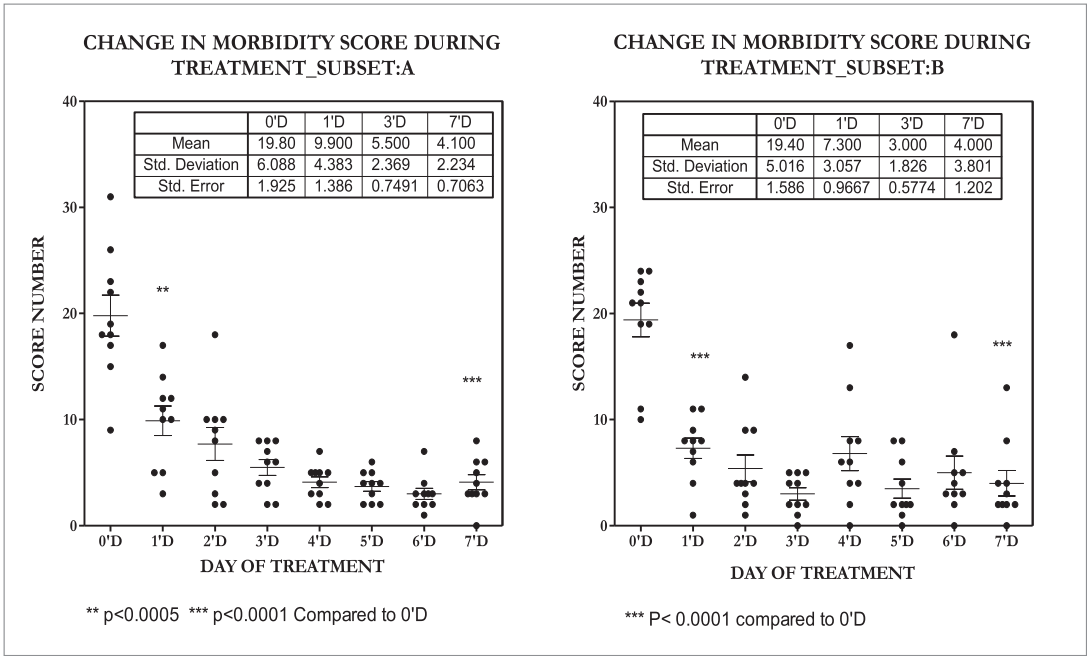

Fig. 3. Shows MS in two subsets A and B at 0'D (baseline) and on every day thereafter for 7 days during the treatment with *N. arbor-tristis*. MS was calculated by adding severity score for 20 malaria related markers in each patient. Drop in MMS was highly significant in both the subsets, suggesting clinical improvement.

