

## SEARCH FOR NEW ANTILEISHMANIAL CHEMOTHERAPEUTICS

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### ABSTRACT

**Objective:** The objective of this work was to screen a number of compounds for their antileishmanial efficacy and cytotoxicity profiling.

**Methods:** Curry leaf oil, cypress oil and spikenard oil were identified by gas chromatography-mass spectrometry (GC/MS) analysis. Betulinic acid, spikenard oil, cypress oil and curry leaf oil were evaluated for their *in vitro* antileishmanial activity against *Leishmania donovani* AG83 wild-type, sodium stibogluconate resistant (SSG-resistant), paromomycin (PMM-resistant) and GE1 field type strains on axenic and cellular amastigote model and compared the results with standard drugs used to treat leishmaniasis.

**Results:** Betulinic acid showed strong antileishmanial activity against wild-type (SI= 192.8), SSG-resistant (SI= 19.3) and GE1 strains (SI= 100), whereas cypress oil has produced highest antileishmanial activity against PMM-resistant strains (SI= 15.09) among all the tested drugs. The data obtained also revealed that cypress oil had the maximum CC<sub>50</sub> value of 452.9  $\mu$ l among all standard and tested drugs.

**Conclusion:** All tested drugs had antileishmanial property but among them, betulinic acid possess strong antileishmanial activity in case of both wild-type and drug-resistant leishmaniasis.

**Keywords:** Betulinic acid, Spikenard oil, Cypress oil, Curry leaf oil, GC/MS, *In vitro* antileishmanial activity

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### INTRODUCTION

Leishmaniasis is a vector-borne parasitic disease caused by a protozoan parasite of the genus *Leishmania* [1]. The parasite is generally transmitted to human beings by the bite of a previously infected phlebotomine sandfly. The parasite of the disease occurs in two distinct forms: the flagellated, extracellular promastigotes that resides in the gut of female sandfly vector; and the nonflagellated, nonmotile amastigote form that exists and multiplies within the phagolysosomal compartment of macrophages [2, 3]. Depending on the causative species involved, human leishmaniasis may manifest in various forms that include cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), diffused cutaneous leishmaniasis (DCL), and visceral leishmaniasis (VL), of which visceral leishmaniasis is the most lethal form of disease caused by the species of *Leishmania donovani* [4].

Pentavalent antimonials such as meglumine antimoniate (Glucantime, Sanofi-Aventis) and sodium stibogluconate (Pentostan, GlaxoSmithKline) are variably effective against both VL and CL and may be administered via intravenous (IV), intramuscular (IM) or intralymphatic (IL) route. Unfortunately, the increased risk of cardiotoxicity, nephrotoxicity and widespread antimonial resistance has limited their use [5]. Recently, four new potential therapies are developed for the treatment of VL, such as an amphotericin B liposomal formulation (AmBisome), oral miltefosine, a parenteral formulation of aminosidine (paromomycin), and oral sitamaquine. However, individual users of these drugs have several drawbacks [6]. According to recent clinical studies, combination therapies possess potential benefits against VL in India, in which short-course multidrug treatment is compared with standard therapy [7]. Nevertheless, inadequate mode of administration of current therapies, resistance and cost-related issues have prevented their widespread use. Therefore, it is imperative to develop new antileishmanial compounds with reduced side effects and toxicity. The terpenoids also referred to as terpenes obtained from the plants might be an alternative source of potent new molecules for the treatment of several critical diseases since they are a rich source of therapeutically active constituents [8, 9].

