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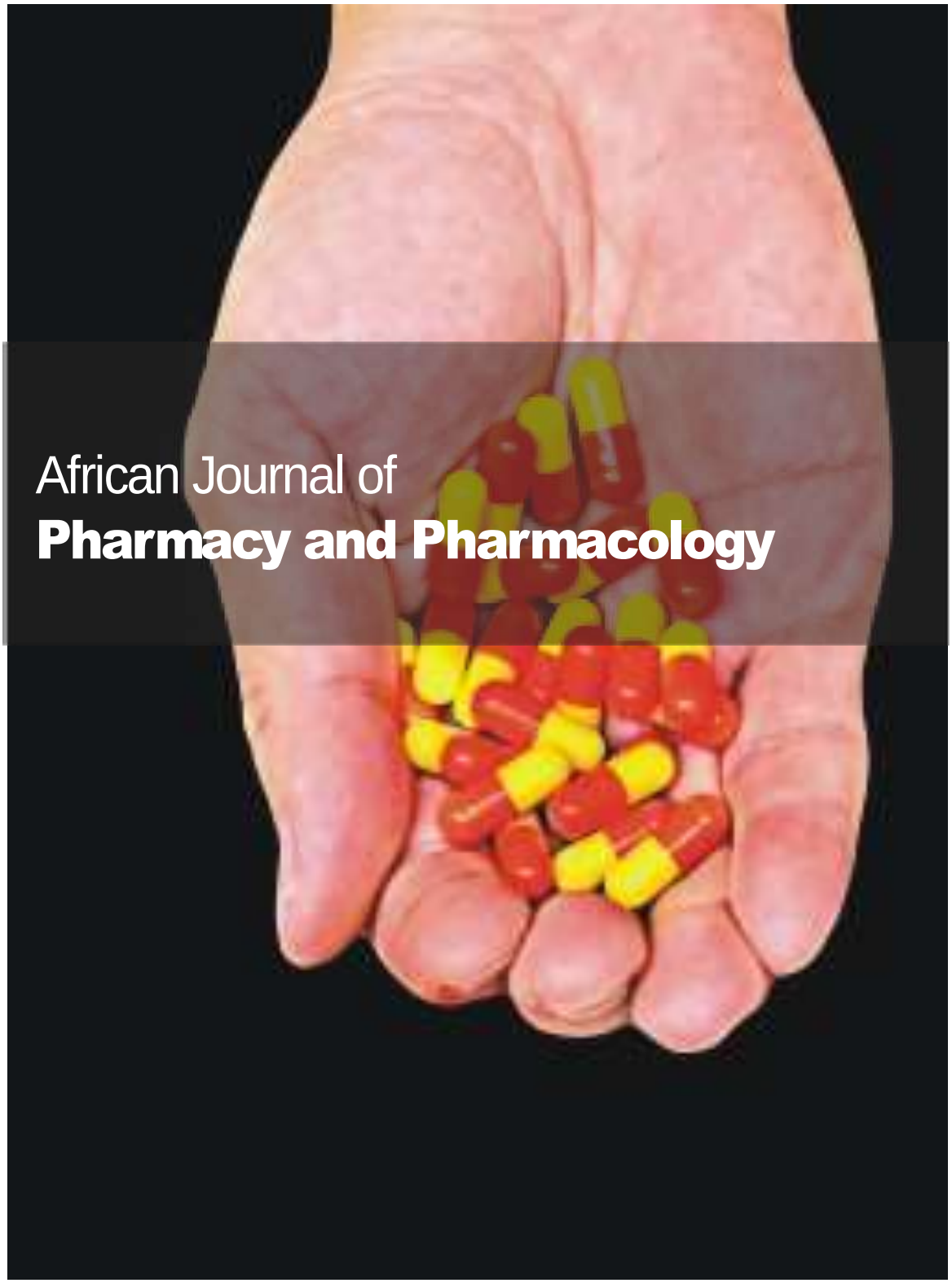

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Full Length Research Paper

Hypoglycemic profile and ameliorative potential of aqueous garlic extract on sperm characteristics in glibenclamide treated diabetic male rats

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This study was carried out to determine the protective effect of aqueous garlic extract on testicular and spermatogenic changes in glibenclamide treated diabetic male rats. Thirty matured male rats were used in this study and were assigned into five groups of six rats each. Diabetes was induced in groups 1, 2, 3, 5 but not induced in group 4. Rats in group 1 were treated with glibenclamide (0.6 mg/kg) daily for 21 days. Rats in group 2 were treated with glibenclamide (0.6 mg/kg) and garlic extracts (*Allium sativum)* at the dose of 300 mg/kg for 21 days. Rats in group 3 were untreated diabetic given distilled water. Rats in group 4 were the normal control, given distilled water. Rats in group 5 were treated with garlic (300 mg/kg) dissolved in distilled water. On day 21 post treatment, there was a significant ($p < 0.05$) decrease in the fasting blood sugar (FBS) level of glibenclamide treated group when compared to garlic treated group and diabetic untreated group but there was a significant ($p < 0.05$) decrease in the FBS level of co-administration of glibenclamide and garlic when compared to glibenclamide alone and garlic alone. There were significant ($p < 0.05$) increases in testicular sperm count, epididymal sperm count and percentage sperm motility of group 2 when compared to groups 1, 3 and 5. From the above result, co-administration of glibenclamide and garlic extract produced optimum hypoglycemic activity and protective effect on testicular sperm and epididymal sperm counts, and percentage sperm motility in diabetic male rats.

Key words: Diabetes, fasting blood sugar (FBS), garlic, glibenclamide, sperm count, sperm motility.

INTRODUCTION

Diabetes is a metabolic disease characterized by high blood sugar (FBS) levels, either, because the body does not produce enough insulin or because the body cells do not properly respond to insulin that is produced (Rother,

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2007). Insulin is a hormone produced by the beta cells of the pancreas. Its primary function is to transport glucose to cells.If this function cannot be met as in diabetic cases glucose accumulates in the blood leading to many complications (Rother, 2007). Diabetes mellitus has classical signs of frequent urination (polynuria), increased thirst (polydypsia), increased hunger (polyphagia) and weight loss. There are two main types of diabetes, which includes Type 1 and Type 2 diabetes mellitus. Type 1 diabetes mellitus which results from the body's failure to produce enough insulin due to loss of insulin producing beta cells of the pancreas. This form is also referred to as "insulin-dependent diabetes mellitus" (IDDM) or "juvenile onset diabetes". Majority of this Type 1 is of immune mediated nature where the beta cells loss is due to a T-cell mediated autoimmune attack (Thomas and Philipson, 2015).

Type 2 diabetes mellitus, begins with insulin resistance. A condition in which cells fail to respond to insulin properly; as the disease progresses a lack of insulin may also develop (James and Luke, 2009). This form is also referred to as "non-insulin dependent diabetes mellitus" (NIDDM) or "adult-onset diabetes".

Diabetes mellitus is a disease of both humans and animals. In animals, diabetes mellitus is more common in dogs and cats (Baker et al., 1983) and laboratory rodents (Thomas et al., 1997). The disease has also been reported in horses, cattle, pigs, sheep and guinea pigs (Thomas et al., 1997).

Male reproductive organ alterations have been widely reported in both man and animals with diabetes. About 90% of diabetic males have changes in testicular and spermatogenic parameters, including decreases in testicular weight, percentage sperm motility, testicular and epididymal sperm reserves due to testicular dysfunction associated with sustained hyperglycemia (AbuAbeeleh et al., 1984; Orth et al., 1979; Paz and Homonnai, 1979; Hurtado de Catalfo et al., 1998). Alloxan induced diabetic male rats exhibit decreases in testicular parameters after 2 weeks of induction of diabetes (Sanguinetti et al., 1995). Zhao et al. (2011) demonstrated that oxidative stress induced by hyperglycemia is the major cause of diabetic testicular damage as oxidative stress is increased in diabetes, due to the overproduction of reactive oxygen species (ROS) and decreased efficiency of antioxidant defences (Ballester et al., 2004). The statement of the problem include: (1) There is no synthetic agent that perfectly improves FBS levels and spermatogenic alterations associated with diabetes in males thus, the need to evaluate concurrent use of synthetic and herbal remedies in the management of diabetes for possible physiological benefits. (2) Growing evidence has shown that diabetes mellitus has negative effect on male reproduction hence the search for a protective agent has become an area of active research. Therefore, the aim of this study was to investigate the hypoglycemic activity and protective effect of aqueous garlic extract on sperm characteristics in glibenclamide treated diabetic male rats.

MATERIALS AND METHODS

Animals

Thirty mature male albino Wistar rats (*Rattus norvergicus*) of 12 weeks old weighing between 180 to 200 g were used for the study. They were sourced from Laboratory Animal Unit of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. They were housed in clean cages at room temperature (37°C) and were fed *ad libitum* on a standard commercial grower feed (Vital feeds, GCOM Nig. Ltd) and clean drinking water. The animals were maintained under a cycle of 12 hof light and 12 h of darkness daily throughout the period of experiment. The ethical rules governing the conduct of experiments with life animals were strictly observed as stipulated by Ward and Elsea (1997) and Zimmermann (1983).

Ethical approval

Guidelines for the care and use of experimental animals complied with the University animal welfare guidelines and policies and were approved by the ethical committee of University of Nigeria, Nsukka (approval ref no. 20170704)

Experimental design

Thirty mature male albino Wistar rats (*R. norvergicus*) of 12 weeks old weighing between 180 to 200 g were used for the study. They were fasted overnight and their blood glucose levels (normoglycaemic levels) were determined using Accu-check glucometer (Roche, Germany). Diabetes was then induced in groups 1, 2, 3 and 5, but not in group 4 and treated as follows daily for 21 days: Group 1: rats in this group were diabetic male rats treated with glibenclamide (0.6 mg/kg) dissolved in distilled water; group 2: rats in this group were diabetic male rats treated with glibenclamide (0.6 mg/kg) and garlic extract (300 mg/kg) dissolved in distilled water; group 3: rats in this group were diabetic untreated rats given distilled water; group 4: rats in this group were the normal control; group 5: rats in this group were diabetic male rats treated with only garlic extract (300 mg/kg) dissolved in distilled water.

Preparation and extraction of plant materials

The plant was acquired from Orba market. Fresh garlic *(Allium sativum*) was dried. The fresh garlic was crushed using mortar and pestle into pasty materials. Cold extraction of the pasty garlic material was performed using distilled water. The extract was filtered using Wattman no 1 filter paper. The filtrate was stored in the refrigerator before it was finally used.

