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In Vitro inhibition of plasmodium falciparum by substances isolated from antimalarial plants.

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***Keywords*— Neosergeolide, Aspidocarpine, 4-nerolidylcatechol, Jurinea dolomiaea, Alstonia scholaris and Datura stramonium.**

***Abstract*—** *In the present study, a neosergeolide, isolated from the roots of Jurinea dolomiaea Boiss (Asteracae), the indole alkaloid aspidocarpine, isolated from the bark of Alstonia scholaris (Apocynaceae) and 4-nerolidylcatechol, isolated from the seed of Datura stramonium Linn. (Solinaceae), all presented significant in vitro inhibition (more active than quinine and chloroquine) of the multi-drug resistant K1 strain of Plasmodium falciparum. Neosergeolide presented activity in the nanomolar range. These compounds are good for pre-clinical tests as novel lead structures with the aim of finding new antimalarial prototypes and provide support to the traditional use of the plants.*

I. INTRODUCTION

Malaria is the main cause of economic loss and high morbidity in the world today. The lack of an effective vaccine and the increasing expansion of strains of Plasmodium falciparum presenting resistance towards commonly used, low-cost antimalarials make control of this disease difficult [1]. As a result, the World Health Organization (WHO 1978, 1995) has been promoting research on natural product based drugs for treatment of disease and many plant species have been evaluated for antimalarial activity [2,3]. In these studies, emphasis has been on the discovery of lead compounds for drug development. The search for active substances in medicinal plants is a very promising and cost-effective discovery for antimalarial drug. This approach benefits from the limited knowledge of the curing capacity of plant inhabitants of malaria in endemic regions and permits the extensive evaluation of natural products [4, 5].

The medicinal knowledge of these traditional plants is useful and effective and is an extremely important source of therapeutic compounds in use today. Important semi-synthetic, low-cost, highly effective antimalarial drugs such as the quinolines (chloroquine, mefloquine, primaquine, etc.) and artemisinin derivatives (sodium artesunate, arteether, artemether, etc.) owe their initial discovery to the isolation and structural identification of antimalarial natural products (quinine and artemisinin, respectively) from traditionally used antimalarial plant species. Recent studies on traditionally used antimalarial remedies have revealed that the plants which produce indole and isoquinoline alkaloids, sesqui-, di- and triterpenes, flavonoids and other substances presenting proven in vitro activity against P. falciparum [6, 7, 8, 9].

Research on new antimalarials from natural products involves coordinated scientific effort on the part of different professionals. These professionals generally represent distinct academic disciplines, most importantly, botany, natural product, and synthetic chemistry, pharmacology, parasitology, and molecular biology. Groups with these characteristics can in the short and long run produce sound knowledge of the chemical, pharmacological, and biological diversity. Based on scientifically sound facts, the most promising agents for

further clinical and industrial development can be identified.

In this work, the pharmacological potential of several substances isolated from traditionally used antimalarial plants was evaluated through screening for in vitro inhibition of human malaria parasite species P. falciparum. The ultimate goal of this work is to identify new classes of antimalarial substance which may serve as prototypes for the development of drug having novel mechanisms of action.

II. MATERIALS AND METHODS

Plant material extraction and chemical constituent isolation

The plants from which the substances under study were isolated and are traditionally used for the treatment of malaria are the roots of Jurinea dolomiaea Boiss (Asteracae), bark of Alstonia scholaris (Apocynaceae) and the seed of Datura stramonium Linn. (Solinaceae). All plant materials were collected in the state of Jammu and Kashmir. Plant materials were collected from Himalayan region of upper Danchigam in south Kashmir about 38- 45 meters above the sea level. The plants were identified taxonomically and authenticated at the Herbarium, Department of Botany, Kashmir University. Plants were washed thoroughly 2 - 3 times with running tap water and then with sterile water followed by shade-dried, powdered and used for extraction. Structural elucidation of isolated compounds was performed by analysis of 1-D / 2-D NMR, mass, infrared and ultraviolet spectral data and comparison to spectral data available in the literature.