The genus *Cupressus* (commonly known as Cypress), belonging to the family Cupressaceae [10] is traditionally known to possess several beneficial activities for the treatment of stomach ache, toothache, diabetes, inflammation, laryngitis and as astringent and contraceptive [11, 12]. *Murraya koenigii*, commonly known as 'Curry patta' in India, belongs to the member of family Rutaceae. Traditionally, the plant has been found to exhibit potent antioxidant [13], antimicrobial [14], hypoglycemic [15], antidiarrhoeal [16], hepatoprotective [17], melanogenesis inhibitory [18], antiobesity and lipid-lowering [19] activities. Spikenard, also known as 'Muskroot' is a class of aromatic amber-coloured essential oil obtained from the dried roots and rhizomes of a flowering plant *Nardostachys jatamansi*, belonging to the Valerian family [20]. Traditionally, the roots of jatamansi exhibited antimicrobial [21], antioxidant and anticholinesterase activity [22], anxiolytic [23], neuroprotective [24], anticancer [25], anti-diabetic [26] and anti-androgenic [27] activity. Betulinic acid (3- $\beta$ -hydroxy-lup-20(29)-en-28-oic acid) is a pentacyclic lupane-type triterpene obtained from various plants such as *Tryphillum peltatum*, *Ancistrocladus heyneanus*, *Diospyros leucomelas*, *Tetracera boliviana*, *Zizyphus joazeiro*, *Syzygium formosanum*, etc. [28]. It exhibits a wide range of biological activities which includes antibacterial [29], antiprotozoal [30], antiviral [31], anticancer [32], anti-inflammatory and immunomodulatory [33], and anti-HIV [34] activity.

Based on the significant biological activities and medicinal properties of several bioactive terpenoids, our present study was aimed to investigate the *in vitro* antileishmanial properties and toxicity profile of few selected terpenoids, which include cypress oil, spikenard oil, curry leaf oil, betulinic acid and to compare their activity against some conventional antileishmanial agents like amphotericin B, sodium stibogluconate (SSG), paromomycin (PMM) and miltefosine.

### MATERIALS AND METHODS

#### Chemicals and reagents

Curry leaf oil (CAS no. 8008-52-4) was procured from Mother Herbs Pvt. Ltd. (India), Spikenard oil (CAS no. 8022-22-8) was procured

from Aromatic and Allied Chemicals (India), Cypress oil (CAS no. 8013-86-3) and betulinic acid (CAS no. 472-15-1) were purchased from Sigma-Aldrich (India). Sodium stibogluconate was a generous gift from Albert David Ltd. (Kolkata, India). Amphotericin B (CAS no. 1397-89-3), paromomycin (CAS no. 1263-89-4) and miltefosine (CAS no. 58066-85-6) were procured from Sigma-Aldrich (India). All solvents used in the experiment were of analytical grade.

## Methods

### Ethics statement

BALB/c mice (20-25 g) of either sex were used to carry out the experiments. Jadavpur University Animal Ethics Committee (AEC/PHARM/1403/2014) has approved the experimental protocols. The procedures were followed in accordance to the committee's guidelines. Animals were kept in polypropylene cages and fed with standard diet and water ad libitum. Animals were exposed to a normal day and night cycle.

### Identification of oils by gas chromatography-mass spectrometry (GC/MS)

Curry leaf oil, cypress oil and spikenard oil were evaluated for the identification of chemical constituents present in them by GC/MS analysis [35]. GC/MS (Thermo Fisher Scientific India Pvt. Ltd.; model: POLARISQ) analysis was performed using ion-trap technology having electron ionization detector with the energy of 70 eV. Helium (He) was used as a carrier gas. Data was acquired by xcalibur software.

### Parasite culture

VL isolated *Leishmania donovani* AG83 (MHOM/IN/83/AG83) was acquired as a gift from Indian Institute of Chemical Biology, Council of Scientific and Industrial Research, Kolkata, India. According to Roy et al. [36], the parasite, *Leishmania donovani* AG83 promastigotes were allowed to grow in the medium 199 supplemented with 10% heat-inactivated fetal bovine serum, 20 mmol HEPES, 2 mmol L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at room temperature.

The axenic amastigotes were derived from promastigotes in MMA/20 (Medium for axenic amastigote), pH 5.5 culture medium. Developed axenic amastigotes were maintained by sub-culturing of  $10^5$  parasites/ml in every five days under 5% CO<sub>2</sub> incubator at 37 °C.