Induction of diabetes

The basal fasting blood sugar of each animal was established using Accu-check active glucose test strip. Diabetesc was induced in rats

Table 1. Hypoglycemic Study.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5
FBS day 0 (mg/dl)	160.33±10.45 [a]	165.66±5.55 [a]	164.66±6.96 [a]	87.00±7.02 [a]	156.67±7.69 [a]
FBS day 7 (mg/dl)	89.33 ±5.21 [ab]	79.09±0.58 [b]	169.37 ±6.36 [c]	86.67±6.77 [ab]	125.33 ±2.60 [d]
FBS day 14 (mg/dl)	91.00±3.79 [a]	73.33±1.45 [b]	169.34±6.36 [c]	86.67±6.56 [ab]	119.67±1.45 [d]
FBS day 21 (mg/dl)	92.33±3.84[a]	72.33±1.45[b]	186.67±6.96[c]	87.33±6.01 [a]	118.67±1.33 [d]

[a,b,c,d]Different superscripts along the same column express significant ($p < 0.05$). FBS, Fasting blood sugar.

using the method described by Venugopal et al. (1998). Diabetes was induced in overnight fasted male rats by a single intraperitoneal injection of freshly prepared solution of alloxan-monohydrate (160 mg/kg body weight). The fasting blood sugar levels of the rats were determined daily until diabetes was confirmed. Rats with blood sugar levels above 126 mg/dl were considered diabetic (Iwalewa et al., 2008).

Determination of testicular weights

At the end of the 21days of treatment (end of study period), the rats in each treatment group as well as the control were euthanized using euthethal (180 mg/kg) intraperitoneally. The testis from each rat was carefully dissected out, trimmed free of extraneous tissues and weighed with a weighing balance.

Determination of gross percentage sperm motility

This was done using the method described by Hotchkiss et al. (1952). A drop of sperm sample from the epididymis was placed on a clean slide, covered with a cover slip and viewed under the microscope at X40 magnification (Olympus xxx). Then, motile sperm cells were determined per 100 sperms seen. Two counters were used: one for a total of 100 sperms, the second for motile sperms.

Determination of epididymal sperm reserve

This was done using the method described by Amann (1986). The left and right caudal epididymis were crushed with ceramic mortar and pestle, 10 ml of normal saline added to each and filtered through a nylon sieve. Each filtrate (0.1 ml) was further diluted with 0.9 ml of white blood cells diluting fluid in a test tube and 20 µl of each diluted sperm solution was used to charge the improved Neubauer chamber (Germany) and viewed under the microscope at ×40 (Olympus xxx). The number of sperm cells were counted on the four corner squares and estimated in 169 squares. As these sperm cells were counted in 2.5×10^{-4} ml, which is the volume of the Neubauer chamber, the number of sperm cells counted in each sample was multiplied by 10 to get the total number of sperm cells in 10 mls of normal saline.

Determination of the testicular sperm reserve

This was done with the two testicles using the method of Amann, 1986 already described.

RESULTS

Hypoglycemic study

The anti-hyperglycemic study reveals that on day 0, there was no significant difference in fasting blood glucose level among the diabetes induced groups. On day 21 post treatment, there was a significant decrease in the FBS level of glibenclamide treated group (group 1) when compared to garlic treated group (group 5) and diabetic control (group 3) but there was a significant decrease in the FBS level of co-administration of glibenclamide and garlic (group 2) when compared to glibenclamide alone (92.33±3.84) and garlic alone (Table 1).

Determination of testicular weight, epididymal and testicular sperm reserves and percentage sperm motility

There were significant ($p < 0.05$) increases in testicular sperm count, epididymal sperm count (Table 2) and percentage sperm motility (Figure 1) of group 2 when compared to group 1.

DISCUSSION

The result of anti-hyperglycemic study revealed that on day 0, there was no significant difference in fasting blood glucose level among the diabetes induced groups. This showed that diabetes was confirmed on day 0 in induced groups. On day 21 post treatment, there was a significant ($p < 0.05$) decrease in the FBS level of glibenclamide treated group when compared to garlic treated group and diabetic untreated group (186.67±6.96) but there was a significant ($p < 0.05$) decrease in the FBS level of co-administration of glibenclamide and garlic when compared to glibenclamide alone and garlic alone. This significant higher glycemic control of co-administration of glibenclamide and garlic may be due to synergistic drug-dietary interactions. The observed decreases in testicular weight, testicular sperm reserve,

Table 2. Testicular weight and sperm counts.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5
TW(g)	1.20±0.06[a]	1.37±0.23[a]	0.83±0.03[b]	1.57±0.12[a]	1.17±0.03[c]
TSR(10^6)	11.33±0.35[a]	17.43±0.64[b]	8.3±0.26[c]	20.23±0.46[d]	11.0±0.40[a]
ESR(10^6)	13.90±0.06[a]	19.97±0.12[b]	8.7±0.35[c]	22.40±0.38[d]	13.63±0.32[a]

[a,b,c,d]-Different superscripts along the same column express significant ($p < 0.05$). TW, testicular weight; TSR, testicular sperm reserve; ESR, epididymal sperm reserve.

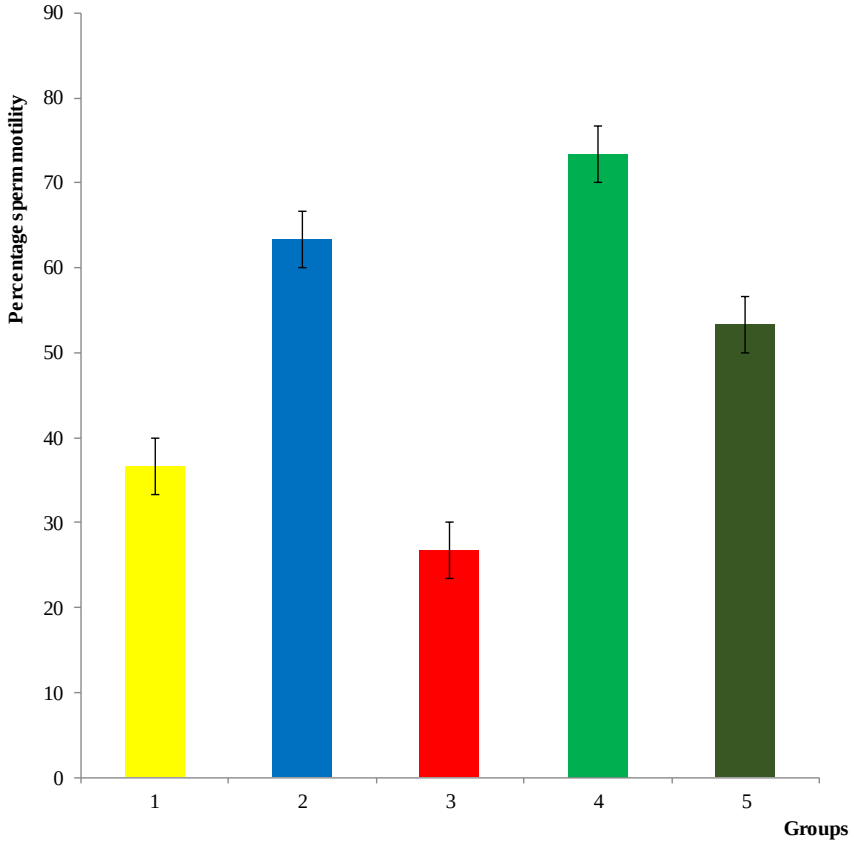

Figure 1. Mean percentage sperm motility.

epididymal sperm reserve and percentage sperm motility in the untreated diabetic male rats in this study agreed with those of earlier reports (Abuabeeleh et al., 1984; Hurtado de Catlfo et al., 1998; Anderson and Thliveris, 1986; Rossi and Aeschlimann, 1982; Orth et al., 1979; Paz and Homonnai, 1979).The significant ($p<0.05$) increases in testicular sperm count, epididymal sperm count and percentage sperm motility of group 2 when compared to group 1- testicular sperm count, epididymal sperm count and percentage sperm motility may be due to the antioxidant activity of aqueous garlic extract (Borek et al., 2001).

Oxidative stress and lipid peroxidation is known to play a major role in the etiology of the defective reduction of sperm count and decline in cell quality which results in insufficient numbers of viable spermatozoa and infertility in diabetic males (Boonsorn et al., 2010).

Garlic contains antioxidant, selenium which scavenges reactive oxygen species and inhibits lipid peroxides in the body (Borek et al., 2001; Chang et al., 1980). In this study, garlic improved sperm count and cell quality in glibenclamide treated diabetic male rats.

Conclusion

In conclusion, co-administration of glibenclamide and aqueous garlic extract produced optimum hypoglycemic activity and protective potential on spermatogenic changes in alloxan induced diabetic male rats. Since garlic is available in our environment, its consumption in addition to glibenclamide (antihyperglycemic agent) will be an effective way to reduce the toxic effects of diabetes on the reproductive system of males, and by so doing, will help in improving fertility in diabetic males.

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CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

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Full Length Research Paper

Plasmodium falciparum drug resistance gene status in the Horn of Africa: A systematic review

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Antimalarial drug resistance monitoring is the key factor in malaria control policy for early detection and subsequent prevention of drug resistance spread. This review was performed to collate all available data of *P. falciparum* resistant genes in the Horn of Africa as a baseline for future appraisal of the regional malaria control policy. The search of this review was performed in January 2018 using the scientific databases Pub Med and Google Scholar. The search terms used included: *Plasmodium falciparum* AND drug resistance genes OR molecular marks AND Somalia OR Ethiopia OR Eritrea OR Djibouti. The majority of studies (9 of 18 studies, 50%) examined *pfdhfr*, *pfcrt* and *pfmdr 1* genes. Eight (44%), 4 (22%), and 2 (11%) studies analyzed *pfdhps*, *pfk 13* and *pfatp 6 gene*s, respectively. The *Pfcytbc1* associated with atovaquone resistance is the only gene with no mutation detected. High frequencies of *pfdhfr* and *pfdhps* mutations were reported with an association to treatment failure after the artemisinin-based combination therapy (ACT) - artesunate + sulfadoxine/pyrimethamine. The aminoquinoline resistance genes such as *pfmdr1,* and *pfatp 6* were only reported with low frequency. The 76T mutation of *pfcrt* ranged from 4 to 100%, while *pfmdr1* mutations at codon 86 and 184 varied depending on geographical locations. The 402V and 431K mutations of *pfatp 6* were found highly prevalent at 93 % and 58 % in Southwestern Ethiopia, respectively. The *pfk13* gene mutation at codon 622I was 2.4%, with an association to artemether-lumefantrine efficacy and delay of parasite clearance on day 3.

Key words: *Plasmodium falciparum*, Drug resistance gene, Molecular marker, Somalia, Ethiopia, Eritrea, and Djibouti.