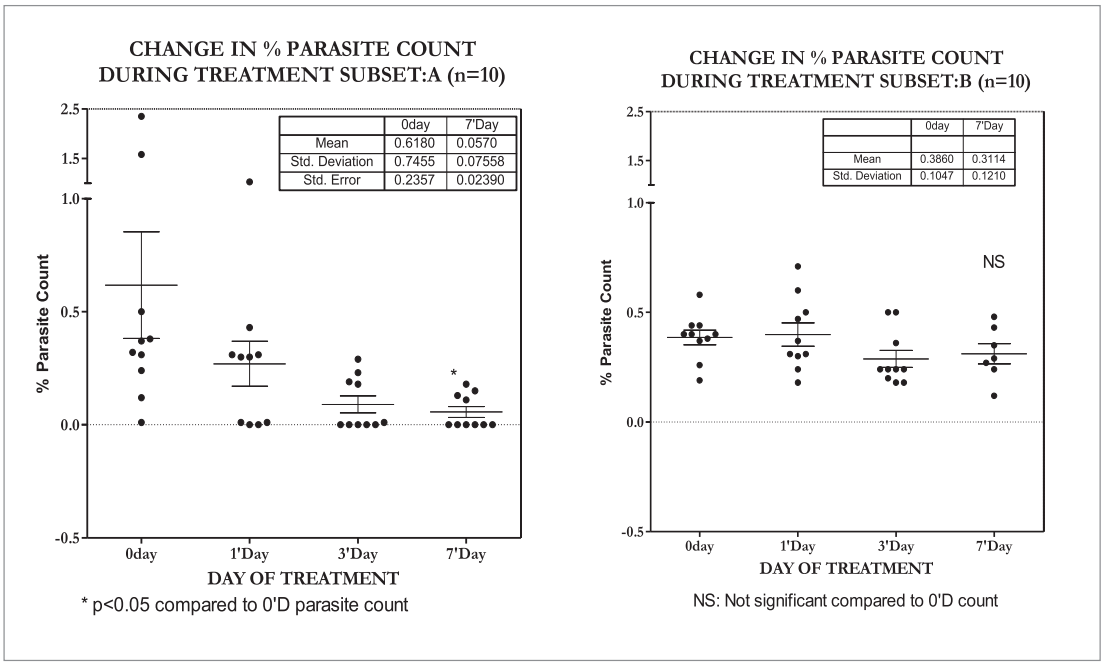

Fig. 4. Shows parasite count in subset A and B that was monitored by microscopy on day 0, 1st, 3rd and 7th day of treatment with *N. arbor-tristis*. Parasite count was done by counting infected RBCs, against 2000 RBCs. Subset A showed significant drop in parasite count by 7th day, with complete clearance in six patients.

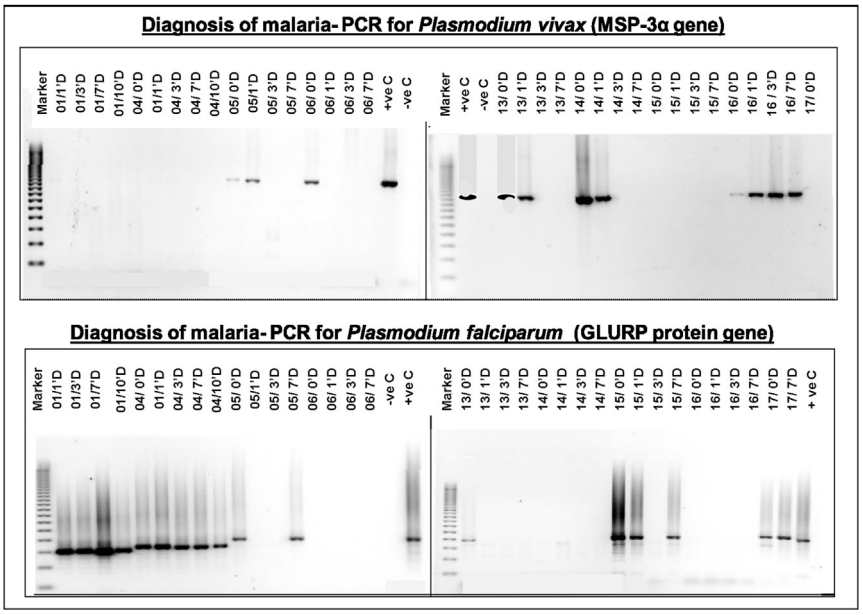

Fig. 5. Shows representative electrophoretic run of PCR product: PCR was done for diagnosis and monitoring of parasite clearance in patients treated with *N. arbor-tristis*. Specific primers for gene – MSP-3α for *P. vivax* (Top) and GLURP for *P. falciparum* (Bottom) were used. Lane i.d. is indicated on the top of each lane as Patient #/day of sampling. The presence of band indicates that sample was positive and absence of band indicates negative for parasite. Note two mixed infections Pt. 05: +ve for *P. vivax* on 0 day and 1'day of treatment then became negative ; the *P. falciparum* infection came up by day 7, which cleared on day 15 (data not shown), this patient was clinically normal by day 7. In other patient (#13), mixed infection was detected on 0 day – *P. vivax* got cleared by day 3 and *P. falciparum* got clear by day 1, clinically this patient was normal by day 2.

