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Biological activities of the essential oils of *Cupressus macrocarpa*, *Lantana camara* and *Psidium littorale* against *Plasmodium falciparum* welch, 1897 and *Anopheles gambiae* giles, 1902

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Abstract
This study assessed the *in vitro* anti-plasmodial on and larvicidal activity of essential oils from the leaves of *Cupressus macrocarpa*, *Lantana camara* and *Psidium littorale*. The chemical compositions of the different oils were determined. The toxicity of the oils was tested on *An. gambiae larvae* according to WHO protocol and their *in vitro* anti-plasmodial activity was assessed by radio-isotopic method. α-pinene (20.78%), β-caryophyllene (20.37%) and 1,8-cineole (eucalyptol) (39.55%) were the major compounds in *Cupressus macrocarpa*, *Lantana camara* and *Psidium littorale* oils respectively. The *in vitro* anti-plasmodial activity showed that *Lantana camara* essential oil is more effective (IC50=12.34 ppm) than *Cupressus macrocarpa* (147.29 ppm) and *Psidium littorale* (115.45ppm). Essential oil of *Lantana camara* showed higher activity on larvae from Yassa (DL=7.37 ppm) while that of *Psidium littorale* was more active on larvae from Youpwe (DL=49.2 ppm). These oils can be used for the development of new biocides and natural anti-malarial drugs.

Keywords: *Cupressus macrocarpa*, *Lantana camara*, *Psidium littorale*, *Anopheles gambiae s.l.*, larvicidal activity, anti-plasmodial activity

Introduction
More than a century after the discovery of its causal agent and the role of the mosquitoes belonging to the '*Anopheles*' genus in its transmission, malaria remains one of the most dreaded diseases [1]. Approximately 154-289 million people are infected each year, with 80% of cases occurring in sub-Saharan Africa. Children less than 5 years old, pregnant women, people living with HIV/AIDS, naive migrants, mobile populations and travellers are the most vulnerable [2]. Endemic countries are deploying various malaria control means including vector control activities and adequate patient management based on early diagnosis and administration of effective therapies. Despite the 21% drop in prevalence recorded worldwide between 2010 and 2015 [3], the situation remains a cause for concern. This may owe to rough application of the recommended preventive and curative measures, and above all, in the dual resistance of vectors to insecticides and *Plasmodium* to antimalarial drugs. With regard to vectors, use of DDT and pyrethroids in both agriculture and public health has resulted in the selection of resistant strains [4, 5]. In recent years, the emergence of *Anopheles* strains resistant to synthetic insecticides has been reported in many African countries including Benin [6], Côte d'Ivoire [7], Niger [8], Nigeria [9], Equatorial Guinea [10] and Cameroon [11, 12]. With regard to the parasite, it is known that self-medication, utilization of drugs from street vendors and non-compliance with prescribed doses are responsible for the development of resistant strains of *Plasmodium* [13]. The emergence and spread of resistant strains of *Plasmodium* and vectors in sub-Saharan Africa is jeopardising malaria control efforts in endemic countries. In this context, the search for natural molecules with effective biological properties is essential. Plants from the Cameroonian flora have for millennia been an inexhaustible source of new molecules that simply need to be explored. Numerous studies have been carried out, highlighting the insecticidal activity of plant species from Cameroonian flora [14, 15, 16, 17].

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They demonstrated that plants insecticidal activities boosted when used in the form of essential oils [18]. Volatile essences have several functional groupings and can easily diffuse across the cell membranes [19]. Essential oils can therefore be useful for malaria treatment and vector control [20, 15].

Cupressus macrocarpa, *Lantana camara* and *Psidium littorale* are plants of the Cameroonian flora traditionally used by the population as insect repellents and treatment of many diseases including amoebiasis and malaria [21]. The traditional use of these plants attest to their potential as reservoirs of active molecules against malaria parasites and vectors. However, this is still to be investigated. The present study aims to evaluate the *in vitro* insecticidal and antiplasmodial activities of essential oils from *Cupressus macrocarpa*, *Lantana camara* and *Psdium littorale* leaves.

Materials and Methods

Collection of plants and extraction of essential oils

The plants were chosen because of their traditional use as insect repellents and treatment of certain diseases in many villages of the West region of Cameroon. Plants were collected in September 2018 in Bandjoun and Douala, free of insecticide treatment. The specimens were then identified by botanists from the Department of Plant Organism Biology of the University of Douala. The leaves of each plant species were washed with spring water, cut into small units and then subjected to hydrodistillation for 5 hours using a Clevenger-type equipment. The essential oil collected at the end of the distillation process was filtered on an anhydrous sodium sulphate column, then stored in a dark, hermetically sealed glass bottles at 4°C.