### Development of drug-resistant amastigote strains

According to the Das et al. [37], drug-resistant strains of *L. donovani* amastigotes were developed. Briefly, in the presence of drug concentrations respective of their IC<sub>50</sub> values of the strain, the promastigote cells were cultured in medium 199 with required supplements. The drug concentrations were increased after three subcultures. The concentrations were increased in such a manner that the population of the promastigote cells was decreased by 20% for each batch. At the time point when 10% promastigote cells were survived, the strains were cultured on medium 199 agar plates in the presence of a similar concentration of drugs. The generated strains were then cultured in medium 199 liquid media. After that drug pressure was removed slowly and stability was checked at different time points.

### Drug sensitivity study

Drug sensitivity to axenic amastigote study was done by determining the 50% inhibitory concentration (IC<sub>50</sub>) of selected drugs. So, the IC<sub>50</sub> values suggest that it was the concentration where 50% cells of the total population were inhibited. RI is an index which can be calculated as IC<sub>50</sub> of a drug for a particular strain/IC<sub>50</sub> of that drug for the wild-type strain. To perform the study, a pre-counted amastigote cells ( $1 \times 10^5$  amastigotes/ml) of wild-type, drug-resistant and field isolates were seeded at 96 culture plate. Different concentrations of drugs were added to the respective wells and the well plate was kept in the 5% CO<sub>2</sub> incubator for 72 h. After that cell counts were taken using

hemocytometer under an inverted microscope. Numbers of amastigotes of untreated controls were compared with the drug treated wells to determine the IC<sub>50</sub> values [38].

### Drug sensitivity assay of axenic amastigote in macrophages

Mice peritoneal macrophages were isolated by the similar technique adopted in our previous work [39]. For this study, sterile coverslips were kept in each well of the culture plate. 0.5 ml of RPMI-1640 media having isolated macrophage cells at a concentration of  $4 \times 10^5$  cells/ml were added to each well.

Then 0.5 ml of medium containing axenic amastigotes were added to the wells so that the ratio between the macrophage to amastigotes should be 1:10 and kept into the 5% CO<sub>2</sub> incubator at 37 °C for 4h. After the internalization period, the old media was replaced with fresh RPMI-1640 medium with or without drug at the predetermined concentrations in triplicate wells. Then well plates were kept into 5% CO<sub>2</sub> incubator at 37 °C for 72 h. After 72 h, the medium was decanted and the coverslips were removed, fixed on the glass slides and air dried. Before counting the amastigotes in 100 macrophage cells, Giemsa staining was done. Results were calculated by comparing the number of amastigotes in 100 macrophage cells in treated and untreated control.

### Cytotoxicity assay and selectivity index

Cytotoxicity of macrophage cells to the drugs were represented as CC<sub>50</sub> values. CC<sub>50</sub> is the concentration in which 50% of the macrophage cells were killed or damaged in respect of the total population of macrophage cells. The experiment was carried out in 96-well culture plates in presence of RPMI-1640 medium with supplements. The wells were seeded and predetermined concentrations of drugs were given to the respective wells and the viable macrophages were counted under an inverted microscope using hemocytometer.

The antileishmanial potency of the drugs was evaluated by the selectivity index (SI). The parameter SI is considered as the highest exposure of the drug that will not result in any toxicity to that particular exposure and produces the preferred efficacy [40]. The selectivity indexes of the drugs are determined as CC<sub>50</sub> of the drugs in macrophages/IC<sub>50</sub> of the drugs against cellular amastigotes. It is assumed that drug would have the promising activity when the SI value is  $\geq 10$  [41].

### Statistical analysis

Experimental data were expressed as mean  $\pm$  standard deviation. Student t-test was used to calculate the statistical significances. Differences were considered to be significant when  $p < 0.01$  and  $p > 0.05$  was considered to be statistically not significant.

## RESULTS

Curry leaf oil, cypress oil and spikenard oil were evaluated for the identification of the potential components by GC/MS analysis. Table 1 revealed the identified components. From fig. 1A it was found that the curry leaf oil possessed 21 numbers of components present in it where 5 components were chemically identified.

