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Review

DNA methylation as the most important content of epigenetics in traditional Chinese herbal medicine

Mohamad Hesam Shahrajabian[1,2#], Wenli Sun[1,2#] and Qi Cheng[1,2#*]

[1]Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China.
[2]Nitrogen Fixation Laboratory, Qi Institute, Building C4, No.555 Chuangye Road, Jiaxing 314000, Zhejiang, China.

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Traditional Chinese medicine is commonly used in China and so many other Asian and western Countries. Epigenetics relates to heritable alternations in chromatin architecture that do not involve changes in the underlying DNA sequence but profoundly affect gene expression and impact cellular function. Epigenetic regulation is attained by specific mechanisms involving DNA methylation, histone posttranslational modifications and the action of noncoding RNAs. Epigenetic variations also involved in the control of plant developmental processes and contribute in shaping phenotypic plasticity to the environment. Epigenetics has considerable impact on evolution and epigenetic epidemiology which has shown the intricate function between the environment and epigenetics. DNA methylation is an epigenetic mechanism that regulates gene expression and may affect plant growth, development and acclimation. DNA methylation is associated with gene expression and morphological variation. Plants, utilization of the epigenetic approach are used to manage and resist the fungal, bacterial and others biotic stresses; microbes, also employ epigenetic mechanisms to modify growth and pathogenicity, leading to resistance against plant-host immune system. DNA methylation is a chemical modification process where the methyltransderass (DNMTs) are catalyzed by selective addition of methyl groups to form 5-methylcytosine in CpG sequences. A mixture of herbs and fruits used in traditional Chinese medicine maybe use to decline diseases by adding and removing epigenetic marks on DNA. Epigenetics has been introduced to the area of TCM recently, which resulting in the hypothesis of an epigenetic role in the modern pharmacology of TCM prescriptions. Epigenetics is the partial material basis of TCM syndrome diversity, and the microscopic index of epigenetics can be an important supplement to the macroscopic syndrome differentiation of TCM syndromes. The role of epigenetic information is in developmental gene regulation, natural variation of gene expression levels, and response to the environment. The significant attention has started about the potential for epigenetic information to contribute to heritable variation in many species, even traditional Chinese herbs.

Key words: DNA methylation, epigenetics, traditional chinese medicine.

INTRODUCTION

Traditional Chinese medicine, epigenetics and DNA methylation

Traditional Chinese medicine (TCM) uses natural products, chiropractic, acupuncture and a combination of these subjects in a therapeutic course (Ogbaji et al., 2018; Shahrajabian et al., 2018; Shahrajabian et al., 2019a; Shahrajabian et al., 2019b; Shahrajabian et al., 2019c). The recognizing of the molecular mechanisms of epigenetic inheritance is growing significantly. It has been reported that epigenetic modifications, including DNA methylation, histone post-transcriptional modifications,

micro RNA interference and etc, may help explore the molecular basis of Chinese medicine (CM) syndrome classification, and mechanisms of Chinese herbal medicine (CHM), CHM compounds and Chinese herbal formulae activities (Hu and Su, 2016). The mutual characteristics of Chinese medicine (CM) theory and epigenetic cognition is shown in Figure 1. The mutual characteristics include of 1) holism-integrity of environment, emotion, and diet with human body; 2) reversibility and balance of yin-yang and epigenetic modifications; 3) dynamic natures and epigenetic changes; 4) visceral manifestation and CM syndrome originated from epigenetic genotype. Epigenetics is considered to be a potential link between postnatal environmental factors and diseases, which refers to a reversible and heritable change that handles gene expression without any alter in the DNA sequence (Yao et al., 2018). An increasing number of epigenomes are now being investigated on crops of high economic value. Three types of known epigenetic mechanisms consist of DNA methylation, histone modifications, and small non-coding RNAs that regulate gene expression (Jirtle and Skinner, 2007). DNA methylation changes have been reported to be involved in the onset of a variety of metabolic diseases, like diabetes mellitus, fatty liver, and metabolic syndrome (Wang et al., 2015; Yara et al., 2015; Marfil et al., 2019). DNA methylation is one of the most important modifications of DNA nucleic acids, and it is considered as one of the most important epigenetic control mechanisms of gene expression (Serman et al., 2006). DNA methylation happens in many key physiological processes that including X chromosome inactivation, imprinting and the silencing of germline-specific genes and repetitive elements (Zhang, 2015). Plants are a powerful system for studying DNA methylation and epigenetic phenomena (Niederhuth and Schmitz, 2017). In plants, as in mammals, changes in DNA methylation are remarkable during reproduction and in reproductive tissues, a topic that has been reviewed elsewhere (Kawashima and Berger, 2014). Most important DNA methylation in plants comes from the study of one species, *Arabidopsis thaliana*, but it should be rapidly expanding to other species, especially traditional Chinese herbs and fruits (Zhang et al., 2018). Methylation has been found in all plant genomes, and certain pathways have been found to predominate and others to be defective (Niederhuth and Schmitz, 2017). DNA methylation is medicated by the family of DNA methyltransferases (DNMTs) that catalyze the transfer of a methyl group from S-adenosyl methionine (SAM) to cytosine of DNA (Wirbisky-Hershberger et al., 2017). DNA methylation suppresses the expression of harmful DNA sequence such as endogenous retroviral genes that have been integrated into the host genome during evolution (Jaenisch and Bird, 2003). Cyotosine methylation plays important roles in development and stress resistance in plants (Yang et al., 2018). The levels of genomic DNA methylation in plants are coordinately regulated by both methylation and demethylation (Chinnusamy and Zhu, 2009; Deleris et al., 2016). Methylation levels can change within repeats and transposons themselves depending on the particular transposon family (Eichten et al., 2012) and potentially also its age (Maumus and Quesneville, 2014). Mutual characteristics of CM theory and epigenetic cognition is shown in Figure 1. A model of the holistic and long-term therapeutic effect of TCM prescriptions is presented in Figure 2. Patterns of epigenetic marks such as DNA methylation (red) and histone acetylation (green) help maintain the gene expression profile for normal cell functioning. Aberrations in the epigenetic pattern, due to endogenous or exogenous factors, lead to disorders. Compounds in TCM medicinal (TCMFs), in particular the Monarch, interact with the epigenetics-related protein and the epigenetic pattern, once restored, is passed on to the daughter cells, the holistic (in contrast to reductionist's single gene or pathway) an long-term effects of TCM prescriptions are realized (Hseih et al., 2011).

ROLE OF EPIGENETICS AND DNA METHYLATION IN CROP IMPROVEMENT

There is the potential for epigenetics to play an important part in crop improvement strategies including the selection for favorable epigenetic states, creation of novel epialleles, and regulation of transgene expression (Springer, 2013). Cabej (2019) mentioned that epigenetic factors are determinants of the transgenerational plasticity in plant. He has concluded that a correlation is observed between the new acquired traits that appear as a result of transgenerational inheritance and specific epigenetic modifications, but it is not easy to determine whether these modifications are cause or effect of another unknown cause. Gallusci et al. (2017) stated that intense breeding has eroded genetic diversity, and epigenetic diversity now emerge as a new source of phenotypic alterations to improve adaptation to changing environments and ensure the yield and quality of crops. In spite the fact that, the mechanism of epigenetic regulations is a complex process, because of advances in gene expression mechanisms, many molecules for modulation of epigenetics will be recognized. Huang et al. (2017) found that epigenetic control of gene expression

*Corresponding author. E-mail: chengqi@caas.cn. #All authors contributed equally to this research.

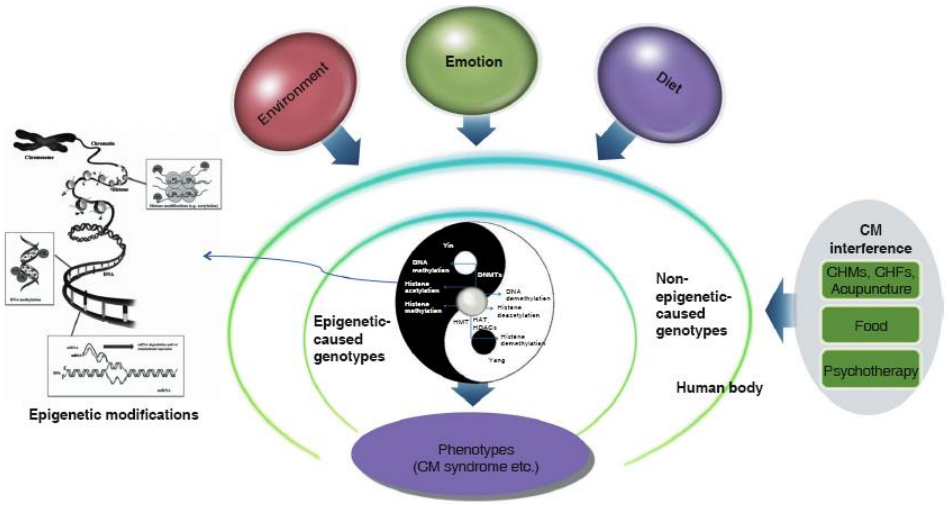

Figure 1. Mutual Characteristics of CM Theory and Epigenetic Cognition.
Source: Hu and Su (2017).

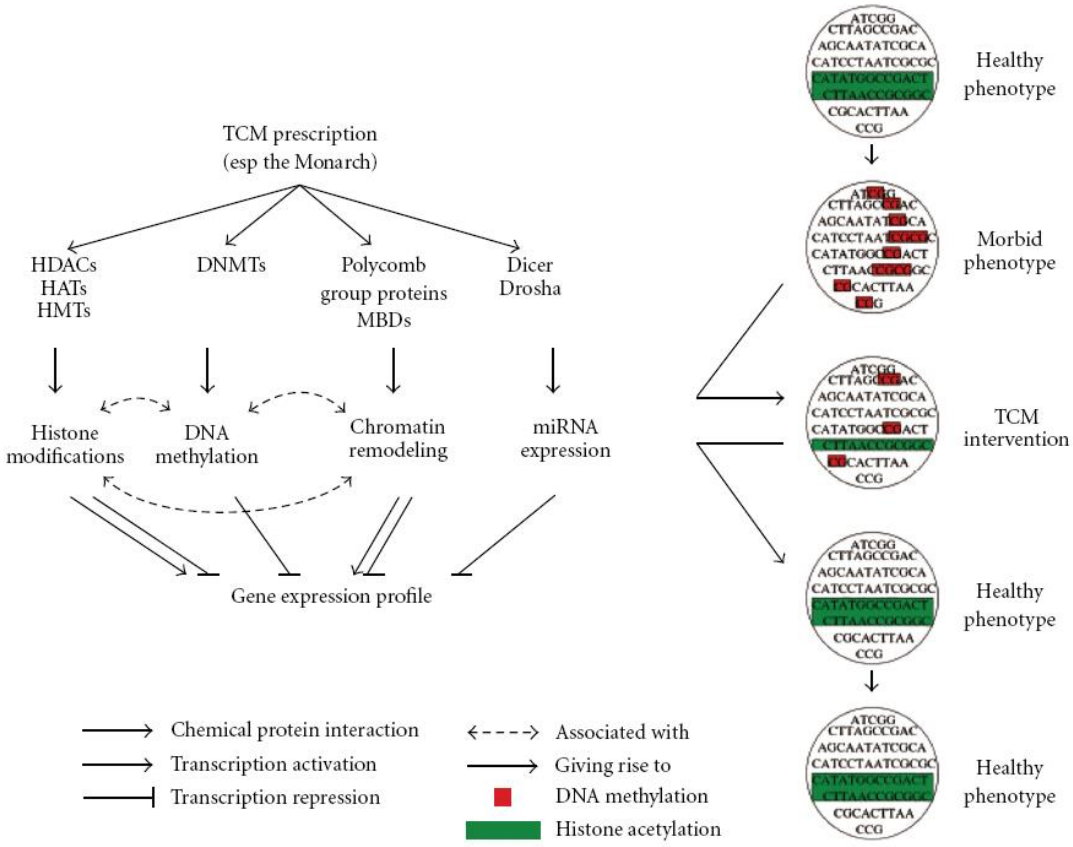

Figure 2. A model of the holistic and long-term therapeutic effect of TCM prescriptions.

encompasses the context of genes relative to DNA methylation, chromatin structure, and transposable element content. The epigenetic changes do not consist of changes in the actual DNA sequence and consist of an ever-expanding array of other epigenetic processes mediated by prions, Polycomb protein, and higher-order

Table 1. Key terminologies.