Isolation of neosergeolide (1) from J.dolomiaea

Roots and stems (6.5 kg) were degreased with hexanes in a soxhlet apparatus then repeatedly extracted with water using the same equipment. Continuous liquid-liquid extraction of the resulting concentrated H2O extract with CHCl3 was then performed. The procedure was essentially described by [10] for the isolation of other quassinoids. We developed a method which obviates the need for a chromatography step. The concentrated chloroform extracts (35.1 g) were dissolved in a minimum of hot water and acetone (2:1). The resultant precipitate was fractionally recrystallized to give pure neosergeolide (1) (685.4 mg, 0.011% based on dry weight of plant).

Isolation of aspidocarpine (2) from Alstonia scholaris (Apocynaceae)

Isolation and purification of 2, was essentially the same as described above for 1, wherein bark (1.2 kg) yielded ethanol extract (35 g) which was partitioned and yielded an alkaloid rich pH 8 fraction (1.45 g). Sequential normal-phase chromatography on a portion (1.40 g) of this fraction yielded aspidocarpine (68.5 mg, 0.0057% based on dry weight of plant).

Isolation of 4-nerolidylcatechol (3) from Datura stramonium

Roots (150 g) were extracted with a 1:1 mixture of CHCl3/EtOH (3 × 150 ml; 15 min each) in an ultrasound bath. After total evaporation, the extract (19.5 g; 13%) was chromatographed on silica gel using a 9:1 mixture of CHCl3/EtOH 9:1 which yielded pure 1 (8.6 g, 44.1% w /w based on extract, 5.7% based on dry weight of plant.

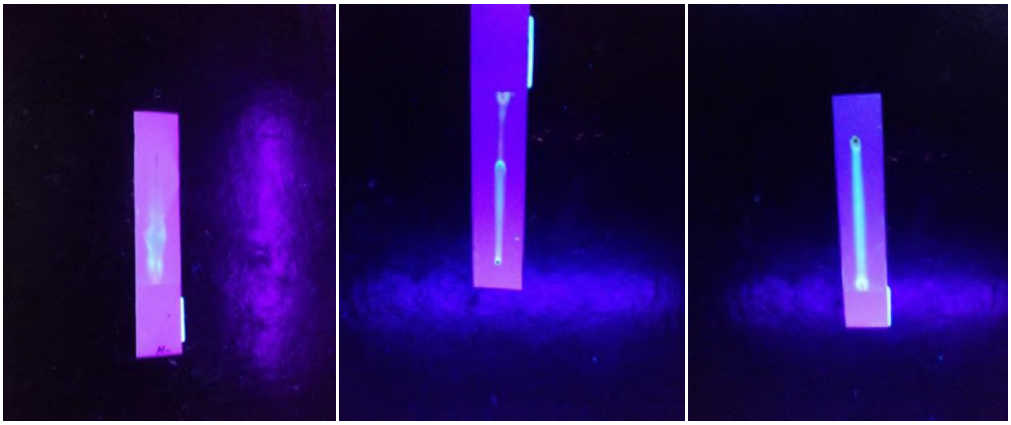


Fig.1 TLC plates of crude extracts of J.dolomiaea, Alstonia scholaris and Datura stramonium

Parasite culture and in vitro antimalarial tests

Chloroquine, pyrimethamine, and cycloguanil resistant P. falciparum strain K1 was acquired from MR4 (Malaria Research and Reference Reagent Resource Center, New Delhi, India) and was used in the in vitro tests. Parasites were maintained in continuous culture in A+ human erythrocytes, using RPMI medium supplemented with 10% human serum, as described by [11]. The antiparasitic effect of the compounds was measured by growth inhibition

percentage as described by [12]. Briefly, trophozoitestages in sorbitol-synchronized blood [13] were cultured at 1-2% parasitaemia and 2.5% hematocrit and then incubated with the plant extracts or isolated compounds (maximum 1 mg/ml in serial dilutions), diluted with 0.02% final concentration of DMSO in culture medium (RPMI 1640) for a total of 48 h at 37ºC. A positive control with reference antimalarial drug (chloroquine and quinine) in standard concentrations [14] was used in each experiment. The stock solutions were further diluted in complete medium (RPMI 1640 plus 10% human serum) to each of the used concentrations (0.0001 up to 100 µg/ml in seven dilutions). The half-maximal inhibitory (IC50) responses as compared to the drug-free controls were estimated by interpolation. Each duplicate experiment was repeated three times and blood smears were read blind.

