Antibacterial and antimalarial activity of Angolan *Cymbopogon citratus* essential oil

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Bacterial infections and malaria remain a major public health problem due to the emergence and spread of drug resistant strains. There is an urgent need to investigate new sources of antibacterial and antimalarial drugs, which are more effective. One of the potential sources of antibacterial and antimalarial drugs is traditional medicinal plants. Our ethnopharmacological studies, in several Angolan regions, showed that *Cymbopogon citratus* (DC) Stapf., has high bio-activity against bacterial infections and malaria.

The constituents of *Cymbopogon citratus* essential oil (CCEO), obtained by hydro-distillation, were analysed by GC and GC-MS and identified from their retention indices and mass spectra [1]. The antimicrobial activity of CCEO and major natural volatile compounds were tested against several bacterial strains. The antimalarial assays where performed in continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* using the parasite lactate dehydrogenase assay [2].

CCEO exhibits broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria. Our results showed that multi-resistant *Stafilococus aureus* (MRSA) isolates were more sensitive to CCEO than non-MRSA. When tested against MRSA resistant to amoxicillin-clavulanic acid combination, penicillin G and methicillin, CCEO shows a significant increase in bactericidal activity when compared with the commercial antibiotics. The same results were obtained using vancomycin resistant *S. epidermidis* and other strains.

Regarding the anti-malaria activity, test samples where considered active for *in vitro* antimalarial activity exhibiting IC_{50} values of 5.34±1.01 μg/ml and 7.06±0.47 μg/ml.

Our work shows that CCEO has higher antibacterial activity than commercial antibiotics against MRSA strains, as well as antimalarial activity. This offers opportunities for clinical treatments since preliminary *in vivo* studies showed no toxic effects and high antimalarial activity.

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Keywords: *Cymbopogon citratus; essential oil; antibacterial activity; multi-resistant bacteria. P. falciparum;* malaria

1. Introduction

The indiscriminate use of antimicrobial and antimalarial agents resulted in the emergence of drug-resistant bacteria, and *Plasmodium* species *P. falciparum*, *P. vivax* and *P. malariae* [3]. The antimalarial drug resistance is compound by drug cross-resistance, in which resistance to one drug confers resistance to other drugs that belong to the same chemical family or which have similar modes of action [3]. Various populations in developing countries are using medicinal plants against infectious diseases by accidental discovery, and trust in the benefit of their use. To overcome the increased resistance of pathogenic microbes, researchers are using traditional knowledge as source of development of new drugs with high antibacterial/antimalarial potential.

According to previous results obtained by our research group [4] *C. citratus* is widely used in folk medicine to treat some skin infections, feverish and anti-inflammatory conditions, digestive disorders, as well as other health problems, in several Angolan regions.

Since the last decade, particular attention has been given by the researcher to the pharmacological properties of essential oils against several pathogens. Polyphenols and mono- and polymeric flavonoids, such as luteolin, quercetin,

kaempferol, and apigenin glycosides and proanthocyanidins, were thought to be partially responsible for its therapeutic potential [5-7].

This chapter discusses the antibacterial activity of *Cymbopongus citratus* leaves extracts in multi-resistant drug strains of *S aureus*, *S. epidermidis*, *E. colli*, *K. pneumoniae* and *P. mirabillis*.

Although, some studies have demonstrated that several constituents of essential oils have antibacterial activities [8], the antibacterial mechanism thereof is still unknown. We considered the possibility that permeabilization of bacterial cells' plasmatic membrane will be the major mechanism for bacterial death. In support of this hypothesis, our results indicate that the composition of the essential oil of *Cymbopungus citratus* reveal the presence of several hydrophobic compounds. Permeabilization studies, using ethidium bromide as a fluorescent probe, were performed in order to validate these hypotheses, as described by Sato *et al.* [9].