Analysis of the chemical composition by GC and GC/MS

The chemical analysis of essential oils was carried out using a Varian CP-3380 type chromatograph equipped with a flame ionisation detector and a capillary column (length 30 m, internal diameter 0.25 mm) with an apolar stationary phase of the methylsilicone type (DB-1, film thickness 0.25 µ). Nitrogen was used as carrier gas with 0.8 ml.min-1 flow rate. The temperature of the injector was 220°C; the detector at 250°C. The furnace was programmed from 50°C to 200°C with a temperature gradient of 5°C.min-1. The retention indices of the different constituents was calculated in relation with the retention times of a series of n-alkanes and their relative percentages calculated by electronic integration, considering that their response factors are all equal to 1.

The gas chromatography-mass spectrometry coupling was done using a Hewlett Packard HP 5970 A apparatus, equipped with an apolar capillary column (30 m x 0.25 mm) in HP-1 fused silica (film thickness 0.25 µ) and a quadrupole detector (ionisation energy 70 eV). The temperature of the injector was 220°C and that of the interface area was 210°C. Injection in split mode (1/100) of 1 µl of a 10% solution of essential oil in dichloromethane. The furnace temperature was programmed from 70°C to 200°C with a gradient of 10°C.min-1. Helium was used as carrier gas with 0.6 ml.min-1 flow rate. The acquisition was performed in scan mode [35-300 amu] at 2.96 scan.sec-1.

The identification of the constituents of the essential oils was made on the basis of their retention indices and mass spectra by comparison with the data from literature [22].

Collection sites for larvae of *Anopheles gambiae* s. l.

Larval collection took place in 2 districts of Douala, namely Yassa and Youpwe. Yassa (3°58 N, 9°49 E) is a peri-urban district in the east of the city. Main activities are agriculture, animal husbandry and trade. This district is also characterised by the presence of soap and oil companies. Pot holes and old vehicle tyres, are main breeding sites for mosquitoes, especially in the rainy season. Youpwé (04°00'N, 09°42'E) is a densely populated district located in the Wouri river estuary. Urbanisation is poorly controlled and the population lives mainly from fishing and petty trade. The Wouri River is the only permanent breeding ground for mosquito larvae in this area, although some temporary breeding sites may be visible during the rainy season.

Collection and rearing of Anopheles larvae

Populations of *Anopheles* species for testing were collected in the larval stage in natural deposits (sewers, gutters, drums, pits, old tyres, pits and tracks, pot holes) using the dipping method [23]. Collection took place during the short rainy season (May to July 2019) at the rate of five consecutive days per month, simultaneously in the Youpwe and Yassa. Anopheles larvae were reared in water from the lodges and fed on Tetrababy fish food [24]. The adults obtained were morphologically identified [25, 26]. The males and females of *An. gambiae* s.l. were crossed to obtain F1 generation larvae that were later on tested.

Cultivation of *Plasmodium falciparum*

The chloroquine-resistant FcB1/Colombia strain of *P. falciparum* was maintained on human red blood cells in RPMI 1640 medium, containing 25 mM HEPES, pH 7.3, 2 g/L sodium bicarbonate, 2 g/L glucose, penicillin and streptomycin [27]. The medium was enriched with 10% heat-activated human serum. The RBCs and serum used came from the *Etablissement Français du Sang*. The culture was conducted at 2% haematocrit, in 25 and 75 mL vials and maintained in an oxygen-deficient atmosphere at 37°C. The culture medium was renewed once a day. A Giemsa stained blood smear was taken daily to control parasitaemia.

Larvicidal tests

These tests consisted of evaluating the mortality of mature stage larvae (L3 and L4) of Anopheles in the presence of diluted solutions of essential oils according to WHO protocol [28]. Twenty larvae were sampled using a Pasteur pipette and placed in bowls of 8 cm diameter each containing 99 ml of well water to which 1 ml of diluted test solution was added. Preliminary experiments enabled the selection of a range of concentrations for the tests. Stock solutions of essential oils from each sample were prepared in 90° ethanol. From these, dilutions were made to obtain final experimental concentrations of 50, 100 and 150 ppm. Three repetitions were carried out for each dilution. Two control bowls were also prepared under the same conditions as the test bowls. These negative control contained only ethanol (in the same proportions as for the tests, i.e. 1%) with no trace of essential oil. Larval counts were carried out every 5 minutes for 1 hour; then every hour for 10 hours and finally after 24 hours of exposure to volatile extracts solubilised in water.