Epigenetics: Hereditable modifications in gene function without any involvement of the DNA sequence.
Epigenome: Set of chemical molecules and proteins that can bind to DNA to lead to activities turning genes on/off and regulating the proteins synthesis in certain cells.
Epigenomics: Study of the whole set of epigenetic alterations (epigenome) on the hereditary material of a cell.
Epigenetic marks: The modifications to the DNA and/or histones that causes epigenetics impacts including methylation, acetylation, ubiquitination, phosphorylation, sumoylation and poly (ADP) ribosylation.
Priming: memory of an internal and/or external cellular stimulus exposure that enables a plant to respond in quick and effective manner during future encounters.

Source: Barozai and Aziz (2018).

chromatin organization, as well as many others (Tollefsbol, 2017). Yao et al. (2018) suggested that the potential risk for metabolic disorders in individuals with phlegm-dampness constitution (PC) and also they did explain the clinical features of PC with DNA methylation features. Sorghum tissue methylation profiles produced at lower resolution using methylation-sensitiv amplified polymorphism revealed similar findings, with insignificant methylation changes across seven tissues except for the endosperm (Zhang et al., 2011). The endosperm possessed genome-wide hypomethylation, which concurs with studies on *A. thaliana*, rice, and maize endosperm. Cao et al. (2019) demonstrated that DNA methylation influenced the transcriptional expression of Sclerostin (SOST) gene, which probably may play a vital role in the pathogenesis of primary osteoporosis. More et al. (2016) concluded that DNA methylation plays a meaningful role in gene regulation during growth, development and also different stresses. They have also indicated that DREB (Dehydration-responsive element binding) gene might be part of methylation events in transgenic plants by regulating certain regions of the plant genome that plays important role during stress conditions. DNA methylation mainly depends on two pathways: maintenance and de novo methylation. DNA methyltransderase (MET) and chromomethylase (CMT) are two key enzymes for maintenance methylation (Zhang et al., 2018). DNA methylation has been considered an underlying mechanism for temporary changes in plant phenotypes; also, DNA methylation is carried out by DNA methyltransferases that catalyse the transfer of a methyl group from S-adenosyl-L-methionine to cytosine bases in DNA, and cytosine methylation primarily appears in both CpG and CpNpG sequences (Hu et al., 2014). Key terminologies is presented in Table 1.

Gady et al. (2017) reviewed four epigenetic-based regulations in each phase of the tomato fruit set, development and ripening. Those regulations were included: (1) modifications in histone proteins, (2) methylation and demethylation of DNA, (3) regulation of significant locus by small RNAs through post-transcriptional processes, and (4) lncRNA (long non-coding RNA)-associated regulatory pathways. Cytosine DNA methylation levels have been found positively correlated with the root growth through determining the role of salicylic acid (SA) on the methylation patterns and plant development in four pearl millet (*Pennisetum glaucum*) varieties (Ngom et al., 2017). Farinati et al. (2017) reviewed in detail the role of significant epigenetics parameters in rosaceae for about three important fruit developmental processes. Crisp et al. (2016) presented a detailed review on the plants' response processes involved in recovery from stresses through epigenetics regulations including resetting to memory development. Recently, Secco et al. (2017) reviewed in detail some of the advancement on involvement of histone modifications, histone variants and DNA methylation in response to nutrient stresses. Epigenetics' regulatory tools of gene expression are also vital for plants survival especially when they are exposed to biotic stresses such as bacteria, viruses, fungi, parasites, insects and weeds (Barozai and Aziz, 2018). Dubey and Jeon (2017) explained in detail the host epigenetic modifications during *Magnaporthe oryzae* pathogenesis progress and host-pathogen interactions, while suggesting the role of epigenetics in the epidemiology for future research and studies. A mode depicting in the involvement of DNA methylation in fruit ripening is shown in Figure 3. In immature fruit, numerous genes associated with ripening have methylated promoters, which inhibit RIN targeting and subsequent transcriptional activation. In an uncharacterized manner, the promoter regions become demethylated during ripening stages and corresponding genes experience binding by RIN and active transcription, triggering fruit ripening. The cnr mutant inhibits the demethylation process and prevents fruit ripening (Ji et al., 2015). The model of regulation of secondary metabolism by DNA methylation is shown in Figure 4.

Wei et al. (2014) proved that the correlating changes of cytosine methylation and proteomic expression were evidenced under cold treatment in *Brassica napus*. Duan et al. (2018) found that all of the major epigenetic mechanisms in mammalians also occur in plants. Niederhuth and Schmitz (2017) concluded that methylation as well as the pattern of that methylation

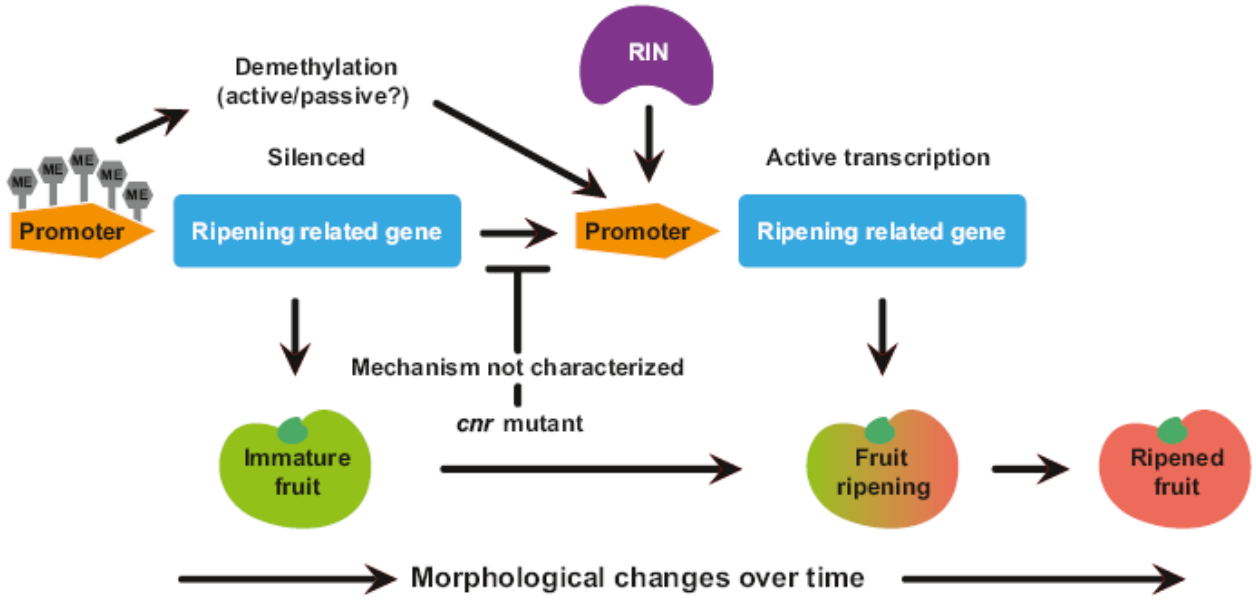

Figure 3. A mode depicting the involvement of DNA methylation in fruit ripening.

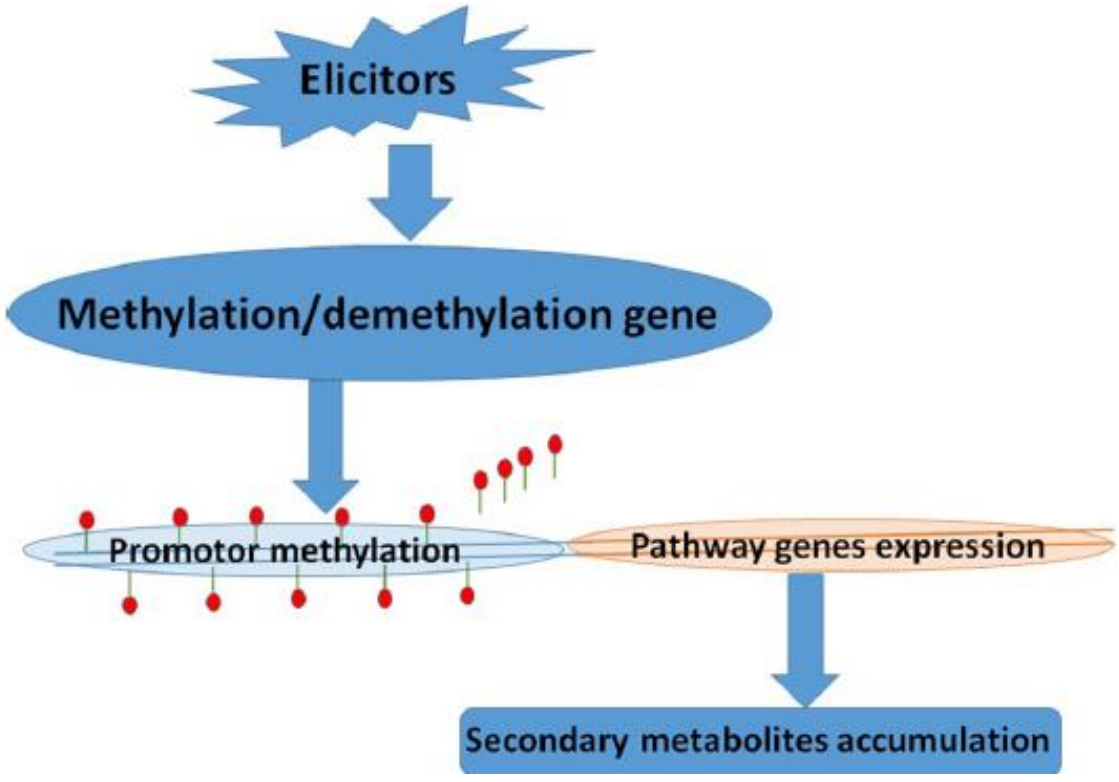

Figure 4. The model of regulation of secondary metabolism by DNA methylation.
Source: Yang et al. (2018).

within or outside of the gene is highly dependent upon the type of methylation. Hseih et al. (2011) reported a further support for the notion of the epigenomes as a drug target and a new set of tools for the design of TCM prescriptions. Amarger et al. (2014) found that dietary compounds containing methyl group donors are important