3.4. *N. arbor-tristis: effect on biochemical markers*

Decrease in platelet count and increase in lactic acid levels is associated with the pathogenesis of malaria. These pathological markers were monitored in patients of subset A and B. Figs. 6 and 7 show comparative data. Low baseline mean platelet count was seen in both the subsets – subset A: 64.2 ± 11.9 × 10^3/mm^3 and in subset B 80.38 ± 19.1 × 10^3/mm^3 (Normal range 150 × 10^3/mm^3–450 × 10^3/mm^3). Both the subsets showed increasing trend in platelet count by 7th Day as 188.4 ± 37.2 × 10^3/mm^3 and 105.7 ± 24.8 × 10^3/mm^3. However, the increase was significant ($p < 0.005$) in subset A (Fig. 6). Out of total 18 patients, in whom platelet count was available, 16 had baseline count below normal. Irrespective of MS, parasite type and count, out of these 16 cases 11

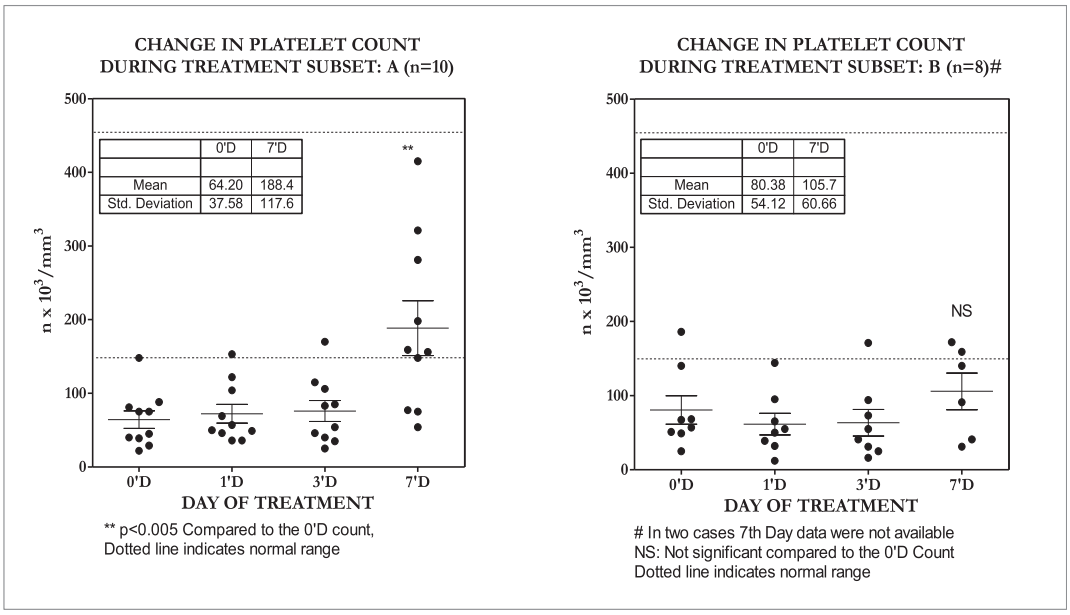

Fig. 6. Shows platelet count in subset A and B. The dotted lines in the graphs show normal range. Increasing trend was seen in 11 patients and 8 returned to normal platelet count by 7th days.