2. Antibacterial activity of Angolan *Cymbopogon citratus* essential oil

Infectious diseases were responsible for 19,3 million global deaths annually [10] among them bacterial infections being a major threat [10]. The only solution to the problem is the use of antibiotics or chemicals. However, the increasing failure of chemotherapy and antibiotic resistance exhibited by bacterial pathogens has prompted researchers to screen plants for their antimicrobial activity.

In Angolan folk medicine, *C citratus* has been used to treat infected wounds due to its antibacterial activity. This chapter discusses the antibacterial activity of *Cymbopongus citratus* leaves extracts in *S aureus*, *S. epidermidis*, *E. colli*, *K. pneumoniae* and *P. mirabillis*, as well as multi-resistant drug strains.

2.1. Physico-chemical characterization of *Cymbopongus citratus* essential oil

Physico-chemical characterization of *C. citratus* essential oil was carried out by gas chromatography (GC) and by gas chromatography-mass spectroscopy (GC/MS). Using the retention time and the relative proportions of the picks it was possible to identify and quantify the *C. citratus* essential oil (CCEO) compounds. The main compounds from CCEO is geranial (48.4%), neral (32.6%) and myrcene (6.4%) [1].

The results in Table 1 show that the essential oil concentrations between 15 and 80% inhibit the growth of both *S. aureus* and *S. epidermidis*, with diameters ranging from 8 to 37 mm in diameter. The inhibition of the positive controls ranging from 20.6 to 42.2 mm, corresponding to methacycline 5µg and Penicillin G 10mg.

The results of inhibition formed by disk diffusion method of essential oil of *Cymbopogon citratus* on *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* (Table 1) demonstrate that concentrations of 5 and 20% has no effect on the three species tested, and that the maximum effect is obtained when using a figure of 60 to 100%. It should be noted that the halos obtained from the essential oil are always lower than those obtained for the positive controls.

Similar studies were carried out, using the majority constituents present in the essential oil of *Cymbopogon citratus*.

The results show that citral at low concentrations has no inhibitory activity on the growth of *S. aureus*. The dilutions between 20 and 45% *S. aureus* revealed to have only intermediate susceptibility, whereas at concentrations of 60 to 100% its activity is close to that obtained in positive controls. The same is observed for *S. epidermidis*, however above 45% its activity is higher than that obtained with the positive controls, although the difference was not statistically significant, being the same results obtained with the other major constituents of *Cymbopogon citratus* essential oil.

2.2 Antibacterial Activity

The microorganisms used in this study were obtained from the Culture Collections of the IINFACTS /CESPU Paredes Portugal. The antimicrobial activity of CCEO was evaluated using the following laboratory control strains: *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Candida albicans* (ATCC 10231), *Candida parapsilosis* (ATCC 2219) and *Candida tropicalis* (ATCC 750) and the antibiotic resistant strains isolated from Braga Hospital (Portugal, GPS:N 41o34′ W 8o24′): *Staphylococcus aureus 1* (*S. aureus 1*); *Staphylococcus aureus 2* (*S. aureus 2*); *Staphylococcus epidermidis* 1 (*S. epidermis 1*); *Staphylococcus epidermidis* 2 (*S. epidermis* 2); *Escherichia coli* 830 (*E coli* 830);. *Escherichia coli* 986 (*E. coli* 986); *Klebsiella pneumoniae* 822 (*K. pneumoniae* 822).

The *in vitro* antibacterial screening was performed using the disk diffusion method [12], using different concentrations of CCEO (5%, 10%, 20%, 40%, 60%, 80% and 100%). The bacterial assessment was performed after 24 or 48 hours incubation at 37ºC. Methicillin (5 µg/disc); penicillin (10 µg/disc); amoxicillin/clavulanic acid (augmentim) (30 µg/disc); vancomycin (5 µg/disc) discs were used has a positive control in Gram positive strains and ciprofloxacin (5 µg/disc), nitrofurantoin (300 µg/disc), ceftazidime (30 µg/disc); gentamicin (10 µg/disc) in Gram negative strains.