They were linalool,  $\beta$ -ocemene, Allo-ocemene,  $\alpha$ -terpene and geranyl acetate. From fig. 1B it was observed that cypress oil possessed 25 components where 3 components were chemically identified which were  $\alpha$ -terpinyl acetate, D-3-carene and  $\alpha$ -carene and from fig. 1C it was found that spikenard oil possessed 32 components where 5 components were chemically identified and they were aristolene,  $\beta$ -patchoulene, D-cadinene,  $\alpha$ -cadinol and  $\beta$ -gurjunene.

The resistance selection process by increasing the drug pressure in a stepwise manner for AG83-resistant phenotype was found to be restrictive at 35 mg Sb(V)/ml for SSG and 164 mmol for PMM when 10% viable cells among the total cell population was observed in comparison to untreated controls.

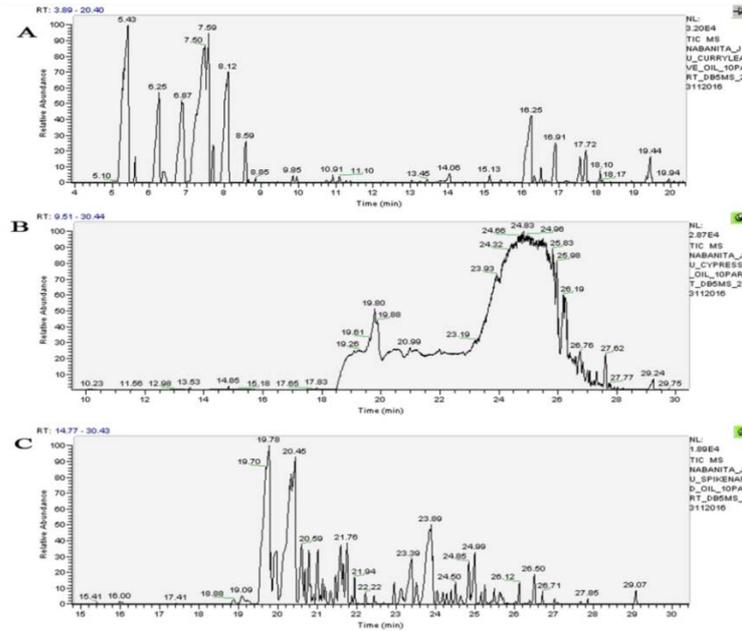


Fig. 1: GC/MS analyses of (A) curry leaf oil, (B) cypress oil and (C) spikenard oil. RT= retention time

Table 1: Identification of compounds from curry leaf oil, cypress oil and spikenard oil by GC/MS analysis

Curry leaf oil			Cypress oil			Spikenard oil		
Competents	RT* (min)	% peak area	Competents	RT* (min)	% peak area	Competents	RT* (min)	% peak area
Linalool	5.46	21.16	α-terpinyl acetate	24.02	1.94	Aristolene	19.85	22.76
β-ocemene	5.63	0.61	D-3-carene	24.85	21.35	β-patchoulene	20.01	4.14
Allo-ocemene	6.46	0.78	α-carene	25.92	6.12	D-cadinene	20.46	24.53
α-terpene	6.95	8.82	-	-	-	α-cadinol	20.65	3.37
Geranyl acetate	7.61	32.55	-	-	-	β-gurjunene	26.14	0.54

\*RT= Retention time

When the adequate growth of the resistant strain was achieved, promastigotes were transformed to amastigotes and the IC<sub>50</sub> values of parent and the resistant-strain were compared to reveal the significant differences among them. Table 2 showed that SSG was resistant at about 35 to 40 fold for AG83-resistant strain, whereas, it showed about 27 to 31 fold for PMM resistance.

In case of field isolate GE1 strain, it showed 3.52 fold SSG resistance and 2.25 fold PMM resistances. Table 4 revealed interesting results, where the test drugs spikenard oil and curry leaf oil showed minimal and similar resistance index for both the resistant strains. However, betulinic acid showed higher resistance against SSG resistant strain (RI= 10.6) and PMM resistant strains (RI= 23.9).