INTRODUCTION

Malaria is an arthropod-borne disease with a major impact on the world's human population health. In 2017, 90% of malaria cases were reported from Africa, 7% from Southeast Asia and 2% from the Eastern Mediterranean

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Figure 1. Map of Horn of Africa (white area).

Region (WHO, 2017). The Horn of Africa consists of four countries, *i.e.,* Djibouti, Ethiopia, Eritrea, and Somalia (Figure 1). In 2015, about 1.9 million malaria cases were reported from Ethiopia, while 20,963 cases were reported from Somalia and 19,372 from Eritrea (WHO, 2016). Malaria exists as hypo-endemic in Djibouti, varies from hypo- to mesoendemic in Somalia, endemic or epidemic in Eritrea, while in Ethiopia, it seasonally fluctuates (WHO, 2007). The influx of refugees is exacerbating the situation of malaria in the Horn of Africa. Drug resistance is the major problem confronting malaria control in the region (Heuchert et al., 2015). *Plasmodium falciparum* has developed resistance to multiple drugs including chloroquine (CQ), sulfadoxine-pyrimethamine (SP) and recently, artemisinin (ART) resistance strains have emerged at the Thai-Cambodian border (Golassa et al., 2014; Heuchert et al., 2015). The situation has also jeopardized the use of safe, cheap and affordable drugs in poor countries where control of malaria has been inefficient (Gebru et al., 2005). The spread of antimalarial drug resistance by human migration from one country to another has to be regarded and neighboring countries need to enforce regional instead of national programs to avoid genetic drug resistance spreading among them (Bridges et al., 2009; WHO, 2007). CQ was the first antimalarial drug used in endemic areas and resistance was reported from Thailand in the late 1950's. Since then, it spread out quickly to South Asia, then to Africa in 1974, and finally reaching East Africa in 1980's (Mekonnen et al., 2014). The dispersion of CQ resistance was a paramount factor in the failure of the first malaria control and elimination efforts in the mid of 20th century (Takala-Harrison and Laufer, 2015). Ethiopia was the first country in the Horn of Africa to document CQ resistance in 1985. At that time, the resistant isolates in Ethiopia were reported from areas bordering Kenya, Somalia, and Sudan, while the central part was apparently free from resistant strains (Alene and Bennett, 1996). In 1998, Ethiopia switched to SP as the first-line treatment for uncomplicated *P. falciparum* malaria. Unfortunately, within a short period, *P. falciparum* developed resistance to SP (Lo et al., 2017; Mekonnen et al., 2014; Schunk et al., 2006). Retrospective analysis confirmed that pyrimethamine resistant strains were present in sub-Saharan Africa before implementation of SP (Alifrangis et al., 2014). In 2004, an artemisinin combination therapy (ACT) called artemether-lumefantrine (AL) became Ethiopia's first-line treatment for uncomplicated falciparum malaria (Heuchert et al., 2015; Mekonnen et al., 2014). Eritrea, which was previously using SP, shifted to artesunate-amodiaquine (AS-AQ) in 2007(Menegon et al., 2016) and Somalia adopted AS plus SP (AS-SP) as

first-line therapy in 2007 (Warsame et al., 2015). In 2016, AL became the first line drug in Somalia's national plan for uncomplicated *P. falciparum* malaria (Warsame et al., 2017).

Drug resistance monitoring is the key factor in malaria control policy for early detection and subsequent prevention of the spread of drug resistance (Takala-Harrison and Laufer, 2015). The three basic approaches used to detect antimalarial drug resistance are *in vivo* test, *in vitro* test, and analysis of molecular markers. *In vitro* test allows investigation of parasite susceptibility to antimalarial drugs in artificial culture media without the influence of host's factors. Numerous tests are available and different drugs can be simultaneously assessed with a single isolate. Nevertheless, the requirement of live parasites and highly skilled personnel are the drawbacks of this approach Bloland et al, 2003; WHO, 2010). *In vivo* test is the gold standard for guiding malaria control policy since the test accounts for the influence of parasite, host, and drug factors. However, this approach is expensive and difficult to apply in clinical settings in most endemic areas due to limited supplies and equipment, long patient's follow-up for 28 or 48 days, repeated biological samples, and requirement of experienced staff (Bloland et al., 2003). An analysis of validated molecular marker of antimalarial drug resistance is an alternative approach for detecting antimalarial drug resistance associated with previously documented mutations. These markers are useful for monitoring of antimalarial drug resistance and obtaining a picture of the situation at a particular time (Mvumbi et al., 2015). Single or multiple nucleotide mutations in diverse *P. falciparum* gene alleles are related to a wide range of antimalarial drugs resistance. The prominent genes identified up to now include *P. falciparum dihydropteroate* synthase gene (*pfdhps*), *P. falciparum* dihydrofolate reductase gene (*pfdhfr*), *P. falciparum* multidrug resistance 1 gene (*pfmdr 1*), *P. falciparum* chloroquine resistance transporter gene (*pfcrt*), *P. falciparum* Klech 13 propeller gene (*pfk13*), *P. falciparum* adenosine triphosphatase 6 (*pfatp*6), and *P. falciparum* cytochrome b1 (*pfcytb1*).

The decline in SP sensitivity is a blend of single-nucleotide polymorphisms (SNPs) in two different genes encoding enzymes involved in the synthesis of the folate cofactor which is essential for parasite growth and survival. Mutation of *the pfdhps gene* is known to be related to sulfadoxine resistance, while that of the *pfdhfr gene* is associated with pyrimethamine resistance (Hailemeskel et al., 2013; Tessema et al., 2015). Both are often investigated in combination because of their correlation to SP resistance. The resistance level depends on the number of point mutations in these genes. Thus, multiple changes in the genes are accountable for SP treatment failure in *P. falciparum* malaria (Gebru et al., 2005). Mutations related to SP treatment failure have been identified at codons 613, 540, 437, and 436 in the *pfdhps* gene and at codons 164, 108, 59, 51, and 16 in the *pfdhfr* gene (Gebru et al., 2005; Warsame et al., 2015). The presence of the *pfdhps* double mutant A437G and K540E along with the *pfdhfr* triple mutant N51I, C59R, and S108N, known as a quintuple mutant, strongly correlates with SP resistance (Warsame et al., 2017).

The mutation at K76T of the *pfcrt* gene is strongly related to CQ resistance (Golassa et al., 2014) and is also proposed to affect the susceptibility of *P. falciparum* to ART, quinine, and amodiaquine (Heuchert et al., 2015). *Pfmdr 1* mutation has been associated with resistance of *P. falciparum* to several antimalarial drugs including CQ, mefloquine, halofantrine, quinine, and ART (Eshetu et al., 2010; Heuchert et al., 2015). Mutations in both *pfcrt* and *pfmdr1* alleles are associated with CQ resistance, although mutation of the *pfcrt* gene is a stronger indicator for CQ resistance (Golassa et al., 2014; Mekonnen et al., 2014). The *pfcrt* K76T together with other *pfcrt* mutations(C72S, M74I, N75E, A220S, I356K, and R371I) is often used as markers of CQ resistance but the clinical association has not been fully validated (Wurtz et al., 2012). The *pfcrt* CVMNK wild-type can mutate either into the CVIET or CVMNT haplotype, both of which are related to the regional evolution of CQ resistance (Heuchert et al., 2015; Menegon et al., 2016). The *pfmdr1* mutant allele 86Y has been associated with a decline in CQ sensitivity in areas where the prevalence of the parasite is low to moderate (Wurtz et al., 2012). The return of CQ sensitivity was reported after long time abandonment of CQ use in several endemic countries(Mvumbi et al., 2015) and this proposes the possibility of re-introduction of CQ for treatment of *P. falciparum* malaria in the future (Golassa et al., 2014; Golassa et al., 2015). Nevertheless, the decision will require close regional monitoring because studies of CQ resistance after discontinuation of drug pressure are considerably inconsistent among parasitic populations (Golassa et al., 2015). In Ethiopia for instance, the prevalence of *P. falciparum* clinical isolates carrying CQ resistant *pfcrt* K76T varied from 16% (Mekonnen et al., 2014) to 100% (Golassa et al., 2015).

The recent emergence of *P. falciparum* ART resistance strains in Greater Mekong Sub-region (GMS) represents a challenge to the efficacy of ART and also will postpone the goal of malaria elimination by 2030 in the region (WHO, 2015). The dissemination of these strains to Africa, where the majority of deaths due to malaria occurs, will have catastrophic results (Bayih et al., 2016). Mutation of *pfatp* 6 (encodes *P. falciparum* SERCA-type ATPase 6) has initially been linked to resistance of *P. falciparum* to artemether (Heuchert et al., 2015). Currently, the *pfk13* gene mutation, firstly reported at the Thai-Cambodian border (Heuchert et al., 2015), is suggested as the accurate marker of ART resistance. The *pfcyt b1* mutation at codon 268 was associated with

delay recrudescence of parasites after atovaquone-proguanil therapy (Musset et al., 2006; Sutherland et al., 2008; Wichmann et al., 2004).

In the present review, a qualitative analysis was systematically conducted to obtain relevant data available on *P. falciparum* molecular markers of antimalarial resistance (*pfdhps*, *pfdhfr*, *pfcrt, pfmdr 1*, *pfk13, pfatp*6, and *pfcyt b1*) in the Horn of Africa as a baseline for future assessments. Knowing how malaria parasites disseminate along with monitoring the prevalence of drug-resistant markers in the high-risk endemic areas is imperative for antimalarial policymaking.

MATERIALS AND METHODS

Search strategy

The search of relevant research articles was performed in January 2018 through the two scientific databases, namely, PubMed and Google Scholar. The following medical subject heading (MeSH) terms were used: *Plasmodium falciparum* AND drug resistance genes OR molecular marks AND Somalia OR Ethiopia OR Eritrea OR Djibouti. The references of retrieved articles were searched to obtain additional relevant articles. The inclusion criteria were (a) studies related to *P. falciparum* malaria in the Horn of Africa, (b) studies that analyzed *P. falciparum* resistance molecular markers, and (c) studies presented as original research articles. The exclusion criteria were: (a) studies that only assessed the clinical efficacy of antimalarial drugs, (b) non-molecular studies, (c) studies conducted outside the Horn of Africa (d) studies without full-text articles available online, (e) review articles or case reports, or (f) articles in language other than English. Title and abstract of each article were initially screened, followed by a full-text assessment.

Data extraction

The following information was extracted from the eligible articles: first author, publication year, *P. falciparum* molecular markers (type and frequency), techniques used to detect these markers, sample size, year of sample collection, and geographic location of the study. The frequencies of mutant alleles for *pfdhps, pfdhfr, pfcrt, pfmdr1, pfk13, pfatp 6,* and *pfcyt b1* genes were then extracted. Screening, selection, and extraction of data were performed by two independent researchers. When disagreement arose at any stage of the process, a higher professional person was consulted for a final decision.