The antibacterial activity of all the tested antibiotics was interpreted according to the CLSI guidelines (2012).

Table 1 - Antibiotic sensitivity of multidrug resistant hospital isolated strains and their respective ATCC control strains

Bacteria (Gram +)	S aureus	S aureus 1	S. aureus 2	S epidermidis	S epidermidis 1	S epidermidis 2
MET	S	R	R	S	R	R
P	S	R	R	R	R	R
AMC/AUG	S	R	R	S	R	S
VA	-	-	-	S	R	R
CCEO concentration (%)	S≥20	S≥40	S≥60	S≥20	S≥20	S≥20

Sensitivity values: R-Resitant; I- intermediate; S-sensitive
MET- Methicilin; AMC/AUG-Amoxicilin/Clavulamic acid; VA-Vancomycin (from CLSI guidelines 2012)

Hospital isolated strains of *S. aureus* 1 and 2 present resistance to methicillin, penicillin and amoxicillin-clavulamic acid, consistent with the profile of a methicillin resistant *S. aureus.* ATCC 25923 *S. aureus* presents the sensitivity to the antibiotics used in these assays (Table 1). On the other hand, *S. epidermidis* 1 was resistant to methicillin, penicillin and amoxicillin-clavulamic acid and vancomycin consistent also with the methicillin resistant profile. *S. epidermidis* 2 was sensitive to amoxicillin-clavulamic acid and resistant to penicillin, methicillin and vancomycin. By comparison, *S. epidermidis* ATCC 12228 was sensitive to all antibiotics tested for Gram positive bacteria.