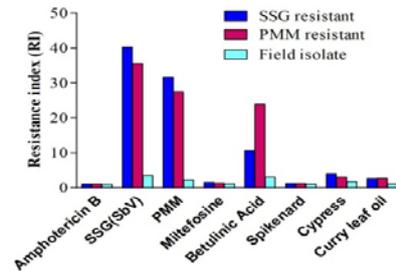


Fig. 2: Comparative study of resistance index (RI) of amphotericin B, sodium stibogluconate (SSG), paromomycin (PMM), miltefosine, betulinic acid, spikenard, cypress, curry leaf oil

Table 2: Drug sensitivity profiles against *L. donovani* wild-type, drug-resistant and field isolate axenic amastigote cell lines

Drug	IC <sub>50</sub> (mean±SD, n=4) μM <sup>a</sup>						
	Axenic AG83 evaluation model				Axenic GE1 evaluation model		
	Wild-type	SSG resistant <sup>b</sup>	RI <sup>c</sup>	PMM resistant <sup>b</sup>	RI <sup>c</sup>	Field isolate	RI <sup>d</sup>
Amphotericin B	0.27±0.02*	0.29±0.02*	1.07	0.30±0.03*	1.11	0.25±0.03*	0.92
SSG(SbV) <sup>e</sup>	3.4±0.37	137±15	40.29	121±13.3	35.58	12±1.6	3.52
PMM	12±1.56*	379±49.2*	31.58	329±39.5*	27.41	27±3.24*	2.25
Miltefosine	0.42±0.06*	0.64±0.09*	1.52	0.54±0.07*	1.3	0.50±0.07*	1.2

Data represented as mean±SD, n=4; the presented results are the average of four (4) determinations, IC<sub>50</sub>= 50% inhibitory concentration, <sup>a</sup>Assays are described in Materials and Methods, <sup>b</sup>SSG and PMM resistant strains were sodium stibogluconate resistant strains and paromomycin resistant strains, respectively, generated *in vitro* as given in Materials and Methods section, <sup>c</sup>RI, Resistance index was IC<sub>50</sub> of AG83 phenotype generated at maximum drug pressure/IC<sub>50</sub> of wild type, <sup>d</sup>RI, Resistance index was IC<sub>50</sub> of field isolate/IC<sub>50</sub> of wild type, <sup>e</sup>Values for antimonial agent was in μg Sb/ml, \*p<0.001, significant difference compared with SSG, \*\*p>0.01, no significant difference compared with SSG.

**Table 3: Drug sensitivity profiles of intracellular amastigotes against wild-type, drug-resistant and field isolate *L. donovani* cell lines**

Drug	IC <sub>50</sub> (mean±SD, n=4) µM <sup>a</sup>							Cytotoxicity CC <sub>50</sub> (µM) (Macrophage cells)	
	Cellular AG83 evaluation model					Cellular GE1 evaluation model		Field isolate	SI <sup>c</sup>
	Wild-type	SI <sup>c</sup>	SSG resistant <sup>b</sup>	SI <sup>c</sup>	PMM resistant <sup>b</sup>	SI <sup>a</sup>			
Amphotericin B	0.18±0.02*	88.88	0.23±0.03*	69.56	0.24±0.03*	66.66	0.19±0.02*	84.21	16±2.1*
SSG	2.1±0.27	13.8	20.2±2.02	1.4	18.3±2.19	1.58	8.7±1.22	3.33	29±4.35
PMM	8.4±1.1*	28.9	128.9±18.04*	1.88	117±11.7*	2.07	20.0±2.4*	12.15	243±29.76*
Miltefosine	0.36±0.05*	96	0.60±0.09*	57.5	0.46±0.05*	75	0.39±0.04*	88.5	34.5±4.48**

Data represented as mean±SD, n=4; the presented results are the average of four (4) determinations, IC<sub>50</sub>= 50% inhibitory concentration, <sup>a</sup>Assays are described in Materials and Methods, <sup>b</sup>SSG and PMM resistant strains were sodium stibogluconate resistant strains and paromomycin resistant strains, respectively, generated *in vitro* as given in Materials and Methods section, <sup>c</sup>Selectivity index SI was calculated by dividing the CC<sub>50</sub> by IC<sub>50</sub>, <sup>d</sup>Values for antimonial agent was in µg Sb/ml, \*p<0.005, significant difference compared with SSG, \*\*p>0.05, no significant difference compared with SSG.