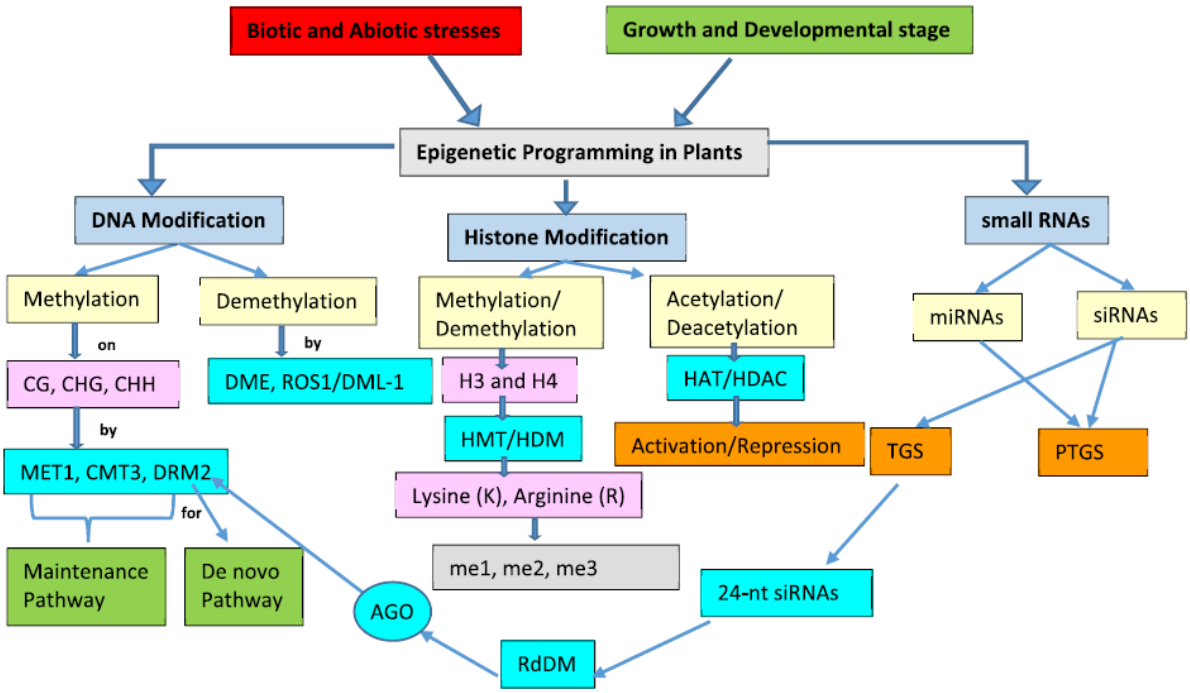

Figure 5. Detail description of epigenetics programming under biotic and abiotic stresses as well as for growth and development in plants. AGO= Argonaut, CG= Cytosine Guanosine, CMT3= chromomethylase 3, DME= Demeter, DML-1= Demeter- like 1, DRM2= domains rearranged methyltransdarase 2, H = A, C or T, H3= Histone 3, H4= Histone 4, HAT= histone acetyl transderase, HDAC= histone acetyl deacetylase, HDM= histone demethylase, HMT= histone methyl transferase, me1= one methyl, me2= two methyl, me3= three methyl, MET1= methyl transderase 1, miRNAs= microRNAs, PTGS = posttranscriptional gene silencing, RdDM= RNA-directed DNA methylation, ROS1= Repressor of silencing 1,siRNAs= small interfering RNAs, TGS= transcriptional gene silencing.
Source: Barozai and Aziz (2018).

regulators of nuclear DNA methylation. Wang et al. (2016) revealed that sequence analysis of the fragments that underwent changes in DNA methylation or gene expression indicated that these changes were associated with various biological pathways. They have suggested that alternations in DNA methylation caused by hybridization and polyploidization may induce changes in gene expression and lead to new agronomic traits in polyploids. Liu et al. (2018) indicated DNA methylation-regulaed auxin pathways plot a role in establishing inbred depression phenotypes in plant. Vargeer et al. (2012) reported that DNA methylation appears to have a direct effect on the severity of negative inbreeding phenotypes. Xiao et al. (2006) stated that DNA methylation regulates gene transcription over large regions of the genome, and can be maintained throughout life cycles and over various generations. Yang et al. (2018) announced that DNA methylation regulates the expression of key genes involved in phenolic acids biosynthesis to affect phenolic acids production in *Salvia miltiorrhiza*. They have also reported the roles of DNA methylation on phenolic acids accumulation will provide new perceptions on the regulation of secondary metabolism in plants. Detail description of epigenetics programming under biotic and abiotic stresses is presented in Figure 5. Plant epigenetics programming under stress managements and growth related stages are shown here, at three levels: DNA modification, Histone modification and small RNAs. The boxes colors are showing symmetry in explanation, mode of action, epigenetics types, processes and players involved in these processes. The plasticity during growth and development as well as under biotic and abiotic stress management is depicted via the routes of DNA modification, Histone modification and generating small RNAs (Barozai and Aziz, 2018).

Mastan et al. (2012) revealed that the (Methylation sensitive amplification polymorphism (MSAP) analysis showed that under salt stress homologous nucleotide sequences in genome from control and salt treated plants of *J. curcas* showed different patterns of methylation; which suggest that these fragments probably an important role to induce immediate adaptive responses in *Jatropha* under salinity stress. Li et al. (2015) revealed that $NiCl_2$ application caused variation of DNA methylation of the *Arabidopsis* genomic and offspring's. $NiCl_2$ also resulted in nucleolar injury and deformity of root tip cells; the methylation rate of 18S rDNA also changed by adding $NiCl_2$. Dossa et al. (2018) observed

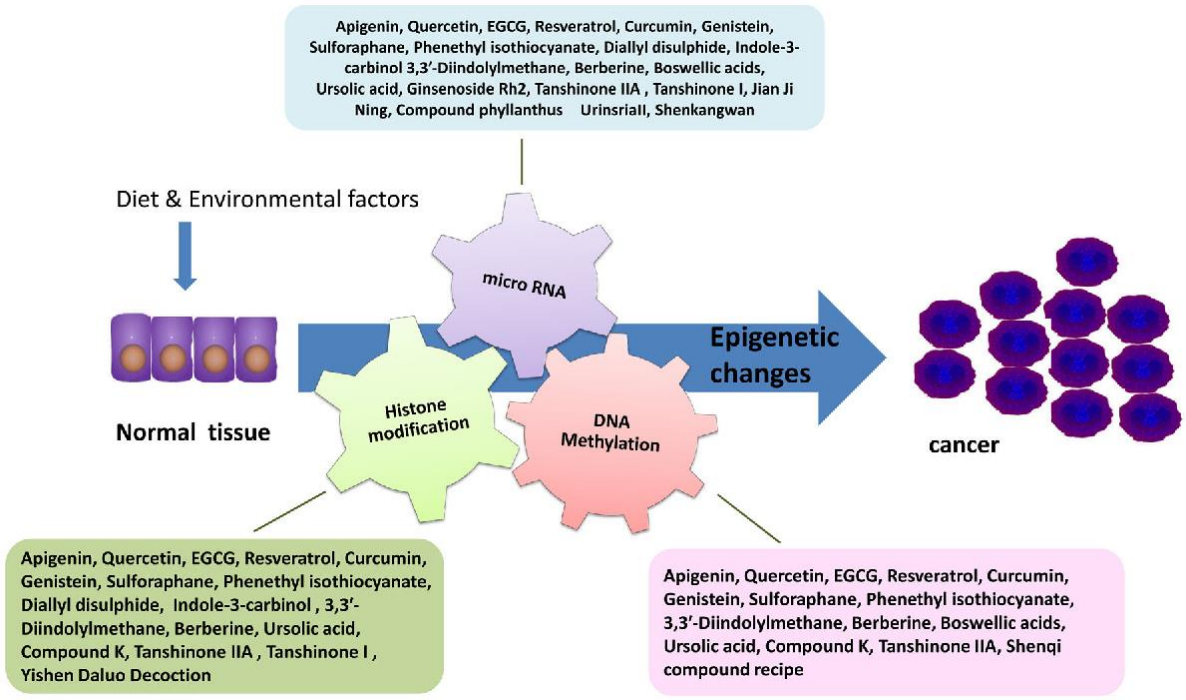

Figure 6. Potential epigenetic mechanism of phytochemicals in traditional Chinese herbal medicine for cancer prevention.
Source: Zhou et al. (2017).

that sesame has divergent epigenetic programs that respond to drought and waterlogging stresses and an interplay among DNA methylation and transcript accumulation may partly modulate the contrasting responses to these stresses. Wang et al. (2011) indicated that demethylation of genes is an active epigenetic response to salt stress in roots at the seedling stage, and helps to further elucidate the implications of DNA methylation in crop growth and development. Arikan et al. (2018) demonstrated the mutual interaction between morphophysiology, DNA methylation and the associated gene expression, and provide insight into the abiotic stress response of Arabidopsis. Li et al. (2015) described that cultivated ginseng maintained high levels of genetic and epigenetic diversity, but showed distinct cytosine methylation patterns compared with wild ginseng high methylation level and polymorphism reported could be related with the high structural genome plasticity which has been reported in the *Brassica* species to explain the phenotypic variability of this species. Zhao et al. (2010) suggested that the demethylation positively contributed to salt tolerance and the hypermethylation had negative influence on salt tolerance in cotton. Peng et al. (2007) showed that the methylation mutation involved into the whole rice genome and the 12 pairs of chromosomes; the mutation trend was site-related and there were different mutation loci for different triploids, which foretold that SARII-628 would have different evolution fates after natural homologous triploidization. Chen et al. (2014) suggested that DNA methylation maybe involved in the sterility-fertility transition of PA64S under two different environmental conditions. Underwood et al. (2017) concluded that DNA methylation profiling of hundreds of natural Arabidopsis accession has revealed that transposable elements exhibit significant intraspecific genetic and epigenetic variation, and that genetic variation often underlies epigenetic variation. Lu et al. (2008) demonstrated the power of the MSAP (methylation sensitive amplification polymorphism) technique for large-scale DNA methylation detection in the maize genome, and the complexity of DNA methylation change during plant growth and development. They have concluded that different methylation levels maybe related to specific gene expression in various tissues. Epigenetics has the ability to impose flexible growth in plants depending on the conditions prevailing so that deciphering such regulation may enable designing stress-tolerant crops (Banerjee and Roychoudhury, 2017). Potential epigenetic mechanism of phytochemicals in traditional Chinese herbal medicine for cancer prevention is shown in Figure 6. Correlation of epigenetic regulators with salinity and drought is shown in Table 2.

Epigenetic modifications, including DNA methylation, histone post-transcriptional modifications, micro RNA interference and etc. may help explore the molecular basis of Chinese medicine syndrome classification, and

Table 2. Correlation of epigenetic regulators with drought and salinity.