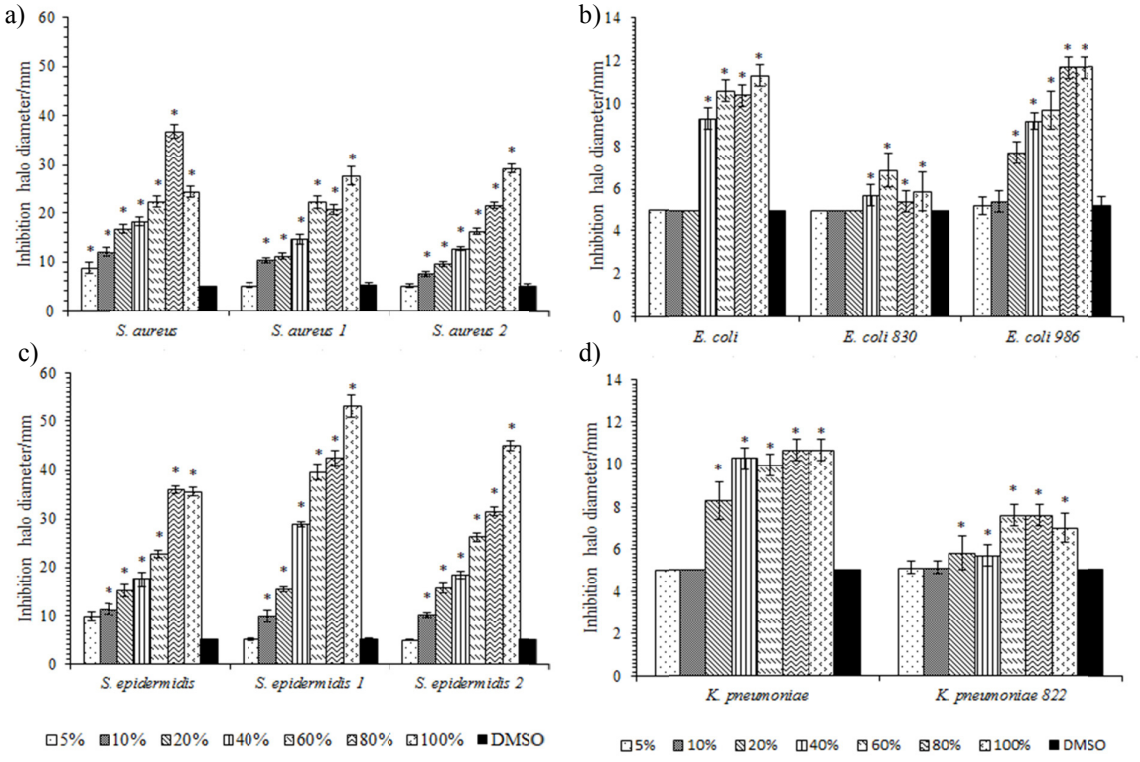

Fig. 1 a) and c) Antibacterial activity of different EO concentrations of *C. citratus* against Gram positive ATCC and multidrug resistant strains. *Statistically different (95% significance) relatively to negative control DMSO; b) and d) Antibacterial activity of different EO concentrations of *C. citratus* against Gram negative ATCC and multidrug resistant strains. *Statistically different (95% significance) relatively to negative control DMSO

Figure 1 shows that gram negative *E. coli* 830, *E. coli* 986 and *K. pneumoniae* 822 presented an intermediate resistance pattern, with growth inhibition at *C. citratus* EO concentrations equal or superior to 40%. *E. coli* 986 and *K. pneumoniae* are more sensitive to the inhibition effect of the EO started at inferior concentrations (≥ 20%).

The results thereby suggest that the oil is more effective on Gram positive bacteria. The higher resistance manifested by the Gram negative bacteria may be associated to the constitution of its outer membrane which acts as a relatively impermeable barrier [11]. Gram negative bacteria are inherently resistant to hydrophobic antibiotics, as their outer membrane limits the entry of these antibiotics into the cell [13]. Thus we can hypothesize that the CCEO is less effective against Gram negative bacteria due to the same reason [14].

3. Antimalarial activity of Angolan *Cymbopogon citratus* essential oil

Malaria remains one of the most widespread infectious diseases and a major global health problem. In 2015, there were an estimated 214 million malaria cases, with 438,000 deaths [15]. Global efforts to eliminate malaria have achieved success in Europe and North America, but the disease remains a major health problem in sub-Saharan Africa [16]. To achieve total elimination of malaria, new drugs inhibiting liver stage reproduction or gametocyte development are a necessary element. Parasite asexual stages cause the clinical symptoms of malaria, while the sexual stages (gametocytes) allow transmission of the parasite from human to mosquito.

The in vitro antimalarial activity of *Cymbopogon citratus* essential was evaluate against the 3D7 and NF54 sensitive strains of the malaria parasite, *Plasmodium falciparum*. The organisms were obtained from the Department of Pharmacology, University of Cape Town. Continuous in vitro cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen (1976). Parasitemia was kept at 2% in complete medium (RPMI 1640). Samples of the essential oil were prepared from two separate batches of the raw plant material.

The *C.citratus* essential oil was evaluated at a starting concentration of 100μg/ml which was then serially diluted 10-fold in complete medium to give six concentrations; with the lowest concentration being to 0.001μg/ml. Chloroquine (CQ) and artesunate were used as the reference drugs in all experiments. Quantitative assessment of antiplasmodial activity determined via the parasite lactate dehydrogenase (pLDH) assay using a modified method described by Makler (1993).

Gametocytogenesis was induced by a combination of nutrient starvation and a drop in haematocrit, using a method adapted from Carter (1993). Asexual culture of NF54 were allowed to reach >5% parasitaemia in glucose-free RPMI 1640 medium containing 0.65mM hypoxanthine, 10% (v/v) Human Serum (blood type A+), 6% haematocrit, with medium change every 48h for 21 days until culture was anaemic, and starved asexual parasites were detectable. Culture was treated for 48–72 h with N-acetylglucosamine (NAG) to obtain a pure gametocyte culture at stage IV and V of maturation. Gametocytes were maintained in a nutrient rich environment at 2% gametocytaemia and 5 % haematocrit. The *C.citratus* essential oil was evaluated at three concentrations of 100, 10, and 1μg/ml in complete medium, while dihydroartemisinin (DHA) was evaluated at 10 μM – 1.0 x 10^{-3} μM concentrations. Gametocyte viability was determined by PrestoBlue cell viability assay [17].