In process of drug development or drug discovery for any particular indication, the more crucial part is the balance between safety and efficacy of the drug candidate. The window between the safety and efficacy should be adequate enough to use them as a drug. Here comes the term selectivity index (SI). SI is an index which demonstrates the highest exposure to drug which should provide

the highest efficacy with no toxicity. SI is calculated as CC<sub>50</sub> of a drug/IC<sub>50</sub> of that drug. 50% Cellular Cytotoxicity (CC<sub>50</sub>) is the concentration at which 50% of the cell population should be killed and IC<sub>50</sub> is the 50% inhibitory concentration. It is considered that when SI value is equal to or more than 10, the drug candidate have the promising activity for its intended purpose [38].

**Table 4: Drug sensitivity profiles against *L. donovani* wild-type, drug-resistant and field isolate axenic amastigote cell lines**

Drug	IC <sub>50</sub> (mean±SD, n=4) [µM/µl] <sup>a</sup>						
	Axenic AG83 evaluation model				Axenic GE1 evaluation model		
	Wild-type	SSG resistant <sup>b</sup>	RI <sup>c</sup>	PMM resistant <sup>b</sup>	RI <sup>c</sup>	Field isolate	RI <sup>d</sup>
Betulinic acid	2±0.22*	21.2±2.33*	10.6	47.8±4.78*	23.9	6±0.6*	3
Spikenard oil <sup>†</sup>	33±4.62*	42.5±5.95*	1.28	42.5±4.67*	1.28	35.5±4.26*	1.07
Cypress oil <sup>†</sup>	11.5±1.49*	45.5±5.46*	3.96	35±3.85*	3.04	20±2.6*	1.74
Curry leaf oil <sup>†</sup>	15±1.5*	39±5.85*	2.6	40±5.6*	2.66	18±2.16*	1.2

Data represented as mean±SD, n=4; the presented results are the average of four (4) determinations, IC<sub>50</sub>= 50% inhibitory concentration, <sup>a</sup>Assays are described in Materials and Methods, <sup>b</sup>SSG and PMM resistant strains were generated *in vitro* as given in Materials and Methods, <sup>c</sup>RI, Resistance index was IC<sub>50</sub> of AG83 phenotype generated at maximum drug pressure/IC<sub>50</sub> of wild-type, <sup>d</sup>RI, Resistance index was IC<sub>50</sub> of field isolate/IC<sub>50</sub> of wild type, <sup>e</sup>Values for antimonial agent was in µg Sb/ml.

Table 5 and fig. 3 revealed that in case of cellular AG83 evaluation model, betulinic acid showed highest SI values which were 192.8 and 19.3 for wild-type and SSG resistant strains, respectively and highest in case of cellular GE1 evaluation model that was 100.

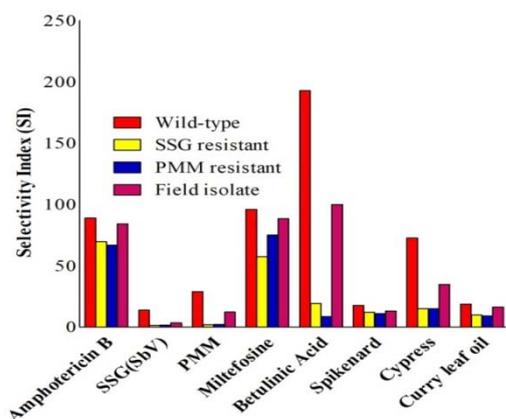
Whereas in case of PMM resistant strains, cypress showed highest SI value (SI= 15.09). When the results against the cellular models of

test drugs were compared to the standard drugs conventionally used for the treatment of visceral leishmaniasis, showed interesting results. Betulinic acid showed the highest activity against wild-type, SSG resistant and GE1 field isolated strains compared to all the standard drugs. From table 5 and fig. 4 it was also observed that cypress oil had the maximum CC<sub>50</sub> value among all standard and tested drugs.