Stress	Epigenetic regulators	Functions	Relation with stress tolerance
Salinity	ADA2b	Transcriptional adaptor	Positive
	HDACs (HD2C, HDA6, HDA19)	Histone deacetylase	Positive
	HDA705	Histone deacetylase	Negative (seedlings)
	HDT701	Histone deacetylase	Positive (seedlings)
	AtROS1	Demethylase	Positive
	SW13B	Chromatin remodeler	Positive
Drought	ATX1	Histone methyltransderase	Positive
	MSI1	Silencer	Negative
	MYST, ELP3, GCN5	Histone acetyltransferase	Positive
	AtHD2C	Histone deacetylase	Positive
	CHR12	Chromatin remodeler	Negative
	BRM	Chromatin remodeler	Positive

Source: Banerjee and Roychoudhury (2017).

mechanisms of Chinese herbal medicine (CHM), CHM compounds and Chinese herbal formulae activities (Hu and Su, 2017). Hu and Su (2017) concluded that Chinese medicine (CM) and epigenetics might promote each other and jointly develop following the continuous progress of epigenetics in Chinese medicine researches. Zhou et al. (2017) stated that the progress of epigenetic alternations in cancer, emphasizing the role of traditional Chinese herbal medicines (TCHMs) as potential preventive/ therapeutic agents for cancer management, and provides a basis and potential future direction for the development of novel therapeutic drugs. Zhai et al. (2016) reported that epigenetic regulation may provide some innovative research ideas for finding new drugs of the treatment of diabetes from traditional Chinese medicine and natural medicine. DNA methylation is a reversible process by the removal of methyl groups and this property makes DNA methylation a promising drug target to treat cancer (Easwaran et al., 2014). Ou et al. (2009) found that a set of genes encoding for the various putative DNA methyltransferase, 5-methylcytosine DNA glycosylases, the *SWI/SNF* chromatin remodeler (DDM1) and siRNA-related proteins are extremely sensitive to perturbation by spacefliht, which might be an underlying cause for the altered methylation patterns in the space-flown plants. Hseih et al. (2013) concluded that TCM prescriptions' modulation of the human epigenome help elucidation of phyto-pharmacology and discovery of epigenetic drugs. Furthermore, as TCM medicinals' properties are closely tied to patient TCM syndromes, results of this material-medica-wide, bioinformatic analysis of TCM medicinal may have implications for molecular differentiation of TCM syndromes. Different traditional Chinese medicine has been shown to be safer and more fruitful for preventing cancer by targeting epigenetic landscape (Hun Lee et al., 2013). Yang et al. (2016) expressed that targeting epigenetic genes can be a promising plan to treat hematopoietic system malignancies and TCM is attractive to explore drugs targeting epigenetic modifiers.

TRADITIONAL CHINESE MEDICINE AND DNA METHYLATION

Some scientists have proposed that epigenetics is the partial material basis of TCM syndrome diversity, and the microscopic index of epigenetics can be a necessary supplement to the macroscopic syndrome differentiation of TCM syndromes. So, the correlation between epigenetics and TCM syndromes phenotypes is considered as one of the key scientific problems to realize the breakthrough of TCM clinical practice. Ma et al. (2018) indicated that DNA methylation-miRNA-Target gene is the main line, which further reveals the essence of TCM syndrome. To improve the level of TCM clinical syndrome differentiation and the clinical efficacy of TCM, especially in the study of RCM syndromes of chronic hepatitis B (CHB), discovering its underlying biological signature is necessary. Correlation between TCM syndromes and epigenetics is shown in Figure 7. Epigenetics is the partial material basis of TCM syndrome diversity, and the microscopic index of epigenetics can be a necessary supplement to the macroscopic syndrome differentiation of TCM syndromes (Ma et al., 2018). Integrative medicine (IM), routes and effects is presented in Figure 8. The figure represents how IM works at different levels such as the psychological, physiological, biochemical and/or epigenetic levels. The red arrows indicate the different initial levels at which IM can exert its action so that the final action is exerted at the epigenome. An altered epigenome then produces beneficial effects at different levels indicated by green arrows. The epigenetic regulations of TCM on tumors have been shown in Table 3. An IM approach can

Table 3. The epigenetic regulations of TCM on tumors.

Targets	TCM or TCM active ingredients	Effects
DNMTs	Trichosanthin	↓DNMTs activity in human breast cancer MDA-MB-231 cells
	Tanshinone IIA	↓DNMT1 in HepG2 human hepatoma cells
	Arsenic Trioxide	↑DNMT1 and ↓p16 in human hepatoma cells
		↓↓DNMT3B in leukemia HL-60 cells
	Yugan granule	↓DNMT1, ↓DNMT3A and ↓DNMT3B in mice hepatoma cells
	Genistein	↓DNMTs activity and ↑ p16 in KYSE 510 cells
Histeon modifications	Curcumin	↓Histone H3 acetylation in Raji, HL-60 and K562 cells
		↓HDAC1 activity in HepG2 human hepatoma cells
	Triptolide	↓SUV39H1 and EZH2 in multiple myeloma RPMI8226 cells
	Epigallocatechin-3-gallate (EGCG)	↓HAT (histone acetyltransferase) activity in androgen-dependent prostate cancer cells
	Gernistein	↑Tumor suppressor genes (PTEN, CYLD, p53 and FOXO3a), remodeling the heterochromatic domains of their promoters in prostate cancer cells

Source: Yang et al. (2016).

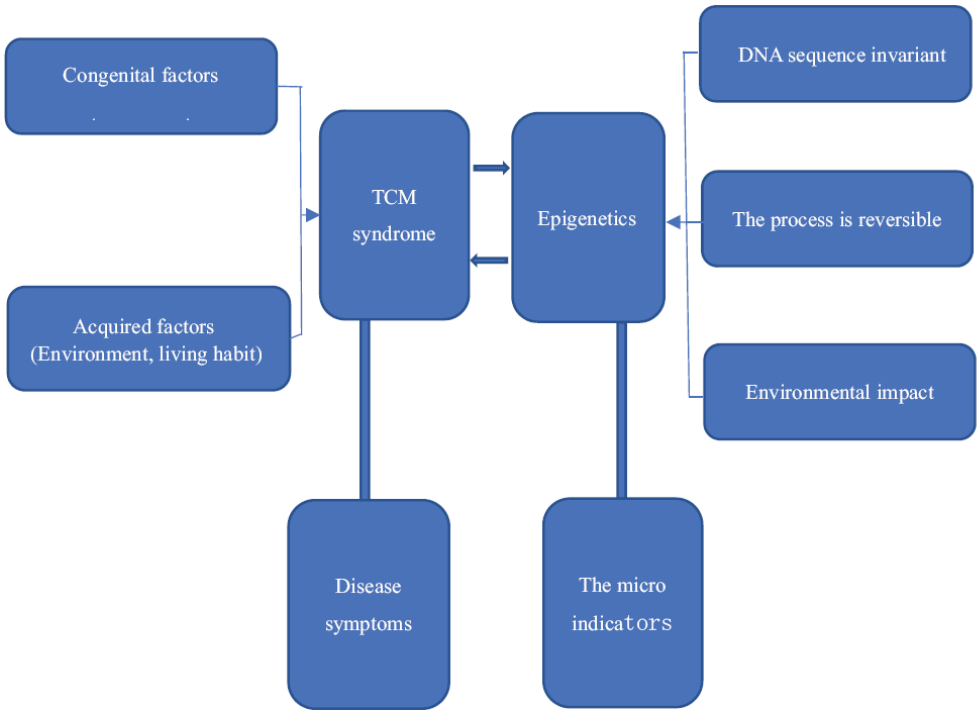

Figure 7. Correlation between TCM syndromes and epigenetics.
Source: Ma et al. (2018).

work at these different levels through a specific hierarchical sequence, or at different levels simultaneously. Some approaches can first act directly at the epigenetic level, surpassing the psychological and physiological levels. After the alteration of the epigenetic profile, the effect can manifest in the form of gene expression and biochemical changes, physiological and/or psychological changes (Kanherkar et al., 2017). Direct, indirect and combined epigenetic pathways of integrative medicine is shown in Figure 9. The figure represents a summary of the epigenetic mechanisms underlying different IM approaches. The cell is represented as oval pink structure with a yellow nucleus. The fine structure of chromatin comprised of DNA

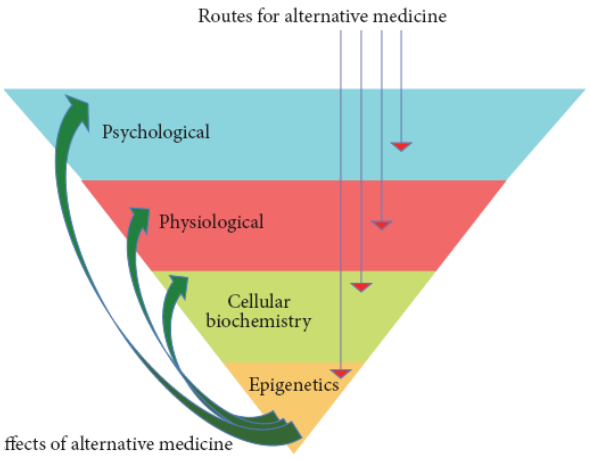

Figure 8. Integrative Medicine (IM): routes and effects.
Source: Kanherkar et al. (2017).

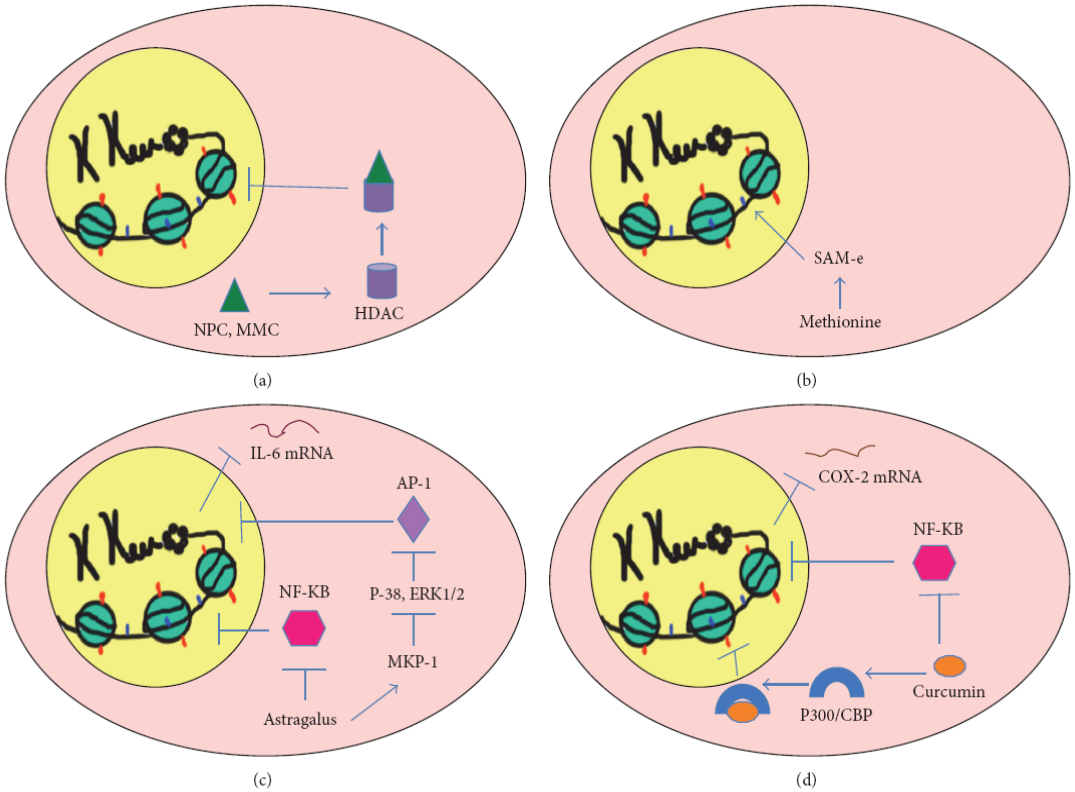

Figure 9. Direct, indirect, and combined epigenetic pathways of integrative medicine (a) Type 1 direct pathway: Traditional Chinese Medicine (b) Type 2 direct pathway: herbal methionine (c) Indirect pathway: Astragalus (d) Combined pathway: curcumin.
Source: Kanherkar et al. (2017).