Dose-response curves were obtained by plotting percentage parasite or gametocyte survival against the logarithm of the concentration using the GraphPad Prism software package (GraphPad software, Inc, California, USA). IC50 values were calculated using Prism software and graphically by interpolation from these curves. The performance and quality the screening assay was determined by the Z'-factor.

Table 2. In vitro antiplasmodial and gametocytocidal activity against *P. falciparum* (CQS) NF54 and 3D7 strains

Sample	NF54: IC50 (μg/ml)	3D7 IC50 (μg/ml)	Gametocytocidal at (1μg/ml)
Essential oil-1	7.06±0.47	9.22±0.31	65% inhibition
Essential oil-2	5.34±1.01	7.66±0.2	~75% inhibition
Chloroquine	0.005±0.002	0.0093±0.05	ND
Artemisinin	0.002±ND	ND	ND
Dihydroartemisinin (DHA)	ND	ND	11±0.4nM

ND= Not done

The results in Table 2 show the highest IC50 values of 5.34±1.0 and 7.66±0.2 μg/ml against the NF54 and 3D7 strains of *P. falciparum* respectively. Two batches of the essential oil were evaluated. The second batch, which was tested few days after extraction, exhibited higher activity on both strains of the parasite. The essential oil inhibited 80% and 60% growth of NF54 gametocytes when evaluated at 100 and 1μg/ml respectively, suggesting that the IC50 is < 1μg/ml.