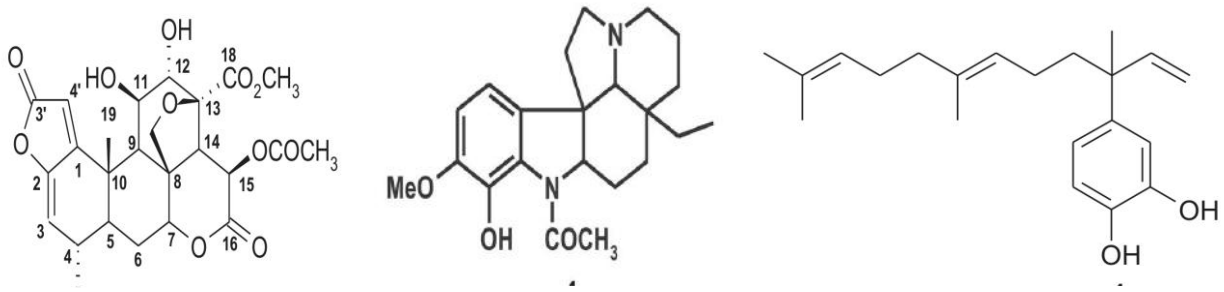
Statistical analysis - The data of in vitro antimalarial tests were analyzed with the Biostat 1.0 MCT-CNPq software package using Anova and Students t-test.

III. RESULTS

The results of the in vitro tests with compounds obtained from plant extracts against multidrug-resistant P. falciparum K1 strain are presented in the Table1. The IC50 of compounds ranged from 2.0 nM to 0.67 µM. Neosergeolide (1), a known quassinoid which has previously been isolated from Jurinea dolomiaea but for which no data on antimalarial activity has been previously reported, showed significantly higher activity (IC50 = 2.0 nM) than did the other compounds tested. Fig. 1 illustrates the dose-response curve for this quassinoid showing a tendency of standard curve; this analysis was performed for all tested compounds. Aspidocarpine are known indole alkaloids for which antimalarial activity has not apparently been previously described. A significant inhibition of parasite growth (IC50 = 73nM). 4-Nerolidylcatechol, a metabolite found in Datura Stramonium for which no data is available as to antimalarial activity, presented good parasite inhibition (IC50 = 0.67 µM). The in vitro sensitivity of the P. falciparum strain to the compounds tested was similar and reproducible in assays in duplicate on separate occasions.

Table1. The half-maximal inhibitory concentrations (IC50) of isolated substances from Jurinea dolomiaea, Alstonia scholaris and Datura Stramonium towards Plasmodium falciparum (K1 strain). a: mean values in representative assay.

Compound Name	Structural class	Plant species/source	Mean IC50 values[a]	
			µg/ml	µM
Neosergeolide	Quassionoid/terpenoid	Jurinea dolomiaea	0.001	0.002
Aspidocarpine	Indole alkaloid	Alstonia scholaris	0.007	0.019
4-Nerolidylcatechol	Phenylpropanoid/terpenoid	Datura Stramonium	0.21	0.67
Chloroquine diphosphate salt	Quinoline	Synthetic commercial standard	0.46	0.89
Quinine salt	Quinoline alkaloid	Natural commercial standard	0.004	0.012



(1) (2) (3)

Fig.2 Chemical structure of antimalarial isolated compounds Neosergeolide, Aspidocarpine and 4-Nerolidylcatechol.