Antiplasmodial test

The *in vitro* anti-plasmodial activity of essential oils has been evaluated by the radioisotopic method [29]. This method determines the inhibition of parasite growth in culture in the presence of various concentrations of molecules by measuring

the incorporation of [3H] hypoxanthine in the parasite nucleic acids. The experiment was carried-out in 96-well plates as described by Guillon *et al.* [30]. Briefly, serial dilutions of essential oils were prepared in culture medium and added to asynchronous parasite cultures (1% parasitaemia, 1% final haematocrit, 200 µL final volume per well) for 24 h, at 37°C, before addition of 0.5 µCi of [3H] hypoxanthine (1-5 Ci/mmol; Amersham, Les Ulis, France) per well for 24 h. The plates were incubated at 37°C in a humid, oxygen-deficient atmosphere. Freezing plates at -80°C interrupted the experiments. After thawing, the contents of the wells were collected on glass fibre filters (Wallac®, USA) using a cell collector (Filter Harvester, USA). After adding scintillation fluid (Perkin Elmer®, USA), the radioactivity (counts per minute) was measured using a spectrophotometer (450-Microbeta Trilux, USA). The growth inhibition for each concentration was determined by comparing the radioactivity incorporated in the treated culture with that of the control (essential oil-free) culture on the same plate. The concentration causing 50% inhibition (IC50) was obtained from the drug concentration-response curve and the results were expressed as the mean ± standard deviations determined from at least three independent experiments.

To avoid inhibition of parasite growth due to diffusion of essential oils from neighbouring wells, preliminary tests were conducted to determine the highest essential oil concentration for which such inhibition was not measured. For this purpose, the crude essential oil was only diluted in series with culture medium in one row of a plate, the other row containing only culture medium. Parasites were added to all wells of the plate and the plate was treated as described above. The highest concentration of essential oil showing no inhibition of parasite growth in the surrounding wells was used as a starting concentration to further determine the intrinsic anti-plasmodial activity of the essential oil.

Statistical analysis

Statistical analyses were carried out using Statview version 5.0 software (SAS Institute, Inc, USA). Kruskal Wallis H-tests was used to compare larval mortalities. The regression curve from Henry's simplified table which transforms the mortality percentages into probits [31] made it possible to determine the LC_{50} and LC_{95}. Statistical significance was set at a probability value of less than 0.05.

Results and Discussion

The present study shows that the leaves of *Cupressus macrocarpa* (0.673%) have a higher essential oil content than those of *Psidium littorale* (0.118%) and *Lantana camara* (0.076%) (Table 1). These yields contrast with results from Sousa *et al.* [32], Lídia *et al.* [33], and Nacira & Yousra [34]. The difference in yields can be related to extraction method, climatic conditions, geographical location of the harvest site, harvest period, and physiopathological state of the plant at the time of harvest [35, 14].

Table 1: Data on essential oils from plants collected in Cameroon

Plant			Collection		Essential oil	
Family	species	Organ	Area	Date	Color	Outputs
Verbenaceae	*Lantana camara*	Leaves	Douala	24.09.2018	yellow	0.076 %
Cupressaceae	*Cupressus macrocarpa*	Leaves	Bandjoun	18.09.2018	yellow	0.673 %
Myrtaceae	*Psidium littorale*	Leaves	Douala	26.09.2018	yellow	0.118 %

Chemical composition

The chemical composition of the essential oils of the three plants is given in Table 2. Monoterpenes constitute the major fraction (50.27% - 89%). These monoterpenes were dominated by α-pinene (20.78%) in the oil of *Cupressus macrocarpa*. Similar results were obtained with samples from Argentina and Greece [36, 37, 38]. In addition, it should be noted that α-pinene has been reported to be the major component of essential oils of other species of the genus *Cupressus*, notably *Cupressus arizonica* from Tunisia, *Cupressus atlantica* from Morocco and *Cupressus simpervirens* from Algeria [39, 40]. From the above, it should be suggested that α-pinene is the characteristic molecule of the essential oils of Cupressus, although it was shown that neral and α-terpineol were the major compounds in *Cupressus macrocarpa* from Egypt had [41, 42]. β-Caryophyllene (20.37%) was the majority compound in the essential oil of *Lantana camara*. Some works have shown a certain variability in the chemical composition of this plant species according to the collection site with β-caryophyllene as the majority compound for the sample from Egypt [43]; (E)-nerolidol for the sample from Cuba [44]; davanone for the sample from Nepal, sabinene for the sample from Yeme [45].