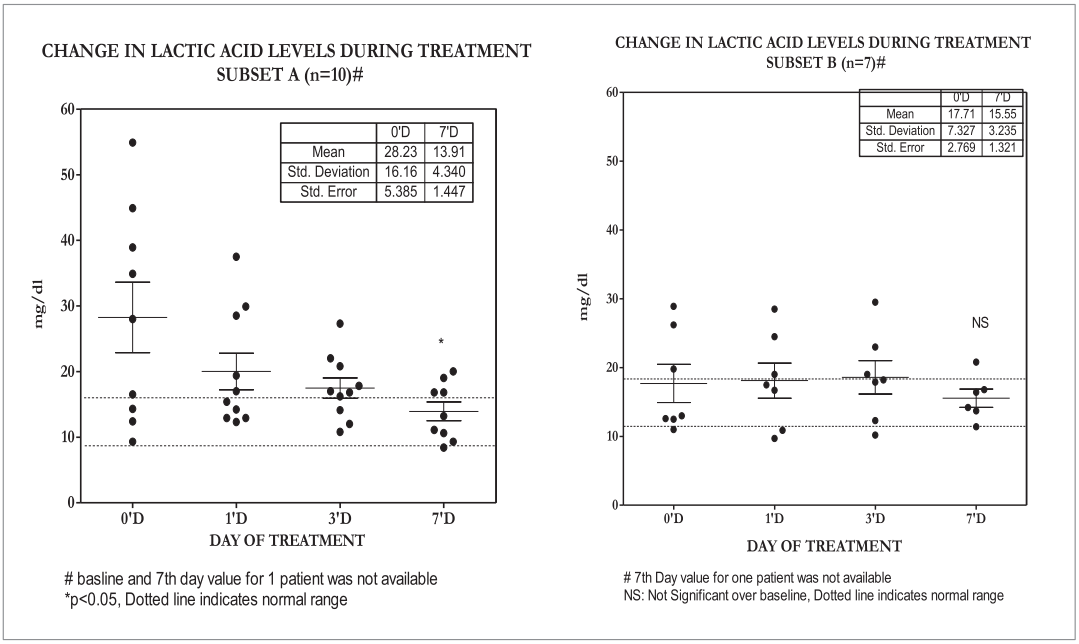

Fig. 7. Shows lactic acid levels in subset A and B during treatment. Significant drop in mean lactic acid levels was seen in Ssubset A. The dotted lines in the graphs show normal range. Eight patients from both the subsets had high lactic acid levels, above normal range, all of them showed significant decrease by 7th day.

showed prompt increase and 8 of them reached to normal level by 7 days.

Mean lactic acid measured at baseline was high (Fig. 7): 28.2 ± 5.4 mg/dl and 18.2 ± 3.8 mg/dl in subset A and B respectively (normal range 9–16 mg/dl). Both the subsets showed decrease in lactic acid by 7 days, though not significant. Out of total 17 patients, in whom lactic acid levels were available, 8 had baseline high value (34.2 ± 4.2 mg/dl). All these 8 cases (6 from subset A and 2 from subset B) showed significant decrease to 13.3 ± 1.4 mg/dl by day 7 ($p < 0.005$).

3.5. *N. arbor-tristis: effect on organ function*

Serum levels of ALT, AST, alkaline phosphatase, protein and albumin were measured to monitor liver function. Blood urea nitrogen (BUN), uric acid, creatinine were measured to monitor kidney function in 17 patients during the treatment. All these 17 patients, irrespective of their clinical response showed baseline abnormal levels of one or more of these markers, indicating liver &/or kidney dysfunctions. All the patients showed improvement in organ function markers (*vide infra*).

3.5.1. Liver function

Four patients had high ALT (58–501 IU/L) and 10 had high AST (44–409 IU/L) at the time of admission. Both these liver enzymes showed decreasing trend from day 1 itself. On the 7th day ALT levels were in the range of (25–60 IU/L) and AST levels were (19–69 IU/L). The synthetic function of the liver was found to be affected either at the baseline as albumin was low in all 15 patients and protein was low in 10 patients. During the treatment with *N. arbor-tristis*, 11 patients showed an increase in albumin and 8 patients showed increase in protein by 7th day. Alkaline phosphatase measured in all these 15 patients was within normal range (supportive data is given in a Supplementary File).

3.5.2. Kidney function

Kidney function was found to be affected in 8 patients as one or more marker of kidney function showed abnormal levels. Six patients had high BUN (4–87 mg/dl) and 5 patients had high uric acid (6.9–9.4 mg/dl). All of them showed normal levels by 7th day of treatment as BUN (19–26 mg/dl) and uric acid (3.9–6.6). One patient had high baseline creatinine (2.5 mg/dl) which reduced to 1 mg/dl by day 7 (supportive data is given in a Supplementary File).

None of the 17 patients showed aggravation of liver or kidney functions during the treatment.