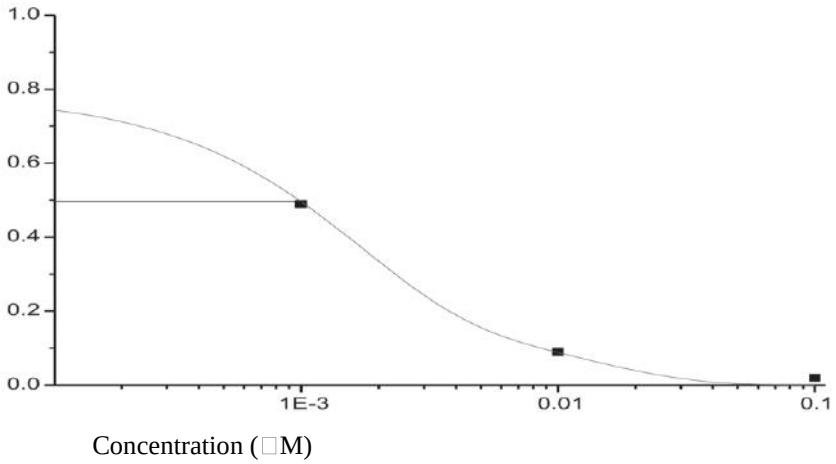


Fig. 3: Illustrative dose-response curve for multi-drug resistant Plasmodium falciparum K1 strain in the presence of different concentrations of the quassinoid neosergeolide (1) tested with IC50 value in representative experiment; confidence interval (95% CI) = 0.00 – 0.01. Statistical analysis among additional assays showed: standard error = 0.0007; p = 0.10.

IV. DISCUSSION

Plants of the family Asteracae are widely used in traditional medicine for the treatment of malaria, cancer, dysentery, and other diseases in countries around the world [15]. Quassinoids are a group of degraded triterpenes found in the family Asteracae, that show many biological activities such as antitumor [16], antifeedant [17], phytotoxic [18], antiviral [19] and antihelmintic [20]. The antimalarial activity of some quassinoids like brusatol, glaucarubinone and quassin has been demonstrated previously [21].

An ethnopharmacological study in French Guyana showed that Alstonia schlaris root, stem and bark alcohol extract is used in local traditional medicine as a curative treatment of malaria. In a subsequent study [22] demonstrated that A. schlaris water extract can inhibit hemozoin formation. In vitro assays demonstrated the antimalarial activity of this extract against the chloroquine resistant P. falciparum strain W2. Sergeolide and isobrucein B are quassinoids which have been isolated previously from A.schlaris and exhibit high antiplasmodial activity against chloroquine-sensitive FUP strain, sergeolide exhibited an IC50 which was five times less than that of isobrucein B and three times less than that of chloroquine in the same strain. In vivo assays demonstrated that sergeolide was capable of inhibiting P. berghei strain NK65 with an ED50 of 0.2 mg/kg/day, five times less than chloroquine [23].

Our data show that the quassinoid isolated from the roots and stems of the A.schlaris was more active than quinine and chloroquine; with activities in the micromolar ranges comparable to recently reported results. Several quassinoids are known to inhibit the growth of P. falciparum in culture at nanomolar concentrations [24]. The quassinoids orinocinolide and simalikalactone D, isolated from the root bark of Simaba orinocensis were found to be potent in vitro against P. falciparum clones D6 and W2 [25]. Research has also revealed quassinoids which are 4 and 12 times more active in vivo (via oral) against rodent malaria parasite [26] than chloroquine and artemisinin, respectively [27]. Despite these antimalarial activities, quassinoids usually present toxicity due principally to protein synthesis inhibition and it is likely that parasite and host cell ribosomes are too similar to allow for the development of selective inhibitors [28]. Some structural requirements, like and α, β-unsaturated ketone in the A ring, an epoxymethylene bridge in the C ring and an ester function in C-15 are considered very important for the antimalarial activity presented by quassinoids [29, 30].

Datura stramonium plant produce the secondary metabolite of mixed terpene and phenylpropanoid biosynthetic origin, 4-nerolidylca-techol, which was evaluated in the present study. Qualitative tests have shown the presence of this compound in the seed, and leaf. Reductions in parasitemia of 66, 55, and 28% were observed for the D. stramonium [31] evaluated seed ethanol extracts of the same species by subcutaneous and oral administration and found in the seed extract significantly reduced blood parasite levels at different doses. On the other hand, [32] evaluated D.stramonium seed hexane and methanol extracts in vivo through oral and subcutaneous administration in P. berghei infected mice and found that these extracts were inactive against blood forms of P. berghei. These results lead [33] to conclude that the oral or subcutaneous administration of plant extracts in Plasmodium berghei infected rats was not effective at detecting the antimalarial activity of these plants [34]. Tested the in vivo and in vitro antimalarial