RESULTS

Study selection

A total of 81 articles were obtained from Google Scholar and PubMed databases. Six duplicate articles were removed using EndNote X7; 39 articles were excluded after screening their titles and abstracts against the inclusion criteria; and 19 articles were excluded from the review after full-text assessment due to following reasons: *in vivo* studies, studies conducted outside the Horn of Africa, studies focusing on treatment adherence, studies with repeated sample analysis, and studies with unsuitable findings. Eighteen eligible articles were therefore selected for this review (Figure 2). Seven *P. falciparum* genes were investigated in these articles: *pfdhps*, *pfdhfr*, *pfcrt, pfmdr1*, *pfk13, pfatp*6, and *pfcyt b1*. The majority of studies (9 of 18 studies, 50%) examined *pfdhfr, pfcrt*, and *pfmdr1*genes. Eight (44%), 4 (22%), and 2 (11%) studies analyzed *pfdhps*, *pfk13* and *pfatp 6 gene*, respectively (Table 1).

Antimalarial drug resistance genes

Pfdhps and pfdhfr

Of the 16 studies, 8 studies analyzed *pfdhfr* and *pfdhps* together, while 1 study analyzed only *pfdhfr* along with other genes. Mutation at codons 108N, 59R and 51I of the *pfdhfr* and at codons 540E and 437G of the *pfdhps* were reported. Eight studies reported the frequency of either *pfdhps* double mutants (K540E andA437G) or the *pfdhfr* triple mutant (S108N, C59R, and N51I) or both (Table 2).

Pfcrt

Of the 18 studies, 9 studies investigated K76T mutation, of which 3 studies, also investigated C72S mutation with 3.6% prevalence reported in 1 study. The SVMNK and CVIET at positions72-76 of the *pfcrt* gene were reported in 4 studies, while the SVMNT haplotype was reported in 2 studies. A high-frequency rate of CVIET haplotype was reported in 3 studies (Table 3).

Pfmdr1

Nine studies examined *pfmdr 1*mutation at codons 86, 184, 1034, 1042, and 1046. Of these, the N86Y was analyzed in the 9 studies and mutations were detected at codons 86, 184, and 1042. The 1042Y was the least frequently detected mutation (17% reported in one study) (Table 4).

Pfk 13

The analysis of gene mutation was performed in 4 studies; 2 studies reported mutation at codons N531I (4.0%) and R622T (2.5%), while the other 2 studies reported wild-type alleles (Table 5).

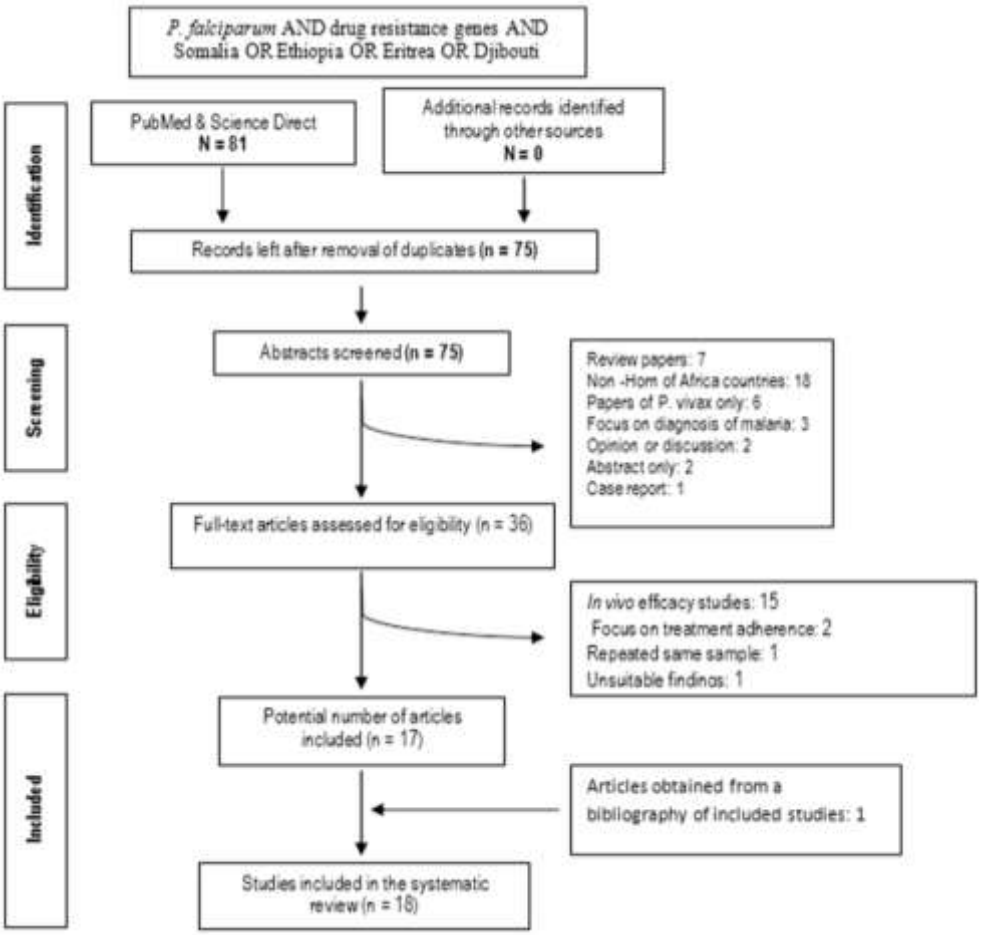

Figure 2. Flow chart showing article selection process.

Pfatp 6

Of the 18 studies, 2 investigated *Pfa*tp6 gene at different codons and a total of 12 synonymous and non-synonymous mutations (E237A, L263L, R682R, K766K, K767E, and K767R) were reported; 6 of these had not been previously depicted as resistance alleles (Table 6).

Pfcyt b1

Two (both from Ethiopia) out of 18 studies analyzed *pfcytbc1* gene mutation and none of them showed gene variation from the reference strain.

Association of molecular markers and clinical efficacy

Association of molecular markers and clinical efficacy of antimalarials was evaluated in 3 studies (2 from Somalia and 1 from Ethiopia). The clinical efficacy of AS-SP and AL were investigated with 28 days follow-up period. The PCR-corrected treatment failure was observed in 33 and 3 patients following treatment with AS-SP and AL, respectively. The cumulative efficacy of AS-SP and AL were 78 and 98%, respectively (Table 7).

The association between AS-SP clinical efficacy and the molecular markers *pfdhps* and *pfdhfr* were reported in 2015 and 2017 in Somalia (Warsame et al., 2017; Warsame et al., 2015), with failure rates of approximately 22.2 and 12.1%, respectively. For the 2015 study, the quadruple 51I/108N+437G/540E and quintuple 51I/59R/108N+437G/540E mutations of *pfdhfr+pfdhps* were shown to be associated with high risk of treatment failure with odd ratios of 5.5 and 3.5 respectively. The double 437G/540E mutation of *pfdhps* was associated with the highest risk of treatment failure (OR =22.4). On the other hand, two years later, all parasites isolated from patients with treatment failure after AS-SP carried the quintuple mutation 51I/108N + 437G/540E/581G.

The efficacy of AL was investigated in two studies in

Table 1. Characteristics of the 18 studies included in the current systematic review.

References	Study location	Study year	Sample size *	Genotyping Technique	Analyzed genes
(Heuchert et al., 2015)	Southwest Ethiopia	2013	177	Nested PCR- RFLP, Real-time PCR, Sequencing	*pfcrt, pfmdr, pfatp 6, Pfk13*
(Gebru et al., 2005)	Southwest Ethiopia	ND	124	Nested PCR, Sequencing	*pfdhps, pfdhfr*
(Golassa et al., 2014)	South-central Oromia	2012	99	Nested PCR-RFLP, Sequencing	*pfcrt-CVIET*
(Mekonnen et al., 2014)	Southeast Ethiopia	2011	195	Nested PCR, Sequencing	*pfmdr1, pfcrt*
(Lo et al., 2017)	South Ethiopia Eastern Ethiopia North Ethiopia	2014	226	Nested PCR, Real-time PCR, Sequencing	*pfcrt,pfmdr1, pfdhps, pfdhfr, pfk13*
(Schunk et al., 2006)	Southern Ethiopia	2004	69	Nested PCR-RFLP	*pfdhs,pfdhfr, pfmdr 1*
(Menegon et al., 2016)	Eritrea	2013-14	180	PCR, Sequencing	*pfcrt, pfmdr-1, pfk13*
(Warsame et al., 2015)	Somalia	2011	283	PCR, Sequencing	*pfdhfr/pfdhps*
(Warsame et al., 2017)	Somalia	2013-15	90	Nested PCR, Sequencing	*pfdhps, pfdhfr*
(Golassa et al., 2015)	East Shoa, Gambella and West Arsi of Ethiopia	2012-2014	152	PCR–RFLP, Sequencing	*pfcrt, pfmdr-1*
(Bayih et al., 2016)	Northwest Ethiopia	2013-2014	148	Nested PCR, Sequencing	*pfk 13*
(Hailemeskel et al., 2013)	Northwest Ethiopia	2005 2008	78 87	Nested PCR-Dot plot hybridization	*pfdhps,pfdhfr*
(Tessema et al., 2015)	Northwest Ethiopia	2005 2007-08	80 79	Nested PCR-Dot plot hybridization	*pfdhps,pfdhfr*
(Eshetu et al., 2010)	Southwest Ethiopia	2006	97	Nested PCR-RFLP	*pfdhfr, pfmdr1,pfatp 6,pfcytbc1,*
(Tajebe et al., 2015)	Gondar-Ethiopia	2014	133		*pfcrt, pfmdr1*
(Mula et al., 2011)	Southern Ethiopia	2007-09	66	Nested PCR-RFLP	*pfdhps,pfdhfr, pfmdr1*
(Rogier et al., 2005)	Djibouti	1998-02	139	Nested PCR, Sequencing	*pfcrt*
(Gebru et al., 2006)	Southwest Ethiopia	ND	141	PCR, Sequencing	*pfcytbc1*

*****Considering falciparum malaria samples only and samples collecting from the Horn of Africa.
ND: no data available, **PCR:** Polymerase chain reaction**, RFLP:** Restrict fragment length polymorphism.