wrapped around histones in the nucleus is the ultimate regulatory components through which IM approaches manifest their outcomes. Red marks on histones represent acetyl groups on histone tails (histone acetylation) and blue marks represent methyl groups on DNA (DNA methylation). (a) Type 1 direct pathway: Traditional Chinese Medicine. Traditional Chinese Medicine (TCM) compounds like Ningposides C (NPC) and Monomethylcucumin (MMC) (represented by green triangles) act as HDAC2 (represented by purple cylinder)

inhibitors that inhibit deacetylation of histones and relaz the chromatin structure. The mode of action of NPC and MMC is through a Type 1 direct pathway since they directly interfere with the epigenetic enzyme HDAC. (b) Type 2 direct pathway: herbal methionine. Dietary compounds like herbal methionine (HM) that donate methyl groups and increase SAMe levels in the body are important regulators of nuclear DNA methylation. An increased production of SAMe from HM increases the bioavailability of methyl groups (metabolite) that contribute to the constitution of epigenetic tags, specifically affecting DNA methylation levels. Thus, HM follows the Type 2 direct pathway by interfering with the bioavailability of compounds that constitute epigenetic tags. (c) Indirect pathway: Astragalus. Astragalus extract has anti-inflammatory properties and can promote its effects through two different pathways. Firstly, it inhibits p38 MAPK and ERK1/2 via stimulation of MPK that in turn blocks the nuclear translocation of AP-1 (lavender diamond) responsible for expression of proinflammatory cytokine IL-6. Secondly, it interferes with the nuclear translocation of NF-κB (pink hexagon) and inhibits NF-κB-mediated transcription that in turn activates proinflammatory genes. Thus, Astragalus follows an indirect epigenetic pathway by interfering with cellular signaling pathways. (d) Combined pathway: curcumin. Curcumin (Orange oval) possesses anti-inflammatory activity that operates through a combination of direct and indirect pathways. Through the direct epigenetic pathway, it specifically inhibits a specific p300/CBP HAT (blue semicircle) thereby reducing histone acetylation and through the indirect epigenetic pathway it blocks pathways involving the transcription factior NF-κB (pink hexagon) that in turn block the production of COX-2. Thus, curcumin operates through a combination of direct and indirect pathways (Kanherkar et al., 2017). Hao and Xiao (2018) reported that the epigenetic and epigenomic mechanisms should be highlighted in the study of specific phenotype and indigenousness of geoherbalism. They have stated that revealing the correlation between epigenetics and geoherbs could shed light on the quality assessment, authentication, molecular breeding and sustainable utilization of medicinal plants and the associated microbes. Vidalis et al. (2016) indicated that the concept of epigenetics should be introduced into the geoherb studies, and the role of DNA methylation. China and adjacent regions possess spectacular ecosystem diversity, which partially determines the epigenetic diversity and is the ecological basis of geoherb diversity (Huang and Chen, 2017).

CONCLUSION

Traditional Chinese medicine has contributed to human health care for thousand years and it is still popular not only in Asian countries, but also in western societies. TCM is a system of both theories and therapies that was first documented in ancient Chinese classics dating back 2100 years. Epigenetics, combining genetics and environment contributes to not only the stability of organisms but also their adaptability to the environment, which is consistent with the theory of human-environmental inter relation of TCM. Epigenetics consists of hereditable modifications in gene function without involvement of the DNA sequence. Epigenetic mechanisms are involved during all stages of a plant, from flower to mature seed for reproductive organs, development that determined crop productivity and seed nutritive traits. Epigenetics is a hot research topic in recent years and DNA methylation is the most common and the most studied epigenic content. DNA methylation may lead to variations in gene expression without changing its DNA sequence. In plant, may epigenetic changes, mainly at the level of DNA methylation, are transgenerational stable and contribute to formation of epialleles, affecting developmental and agronomical traits. Plants traditional Chinese herbs and fruits are suitable for studying epigenetics, and also opens the possibility for utilizing or inducing epigenetic differences for purpose of plant breeding and improvement. Epigenetics of traditional Chinese herbs offers a strong support for the proposition of an epigenetic role in TCM pharmacology. Epigenetic regulation has been recognized as an important player in the response to changes in key environmental conditions such as light, temperature and drought. Epigenetics' regulatory tools of gene expression are important for plants survival especially when they are under various stresses like bacteria, viruses, fungi, parasites, insects and weeds. The epigenetic-based programming in plants under biotic and abiotic stresses as well as during growth and development is carried out by methylation-demethylation, acetylation-deacetylation and small RNAs, which are involved in the regulation of gene expression without any change in the DNA sequences. Various traditional Chinese medicine has been shown to be safer and more effective for preventing cancers. Plants' utilization of the epigenetic approach are used to manage and resist the fungal, bacterial and others biotic stresses; microbes, also employ epigenetic mechanisms to modulate growth and pathogenicity, leading to resistance against plant-host immune system. DNA methylation is a chemical modification process where the methyltransderass (DNMTs) are catalyzed by selective addition of methyl groups to form 5-methylcytosine in CpG sequences. A mixture of herbs and fruits used in traditional Chinese medicine maybe use to alleviate diseases by adding and removing epigenetic marks on DNA. Epigenetics has been introduced to the area of TCM recently, which resulting in the hypothesis of an epigenetic role in the modern pharmacology of TCM prescriptions. Epigenetics is the partial material basis of TCM syndrome diversity, and the microscopic index of epigenetics can be a

necessary supplement to the macroscopic syndrome differentiation of TCM syndromes. All in all, in conclusion, new agronomical plans and adaptive agronomical practices are needed to face the future increase in food demand resulting from expanding population.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Ethnobotanical studies of medicinal plants used in traditional treatment of malaria by some herbalists in Ghana

Tonny Asafo-Agyei*, Heron R. Blagogee, Susana Oteng Mintah, Mary-Ann Archer, Frederick Ayertey, Alexis Conrad Sapaty, Peter Atta-Adjei Jnr, Daniel Boamah, Jerry Asiedu-Larbi and Alfred Ampomah Appiah

Centre for Plant Medicine Research, P. O. Box 73, Mampong Akuapem, Ghana.

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The use of medicinal plants for the treatment of diseases including malaria is a common practice in Ghanaian traditional medicine. The objective of this study is to document indigenous knowledge of medicinal plants used for the treatment of malaria through ethno-botanical studies to facilitate the discovery of new sources of drugs. The study was carried out in 2018 at the Centre for Plant Medicine Research (CPMR) among 36 registered herbalists of the Ghana Federation of Traditional and Alternative Medicine (GHAFTRAM). Data was collected based on oral interview with each of the 36 registered herbalists with the aid of a well-structured questionnaire. Only data from willing respondents were documented after obtaining their consent to participate in the study. 42 different plant species belonging to 27 families were identified as being used by GHAFTRAM herbalists in treating malaria. Among the various plant parts used, the leaves were the most reported (41%), and all of the medicinal preparations were decoctions prepared by boiling the plant parts. About 93% of the herbalists collected plants from the wild, whereas the 7% were collected from their immediate surroundings (within 100 m of their homes). Major threats to the continues availability of medicinal species of plants as indicated by the respondents included: farming activities (40%), bushfires (33%), over-harvesting (14%), and drought (13%). Majority (56%) of the herbalists reported uprooting whole plants as their method of collecting medicinal plant parts. The results of the study suggest a need for conservation and sustainable harvesting strategies to conserve plant wealth in Ghana.

Key words: antimalarial, conservation, medicinal plants, Ghana Federation of Traditional and Alternative Medicine (GHAFTRAM), traditional medicine

INTRODUCTION

The use of herbal medicine is on the increase globally (Asiimwe et al., 2014; Joshi and Joshi, 2000; Kamatenesi-Mugisha, 2005). In Africa, the situation is not different, over 80% of the population particularly in developing

*Corresponding author. E-mail: asafoagyeitonny18@gmail.com.

countries depend directly on plants for their primary healthcare requirements (Senthilkumar et al., 2013). In Ghana, where malaria is a major developmental and socioeconomic issue, many indigenes especially in rural communities rely on medicinal plants, solely and sometimes in combination with orthodox antimalarial drugs, for the treatment of the disease (Abbiw, 1990; Mshana et al., 2000). The Ghana government has specifically boosted the use of herbal medicines by integrating it into the main health care system by establishing herbal units in most of the government hospitals across the country. This has been necessitated by the consequences of limited access to modern health services in most developing countries including Ghana, high cost of modern medicine compared to the indigenous herbal medicines and wide socio-cultural acceptance of traditional medicine (Kamatenesi-Mugisha et al., 2005; Oreagba et al., 2011; Van Andel and Carvalheiro, 2013).

Malaria is the single most important cause of death, ill health and poverty in sub-Saharan Africa (Sachs and Malaney, 2002). Estimates suggest as many as 300 million acute cases of malaria occurring worldwide each year, resulting in 1 million deaths. Approximately 90% of these deaths occur in sub-Saharan Africa, and most of the victims are children younger than 5 years of age (Murphy and Breman, 2001). The disease is caused by members of the parasitic protozoa of the *Plasmodium* genus, which are transmitted by the female *Anopheles* mosquito to the human host. The five species of *Plasmodium* that infect humans include the deadly *Plasmodium falciparum*, which causes malignant malaria responsible for the severe symptoms and death in humans. It is also the most common in sub-Saharan Africa. Others are *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi* which cause the milder, benign malaria (WHO, 2010).

Currently, the World Health Organization (WHO) has adopted an integrated approach in the control of the disease, and this involves case management and disease prevention. Together, they work against the transmission of the parasite from the mosquito vector to humans, and the development of illness and severe disease (WHO, 2012). Case management, which involves treatment with antimalarial drugs, continues to be the most extensive approach to malaria control. Preventive measures encompass intermittent preventive treatment (IPT) and malaria vector control. IPT involves the administration of a full course of an effective antimalarial treatment at specified times, and targets a defined population at risk of malaria, e.g. pregnant women, regardless of whether the recipients are parasitaemic or not, with the objective to reduce the malaria burden in the target population (WHO, 2012). Despite the gains made in malaria control, there are some key challenges in the fight against malaria. A growing concern is the emerging parasites (*Plasmodium* sp.), developing resistance to the most widely available, affordable, and safest first-line treatments such as Chloroquine and Sulfadoxine and pyrimethamine (Khalid et al., 1989; Sarker et al., 2000) ; resistance to a wide range of insecticides rendering mosquito control programmes unsuccessful ; the widespread production and marketing of “new” ineffective antimalarial drugs, such as Artesunate blister packs that contain no active ingredients (Newton et al., 2006); and the unavailability of needed infrastructure and resources to manage malaria in many African countries (Murphy and Breman, 2001) .

In light of the foregoing, the call for new chemical entities, probably with new mechanisms of action, for the treatment of malaria or alternative approaches to malaria treatment remains a priority. Indeed, some medicinal plants with antiplasmodial activity have shown enhanced activity against the parasite when formulated as nanoparticles (Murugan et al., 2016; Rajakumar et al., 2015). WHO, in fact, has put in place a strategy (Traditional Medicine Strategy, 2014-2023) to support the promotion of safe and effective use of traditional and complementary medicine (WHO, 2013).