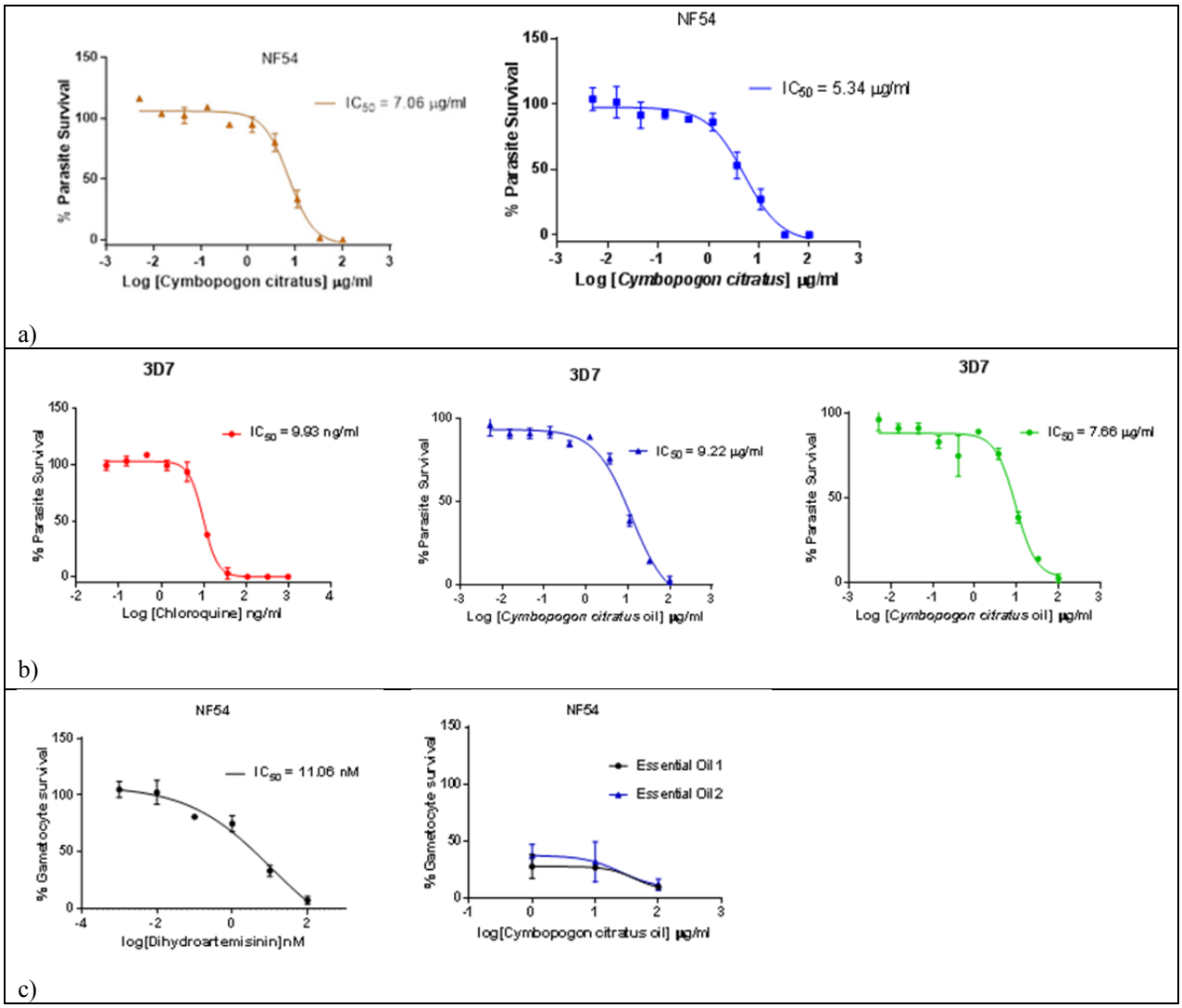

Fig. 2 Antimalarial activity of different EO concentrations of *C. citratus* against a) 3D7 b) NF54 and c) NF54 gametocyte sensitive strains of the malaria parasite, *Plasmodium falciparum.* The *C.citratus* essential oil was evaluated at a starting concentration of 100µg/ml which was then serially diluted 10-fold in complete medium to give six concentrations; with the lowest concentration being to 0.001µg/ml. Chloroquine (CQ) and artesunate were used as the reference drugs in all experiments. Quantitative assessment of antiplasmodial activity was determined via the parasite lactate dehydrogenase (pLDH) assay using a modified method described by Makler (1993). Gametocytogenesis was induced by a combination of nutrient starvation and a drop in haematocrit, using a method adapted from Carter (1993). Results represent the mean of at least two independent experiments in triplicate

Figure 2 illustrate the antimalarial activity of *C citratus* EO. The essential oil exhibited activity with IC50 values of 5.34±1.01 µg/ml and 7.06±0.47 µg/ml against NF54, and 7.66+0.24µg/ml and 9.22+0.31µg/ml against 3D7 strains of *Plasmodium falciparum*. The IC50 values obtained for the reference drugs were 5.0-9.9ng/ml and 2ng/ml for chloroquine and artesunate respectfully (graphs not shown), which are within the reported range for the two drugs. The essential exhibited antiplasmodial activity against the 3D7 strain (b) with IC50 values of 7.66-9.22 µg.ml. Test samples that showed *in vitro* antimalarial activity of ≤ 10 µg/ml were considered active. The essential oil showed gametocytocidal activity at three tested concentrations (c). The lowest concentration of 1µg/ml exhibited 65%-75% inhibition. Generally the second batch of showed higher activity.

4. Conclusions

The CCEO exhibited high antibacterial and anti-yeast properties including against multidrug resistant strains of *S. aureus*, *S. epidermidis, E. coli, K. pneumonia*. The essential oil showed moderate activity against the NF54 and 3D7 strains of *P. falciparum*, and inhibited plasmodium gametocytes growth by 75% at the lowest tested concentration of

1μg/ml. Taking into account the results of this study and the already reported antimicrobial activity of the CCEO against many other pathogenic or spoilage microorganisms, it can be concluded that it meets several features that make it a good candidate for a possible human use and antibacterial and antimalarial agent.