3.6. N. arbor-tristis: effect on inflammatory markers

TNF-α; an inflammatory cytokine, showed drop from baseline in all patients (n = 16) from both the subsets. The drop within 24 h of treatment was significant in patients from subset A. Similar drop was also seen in subset B, even though not significant (Fig. 8). Similarly IL-10, an anti-inflammatory cytokine, studies in these patients showed drop by 7 days, significantly in both the subset ($p < 0.005$ and < 0.05 respectively) (Fig. 9). It was observed that the drop in IL-10 was gradual and higher levels were maintained for a longer period, compared to the drop on TNF-α. CRP measured in all these 16 patients also showed baseline high value. Baseline mean CRP in subset A was 85.1 ± 13.5 mg/L and in subset B was 77 ± 15.3 mg/L. Both subsets showed decrease in CRP on 7th day as 46.5 ± 16.3 mg/L and 52.7 ± 14.2 ($p < 0.05$) (supportive data is given in a Supplementary File).

3.6.1. Tolerability

Not withstanding the bitter taste, the acceptance of the formulation was good and the baseline symptoms of nausea and bitter test due to malaria were relieved. Organ function test did not show any adverse reaction in any of the patient. The safety and tolerability of *N. arbor-tristis* in all these patients were closely monitored clinically as well as with the laboratory markers of organ functions. None of them showed any adverse event except one patient who developed, on the 7th day mild but self-limiting diarrhoea.

4. Discussion

Medicinal plants, used in Ayurveda and other traditional systems of medicine, have been of continuous interest as a potential resource of chemical scaffolds for new antimalarial drugs [16–19]. However a long history of missed opportunities; until quinine and artemisinin emerged, depicts the challenges involved in the process [20]. The drug discovery and development from traditionally used medicinal plants lacked a fast track path. RP; now an organized trans-discipline, delineates a rapid path for the drug discovery from traditional medicine. It is not only cost effective but also meets the challenges of human safety and efficacy [21,22]. RP is, gradually but steadily, finding its pace in the global quest for new drugs [23–25]. The antimalarial potential of *N. arbor-tristis* has been investigated at traditionally used fixed dose, for the rigorous scientific evidence, for its antiparasitic activity and safety in patients through the present observational study. Such an observational study to confirm clinical hits is a prerequisite of RP [26].

The information of *N. arbor-tristis* in the Ayurveda texts and vaidyas' notes and the data in an earlier fairly large experiential study in 120 patients of malaria with the plant [10,11] prompted the present in-depth observational study. Twenty patients of malaria (*P. falciparum*, *P. vivax*. or mixed infection); treated with the traditional fixed dose of leaves of the plant were observed with more

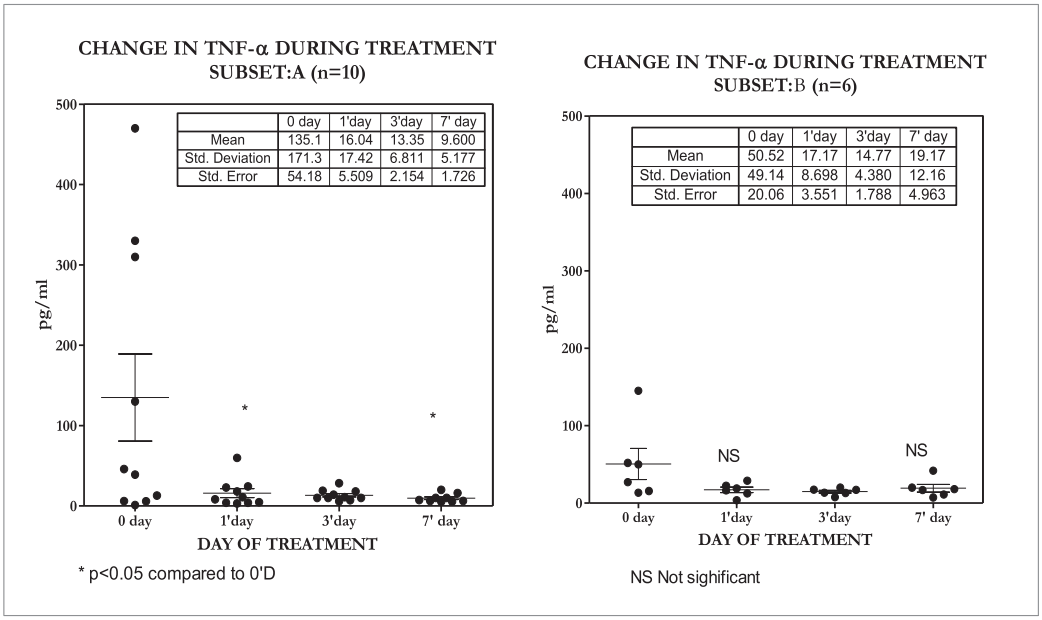

Fig. 8. Shows levels of TNF-α; an inflammatory cytokine in subset A and B during the treatment. Drop in the levels of TNF-α within 24 h was remarkable in all the patients, though statistical significance was seen in subset A.