The first step in conservation and sustainable usage of medicinal plants is to document material traditionally used to treat an ailment (Hamilton, 2004; Ssegawa and Kasenene, 2007). A larger number of medicinal plants and indigenous uses have not yet been documented. The rich history of African cultures and their innovative utilisation of plants as a source of remedies have been passed down through generations largely by oral tradition (Soelberg et al., 2015). This knowledge is gradually being lost (Tabuti et al., 2012) as the custodians die before passing on information to the younger generations. Besides the gradual loss of ethnobotanical knowledge due to lack of documentation, overharvesting of medicinal materials from their natural habitat and destruction of habitats have been two of the major threats to traditional medicine. In order to conserve wild plant species, there is need for reliable data on their distribution and level of use (Ahrends et al., 2011). The documentation of indigenous knowledge through ethnobotanical studies is therefore important in conservation and utilization of biological resources (Munthu et al., 2006).

MATERIALS AND METHODS

Ethno-botanical data on plant species used for the treatment of malaria were collected during a two-week training course from 10th to 21st September 2018. This was achieved by interviewing 36 registered herbalists belonging to the Ghana Federation of Traditional and Alternative Medicine (GHAFTRAM). A well-structured questionnaire was used to obtain and document data during the training course organized by the Centre for Plant Medicine Research (CPMR), Mampong-Akuapem, Ghana. The purpose of the training was to educate the herbalists on best practices in cultivation of medicinal plants, sustainable harvesting or utilization of medicinal plants and best practices in the preparation of herbal medicines. Prior to conducting interviews, the objectives of the study were explained to the herbalists to obtain their consent

to participate. When asked, the herbalists associated malaria with a bite from a mosquito without knowledge of the specific species of mosquito that causes malaria. Symptoms of the disease they described included fevers, chills, and strong headaches. The questionnaires were pretested to 20 individuals randomly in the Mampong township and adjustments were made to validate it before detailed interviews were conducted. After being validated, the questionnaires were used to collect data on the socioeconomic status of the herbalists, common or vernacular names of plants, plant parts used, preparation methods, mode of administration, collection sites, and plant threats. The herbalists were interviewed individually by selected staff of CPMR who were trained purposely for this study. The herbalists were asked to present specimens of all plant species they reported from locations where they make collections for their malaria preparations. The reported local names of plants by the herbalists who mostly rely on sight as identification method, were confirmed using the Flora of West Tropical Africa (Hutchinson et al., 1963) and by comparison with herbarium vouchers at the herbarium of CPMR. The nomenclature of the species was confirmed using the International Plant Names Index (Croft et al., 1999) and Catalogue of Life (Bisby et al., 2009). Voucher specimens of all plant species reported were prepared and deposited in the CPMR's herbarium.

Data analysis

The variation in the knowledge (mean ± standard error) of medicinal plants used for the treatment of malaria due to gender of the respondents was compared using Student's t-test (p =0.3). A one-way analysis of variance (ANOVA) was used to compare differences within age groups, religious groups, educational background, and ethnic groups. The diversity of species used for the treatment of malaria was evaluated using the Shannon-Wiener index ($H = \sum_{i=1}^{S} pi \ln pi$) where s is the total number of species and p is the relative abundance of species (Macías et al., 2008). The relative abundance of each species was estimated from the total citations from among the total number of interviews. Frequency of citation (FC) of the species of plants being used for the treatment of malaria was evaluated using the formula: (number of times a particular species was cited/total number of citations of all species in the study) × 100%. The percentages of threats to medicinal plants, plant parts used, habits and sources of plant materials were also calculated.

RESULTS AND DISCUSSION

The treatment of malaria using plant materials was inquired into because in many parts of the world, including Ghana, the *Plasmodium* sp have developed resistance to frontline antimalarial drugs such as chloroquine, antifolates and recently artemisinin (Sebisubi and Tan, 2010). Resistance to these drugs has been reported to be as high as 40 to 60 percent in some African and Asian countries (Builders et al., 2011). There are also reported cases of debilitating adverse effects of the conventional antimalarial drugs (Builders et al., 2011). This calls for an urgent need to discover new active agents that can overcome these pitfalls. Although hundreds of plants species are being used as a folkloric remedy for malaria and fever, the vast majority have not yet been adequately evaluated. The Ghana Health Service have recently endorsed artesunate-amodiaquine combination drug for the treatment of malaria. However, amodiaquine (4-aminoquinoline) has been linked with hepatic toxicity, agranulocytosis, and other refutations (Asase and Asafo-Agyei, 2011). The combination drug is relatively high-priced, rendering the medication unobtainable to low income earners in endemic communities. The herbalists interviewed reported that most of the patients they treat, give account of how they use both the traditional medicines together with conventional medicine to treat themselves of malaria. This is similar to report of Vigneron et al. (2005). The probable complication of plant-drug interactions when conventional medicines are used together with traditional medicines may arise and ought to be researched into.

Sociodemographic impacts

Considering gender and the number of medicinal plants reported, there was no significant variation with a score of 13.06 ± 1.29 for males and 13.88 ± 2.29 for females. Most of the herbalists beyond 58 years (18.21 ± 1.73) reported on antimalarial uses of plants than within the age group 18 to 37 years (11.40 ± 2.77) and in the age group 38 to 57 years (11.36 ± 1.50) (Figure 1). The herbalists interviewed belonged to different religious groups and were considered knowledgeable in antimalarial uses of plants although the number of plant species mentioned differed. For Christians, 13.43 ± 1.28 plants were reported; for Muslims, 15.67 ± 2.03 plants were reported; for traditional spiritual believers, 12.75 ± 5.45 were reported (Figure 2).

Majority of the herbalists were well informed about antimalarial uses of plants and had university education (36.5± 1.6) or only primary education (19.9± 3.6), whereas only a few have had secondary (17.2± 1.8), no formal education (14.0± 2.9) and college education (12.0± 3.8) indicating differences in familiarity of antimalarial plants due to differences in the level of education achieved (Figure 3).

Religious background and gender did not have any significant impact on the knowledge and usage of herbal preparations for malaria treatment by the herbalists, although educational background influenced their perception of alternatives. The knowledge on traditional uses of plants was possessed by the older generation (beyond fifty-eight years), indicating the lack of cultural and conservational importance of the medicinal species.

Plant species identified from the herbalists' reports for the treatment of malaria

A total of 42 plant species belonging to 27 families were reported as being used for malaria treatment by Ghanaian herbalists (Table 1). Trees were the most dominant habit (47.6%) of plants used in the treatment of malaria. The other species of plants identified included

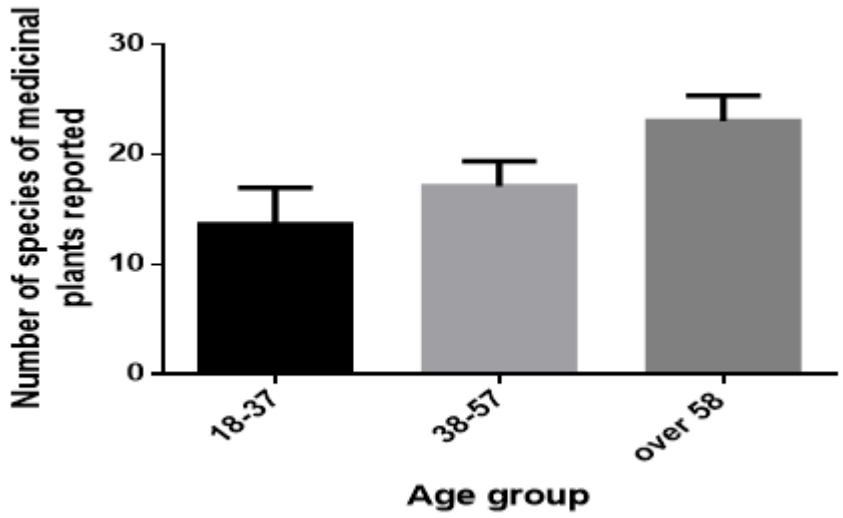

Figure 1. A distribution showing the difference in knowledge of medicinal plants used for the treatment of malaria due to age groups. Data are presented as means±S.E.M.

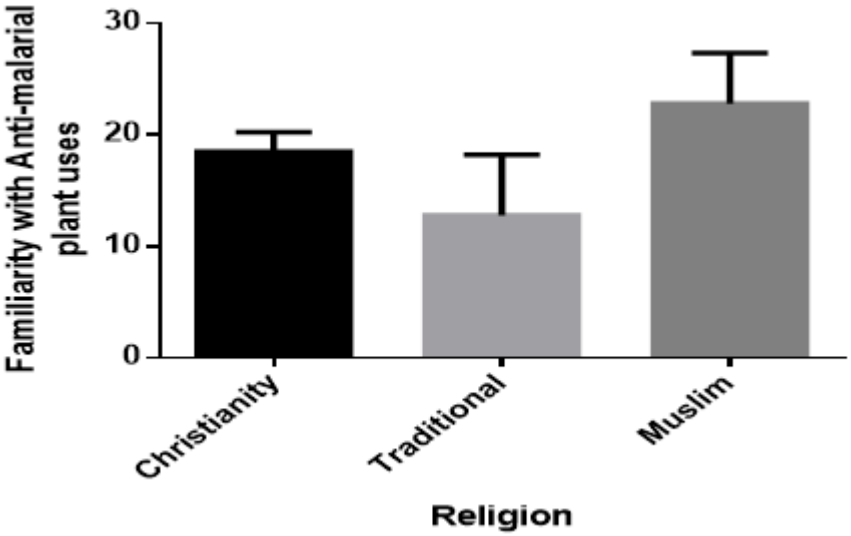

Figure 2. A distribution showing the familiarity with anti-medicinal uses of plants due religious affiliation. Data are presented as means±S.E.M.

shrubs (28.6%), herbs (16.7%), grasses (4.8%) and climbers (2.4%) (Figure 4). The findings of this study indicate that, a considerable high number (42 species) of medicinal plants are being used for treatment of malaria by Ghanaian herbalists. The dominance of tree species used for the treatment of malaria was also reported in Dike et al. (2012).

About 93% of the herbalists interviewed collected plant materials from the wild, whereas the remaining 7% collected plant materials from their immediate surroundings, that is, within 100 m from their homes. The frequency of collection was irregular, because plant parts were collected only when needed to treat malaria. According to the informants, farming activities pose a major (40%) threat to the survival of plant species although some informants also stated bush fires (33%), over-harvesting (14%), and drought (13%) as potential threats (Figure 5). Majority of the herbalists collected plant materials from the wild, similar to the results obtained by Boyom et al. (2011). Conservational strategies for sustainable utilization of medicinal plants are therefore needed in Ghana to protect plant wealth.