Psidium littorale is mainly composed of 1,8-Cineole (eucalyptol) (39.55). Although this result corroborates those recorded by Scur *et al.* [46] and Marques *et al.* [47], it contrasts with the results recorded by Soliman *et al.* [48] and Adam *et al.* [49]. The later showed that β-Caryophyllene was the major compound in the samples of *Psidium littorale* originating from Egypt and French Polynesia. Thus, there is a certain variability in the chemical composition of *Psidium littorale* oil according to the collection site.

Table 2: Chemical composition of essential oils of *Cupressus macrocarpa*, *Lantana camara* and *Psidium littorale.*

IK	Compounds	% Composition		
		Cupressus macrocarpa	*Lantana camara*	*Psidium littorale*
Monoterpenes				
Monoterpenes hydrocarbon				
911	Tricyclene	--	--	--
928	α-thujene	1.51	0.52	4.36
936	α-pinene	20.78	3.9	--
949	α-fenchene	0.45	--	--
951	camphene	--	1.46	--
963	Furfural <S-methyl	--	--	1.37
978	β-pinene	8.81	17.99	0.32
990	myrcene	3.64	1.93	0.41
1,006	p-Terpinène	--	0.18	--
1,007	α-phellandrene	--	--	0.81

1,013	δ-3-carene	4.97	1.5	--
1,019	α-terpinene	1.8	0.28	--
1,026	P-cymene	0.95	--	--
1,033	Limonene	9.96	--	--
Oxygenated monoterpenes				
1,037	1,8-Cineole (eucalyptol)	--	--	39.55
1,046	cis-β-ocimene	--	0.89	--
1,059	trans-β-ocimene	--	0.56	--
1,060	G-terpinene	2.95	--	--
1,070	γ-terpinene	--	0.58	--
1,091	Terpinolene	3.38	--	--
1,101	Linalool	4	14.68	--
1,102	2-Methylbutyl 2-methylbutyrate	--	0.29	--
1,125	Cis-p-menth-2-en-1-ol	2.56	--	--
1,128	cis-Menth-2-en-1-ol	--	--	0.68
1,150	Cis-β-terpineol	0.27	--	--
1,151	Lilac aldehyde D	--	1.06	--
1,163	Trans-β-terpineol	0.44	--	--
1,166	Terpineol <5->	--	--	1.17
1,172	terpinen-4-ol	9.21	0.76	--
1,183	neo-dihydro carveol	--	1.35	--
1,187	α-terpineol	6.01	--	--
1,196	Methyl salicylate	--	0.89	--
1,196	Cis-Piperitol	0.83	--	--
1,252	Piperitone	0.56	--	--
1,270	Cinnamaldehyde <(E)'>	--	--	0.15
1,287	Bornyl acetate	0.13	--	--
1,299	2-cis-dihydro acétate de tepinyl	0.2	--	--
1,315	Déca-(2E,4E)-dien-1-ol	0.18	--	--
1,322	Hexyl 2-methyl-3-pentenoat	--	--	0.48
1,352	α-acétate-terpinyl	2.18	--	--
1,363	Cyclosativene	1.32	--	--
1,364	Anisaldehyde <dirnethylacetal'p->	--	--	0.97
Sesquiterpenes				
hydrocarbonated Sesquiterpenes				
1,383	Longifolene	0.13	--	--
1,384	β-Bourbonene	--	0.57	--
1,388	α -copaene	--	--	5.62
1,399	p-Elemene	--	0.85	--
1,416	β-Caryophyllene	--	20.37	0.72
1,427	(E)-Caryophyllene	--	--	0.69
1,430	ß-Copaene	0.46	--	2.63
1,442	(+)-Aromandrene	--	--	0.28
1,455	α-humulene	0.57	--	0.28
1,474	G-muurolene	1.59	--	--
1,485	D-germacrene	0.51	8.79	0.68
1,489	α-selinene	--	--	1.39
1,494	Bicyclogermacrene	--	2.22	--
1,495	Trans-β-guaiene	--	--	0.7
1,500	β-bisabolene	--	----	1.17
1,508	A-germacrene	1.61	4.7	
1,509	γ-Cadinene	--	--	2.35
1,519	Nookatene	--	--	1.06
1,524	Δ-cadinene	1.42	--	--
1,524	δ-Cadinene	0.3	0.28	0.46
1,532	Davana ether	--	0.21	--
1,533	α-calacorene	--	--	0.21
1,540	trans-Cadina-1,4-diene	--	--	1.49
1,563	7-Hydroxyfarnesene	--	0.8	--
1,564	α-Cadinène	0.16	--	--
Oxygenated Sesquiterpenes				
1,517	Cubebol	--	--	2.94
1,532	10-epi-Cubebol	2.7	--	--
1,568	(E)-Nerolidol	--	--	1.96
1,569	Longipinanol	--	3.65	--
1,592	viridiflorol	--	0.23	--
1,595	Fokienol	--	0.28	--
1,600	Oxyde de caryophyllène	--	--	0.31