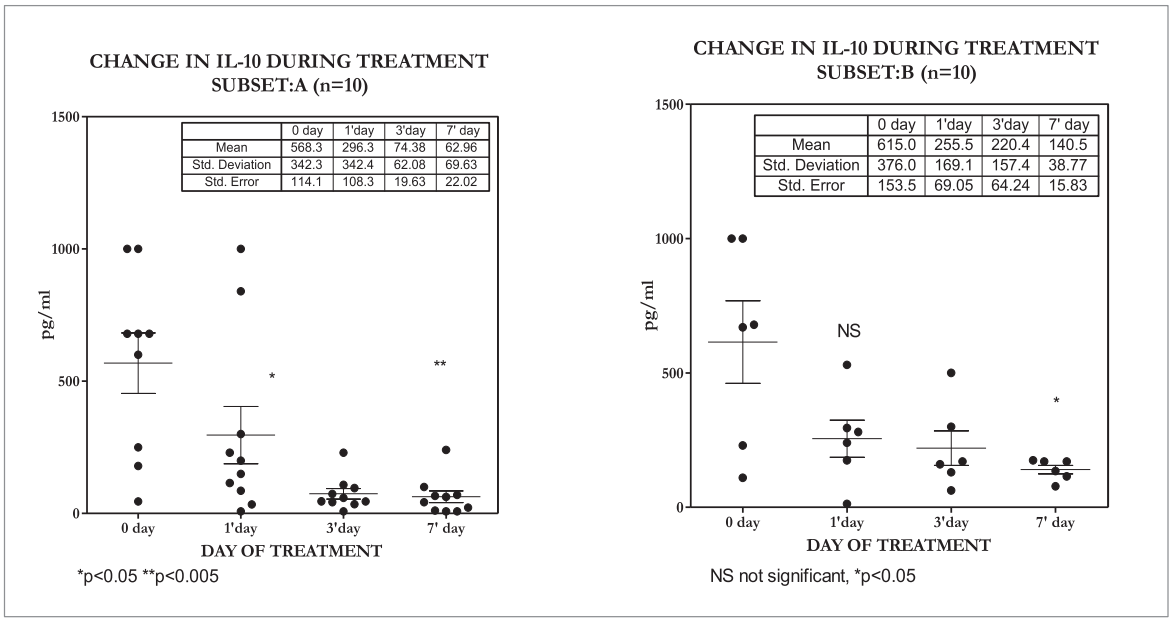

Fig. 9. Shows levels of IL-10, an anti-inflammatory cytokines in subset A and B during the treatment. Both the subsets showed decreasing trend, though the change was significant in subset A. It was seen that IL-10 drop was gradual as compared to TNF-α.

frequent monitoring of safety, apyrexia, parasite clearance and selected markers of the disease severity. The study confirms the earlier clinical hits, through a complete cure in 50% (10/20) patients (subset A). Remaining 10 patients (subset B) showed partial response with early clinical improvement before parasite clearance and gradual decrease in parasite count. Parasite clearance was initially monitored by slide and by RDT. PCR was used to confirm the parasite presence, type and clearance; first time in a clinical study of plant for malaria. The results of slide and PCR were well correlated; whereas occasionally RDT failed to give parasite type accurately. But there was no false + ve or negative either by slide or RDT when compared the data with PCR (data not shown).