Ethiopia and Somalia (Bayih et al., 2016; Warsame et al., 2017)**.** In the first study (Bayih et al., 2016), the association between AL clinical efficacy and the *pfk13* mutation was found. The 622I mutation was found in 2.4% of all samples with association with the delay in parasite clearance on day 3 (all mutant parasites isolated from patients who parasitemic at day 3). In the second study (Warsame et al., 2017), treatment failure rate of AL was <6%; however, no molecular marker was investigated.

DISCUSSION

A total of 18 *P. falciparum* eligible molecular studies conducted in the Horn of Africa were included in the analysis. These studies were conducted 16 years after the completion of malaria genome sequencing in 2002 (Carlton et al., 2004). Three studies particularly addressed the correlation between results of the *in vivo* assessment and molecular markers of antimalarial drug resistance (Bayih et al., 2016; Warsame et al., 2017;

Table 2. The *pfdhps/pfdhfr* mutant allele frequencies reported in the articles included in the analysis.

References	Study location	Study year	Sample size*	*Pfdhfr*mutation (%)				*pfdhps*mutation (%)		*pfdhps/ pfdhfr* Quintuple mutation (%)
				108	59	51	Triple mutation	540	437	
(Gebru et al., 2005)	Southwestern Ethiopia	ND	124	100	54.0	100	54.0	100	100	54.0
(Lo et al., 2017)	North Ethiopia	2014	65	52.3	23.2	52.3	ND	ND	15.6	ND
	South Ethiopia		62	79.0	66.0	77.4	ND	ND	15.0	ND
	East Ethiopia		72	100	59.7	100	ND	ND	14.0	ND
(Schunk et al., 2006)	Southern Ethiopia	2004	69	100	90.0	97.0	87.0	97.0	97.0	86.0
(Warsame et al., 2015)	Jamame, Somalia	2011	88	100	52.3	79.6	31.8	59.3	61.6	24.4
	Janale, Somalia		79	100	35.4	86.1	27.9	7.6	7.6	5.1
	Jowhar, Somalia		102	100	54.9	94.1	49.0	41.2	41.2	15.7
(Warsame et al., 2017)	Bosaso, Somalia	2013-2015	90	100	22.0	100	22.2	ND	ND	11.1
(Hailemeskel et al., 2013)	Northwest Ethiopia	2005	61	98.4	80.3	98.4	78.6	75.4	95.1	60.6
		2008	78	98.7	56.4	98.7	56.4	64.1	97.4	37.2
(Tessema et al., 2015)	Northwestern Ethiopia	2005	63	92.1	82.5	61.9	51.0	80.0	75.4	40.7
		2007-2008	63	74.6	55.6	25.4	16.0	63.1	67.7	13.6
(Eshetu et al., 2010)	Southwest	2006	97	100	87.6	98.8	83.3	ND	ND	_ND
(Mula et al., 2011)	Southern Ethiopia	2007-09	66	100	90.8	97.4	90.8	68.4	92.1	82.9

ND: no data available, *pfdhfr: P.falciparum* dihydrofolate reductase, *pfdhps*: *P. falciparum* dihydropteroate synthase.

Warsame et al., 2015). The development and spread of antimalarial drug resistance is attributed to several factors including inappropriate dosing, poor treatment practice, substandard or counterfeit drugs, the use of ART monotherapy, and the ability of the parasite to modify its genome at any time (Bloland et al., 2003). Even though the usual treatment for malaria has followed the World Health Organization (WHO)'s recommendation Bloland et al., 2003; WHO, 2010), antimalarial drug resistance has continuously been emerging. In this systematic review, the frequencies of *P. falciparum* gene polymorphisms linked with different types of antimalarial drug resistance were analyzed. *Pfcyt bc1* associated with atovaquone resistance is the only gene with no mutation detected since the drug has never been used in this part of Africa.

The high level of *pfdhps/pfdhfr* mutations shown in the majority of the extracted data is likely caused by the wide use of SP prior to AL introduction in the region, or the concurrent use of SP in some countries in the region for intermittent prophylactic treatment for pregnancy (IPTp) and children as recommended by WHO WHO, 2010). In a study conducted in the northern zone of Somalia, the prevalence of the *pfdhfr* mutant alleles 59R, 51I, and 108N were 100, 22 and 100%, respectively. The *pfdhfr* triple mutations (51I,59R, and108N)associated with pyrimethamine

Table 3. The *pfcrt* mutant allele and haplotype frequencies reported in the articles included in the analysis.

References	Study location	Study year	Sample size*	K76 T mutation (%)	Haplotype(*pfcrt*72-76)		
					CVMNK Wild-type (%)	CVMNT mutation (%)	CVIET mutation (%)
(Heuchert et al., 2015)	Southwest Ethiopia	2013	159	4.4	50	0	50
(Golassa et al., 2014)	South-central Oromia	2012	99	100	0	ND_	100
(Mekonnen et al., 2014)	Southeast Ethiopia	2011	195	15.9	95.9	4.1	ND
(Lo et al., 2017)	North Ethiopia	2014	65	57.0	ND	ND	ND
	South Ethiopia		62	54.8	ND	ND	ND
	East Ethiopia		72	62.5	ND	ND	ND
(Schunk et al., 2006)	Southern Ethiopia	2004	69	100	ND	ND	ND
(Menegon et al., 2016)	Eritrea	2013-14	180	84.5	ND	ND	84.5
(Golassa et al., 2015)	East Shoa-Ethiopia	2012-14	31	100	ND	ND	ND
	Gambella-Ethiopia		22	72.7	ND	ND	ND
	West Arsi-Ethiopia		99	100	ND	ND	ND
(Tajebe et al., 2015)	Gondar-Ethiopia	2014	133	54.9	ND	ND	ND
(Rogier et al., 2005)	Djibouti	1988-02	139	93	ND	ND	ND

ND: no data available

resistance was 22% of the isolates. Likewise, the prevalence of *pfdhps* double mutation (437G and 540E) was 24%. The PCR-corrected AS-SP treatment failure rate reported in the study was 12% (Warsame et al., 2017). The findings of this study support previous work conducted in Jamame in the Southern zone of Somalia for the relatively high failure rate (22%) after AS-SP treatment (Warsame et al., 2015). This most likely occurred due to SP resistance since both studies showed high prevalence rates of mutation rate in the *pfdhps/pfdhfr* genes (Warsame et al., 2017; Warsame et al., 2015). In the Jamame study, the odds ratio (OR) of treatment failure within 28-days follow-up period was increased among patients harboring the *pfdhps/pfdhfr* quintuple mutant (OR 3.5,95% CI=1.4-8.8) and those carrying *pfdhps* double mutant (OR 22,95% CI:5.1-98.1) (Warsame et al., 2015). A study from Ethiopia reported mutations at codons R59, I51, and N108 in the *pfdhfr* gene at the frequencies of 100, 91 and 98%, respectively. The frequency of triple mutation (51I, 59R, and 108N) of this gene was 22%, while that of the *pfdhps* mutations at codons 437G, 540E, and both (437G and 540E) was 68, 92 and 91%, respectively (Mula et al., 2011). The observation of high prevalence rate corroborates with findings from Congo and Ghana. This indicates that SP resistance had not yet been declined (Marks et al., 2005; Ndounga et al., 2007). In the above-cited studies from the northern zone of Somalia and Ethiopia, the quintuple (double *pfdhps* plus triple *pfdhfr*) mutation which is used as a marker of SP resistance was 11 and 83% respectively. The SP failure due to *pfdhfr* triple mutation alone has been suggested in other reports from Kenya (Nzila et al., 2000) and Cameroon (Basco et al., 2000). It is worth noting that two studies from northwest Ethiopia investigated *pfdhfr/pfdhps* genes at two different time points (2005/2008) recording a marked reduction in *pfdhfr* triple mutation and *pfdhps/pfdhfr* quintuple mutation in field isolates. Nevertheless, this is not sufficient to conclude the return of SP sensitivity since a high rate of mutation was found (Hailemeskel et al., 2013; Tessema et al., 2015).

Eight out of the nine studies detected a high mutation at the core codon 76T of the *pfcrt* gene, the CQ resistance molecular marker (Mekonnen et al., 2014). The WHO recommends withdrawal of a drug if the prevalence of resistance exceeds 10% Bloland et al., 2003). The 76T mutant often occurred concurrently with other mutants, though their role has not been fully defined (Golassa et al., 2015). In Ethiopia for instance, the frequency of 76T mutation was 100% in a study conducted in South-central Oromia in 2012 (Golassa et al., 2014), while a year later, the frequency of 4.4% was reported in a similar study conducted in Southwest Ethiopia (Heuchert et al., 2015). The recovery of CQ susceptibility is a controversial issue in this country since CQ is still used for management of *P. vivax* malaria in areas with CQ resistant *P. falciparum*, resulting in a persistent selective pressure of *pfcrt* 76T (Golassa et al., 2015). Another reason is the availability of CQ from the drug stores and self-medication without the prescription

Table 4. The *pfmdr1* mutant allele and haplotype frequencies reported in the articles included in the analysis.

References	Study location	Study year	Sample size	Gene Locus						Gene copy number	
				86		184		1246			
				Mutation (%)	Wild-type (%)	Mutation (%)	Wild-type (%)	Mutation (%)	Wild-type (%)	1.5-2.5	>2.5
(Heuchert et al., 2015)	Southwest Ethiopia	2013	163	1.2	98.8	100	0	ND	ND	ND	ND
(Mekonnen et al., 2014)	Southeast Ethiopia	2011	195	14.9	85.1	5.1	94.9	0	100	ND	ND
(Lo et al., 2017)	North Ethiopia	2014	65	5.7	94.3	86	14	ND	ND	ND	ND
	South Ethiopia		62	5	95	85.5	13.5	ND	ND	ND	ND
	East Ethiopia		72	11.5	88.5	100	0	ND	ND	ND	ND
(Schunk et al., 2006)	Southern Ethiopia	2004	69	65	19	_ND	ND	ND	ND	ND	ND
(Menegon et al., 2016)	Eritrea	2013-2014	160	11.2	88.8	85.5	14.5	ND	ND	ND	ND
(Golassa et al., 2015)	Adama this.	2012-2014	30	23.3	76.7	__ ND	ND	ND	ND	ND	ND
	Gambella-Ethiopia		23	26.1	73.9	ND	ND	ND	ND	ND	ND
	West Arsi-Ethiopia		50	2	98	ND	ND	ND	ND	ND	ND
(Eshetu et al., 2010)	Southwest Ethiopia	2006	97	84.5	_ND	ND	ND	ND	ND	ND	ND
(Tajebe et al., 2015)	Gondar-Ethiopia	2014	133	45.9	54.1	ND	ND	ND	ND	25.6	28.6
(Mula et al., 2011)	Southern Ethiopia	2007-09	66	32.9	_ND	ND	ND	17.1	ND	ND	ND

ND: no data available.