1,600	β-atlantol	--	0.59	--
1,619	1,10-di-epi-Cubenol	0.48	--	--
1,622	Cubenol-1-epi	--	--	0.46
1,625	diidromircene,1,6-diol-Z	--	0.67	--
1,643	Cubenol	--	--	1.52
1,645	Humulène époxyde I	1.1	--	--
1,648	α-Muurolol	--	--	0.88
1,650	α-Cadinol	1.25	2.47	0.81
1,661	(E,E)-Farnesol	--	--	0.38
1,668	γ-Eudesmol	1.11	--	--
1,669	14-Hydroxy-9-epi-(E)-caryophyllene	--	1.61	--
1,673	epi-β-Bisabolol	--	--	2.87
1,683	α-Bisabolol	--	--	8.56
1,692	α-Eudesmol	0.68	--	--
1,697	Shyobunol	--	--	2.43
1,722	(E)-Nerolidol acetate	--	0.21	1.18
1,729	(2Z,6E)-Farnesol	--	--	2.21
1,820	(E,E)-Farnesyl acetate	--	--	0.69

Biological Tests

- **Larvicidal tests**

Larvicidal tests have shown that the essential oils of the leaves of *Cupressus macrocarpa*, *Psidium littorale* and *Lantana camara* have important biological properties against *Anopheles gambiae* s.l. However, the level of effectiveness seems to depend on the plant species, the concentration used and the site of collection of the *Anopheles gambiae* s.l. strain (Tables 3,4). LC50 and LC95 values determined from Henry's simplified table were used to classify essential oils according to their toxicity level on *An. gambiae* s.l. larvae. Essential oil of *Psidium littorale* was the most effective, followed by those of *Lantana camara* and *Cupressus macrocarpa* (table 4). According to Pellecuer *et al.* [50], the toxicity of an essential oil is strongly related to its chemical composition. Thus, the larvicidal activity of the essential oils noted in our study would be due to their high monoterpene content. Monoterpenes have long been recognised for their proven larvicidal properties against insects [51, 52, 53]. However, the high toxicity shown by the essential oil of *Psidium littorale* compared to those of *Cupressus macrocarpa* and *Lantana camara* is thought to be due to Cubebol. This molecule is present in *Psidium littorale* oil but is absent in those of *Cupressus macrocarpa* and *Lantana camara*. The work of Hui-Jing *et al.* [54] highlighted the role played by this molecule in its pure state when evaluating the insecticidal activity of the ethanoic extract of *Cryptomeria japonica* on larvae of *Aedes albopictus* and *Aedes aegypti*. Furthermore, our results show that, for certain concentrations and for the same plant species, mean mortality numbers were significantly different for *An. gambiae* larvae from different collection sites (Tables 3 and 4). Thus, larvae collected in Yassa were more sensitive to essential oils than those collected in Youpwé. This result could be explained by the fact that *Anopheles gambiae* s.l. is a species complex [55, 56]. Studies have shown that of the species in the complex, *An. coluzzii* is the one that has developed the most adaptive characteristics to pollutants in poorly urbanised neighbourhoods in African cities, whereas *An. gambiae* s.s prefers peripheral neighbourhoods where the environment is still relatively natural [56, 57]. It should therefore be suggested that the strain collected in Yassa that is more sensitive to essential oils would be *An. gambiae* s.s, while the less sensitive strain from Youpwe would be *An. coluzzii*. However, a molecular analysis should be carried out to confirm this hypothesis.

Table 3: Sensitivity of mature larvae of *Anopheles gambiae* s. l. to different concentrations of essential oils of *Psidium littorale*, *Lantana camara* and *Cupressus macrocarpa*, after 10 hours of exposure (Kruskal Wallis H-test and Mann-Withney Z-test, $P<0.05$).