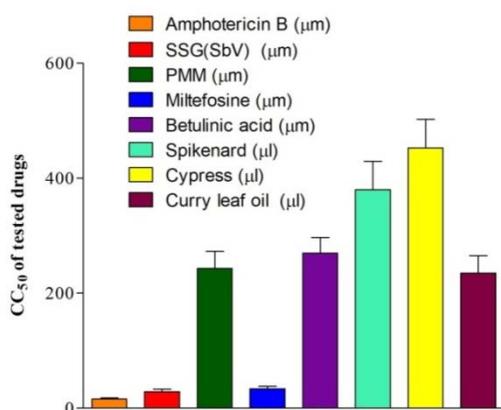
**Table 5: Drug sensitivity profiles of intracellular amastigotes against wild-type, drug resistant and field isolate *L. donovani* cell lines**

Drug	IC <sub>50</sub> (mean±SD, n=4) [µM/µl] <sup>a</sup>							Cytotoxicity CC <sub>50</sub> (µM/µl <sup>†</sup> ) (Macrophage cells)	
	Cellular AG83 evaluation model					Cellular GE1 evaluation model		Field isolate	SI <sup>c</sup>
	Wild-type	SI <sup>c</sup>	SSG resistant <sup>b</sup>	SI <sup>c</sup>	PMM resistant <sup>b</sup>	SI <sup>a</sup>			
Betulinic acid	1.4±0.15*	192.8	14±1.54*	19.3	32±3.84*	8.43	2.7±0.37*	100	270±27*
Spikenard oil <sup>†</sup>	21.5±2.58*	17.67	31.5±3.15*	12.06	35±3.5*	10.85	29±3.19*	13.10	380±49.4*
Cypress oil <sup>†</sup>	6.25±0.93*	72.46	30±3.3*	15.09	30±3.9*	15.09	13.0±1.69*	34.8	452.9±49.8*
Curry leaf oil <sup>†</sup>	12.5±1.75*	18.8	24±2.88**	9.79	26±3.12*	9.03	14.5±1.74*	16.2	235±30.5*

Data represented as mean±SD, n=4; the presented results are the average of four (4) determinations, IC<sub>50</sub>= 50% inhibitory concentration, <sup>a</sup>Assays are described in Materials and Methods, <sup>b</sup>SSG and PMM resistant strains were generated *in vitro* as given in Materials and Methods, <sup>c</sup>Selectivity index SI was calculated by dividing the CC<sub>50</sub> by IC<sub>50</sub>, <sup>d</sup>Values for antimonial agent was in µg Sb/ml, \*p<0.01, significant difference compared with SSG, \*\*p>0.05, no significant difference compared with SSG.



**Fig. 3: Comparative study of selectivity index (SI) of amphotericin B, sodium stibogluconate (SSG), paromomycin (PMM), miltefosine, betulinic acid, spikenard, cypress, curry leaf oil**



**Fig. 4: 50% cellular cytotoxicity (CC<sub>50</sub>) of amphotericin B, sodium stibogluconate (SSG), paromomycin (PMM), miltefosine, betulinic acid, spikenard, cypress, curry leaf oil against isolated peritoneal macrophage cells. Data represented as mean±SD, n=4; the presented results are the average of four (4) determinations**

## DISCUSSION

To outwit the inadequacy of first-line antileishmanial agents is not only the major concern but conquering the emerging problem of drug resistance has high scientific importance in the field of leishmaniasis. To circumvent the hitch of drug resistance, searching for new drug candidate would be the smart approach in the current scenario for the treatment of visceral leishmaniasis.

Curry leaf oil, cypress oil, spikenard oil and betulinic acid had been selected for screening of antileishmanial activity. Curry leaf oil, cypress oil, spikenard oil were subjected to GC/MS analysis for the identification of the chemical components present and it was found that geranyl acetate (32.55% peak area) and linalool (21.16% peak area) were the chemical components majorly present in the curry leaf oil. Whereas in case of cypress oil, it was D-3-carene that acquired 21.35% peak area. D-cadinene (24.53% peak area) and aristolene (22.76% peak area) were the two components majorly present in spikenard oil.