due to poor management of drugs. However, a study from Kenya reported partial re-emergence of CQ sensitive strains after removal of selective pressure for this drug; thus this appears to be a country-specific drug change policy (Mwai et al., 2009). Studies conducted in Ethiopia and Eritrea identified the CVIET haplotype at the frequencies of 50 and 85%, respectively (Heuchert et al.,2015; Menegon et al., 2016), while the frequency reported in another study in Ethiopia was 0% (Mekonnen et al., 2014). Two Ethiopian studies aiming at an investigation of the SVMNT haplotype reported low mutant frequency (4 and 0%) (Heuchert et al., 2015; Mekonnen et al., 2014). The CVIET and SVMNT haplotypes are related to geographic origin of CQ tolerance (Mekonnen et al., 2014), the first haplotype predominates in Africa and Southeast Asia, whereas the other is more prevalent in some South American countries (Mehlotra et al., 2001). Concerning *pfmdr1* polymorphisms, disparate codons (86Y, 184F, 1242D, and 1246Y) with different mutation frequencies were noted in isolates from Eritrea and Ethiopia. The majority of studies showed mutation at codons 86Y and 184F. The 86Y is associated with a decline in the CQ sensitivity. Two studies in Southwest Ethiopia reported the decline in the prevalence of *pfmdr1* 86Yfrom 85% in 2006 to 1.2% in 2013 (Eshetu et al., 2010; Heuchert et al., 2015), while another study in Gondar Ethiopia reported the prevalence of 46% in 2014 (Tajebe et al., 2015). The frequency variation in these studies is likely reflecting the discrepancy of drug pressure in different geographical areas. The prevalence of the *pfcrt* 76T point mutation (55%) and high copy number variability (CNV) at codon *pfmdr1* 86Y from Gondar study, could provoke resistance for the new drug AL and delay the re-introduction of CQ in this location. In this study, high frequency of *P. falciparum* multidrug resistance copy number variants (54.2%) with 25.6% of isolates carried

Table 5. The *pfk13* mutant allele and haplotype frequencies reported in the articles included in the analysis.

References	Study location	Study year	Samples successfully typed at *pfk13*	Mutations found		
				N531I	R622T	Others
(Heuchert et al., 2015)	Southwest Ethiopia	2013	25	4.0%	ND	0.0%
(Lo et al., 2017)	Ethiopia	2014	199	No mutation found from samples analyzed		
(Menegon et al., 2016)	Eritrea	2013-2014	160			
(Bayih et al., 2016)	Northwest Ethiopia	2013-2014	125	ND	2.5%	0.0%

ND: no data available.

Table 6. The *pfatp 6* mutant allele and haplotype frequencies reported in the articles included in the analysis.

References	Study location	Study year	*Pfatp 6* gene, Codon, (N, %)					
			L402V Mutant (n/N, %)	E431K Mutant (n/N, %)	E237A Mutant (n/N, %)	L263L Mutant (n/N,%)	A623E/R682R/N683E/N683K (% for each)	K766K/K767E/K767R/S769N (% for each)
(Heuchert et al., 2015)	Southwest Ethiopia	2013	0/32 (0)	9/48 (18.8)	ND	ND	ND	ND
(Eshetu et al., 2010)	Southwest Ethiopia	2006	14/15 (93.3)	7/12 (58.3)	1/7 (14.3)	1/7 (14.3)	1/23 (4.4)	1/27 (3.7)

ND: no data available.

Table 7. Clinical efficacy of antimalarial drugs used in the Horn of Africa reported in the articles included in the analysis.

References	Study location	Study year	Sample size	Age range (yr)	Treatment regimen	Follow up (days)	Treatment failure rate on day 28 (%)
(Warsame et al., 2015)	Jamame, Somalia	2011	89	0.5-60	AS-SP	28	22.2
	Janale, Somalia		92				4.4
	Jowhar, Somalia		102				1.0
(Warsame et al., 2017)	Bosaso, Somalia	2013-2014	90	1-55	AS-SP	28	12.1
			90	1-60	AL		2.3
	Janale, Somalia	2013-2014	94	1-58	AL	28	0.0
	Jowhar, Somalia		100	2-36			1.0
(Bayih et al., 2016)	Northwest Ethiopia	2013-2014	148	1-69	AL	28	0.0

AL: artemether-lumefantrine, SP: sulphadoxine-pyrimethamine, AS: artesunate.

multi-copies (1.5- 2.5 copies) and 28.6% (> 2.5 copies) were found. This is likely to be a risk factor of resistance development for the artemether (A) partner (lumefantrine), since AL is Ethiopia's first-line treatment for falciparum malaria but the prevalence rate of multidrug resistance CNVs was not statistically significant (P>0.05) (Tajebe et al., 2015). *In vitro* study at the Thai-Cambodian border showed that parasites with higher copy number were

remarkably decreased susceptibility to lumefantrine, mefloquine, and artesunate (Lim et al., 2009).

Artemisinins are potent and rapid-acting compounds against multidrug-resistant *P. falciparum* strains. Eckstein-Ludwig *et al* suggested that artemisinins selectively inhibit *Pfatpase6*, the only SERCA-type Ca^{2+}-ATPase in the *P. falciparum* genome (Ariey et al., 2014). Two studies have targeted this gene; the sequencing of field isolates reported by Jimma *et al* (Eshetu et al., 2010) showed a variety of previously identified (L402V, E431K, A623E, N683, N683K, and S769N) and novel (E237A, L263L, R682R, K766K, K767E, and K767R) mutations in the *pfatp6* gene. Most of these mutations are globally dispersed as reports from South Africa and Asia indicate, but they were not correlated with ART resistance except for the S769N mutant being associated with *in vitro* resistance. An extremely low prevalence rate (3.7%) of S769N mutation was reported (Eshetu et al., 2010). The prevalence of the *pfatp 6* E431K mutation was declined from 58.3% in 2006 (Eshetu et al., 2010) to 18.8% in 2013 (Heuchert et al., 2015)**.** This did not occur under ART selective pressure because the drug was still in use and in addition, E431K was not detected along with A623E mutations which have been associated with a reduction of ART sensitivity. The recent emergence of ART-resistant strains in the Southeast Asian region has led many African countries to adopt ACT as the first-line treatment for uncomplicated *P. falciparum* malaria to become more vigilant since GMS was the previous spreading origin of CQ and SP resistance parasites. However, four studies conducted in Ethiopia showed no mutation at codons580Y, 543T, and 493H in the *pfk13* propeller domain which was previously correlated with artemisinin resistance in Southeast Asia (Ménard et al., 2016). These studies are in accordance with reports from Sub-Saharan Africa (Taylor et al., 2015). Only 1(4%) isolate was found harboring a new mutation at codon N531I in one study (Heuchert et al., 2015)**,** while in another study, 3 (2.5%) isolates were identified as having a novel mutation at R622T. Only one out of the three patients was found malaria positive on day-3 by microscopy with no information on *in vitro* parasite sensitivity. Interestingly, the day-3 positive patients cleared the parasite before day-28 (Bayih et al., 2016). This is unlikely to be resistance strains due to the lack of strong relationship between K13-propeller domain and delayed parasitic clearance after ART treatment as detected in Southeast Asia (Ariey et al., 2014).

Conclusions

This systematic review presents a picture of the geographical distributions of *P. falciparum* drug resistance genes during 1998-2014. The distribution pattern could be changed over time and it will be crucial to maintaining tracking through systematic sampling around lineage distribution boundaries. In this regard, a complete picture of *P. falciparum* resistance allele distribution can be generated. This record will enhance our knowledge of the real challenges on spreading of *P. falciparum* molecular markers at the extremes of the geographical distributions outlined here. Limitation of the current review includes the lack of standardized reporting layout of the data extracted from the published articles. Information on the prevalence of some point mutations was not available. Some articles included in the analysis still require a certain level of interpretation due to variations in methodological and reporting format. Furthermore, molecular studies with high prevalence mutant alleles were more likely to be published than those with wild-type alleles.

ABBREVIATIONS

AL, artemether–lumefantrine, **SP,** salphadoxine-pyramethamine, **AS,** artesunate, **AQ,** amo-diaquine, **CQ,** chloroquine, **ART,** artemisinin, **ACT,** artemisinin based-combination therapy, **SNPs,** single nucleotide polymorphisms, **GMS,** greater Mekong sub-region, **WHO,** world health organization, ***pfdhps*****,** *P. falciparum dihydropteroate* synthase gene, ***pfdhfr*****,** *P. falciparum* dihydrofolate reductase gene, ***pfmdr1*****,** *P. falciparum* multidrug resistance1 gene, *pfcrt*: *P. falciparum* chloroquine resistance transporter gene, ***pfk13*****,** Klech 13 propeller gene, ***PfATPase 6*****,***P. falciparum* adenosine triphosphatase 6, **CNV,** copy number variability, **ND,** no data available, **MeSH,** medical subject headline, **PCR,** polymerase chain reaction, **RFLP,** restriction fragment length polymorphism.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Analysis of patient information leaflets on Artemisinin-based combination therapy

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The objective of this research was to analyze the various patient information leaflets on Artemisinin-based combination therapy (ACTs) sold in Côte-D'Ivoire. A descriptive cross-sectional study was conducted from January 1st to February 20th, 2016, that included all patient information leaflets relating to ACTs registered and marketed in Côte-d'Ivoire. The leaflets were compared to European standards of writing summaries of product characteristics, by focusing particularly on side effects. Regarding artemether-lumefantrine, all leaflets mentioned digestive disorders. As far as endocrine and metabolic systems are concerned, appetite loss and anorexia were outlined in 28.5% and 42.8% of leaflets examined. With regard to skin and annexes, we noticed: rash (100%), pruritus (90%), slate-gray pigmentation (28%) and redness of the face (14%). Finally, only Plasmocid® and Coartem® leaflet reported biological side effects. Regarding artesunate-amodiaquine, side effects involving blood were outlined: agranulocytosis (60%), blood dyscrasia and leucopenia (40%), along with hemolytic anaemia (20%). Side effects affecting gastrointestinal system were nauseas, vomiting and diarrhoea (80%), hepatitis (60%) and fatal hepatitis (20%). Side effects affecting the nervous system include peripheral neuropathy (80%) and extrapyramidal syndrome (20%). Regarding information from pharmaceutical companies differing from one specialty to another for the same molecule, it would be desirable that they harmonize the patient information leaflets contents.

Key words: Side effects, information leaflets, antimalarial drugs, Artemisinin-based combination therapy.