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References

[1] Machado M, Pires P. Dinis, Santos-Rosa AM, Alves M, Salgueiro L, Cavaleiro C, Sousa MC. Monoterpenic aldehydes as potential anti-Leishmania agents: Activity of *Cymbopogon citratus* and citral on *L. infantum*, *L. tropica* and *L. major* *Experimental Parasitology*, 2012, 130 (3) : 223-231

[2] Makler MT1, *et all*., Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. Am J Trop Med Hyg. 1993, 48(6):739-41.

[3] World Health Organization, Antimalaria drug resistance [cited 2012 Jul 9]. Available from: www.who.int/malaria/drug_resistance/overview/en/

[4] M. O. Soares, A. F. Vinha, F. Coutinho e P.C.Pires, "Antimicrobial natural products" In: Microbial pathogens and strategies for combating them: science, technology and education, approved for publication, "Antimicrobial natural products", 946-950, Editor: A. Méndez-Vilas, Publisher Formatex Research Center ISBN (13) Volume 2: 978-84-942134-0-3, December 2013

[5] Miean K and Mohamed S. "Flavonoid (Myricitin, Quercetin, Kaempferol, Luteolin, and Apigenin) Content of Edible Tropical Plants". *Journal of Agricultural and Food Chemistry* 2001; 49.6: 3106-3112.

[6] Faruq M. "TLC technique in the component characterization and quality determination of Bangladeshi lemongrass oil (Cymbopogon citratus (DC) Stapf.)". *Bangladesh Journal of Scientific and Industrial Research.* 1994;29: 27-38.

[7] Torres R and AG R. "Chemical composition of the essential oil of Philippine Cymbopogon citratus (DC) Stapf". *Philippine Journal of Science.* 1996;125: 147-156.

[8] Langeveld WT, Veldhuizen EJ, Burt SA Synergy between essential oil components and antibiotics: a review, Crit Rev Microbiol. 2013;28:.

[9] Sato K, Horibe K, Amano K, Mitusi-Saito M, Hori M, Matsunaga S, Fusetani N, Ozaki H, Karaki H Membrane permeabilization induced by discodermin A, a novel marine bioactive peptide. Toxicon. 2001; 39 (2-3):259-264.

[10] World Health Organization, Top 10 causes of death, Available from http://www.who.int/mediacentre/factsheets/fs310/en/

[11] Nikaido, H. Molecular basis of bacterial outer membrane permeability revisited. Microbiology Molecular Biology Reviews, 2003; 67, 593-656. Available from: http://dx.doi.org/10.1128/MMBR.67.4.593-656.2003

[12] Bauer, A. W., Kirby, W. M., Sherris, J. C., & Turck, M. Antibiotic susceptibility testing by standardized single disc method. American Journal Clinical Pathology, 1996; 44, 493-496. Retrieved from www.ncbi.nlm.nih.gov/pubmed/5325707

[13] Poole, K. Outer membranes and efflux: the path to multidrug resistance in gram-negative bacteria. Current Pharmaceutical Biotechnology, 2002; 3, 77-98. http://dx.doi.org/10.2174/1389201023378454

[14] Saha, S., Savage, P. B., & Bal, M.. Enhancement of the efficacy of erythromycin in multiple antibiotic-resistant gram-negative bacterial pathogens. Journal Applied. Microbiology, 2008; 105, 822-828. http://dx.doi.org/10.1111/j.1365-2672.2008.03820.x

[15] WHO. World malaria report. Geneva: World Health Organization; 2015.

[16] WHO. World Malaria Report. Geneva, Switzerland: World Health Organization; 2013. Google Scholar

[17] Invitrogen, "PrestoBlue cell viability reagent protocol," Product information sheet by Life Technologies, 2012, http://tools.invitrogen.com/content/sfs/manuals/PrestoBlue_Reagent_PIS_15Oct10.pdf.