		Mature larvae		Z-test	P -value
Essential oils	**Concentration (ppm)**	**Yassa**	**Youpwe**		
Psidium littorale	150	20±0.577	20±0.0	-0.655	0.5127
	100	19±1.155	17±1.528	-1.091	0.2752
	50	16±3.215	13±4.041	-1.091	0.2752
	H-test	2.022	7.200	--	--
	P-value	0.368	0.0273*	--	--
Lantana camara	150	19±1.732	17±0.577	-1.528	0.1266
	100	18±1.000	10±2.646	-1.622	0.0495
	50	17±2.309	5±1.732	-1.964	0.0495
	H-test	1.622	7.200	--	--
	P-value	0.444	0.0273*	--	--
Cupressus macrocarpa	150	18±1.528	11±1.528	-1.964	0.0495
	100	17±1.732	09±0.0	-1.964	0.0495
	50	7±3.215	2±1.155	-1.964	0.0495
	H-test	5.600	6.200	--	--
	P-value	0.060	0.045*	--	--

*Statistically significant

Table 4: Lethal doses of essential oils capable of causing 50% and 95% mortality of mature larvae collected in the Youpwe and Yassa districts (Douala).

Plants	Lethals doses			
	DL50		DL95	
	Yassa	Youpwe	Yassa	Youpwe
Cupressus macrocarpa	60.445	125.395	170.827	432.78
Lantana camara	25.088	83.657	167.177	250.939
Psidium littorale	7.337	49.275	97.897	88.407

Antiplasmodial test

The essential oils of the three plants have shown some *in vitro* antiplasmodial activity against *Plasmodium falciparum*. However, the essential oil of *Lantana camara* (IC50=12.34 ppm) appears to be the most active, followed by that of *Psidium littorale* (IC50=115.455 ppm) and *Cupressus macrocarpa* (IC50=147.29 ppm) (figure 1). The strong antiplasmodial activity could be related to the high Linalol content of *Lantana camara* essential oil (14.68%). Terpenes such as farnesol, nerolidol, limonene, and linalol are known for *in vitro* inhibition of the biosynthesis of dolichol in the trophozoite and schizontal cycle of *P. falciparum*. Terpenes also have the ability to inhibit the biosynthesis of the isoprenic side chain of benzoquinone in the schizogonic cycle [58, 59, 60]. Based on their IC50 values, essential oil of *Lantana camara* has a moderate *in vitro* toxicity against *Plasmodium falciparum*, whereas those of *Cupressus macrocarpa* and *Psidium littorale* show no significant activity.

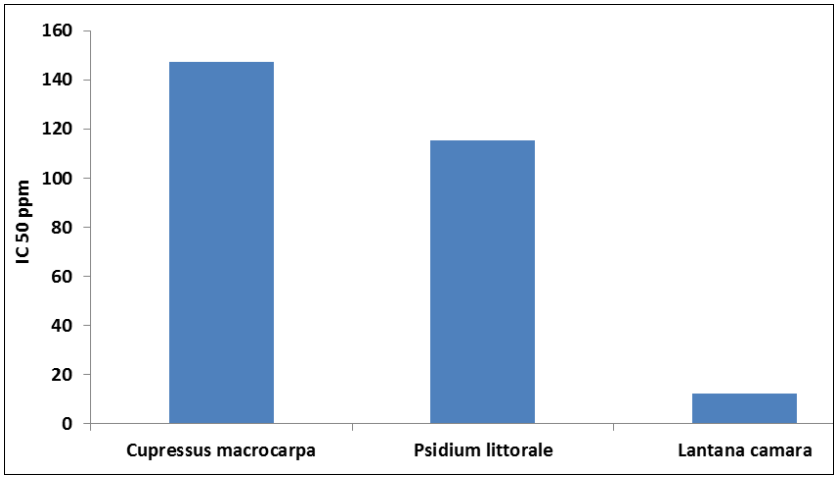

Fig 1: Summary diagram of the anti-plasmodial activity of essential oils on Plasmodium falciparum *in vitro*

Conclusion

The present study has shown that *Psidium littorale* and *Lantana camara* have important larvicidal properties against *Anopheles gambiae* s.l. while *Lantana camara* has moderate activity against *P. falciparum in vitro*. These plants can therefore be considered as a source of new molecules against malaria germs and vectors.

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