activity of D. stramonium seed and leaf water extract using a new method. Briefly, this method involved oral administration in adult rats via gavage tube (6 x 6 ml) for 2 days. After this period of treatment, the rats were bled and blood sera were tested in vitro in microcultures of P. falciparum using tritium-labeled hypoxanthine incorporation for parasite quantification. In vitro P. falciparum inhibition (49%) was observed for serum obtained from rats inoculated with A. schlaris peltata water extract versus controls. From the results of this and other experiments, differences in P. berghei and P. falciparum blood-stage biology might be thought to be responsible for the lack of in vivo activity observed [35]. Also evaluated D.stramonium seed hexane and methanol extracts in vitro in human malaria parasite species P. falciparum. The methanol extract presented greater inhibition of P. falciparum growth than the hexane extracts.

In more recent studies [36] observed the in vitro antiplasmodial activity of A.schlaris bark ethanol extract (IC50 3.7 µg/ml) in chloroquine and pyrimethamine resistant P. falciparum. We obtained a similar result for the alcohol root extract of J.dolomiaea in vitro in the K1 strain of P. falciparum. In preliminary work, 4-nerolidylcatechol (3) was shown to be active against P. falciparum in vitro [37].

The screening of natural products provides the chance to discover new molecules of unique structure with high activity and selectivity which can be further optimized by semi- or fully synthetic procedures [38].

Alkaloids are one of the most fascinating classes of natural products, providing many drugs for human use [39, 40]. In general, indole alkaloids are a class of compound having a range of biological activities, including antibacterial, trypanocidal, leishmanicidal and anticancer [41, 42, 43, 44, 45]. The antiplasmodial activity of monoterpene indole alkaloids has been investigated [46]. Promising results have been obtained previously by others for aspidospermidine structural analogues isolated from A. pyrifoluim and A. megalocarpon to Nigerian chloroquine-sensitive and a Camaroon chloroquine-resistant (FcM2) strain of P. falciparum. In the chloroquine-resistant strain, apidospermine, 10-methoxyaspidospermidina and N-formylaspidospermidine presented, after 24 h, IC50 of 16.3, 19.5 and, 16.1 µM, respectively, whereas after 72 h, IC50 were 3.8, 3.2, and 5.6 µM, respectively. In the chloroquine-sensitive strain, after 24 h, IC50 were 11.0, 13.1, and 22.0 µM, respectively, and after 72 h, 4.6, 5.1, and 5.9 µM, respectively [47]. Here, the isolated monoterpene indole alkaloid aspidocarpine was more active. The activities against K1 strain were of the same order as those observed for the terpeneoid phenylpropanoid compound 4-nerolidylcatechol.

Those compounds or chemical groups have already shown potential as new drug leads or may have an impact on future drugs. Further studies should explore these compounds as a prototype for an antimalarial aimed at the P. falciparum mult-resistant parasites.

Adaptation of the protocol cited above to highthroughput platforms, as well as implementation of modern indirect methods for the quantification of in vitro parasite growth, such as fluorimetry [48] are underway and will be essential for an increase in the scale and dynamism of studies on antimalarial plants, isolated natural substances and their semi-synthetic derivatives, potentializing a process of continuous screening in the near future.

Additionally, stabilization of geographically specific P. falciparum populations in continuous in vitro culture is underway and should permit investigations into the real susceptibility profile of these regional parasites to the active substances and plant extracts which present promising inhibitory concentrations. It is our hope that knowledge of this regional profile can be useful for the identification, based on sound experimental evidence, of the most important and effective medicinal plants for development of new and effective antimalarials for local use. Furthermore, simultaneous studies on the macromolecular profiles of these parasites in association with analysis of genetic resistance markers [49] should contribute to the elucidation of possible mechanisms of resistance of the parasites to the natural products tested as well as aid in the discovery of new targets (and/or new mechanisms of action) for antimalarial chemotherapy.

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