INTRODUCTION

The Ministry of Health and Population of Ivory Coast, since January 2007 has adopted a new treatment protocol for malaria by a Ministerial Order No. 024/CAB /M-SHP of January 12, 2007 [Ministère de laSanté, 2007]. This new protocol includes Atemisinin-based combination therapy (ACTs). Since July 2008, serious

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cases of hepatonephritis and blackwater fever have been reported following the use of antimalarials. That led to a psychosis among population. A working committee has been put in place in the Health Ministry to investigate these cases. Epidemiological and pharmacovigilance surveys were conducted. Hepatonephritis was a new and serious life-threatening event. The role of antimalarials, in particular ACTs, in the occurrence of these serious adverse effects has been highlighted (Bodi et al., 2014; Guevart, 2009; Kamagate, 2004). Factors identified were misuse, short and repeated therapeutic sequences. This situation was related to the lack of knowledge of the tolerance profile of the products by the population and health professionals (Bakayoko, 2009). Although the benefit/risk ratio is always favorable, these serious side effects are mostly preventable and therefore unacceptable. So information or population's training and health professional awareness are useful and crucial. The working committee then deemed it necessary to assess the benefit provided by these substances as against the risk involved. The summary of product characteristics or the patient information leaflet for drug seemed to be a reliable source of data that could provide useful safety and security information. This leaflet is exhaustive and provide clinical, pharmacological and pharmaceutical data according to European standard, adopted by Côte-d'Ivoire. The European standard provides data on efficiency, tolerance profile, security and quality of use. It should also guarantee the authenticity of the products through its administrative information. The objective of this study, was to analyze the information of the package leaflets of the various ACTs marketed in Cote-d'Ivoire to appreciate this information's quality.

MATERIALS AND METHODS

This descriptive cross-sectional study was conducted from January 1st to February 20th, 2016 in ten pharmacies or drugstores selected in the ten municipalities of Abidjan. In this study, any patient information leaflets of ACTs, sulfadoxine/pyrimethamine and quinine were included. The leaflets examined concerned drugs registered and sold in Côte d'Ivoire until 2015. These products also had to be available in drugstores. The selected drugstores were randomised. The study was conducted in the private drugstores mentioned above only with the prior agreement of the pharmacist in charge. The work consisted of collecting and analyzing the various information of the package leaflets written by the pharmaceutical companies on antimalarial drugs, particularly ACTs. The data were collected with record form that consisted of three parts: 1- General data on the active ingredient; 2- Data on the form of the leaflet ; and 3- General information on drugs. This information included indication, dosage, warnings and cautions, interactions, pharmaco-dynamics and pharmacokinetics, side effects, contraindications and other administrative information. The collected leaflets were analysed by comparing to the European model for drafting the Summaries of Product Characteristics (SmPC) (Table 1).

We emphasized on information regarding the pharmacovigilance of ACTs (under side effect section), therapy (indication, contraindication, dosage, warnings and cautions for use), pharmacokinetics and pharmacodynamics (pharmacokinetic, pharmacodynamics, interaction sections).

RESULTS

Form of the patient information leaflets

About 20 leaflets were collected which showed that the European standard plan concerning the drafting of information in package leaflets was not followed in 85% of cases (Table 2).

With regard to the missing headings, in 80% of the cases, the preclinical safety and incompatibility headings were not mentioned. Also, in 70% of cases, the heading pharmacokinetics were missing (Table 3).

Substance or heart of the patient information leaflets

The antimalarial indication in the leaflets was curative in 95% of cases and prophylactic in 5% of cases. This indication of the antimalarial was not mentioned for adults in 20% of cases, for children in 25% of cases and for infants in 60% of cases. In 65% of cases, the daily dosage according to weight of child was not mentioned. However, 4 of the 5 leaflets of artesunate-amodiaquine mentioned it. In most of the cases, the following criteria were missing in the leaflet: ATC classification (anatomical, therapeutic and clinical), metabolites causing enzymes and severity and frequency of adverse effects. The side effect profile for different antimalarial drugs was different. Concerning artemether-lumefantrine (Table 4), digestive disorders have been mentioned in all specialties. The metabolic and endocrine adverse effects described were either a loss of appetite (28.57%) or anorexia (42.86%). Two package leaflets (Plasmocid® and Laritem®) did not mention metabolic and endocrine effects. The Laritem® and Artefan® leaflets described most of the neurological side effects (88%) followed by the Plasmocid® leaflets (66%). In most of the cases, headache. The package leaflets of Artrine®, Lufanter®, Bimalaril®, and Coartem® have almost no neurological side effects mentioned (Table 4). Regarding the side effects affecting the skin by artemether-lumefantrine, the most commonly mentioned side effects were skin rash (100%) and pruritus (85%). The redness of the face and the slate pigmentation were rarely mentioned (14 and 28%, respectively).

Regarding adverse effects affecting the immune system, hypersensitivities were the most mentioned (60%), followed by angioedema (40%). Biological side effects were only mentioned in leaflet of Plasmocid® and Coartem® (Table 4).

Regarding seven antimalarials containing artesunate-amodiaquine (Table 5), Artepal's leaflet described 75% of side effects relating to blood. It was agranulocytosis, blood dyscrasia, and leukopenia. Artediam®'s leaflet

Table 1. European model of summary of product characteristics.

Chapter	Subchapter
Name of the medicinal product	
Qualitative and quantitative composition	
Pharmaceutical formula	
Clinical data	Therapeutic indications
	Posology and method of administration
	Contraindications
	Special warnings and cautions for use
	Interaction with other medicinal products and other forms of interaction
	Fertility, pregnancy and lactation
	Effects on ability to drive and use machines
	Adverse drug reaction
	Overdose
Pharmacological properties	Pharmacodynamic properties
	Pharmacokinetic properties
	Preclinical safety data
Pharmaceutical data	List of excipients
	Incompatibilities
	Shelf life
	Special precautions for storage
	Nature and contents of container
	Special precautions for disposal and other handling
Marketing authorisation holder	
Marketing authorisation number(s)	
Date of first authorisation/renewal of the authorisation	
Date of revision of the text	
Dosimetry (if applicable)	
Instructions for preparation of radiopharmaceuticals (if applicable)	

mentioned a deadly agranulocytosis. Adverse effects relating to blood had not been mentioned in Camoquin's package leaflet. Damage affecting the gastrointestinal system has often been mentioned in Camosunate® and Coarsucam® leaflets. The majority of neurological side effect was mentioned in the Camoquin plus® and Coarsucam® leaflets. The most mentioned adverse effects were peripheral neuropathies (57%) followed by dizziness (28%) and neuromyopathy (28%). Skin damages have been mentioned: pruritus (40%), skin rash (60%) and slate pigmentation (40%). Camosunate® and Coarsucam® package leaflets did not mention any adverse effects relating to the skin. General adverse effects like asthenia and weakness were mentioned only in the package leaflet of Camoquin plus®. Ophthalmological disorders were most often described in the package leaflet of Artediam® and Camoquin plus®. Biological disorders have been mentioned maximally in the package leaflet of Coarsucam®. The most common disorders were the drop in reticulocyte count (100%) and the transient increase in transaminases (100%).

Well described quinine adverse effects were relating to blood, metabolic and endocrine, nervous and skin systems. Side effects relating to cardiovascular and general systems have been poorly described (Table 6).

DISCUSSION

The lack of references in this area made it impossible to confront the findings. It is a princeps work for better understanding of drug security data for the public. This

Table 2. Accordance of pharmaceutical leaflet with European Plan.

DCI	Accordance	No Accordance	N
ART-LUM	2	5	7
ARS-AMO		5	5
QUININE	1	2	3
DHA-PIP		2	2
ARS-SULFM-PYR		1	1
DHA-PIP-TRI		1	1
SP		1	1
Total	3 (15%)	17 (85%)	20 (100%)

ART-LUM: artesunate-lumefantrine; ARS-AMO: artesunate-amodiaquine; DHA-PIP: dihydroartemisinine-piperaquine; ARS-SULFM-PYR: artesunate-sulfamethoxypyrazine-pyrimethamine; DHA-PIP-TRI: dihydroartemisinine-piperaquine-trimethoprime; SP: sulfadoxine-pyrimethamine.

Table 3. Missing headings of SmPC.

Headings of SmPC	SP	ART-PIP-TRI	ART-PIP	ART-SULF-PYR	ART-LUM	ARS-AMO	QUI	Total	%
Pregnancy			2		2	2		6	30
Modality of manipulation	1							1	5
Overdose		1	2		3	2		8	40
Pharmacokinetics		1	2	1	4	4	2	14	70
Pharmacodynamics					3	3	2	8	40
Preclinical safety		1	2	1	5	5	2	16	80
Interaction		1	2		2	3		8	40
Incompatibility	1		2	1	5	5	2	16	80
Driving machine			2		2	3	2	9	45
Warnings			1					1	5
Contraindications						1		1	5
Form and presentation							1	1	5

SP: sulfadoxine-pyrimethamine; ART-PIP-TRI: artesunate-piperaquine-trimethoprime; ART-PIP: artesunate-piperaquine; ARS-SULF-PYR: artesunate-sulfamethoxypyrazine-pyrimethamine; ART-LUM: artesunate-lumefantrine; ARS-AMO: artesunate-amodiaquine; QUI: quinine.

study revealed that records were easily readable, clearly written and easily usable. This measure is intended to avoid any negligence on the part of pharmaceutical companies and aims to protect a sensitive and fragile population: the elderly, the sick, pregnant women who may have impaired understandings and who may find themselves in difficulty due to a mistake in dosage (article 49 de la directive 2004/24/CE). This work showed a lack of information both in the form and in the substance (L'Expert Econome, 2018; Bandello and D'Addario, 2015). The writing plan of the leaflet was not followed for most of them (85% of the cases). In fact, the package leaflets of artesunate-amodiaquine, dihydroartemisin-piperaquine, artesunate-sulphamethopyrazine-pyrimethamine, dihydroartemisine-piperaquine-trimethoprim and sulphadoxine-pyrimethamine were not in conformity with the European Plan for writing package leaflets. Those of artemether-lumefantrine and quinine were also non-compliant in 71.4% of cases. This constitutes a breach from the legal point of view in Cote d'Ivoire. Two to eight sections were missing. It was most often: preclinical safety, incompatibility, pharmacokinetics, pharmacodynamics, overdose, interactions and effects on ability to drive and use machines. This would be an information bias on safety and tolerance. The most often listed sections were general information (denomination, galenic form, presentation and composition) and clinical data (indication, posology and method of administration, contraindications, side effects). These sections remain important for informing about the security of use of medicines to the general public. On the other hand, pharmacological data were missing in most of the cases. The pharmaceutical information given is limited to the conditions of conservation. The administrative information

Table 4. Adverse effects of artemether-luméfantrine.