The previous studies of the selected terpenoids have shown some interesting results. According to Singh *et al.* [42], the extracts obtained from the leaves of *Murraya koenigii* (Linn.), also known as Curry leaf have shown their antileishmanial activity against *L. donovani* promastigotes, however, we have generated the data for the curry leaf oil against *L. donovani* axenic as well as intracellular amastigotes. Similarly, when the extract of the cones of *Taxodium distichum* (Bald cypress) was evaluated for antileishmanial property,

Naman *et al.* [43] have generated the data against *L. donovani* promastigotes and amastigotes of *L. amazonensis*, whereas, we have evaluated the antileishmanial property of cypress oil against *L. donovani* axenic and intracellular amastigotes. A few significant data of the derivatives of betulinic acid have been found. According to Alakurtti *et al.* [44], a betulin derivatives which was the heterocycloadduct between 3,28-di-O-acetyl-lupa-12,18-diene and 4-methylurazine have the IC<sub>50</sub> of 8.9 μM against *L. donovani* amastigotes. Chowdhury *et al.* [45] have published that synthesized betulin derivatives have been found to inhibit the growth of the parasite and it was also found that they can act by relaxing the activity of *L. donovani* topoisomerase I in a reversible manner. No significant data have been found for the antileishmanial activity of spikenard oil.

After reviewing the existing literatures, we have observed that sufficient data is lacking for the comparison of the antileishmanial activities of the selected plant derived terpenoids with that of the standard drugs available in the market. Therefore, in the present work, we have tested four terpenoids like betulinic acid, spikenard oil, cypress oil and curry leaf oil as potential drug candidates against SSG resistant, PMM resistant strains as well as AG83 wild type and GE1 field isolated strains. We have also compared the results of the tested drugs with the standard drugs like amphotericin B, sodium stibogluconate, paromomycin and miltefosine. Among all the tested drugs, betulinic acid showed highest resistance against SSG resistant and PMM resistant cell types (fig. 2). In fig. 2, it was also observed that amphotericin B, miltefosine, spikenard oil, cypress oil and curry leaf oil had almost similar and lowest resistance index than others which depicted that among the test drugs, spikenard oil and curry leaf oil were less resistant when it was compared with their effectiveness against wild type strains. Fig. 3 portrayed that betulinic acid was more effective against wild type, SSG-resistant and GE1 strains among all tested drugs.

The potential antileishmanial activity of betulinic acid might be due to the inhibition of topoisomerases of *Leishmania* spp. and also inducing apoptosis effect in the intracellular amastigotes of *L. donovani* as reported in earlier investigations [45]. Several studies are also reported for the antileishmanial properties of derivatives of betulinic acid [46, 47]. Therefore, further *in vitro* and *in vivo* investigations are required for the exploration of this lead compound with leishmanicidal properties but nontoxic for the human host macrophages.

## CONCLUSION

Since leishmaniasis is largely affecting human population worldwide and is responsible for high rates of morbidity and mortality, there is an urgent need for the search of novel antileishmanial agents. Newer drugs obtained from the plant sources could be a better alternative due to variations in their chemical constituents and fewer side effects. According to our present work, it could be concluded that betulinic acid and cypress oil could be the effective drug candidate against *L. donovani* mediated visceral leishmaniasis and all the four test drugs were significantly more effective than sodium stibogluconate and paromomycin against drug resistant *Leishmania donovani* strains. Moreover, this study further recommends the exploration of active chemical constituents in these terpenoids that are responsible for antileishmanial activity.

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## AUTHORS CONTRIBUTIONS

This research work was carried out in collaboration between all the five authors in the concept and design of the work, collection, assembly, analysis and interpretation of data, drafting the article, critical revision and approval of the final manuscript.

## CONFLICT OF INTERESTS

There is no conflict of interest

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