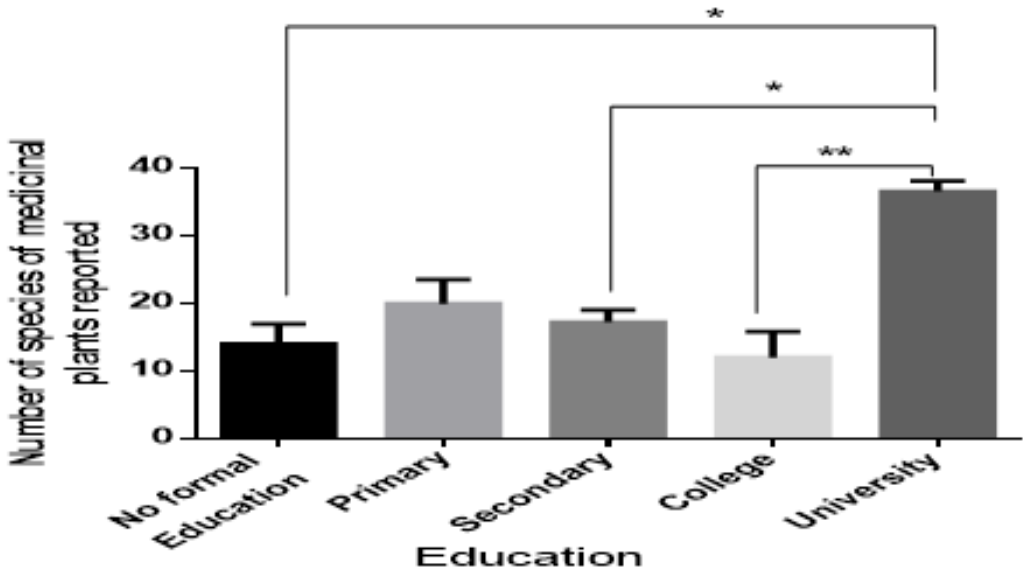

Figure 3. A distribution showing the difference in knowledge of medicinal plants used for the treatment of malaria due to educational background. Data are presented as means±S.E.M.*P<0.05, **P≤0.001.

Three (7.1%) species each belonged to the families Rutaceae, Meliaceae and Fabaceae. Two species each were identified for 9 families -Asteraceae, Cucurbitaceae, Euphorbiaceae, Lamiaceae, Rubiaceae, Zingiberaceae, Annonaceae, Longaniaceae and Poaceae. In contrast, 15 families-Caricaceae, Lauraceae, Meliaceae, Piperaceae, Malvaceae, Sterculiaceae, Bignoniaceae, Combretaceae, Moraceae, Myristicaceae, Sapindaceae, Myrtaceae, Mimosaceae, Amaranthaceae and Bromeliaceae-contributed with only one species to the total number of plant species reported (Table 1). The diversity of species used for malaria treatment by Ghanaian herbalists was high (Shannon-Wiener index = 3.5 ± 0.24). Most of the plants mentioned for the treatment of malaria, in this study, belonged to the families Rutaceae, Meliacea and Fabaceae. In contrast to this result, research from other parts of Africa showed that several species used for the treatment of malaria belonged to the family Rubiaceae (Asase et al., 2005; Iwu, 1994; Van Wyk et al., 2002). This shows that distinct groups of phytochemicals may account for the antimalarial properties possessed by plants being used traditionally for the treatment of malaria (Srisilam and Veersham, 2002). For instance, dissimilar phytochemical groups of compounds such as flavonoids (*Citrus aurantifolia* L.), phenols (*Phyllanthus niruri* L.) and terpenoids (*Momordica charantia* L.) (Berhow et al., 1994; Ishimaru et al., 1992; Chen et al., 2009) constituted different plant species belonging to different families such as Rutaceae, Euphorbiaceae and Cucurbitaceae respectively as reported by other works in Table 1. The most frequently cited plant was *Azadirachta indica* A. Juss (FC = 9.75). Other plant species commonly mentioned as being used for the treatment of malaria were *Nauclea latifolia* (FC=7.31) and *Occimum grattissimum* (FC = 6.09), whereas the least-cited plants were *Acacia nilotica*, *Ananas comosus*, *Alchornea cordifolia , Anthocleista nobilis, Bidens Pilosa, Cassia alata, Cleistopholis patens, Gossypium barbadense, Khaya senegalensis, Mezoneuron benthamianum , Paullinia pinnata, Psidium guajava, Pycnanthus angolensis, Solanum torvum, Occimum basilicum, Persea americana , Piper guineense, Theobroma cacao, Spathodea campanulata, Zanthoxylum zanthoxyloides, Polyalthia longifolia*, Alstonia boonei, *Xylopia aethiopica, Terminalia ivorensis* and *Milicia excelsa* with a FC= 1.21 each. The most frequently cited plant species by the respondents (Azadirachta indica A. Juss (FC = 9.75) could provide a guide for antiplasmodial testing and phytochemical analyses as to their effectiveness in the search for plant materials for the treatment of malaria (Heinrich, 2009). A frequently cited plant, nonetheless, does not indicate the plant's effectiveness for malaria treatment, but its use and frequent citation may be due to its abundance or availability.

Usage and application

The commonest plant parts used for the treatment of malaria were the leaves (40.7%), followed by roots (24.1%), stem bark (24.1%), seeds (5.6%), fruits (3.7%) and whole plant (1.9%) (Figure 6). This corresponds with the findings of Caraballo et al. (2004) conducted in South-eastern Venezuelan Amazon, where they proved

Table 1. Reported species of plants used for the treatment of malaria.

***Genus, species,* authority family Common names- Voucher specimen no.)**	**Growth Form**	**Parts used**	**Preparation**	**Reported phytochemical constituents and antiplasmodial activity**	**Dosage form**	**Frequency of citation (%)**
Acacia nilotica Delile Mimosaceae (Odanwoma- CPMR 4935)	Tree	Stem bark and roots	Boil roots and stem bark with roots of *Citrus aurantifolia* and roots of *Nauclea latifolia* and roots of *Alstonia boonie*and roots of *Butyrospemum parkii*	Antiplasmodial (EI-Tahir et al.1999)	Take two tablespoonsful once a day	1.21
Aframomum melegueta (Roscoe) K.Schum. Zingiberaceae (fam wusa- CPMR 4919)	Herb	Seeds	Boil roots with roots of *Nauclea latifolia* and rhizomes of *Theobroma cacao* and fruits of *Tetrapluera tetraptera*	Genus contains labdane (Duker-Eshun et al., 2006)	Take one ice cream cup three times daily	2.43
Alchornea cordifolia (Schumach.) Müll.Arg. Euphorbiaceae (Ogyama- CPMR 4908)	Shrub	Leaves	Boil leaves with leaves of *Morinda lucida*	Antiplasmodial activity (Valentin, 2000)	Drink three times daily with medium size cup	1.21
Alstonia boonei De Wild. Rutaceae (Nyamedua- CPMR 4924)	Tree	Root	Boil roots with roots of *Citrus aurantifolia* and roots of *Nauclea latifolia* and roots of *Acacia nilotica* and roots of *Butyrospemum parkii*	Terpenoids (Marini-Bettolo et al., 1983), Alkaloids (Oguakwa, 1984), Antiplasmodial (Zirihi et al., 2005a)	Take two tablespoonsful once a day	1.21
Amaranthus spinosus L. Amaranthaceae (Natwibini-CPMR 4938)	Herb	Stem bark and leaves	Boil leaves with leaves of *Cymbopogon citratus,Azadiracta indica and bidens pilosa*	B-cyananins and phenenols Antiplsmodial activities (Hilou et al, 2006)	Drink three times daily with medium size cup.	2.43
Ananas comosus (L.) Merr. Bromeliaceae (Abrobe-CPMR 4940)	Herb	Fruit	Boil fruit peel with fruit peel of *Aframomum melegueta* and stem of *Sacchurum officinale*	Phenols (Litaudon et al., 2009)	Drink one full cup three times daily	1.21
Anthocleista nobilis G.Don Loganiaceae (Owudifo k3t3- CPMR 4930)	Tree	Stem bark	Boil stem bark with stem bark of *Terminalia ivorensis* and stem bark of *Pycnanthus angolensis*	Alkaloids, Flavonoids (Ngwoke et al., 2015)	Take 50 ml three times daily	1.21
Azadirachta indica A.Juss Meliaceae (Nim-CPMR 4913)	Tree	Leaves, and roots	Boil roots with leaves of *Bidens pilosa*	Terpenoids (Siddiqui, et al. 2004)	Take four tablespoonful two times daily	9.75
Bambusa vulgaris Schrad. ex J.C.Wendl. Poaceae (Mpampuro-CPMR 4937)	Grass	Leaves	Boil leaves with leaves of *Alchornea cordifolia, Carica papaya,* and *Persea americana*	Alkaloids, Phenols (Goyal et al., 2010)	Drink decoction three times daily.	3.65
Bidens pilosa L. Asteraceae (Gyinantwi-CPMR 4936)	Herb	Leaves	Boil leaves with roots of *Azadirachta indica*	Antiplasmodial (Brandão et al, 1997)	Take 60 ml three times daily	1.21

Table 1. Contd.

Carica papaya L. Caricaceae (Brofre-CPMR 4906)	Tree	Leaves	Boil leaves and/or stem bark with leaves of *Xylopia aethiopica* and dry leaves of *Morinda lucida* and dry leaves of *Persea americana*	Phenols (Canini, 1994)	Take 150 ml two times daily	4.87
Cassia alata L. Fabaceae (Nsempii -CPMR 4941)	Shrub	Leaves	Biol leaves with leavesw of *Cleistophilis patens and Carica papaya*	Antiplasmodial activites (Zirihi et al., 2005b)	Drink three times daily with medium size cup	1.21
Cassia siamea Lam. Fabaceae (Accasia-CPMR 4945)	Tree	Leaves	Boil the leaves of *Cassia siamea* with the leaves of *Cymbopogon citratus*, *Azadirachta indica* and *Bambusa vulgaris.*	Alkaloids (Smith et al., 1996)	Drink half medium cup three times daily	2.43
Citrus aurantifolia (Christm.) Swingle Rutaceae (Ankaatwasie- CPMR 4917)	Tree	Roots and fruits	Boil roots with roots of *Alstonia boonei* and roots of *Nauclea latifolia* and roots of *Acacia nilotica* and roots of *Butyrospemum parkii*	Flavonoids (Rehm and Espig, 1991): Patil et al. 2010)	Take 150 ml two times daily	4.87
Cleistopholis patens Engl. and Diels Loganiaceae (Ngo ne Kyene- CPMR 4931)	Tree	Leaves and root	Boil leaves with leaves of Azadirachta indica, Spathodea campanulata, stem of Saccharum officinarum and fruits of Citrus aurantifolia.	Antiplasmodial (Addae-Kyereme, et al 2001) Alkaloids (Waterman and Muhammad,1985)	Take four tablespoonful two times daily	1..21
Cymbopogon citratus Stapf. Poaceae (Esre or nantwiwidie-CPMR 4915)	Grass	Leaves	Boil leaves with leaves of *Polyathia longifolia* and roots of *Nauclea latifolia*	Flavonoids (Tapia et al., 2007; Porspi, 1992)	Take decoction as directed	2.43
Gossypium barbadense L. Malvaceae (Asaaba-CPMR 4942)	Shrub	Leaves	Boil the leaves of *Gossypium barbadense* with the leaves of *Paullinia pinnata*, *Cassia alata*, *Psidium guajava* and *Ocimum gratissimum*	No information available	Take one medium cup three times daily	1.21
Khaya senegalensis A.Juss. Meliaceae (Mahogany-CPMR 4946)	Tree	Stem bark	Boil the leaves of *Khaya senegalensis* with the leaves of *Azadirachta indica,Alstonia boonei, Terminalia cartapa* and *Alchornea cordifolia*	Antimalarial (WAHP, 2013)	Take one medium cup three times daily	1.21
Lantana camara L. Cucurbitaceae (Ananse dokono- CPMR 4920)	Shrub	Leaves	Grind and boil with dry leaves of *Momordica charantia* and leaves of *Occimum gratissimum* and dry leaves of *Azadirachta indica*	Terpenoids (Litaudon et al., 2009: Begum et al., 2008a) Flavonoids (Begum et al., 2008b)	Take half a cup two times daily after meals.	2.43
Mezoneuron benthamianum Baill. Fabaceae (Akoobowre-CPMR 4944)	Shrub	Leaves	Boil roots of *Mezoneuron benthamianum* with leaves of T*ectonia grandis, Psidium guajava* and *Carica papaya* and the fruit of *Citrus aurantifolia.*	Antiplasmodial activities (Jansen, 2017)	Drink three times daily with medium size cup of decoction three times daily	1.21
Milicia excelsa C.C.Berg Moraceae (Odum- CPMR 4928)	Tree	Stem bark	Boil stem bark with dry leaves of *Alchornia cordifolia* and stem bark of *Terminalia ivorensis*	Antiplasmodial (Areola et al., 2016.)	Take 50 ml three times daily	1.21

Table 1. Contd.