It is important to note that both subsets showed similar drop in MMS, irrespective of the type of infection and the baseline parasitemia. Prakriti analysis of these patients too did not show any segregation in responders vis-à-vis non-responders (data not shown). The early clinical response in either subset further reflected in an improvement in the various markers of response. Low platelet count; a hallmark of malaria infection, showed increasing trend in both the subsets. Elevated lactic acid level in malaria is a contribution from RBCs, during excessive anaerobic glycolysis by parasite [27,28] and from the muscles during shivering. Increased anaerobic glycolysis (in muscles and RBCs) and impaired hepatic and renal lactate clearance aggravate hypoglycaemia and lactic acidosis in severe malaria [28]. In the present study only mild to moderately severe patients were taken; none of them had hypoglycaemia or lactic acidosis at the time of admission.

In this study all 8 patients having high baseline lactic acid, also had moderate (n = 3) to severe (n = 5) chills on 0'day. Reduction in lactic acid was associated with the relief from chills in all these patients by 7th day. Five out of these 8 patients also had high AST levels which dropped by 7 days. This suggests a high lactic acid and AST level was a contribution from the skeletal muscles during shivering. Six out of these 8 cases (with baseline high lactic acid) also had low platelet count and five of them showed increasing trend.

The safety and tolerability of *N. arbor-tristis* in all these patients was closely monitored clinically as well as with the laboratory markers of organ functions. None of them showed any adverse event except one patient who developed on the 7th day mild but self-limiting diarrhoea. As in the previous study [10,11], the paste was generally well accepted, despite its bitter taste and the unfamiliar paste nature of the formulation. The symptoms of nausea and a bitter taste in mouth were relieved. There were no hepato- or nephro-toxic effects as judged by the laboratory monitoring. On the contrary, 15 patients had abnormal levels of one or more markers of liver and kidney function which improved in all the patients by 7th day. The hepatoprotective activity of the dried leaves of *N. arbor-tristis* has been shown in rats with ethanol extract in carbon tetrachloride (CCl_4)-induced liver damage [29] and with the chloroform extract in streptozotocin (STZ)-induced diabetes [30]. This suggests the putative protection from the free radicals, generated by CCl_4 and STZ.

The striking feature of the present study was an early amelioration of the disease severity even before the complete parasite clearance. This was markedly evident in the delayed clinical responders; n = 10 (4 from subset A and 6 from subset B). This amelioration of disease severity was not markedly associated with the type of infection, degree of parasitemia and baseline MS. The comparative data in two subsets showed significant improvement in all the markers by seven days in subset A. However, the change in subset B was not significant. This may be due to the high baseline value in the patients of subset A for most of the markers compared to subset B, which showed marked improvement. However, in an observational study individual patient needs a meticulous analysis. Such analysis showed similar trend in these markers as to subset A. The study was conducted with a fixed dose regimen. There is a need of dose searching, finding and optimizing in subsequent studies for early parasite recovery in the delayed responders. There is also need to standardise the formulation with concentration of actives. Phytochemical analysis of plant has shown several compounds viz. D-mannitol, β-sitosterole, Astragalin, Nicotiflorin, Oleanolic acid, Nyctanthic acid, Nyctanthin, Tannic acid Ascorbic acid Methyl salicylate, Friedeline, Desrhamnosylverbascoside [31,32].

The marked improvement in clinical markers in the patients can be explained by their cytokine profile. In both the subsets, all the patients showed marked decrease in TNF-α levels within 24 h. of treatment. It was interesting to note that, once TNF-α reduced it did not raise to the baseline level even in the subsequent episodes of fever, which were milder with reduced severity of the symptoms. On

the contrary in both subsets IL-10 drop was gradual. Other pro-inflammatory cytokines viz. IL-6 and IFN-γ measured in these patients also showed significant drop (data not shown). Thus both types of cytokines maintained inflammatory and anti-inflammatory balance.