Organ/System	Type of effect	Artrine	Artéfan	Plasmocid	Lufanter	Bimalaril	Coartem	Laritem	Total
Gastro-intestinal	Nausea	+	+	+	+	+	+	+	7
	Vomiting	+	+	+	+	+	+	+	7
	Diarrhea	+	+	+	+	+	+	+	7
	Abdominal pain	+	+	+	+	+	+	+	7
	Total	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**28**
Metabolic and endocrine	Loss of appetite	+	-	-	+	-	-	-	2
	Anorexia	-	+	-	-	+	+	-	3
	Total	**1**	**1**	**0**	**1**	**1**	**1**	**0**	**5**
Neurological	Headache	+	+	-	+	+	+	+	6
	Dizziness, Vertigo	-	+	-	-	+	+	+	4
	Drowsiness	-	+	+	-	-	+	+	4
	Muscle contraction	-	+	+	-	-	-	-	2
	Muscle cramp	-	-	-	-	-	-	+	1
	Paraesthesia	-	+	+	-	-	-	+	3
	Hypoaesthesia	-	+	+	-	-	+	+	4
	Abnormal gait	-	+	+	-	-	-	+	3
	Ataxia	-	+	+	-	-	+	+	4
	Total	**1**	**8**	**6**	**1**	**2**	**5**	**8**	**31**
Psychiatric	Sleeping disorder	+	+	-	-	-	+	+	4
Heart	Palpitations	+	+	-	-	+	+	+	5
General	Asthenia	-	-	-	-	+	-	-	1
	Tiredness	+	+	-	+	+	-	-	4
	General weakness	+	+	-	+	-	-	-	3
	Total	**4**	**4**	**0**	**2**	**3**	**2**	**2**	**17**
Skin and appendages	Skin rash	+	+	+	+	+	+	+	7
	Redness of the face	+	-	-	-	-	-	-	1
	Pruritus	+	+	+	-	+	+	+	6
	Slate pigmentation	-	-	+	-	+	+	-	3
	Total	**3**	**2**	**3**	**1**	**3**	**3**	**2**	**17**
Musculoskeletal	Arthralgia	-	+	+	-	+	+	+	5
	Myalgia	-	+	+	-	+	+	+	5
	Total	**0**	**2**	**2**	**0**	**2**	**2**	**2**	**10**
Immune	Hypersensitivity	-	+	+	-	-	+	-	3
	Angioedema	-	-	+	-	-	+	-	2
	Total	**0**	**1**	**2**	**0**	**0**	**2**	**0**	**5**
Biological	Increase in liver tests	-	-	+	-	-	+	-	2
	Electrocardiogram QT prolonged	-	-	+	-	-	+	-	2
	Total	**0**	**0**	**2**	**0**	**0**	**2**	**0**	**4**

(+) information mentioned and (-) information not mentioned.

Table 5. Adverse effects of Artesunate-Amodiaquine.

Organ/System	Type of effect	Camoquin Plus	Camosu-nate	Artédiam	Coarsu-Cam	Artépal	Total
	Agranulocytosis	-	+	Mortelle	-	+	3
	Blood dyscrasia	-	+	-	-	+	2
Blood	Leukopenia	-	-	+	+	-	2
	Haemolytic anaemia	-	-	-	-	+	1
	Total	**0**	**2**	**2**	**1**	**3**	**8**
	Nausea	+	+	-	+	+	4
	Vomiting	+	+	-	+	+	4
	Diarrhea	+	+	-	+	+	4
Gastro-intestinal	Abdominal pain	-	-	-	+	-	1
	Hepatitis	-	+	+	+	-	3
	Fatal hepatitis	-	-	+	-	-	1
	Total	**3**	**4**	**2**	**5**	**3**	**17**
	Dizziness	+	-	-	+	-	2
	Peripheral neuropathy	+	+	-	+	+	4
Nervous	Neuromyopathy	-	+	+	-	-	2
	Extrapyramidal syndrome	+	-	-	-	-	1
	Headache	-	-	-	+	-	1
	Total	**3**	**2**	**1**	**3**	**1**	**10**
	Pruritus	+	-	+	-	-	2
Skin and	Skin rash	+	-	+	-	+	3
appendages	Slate pigmentation	-	-	+	-	+	2
	Total	**2**	**0**	**3**	**0**	**2**	**7**
Immune	Allergy	+	-	-	-	-	1
	Total	**1**	**0**	**0**	**0**	**0**	**1**
	Asthenia	+	-	-	-	-	1
General	Weakness	+	-	-	-	-	1
	Total	**2**	**0**	**0**	**0**	**0**	**2**
	Corneal deposit	+	-	-	-	-	1
	Blurred vision	+	-	-	-	+	2
	Retinopathy	+	-	+	-	+	3
Ophtalmic	Corneal opacity	-	-	+	-	-	1
	Ocular disorder	-	-	+	+	-	2
	Accomodation disorder	-	-	+	-	-	1
	Total	**3**	**0**	**4**	**1**	**2**	**10**
	Drop in reticulocyte counts	+	+	+	+	+	5
	Leukopenia	-	-	-	+	-	1
Biological	Anemia	-	-	-	+	-	1
	Transient increase in transaminases	+	+	+	+	+	5
	Total	**2**	**2**	**2**	**4**	**2**	**12**

(+) information mentioned and (-) information not mentioned.

given is limited to the identification of the pharmaceutical companies. Notable missing information could therefore be observed in the leaflet. Indeed, the sections outlined were not sufficient enough to allow patients, assess the benefit-risk ratio of the prescribed drug. Sections for assessing risk, such as preclinical safety, interactions, overdose, incompatibility and cautions for use were missing (Table 2). The cautions for use section, would make it possible to take steps to avoid or lessen the adverse drug reactions of drugs. Apart from quinine and sulfadoxine-pyrimethamine, most medications were contraindicated during pregnancy and in infants. However,

Table 6. Adverse effects of quinine.

Organ/System	Type of effect	Quinimax	Arsiquinoform	Surquina	Total
Blood	Thrombopenia	+	+	+	**3**
	Blackwater fever	+	+	+	**3**
Metabolic and endocrine	Hypoglycemia (faintness)	+	+	+	**3**
Nervous	Tinnitus	+	+	+	**3**
	Decreased hearing	+	+	+	**3**
	Dizziness	+	+	+	**3**
	Headache	+	+	+	**3**
	Blurred vision	+	+	+	**3**
	Nausea	+	+	+	**3**
	Convulsion	+	+	+	**3**
Cardiovascular	ECG changes	-	+	-	**1**
Skin and appendages	Itching	+	+	+	**3**
	Urticaria	+	+	+	**3**
	Rash generalised	+	+	+	**3**
General	Allergy	-	+	-	**1**
	Anaphylactic shock	-	+	-	**1**

(+) information mentioned and (-) information not mentioned.

it can be noted that these important pregnancy and breastfeeding sections were missing.

In the leaflets examined, most of the adverse drug reactions mentioned were benign: gastrointestinal, neurological, and cutaneous. However, in the literature, serious adverse drug reactions have been observed with antimalarials. Serious haematologic effects like blackwater fever and epidermolysis have been noted with sulfadoxine-pyrimethamine and artemether-lumefantrine. These progressed to kidney failure in 80-90% of cases. The mortality rate was 15-30% (Daubrey-Potey et al., 2004b; Hasegawa et al., 2018; Huggan et al., 2018; Olupot-Olupot, et al, 2017; Sweetman, 2007). There were also rare neurological adverse effects like orofacial dyskinesias observed with amodiaquine in children and adults. The onset occurred within 2 days after stopping the drug (Daubrey-Potey et al., 2004a; Kamagate et al., 2004). Hepatitis and fulminant hepatitis have been related to artesunate or to artesunate-amodiaquine. Generally, they were of the cytolytic type (Guevart et al., 2009; Roussel et al., 2017). In addition, recent and new fatal cases of hepatonephritis coinciding with the antimalarial use (in particular ACTs) have been described in several Ivorian pharmacovigilance surveys. The onset occurred within 3 days; with an observed recrudescence of cases in the rainy season when malaria is raging (Bakayoko, 2009; Die-Kakou et al., 2009, 2010; Kamagate et al., 2017). In the leaflets examined, none of these effects have been mentioned. This could be related to the fact that the adverse drug reactions described in the leaflets were the effects observed during pre-commercialization clinical trials of these drugs and without post-commercialization safety data. Since clinical trials are only performed with a limited number of patients, it could be understood why these serious effects with low prevalence were not observed (Organisation mondiale de la sante - OMS, 2004). After commercialization, the consumption of these drugs on a larger scale, allowed the observation of these hepatonephritis and other serious adverse effects. Unlike the SmPC that are regularly updated with pharmacovigilance data, that of the package leaflets seems to be rarely updated. In addition, the severity and frequency of adverse drug reactions were most often not mentioned in the leaflets, leaving the patient in a state of complete ignorance. This could be judged as an ethical problem.

Finally, a disparity in the information provided by different pharmaceutical companies could be observed, for the same active substance. This was the case of serious adverse drug reactions of amodiaquine. Indeed, according to the specialties, they were found or not in the

leaflets. These included agranulocytosis, haemolytic anaemia, and sometimes fatal hepatitis (Sweetman, 2007).

Conclusion

In this work, the package leaflets of ACTs sold in Cote d'Ivoire have been analysed. The analysis concerned both the substance content and the form of the leaflets. Majority of leaflets did not comply with the European Plan for writing leaflets. In addition, the information provided by the pharmaceutical companies differed from one specialty to another, for the same active molecule. This is detrimental to the patient. It would therefore be desirable for the regulatory authorities to ask the pharmaceutical companies to harmonize the content of the different leaflets.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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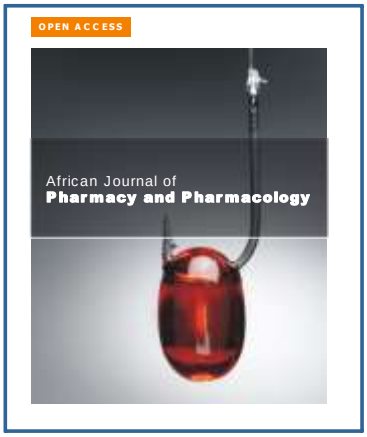

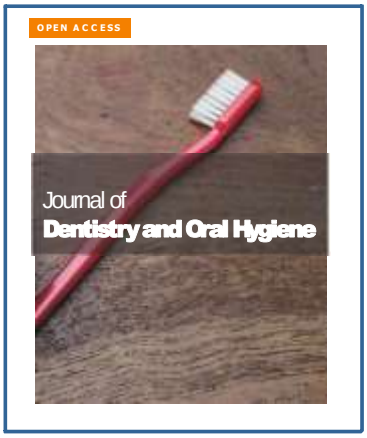

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