The role of pro-inflammatory cytokines in the pathogenesis and severity of malaria is well documented [33–38]. The development of symptoms similar to clinical malaria viz. fever, rigors, headache, myalgia, has been reported in humans with an intravenous infusion of the recombinant TNF-α [36]. Severe malaria, characterized by hypotension, thrombocytopenia, pulmonary oedema, was associated with high levels of TNF-α [37–39]. A burst of TNF-α release occurs after schizonts rupture from RBCs, with the induction of a cascade of immune reaction and inflammation. A marked decrease in TNF-α within 24 h could be one of the responsible factors for an early clinical relief. Immunomodulatory and anti-inflammatory activity of *N. arbor-tristis* earlier reported in *in vivo* and *in vitro* models at the Central Drug Research Institute [40–43], Industrial Toxicology Research Centre [44–46] and by others [47–49]. In the adjuvant arthritis mouse model, the aqueous fraction of the alcoholic extract (100 μg/kg) decreased TNF-α significantly [42–44]. Another study in mice showed inhibition of TNF-α secretion induced *by Staphylococcus aureus*-protein [42]. These experimental data support the present clinical anti-inflammatory findings. In our laboratory too we have observed a significant reduction of TNF-α secretion from LPS-activated macrophage by several fractions of the plant (unpublished data).

This immunomodulation may be reflected in the early disease-modifying activity of the plant seen in this study. The importance of disease-modifying agents to decrease morbidity in severe malaria was emphasized by a group of experts at Dakar [50]. It has also been suggested that a smaller number of residual parasites may even assist the development of the host immunity to malaria. Moreover, this disease-modifying pattern is not seen so commonly when patients are treated with the standard antimalarials; with CQ both apyrexia and parasite clearance are concomitant. Not withstanding a complete parasite clearance with CQ, several symptoms of illness viz. weakness, fatigue, nausea, loss of appetite etc. are often persistent. The extracts of *N. arbor-tristis* have shown antiplasmodial activity *in vitro* cultures (data not shown). This needs to be followed up with phytoactives.

In summary, meticulous monitoring of several clinical and biochemical markers helped proving activity of the plant affecting multiple targets of malarial pathogenesis. It was well tolerated and found to be safe. The striking observation of the study was early clinical improvement despite the presence of parasite; which is partially explained by the marked reduction in inflammatory cytokine – TNF-α. Dose searching – finding and optimizing even with the paste formulation might give the anticipated faster parasite clearance. The study needs to be further explored for the development of standardized formulation with optimised dose and also for the isolation of active compounds to reduce the time of treatment. In this study, markers of response for antimalarial activity were monitored intensively and frequently, which was possible in a smaller sample size. In future, attempts will be made, with a standardized formulation, for a large scale study for disease-modifying activity, parasite clearance and relapse. The history of antimalarial natural products is studied with examples of substantial delays from their traditional use to emergence as modern drugs e.g. quinine, and artemisinin. Such delays can be handled by well-organized and trans-disciplinary RP of natural products use in traditional medicine.

5. Conclusion

The study shows antimalarial potential of *N. arbor-tristis in patients*; confirmed by clinical and parasite markers of response. The paste formulation is well tolerated. The study shows a unique approach, through RP, for drug discovery from an Ayurvedic medicinal plant used for malaria in practice. Further in depth studies for the development of a standardized phytopharmaceutical are required.The plant also can be explored for the discovery of a phytoactive as well as scaffold-based new chemical entities.

Support/funding

Institutional Research Funds and ICMR Centre support.

Conflicts of interest

Nil.

Permissions

Nil.

Ethical committee approval

Yes.

Acknowledgement

(Dedicated to the memory of Vaidya D.S. Antarkar and Vaiyda Mayaram Sundarji.) We acknowledge Dr. A.B. Dharmadhikari, Dean, MA Podar Ayurvedic Hospital, Worli, for permitting the clinical work at the hospital. We are thankful to Dr. Nandu Lad, Chairman and Dr. Gita Gandhe, Pathologist of Sushrusha Hospital, Dadar, who kindly provided laboratory support for haematological study. We are grateful to the donors Dr. Indira K Mehta and Share And Care Foundation for the partial financial support for the study. We thank Sri. Kantibhai Shroff and Sri. Aswinbhai Shroff of Excel Industries, Mumbai for supporting travel to the Drexel University. We thank late Sri. Ramkrishananji Executive Secretary Bhartiya Bhavan and Dhirubhai Mehta President, Kasturba Health Society for their continuous encouragement and support. We are thankful to ICMR for supporting continued research on the plant at the ICMR Advanced Centre of RP in Traditional Medicine. We are also thankful to all the patients for their consent and participation in the study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaim.2016.08.003.

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