Momordica charantia Descourt. Cucurbitaceae (Nyanya-CPMR 4907)	Herb	Leaves	Grind and boil with dry leaves of *Lantana camara* and leaves of *Occimum gratissimum* and dry leaves of *Azadirachta indica*	Terpenoids (Chen et al, 2009: Chen et al., 2008) and phenols (Kubola et al., 2008)	Take decoction as directed	2.43
Morinda lucida A.Gray Rubiaceae (Konkroma- CPMR 4916)	Tree	Leaves, and stem bark	Boil leaves and/or stem bark with leaves of *Xylopia aethiopica* and dry leaves of *Carica papaya* and dry leaves of *Persea americana*	Anthraquinones (Sittie et al., 1999); Antiplasmodial activity (Tona et al. 1999)	Take one medium cup three times daily	3.65
Nauclea latifolia Sm. Rubiaceae (Owintin- CPMR 4921)	Shrub	Stem and root	Boil leaves with dry leaves of *Carica papaya* and leaves of *Vernonia amygdalina* and leaves of *Solanum torvum* and roots of *Occimum gratissimum*	Antimalarial (Prance, 1987; Porspi, 1992)	Take 30 ml three to four times daily	7.31
Ocimum basilicum L. Lamiaceae (Akokomesa-CPMR 4910)	Herb	Leaves	Boil leaves with seeds of *Piper guineense* and leaves of *Azadirachta indica*	Flavonoids (Barua et al., 1978; Grayer et al., 2001), and essential oils (Grayer et al., 1996: Zhang et al., 2009)	Take three tablespoonful three times daily before meals	1.21
Ocimum gratissimum Forssk. Lamiaceae (Nunum- CPMR 4911)	Shrub	Leaves, and roots	Boil leaves with dry leaves of *Carica papaya* and leaves of *Vernonia amygdalina* and leaves of *Solanum torvum* and roots of *Nauclea latifolia*	Flavonoids (Grayer et al., 2001)	Take one medium cup three times daily	6.09
Paullinia pinnata Griseb. Sapindaceae (Toantini- CPMR 4933)	Shrub	Leaves and root	Boil dry leaves and roots with dry stem bark of *Perrsea Americana* and dry stem bark of *Morinda lucida*	Antiplasmodial (Gbeasor et al., 1989)	Take one medium cup three times daily	1.21
Persea americana Mill. Lauraceae (Pear- CPMR 4912)	Tree	Seeds, stem bark and roots	Boil leaves and/or stem bark with leaves of *Xylopia aethiopica* and dry leaves of *Carica papaya* and dry leaves of *Morinda lucida*	Carotenoids (Gross et al., 1973)	Take one medium cup three times daily	1.21
Phyllanthus niruri hort. ex Wall. Euphorbiaceae (Bomma gu makyi-CPMR 4909)	Herb	Whole plant	Boil whole plant	Phenols (Ishimaru, et al 1992) and terpenoids (Singh et al.,1989)	Take 60 ml three times	2.43
Piper guineense Thonn. Piperaceae (Esro wisa- CPMR 4914)	Climber	Seeds	Boil seeds with stem bark of *Zanthozylum xanthoziloids* and leaves of *Azadirachta indica* and seeds of *Aframomum maleggueta*	Alkaloids (Torto and Baxter 1976; Addae-Mensah, et al., 1977) Antiplasmodial activity (Bero et al., 2009)	Take 75 ml three times daily before meals	1.21
Polyalthia longifolia (sonn.) Hook.f. and Thomson Annonaceae (Polalthia- CPMR 4923)	Tree	Leaves	Boil leaves with leaves of *Cymbopogon citiratus* and roots of *Nauclea latifolia*	Diterpenoids (9), Alkaloids (Yang-Chang et al., 1990)	Take decoction as directed	1.21

Table 1. Contd.

Psidium guajava L. Myrtaceae (Guava- CPMR 4934)	Tree	Leaves	Boil leaves with water	Phenols, Flavonoids, Carotenoids, Terpenoids. (Gutiérrez et al., 2008)	Take five tablespoonful three times daily before meals	1.21
Pycnanthus angolensis (Welw.) Exell Myristicaceae (Otie/Otsil- CPMR 4929)	Tree	Stem bark	Boil stem bark with stem bark of *Terminalia ivorensis* and stem bark of *Anthocleista nobilis*	Antiplasmodial (Abrantes et al., 2008)	Take 50 ml three times daily	1.21
Solanum torvum Schltdl. Solanaceae (Konsusua-CPMR 4943)	Shrub	Leaves	Boil the fruits of Solanum *torvum* with leaves of *Morinda lucida* and stem bark of *Trichilia heudelotii*	Flavonoids and Alkaloids (Porspi, 1992)	Take one medium cup three times daily	1.21
Spathodea campanulata Buch.-Ham. ex DC. Bignoniaceae (Kokoanisuo- CPMR 4922)	Tree	Stem bark	Boil stem bark with stem bark of *Pycnanthus angolensis*	Antimalarial (Makinde et al., 1988)	Take 50 ml three times daily	1.21
Terminalia ivorensis A.Chev. Combretaceae (Embre- CPMR 4927)	Tree	Stem bark	Boil stem bark with dry leaves of *Alchornia cordifolia* and stem bark of *Milicia excelsia*	Terpenoids (Ponou et al., 2010)	Take 50ml three times daily	1.21
Theobroma cacao L. Sterculiaceae (Cocoa- CPMR 4918)	Tree	Roots	Boil roots with roots of *Nauclea latifolia* and rhizomes of *Zingiber officinale* and fruits of *Tetrapluera tetraptera*	Alkaloid (Ashihara et al., 2008), proanthocyandin, And polyphenols (Hatano et al., 2002; Vijayakumar et al., 2008)	Take 25 ml three times daily	1.21
Trichilia heudelotii Planch. ex Oliv. Meliaceae (Tanduro-CPMR 4939)	tree	Stem bark	Boil the bark of *Trichilia heudelotii* with the leaves of Persea Americana, Psidium guajava and the leaves and stem bark of *Amaranthus spinosus*	Alkaloids, Flavonoids (Bankole et al., 2016)	Take one medium cup three times daily	3.65
Vernonia amygdalina Delile Asteraceae (Anwonwono- CPMR 4905)	Shrub	Leaves, and Roots	Boil leaves with dry leaves of *Carica papaya* and leaves of *Nauclea latifolia* and leaves of *Solanum torvum* and roots of *Occimum gratissimum*	Antimalarial, Alkaloids, Flavonoids, Analgesia (WAHP, 2013)	Take one ice cream cup three times daily	3.65
Xylopia aethiopica A.Rich. Annonaceae (Hwenteaa-CPMR 4926)	Tree	Root	Boil leaves and/or stem bark with leaves of *Carica papaya* and dry leaves of *Morinda lucida* and dry leaves of *Persea Americana*	Terpenoids (Smith et al.,1996; Harrigan et al.,1994)	Take decoction as directed	1.21
Zanthoxylum zanthoxyloides (Lam.) B.Zepernick & Timler Rutaceae (Okantor- CPMR 4932)	Shrub	Stem bark	Boil bark with seeds of *Piper guineense* and leaves of *Azadirachta indica* and seeds of *Aframomum maleggueta*	Alkaloids (Tatsadjieu et al., 2003) Antispasmodic, Analgesia (Porspi, 1992)	Take one ice cream cup three times daily	1.21

Table 1. Contd.

Zingiber officinale Roscoe. Zingiberaceae (Ginger- CPMR 4925)	shrub	Rhizomes (roots)	Boil rhizomes with roots of *Nauclea latifolia* and roots of *Theobroma cacao* and fruits of *Tetrapluera tetraptera*	Analgesic ((Suekawa et al., 1984; Porspi, 1992)	Take five tablespoonful three times daily before meals	4.87

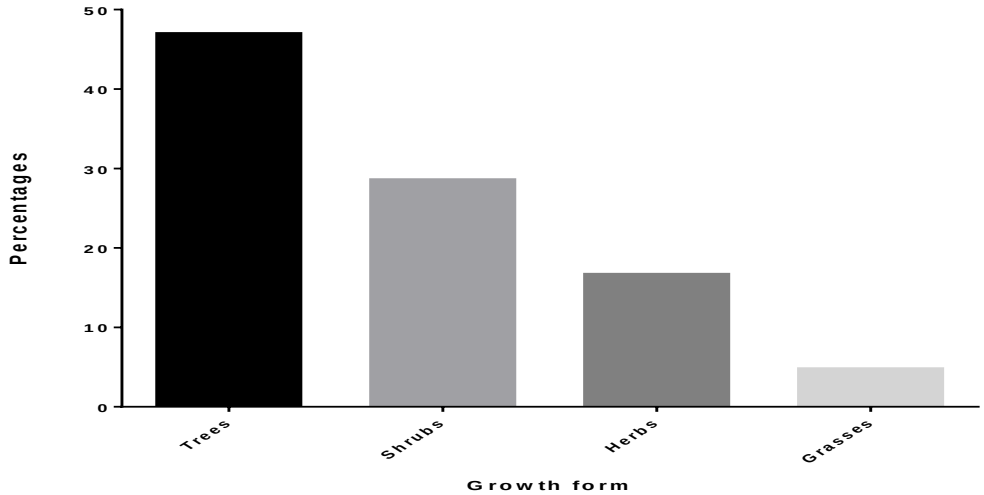

Figure 4. A distribution of growth forms and their percentage.

that the leaves constituted 70% of the parts used. It is likely that leaves are more available and accessible to people and contain highly effective antimalarial substances. The constant use of leaves could also be due to the fact that they are the site of the synthesis of organic substances and, therefore, antimalarial substances. Bhattarai et al. (2010) and Njoroge and Bussmann (2005). In all cases of preparation, the plant parts were boiled alone or together with parts of other plants. Almost all species reported (95.2%) were used as combination therapy with other plant species (Table 1). The mode of administration of the herbal preparations were all by drinking. Titanji et al. (2008) and Asase et al. (2005) by conducting similar studies have found forty-five preparation methods that combine more than one species of plants for the treatment of malaria with mode of preparation and administration being boiling and drinking the infusion respectively. Prescriptions were usually unspecific, and the intake of the herbal preparations continued until recovery. It became evident in this study that, the herbal preparations used by the herbalist's lack consistency and dosage instructions were indeterminate (Table 1). This means the quality of the herbal preparations may differ enormously amid prescriptions. This is not too different from observations reported elsewhere (Asase et al., 2005) and have been prominent also as a main drawback of traditional medicine (Evans-Anfom, 1986; Sofowora, 1993). The inclusion of certain plants, for instance *Citrus sinensis* fruits to herbal preparations might be to sweeten the decoction despite the fact that, *C. sinensis* has been mentioned in Bhat and Surolia (2001) as a

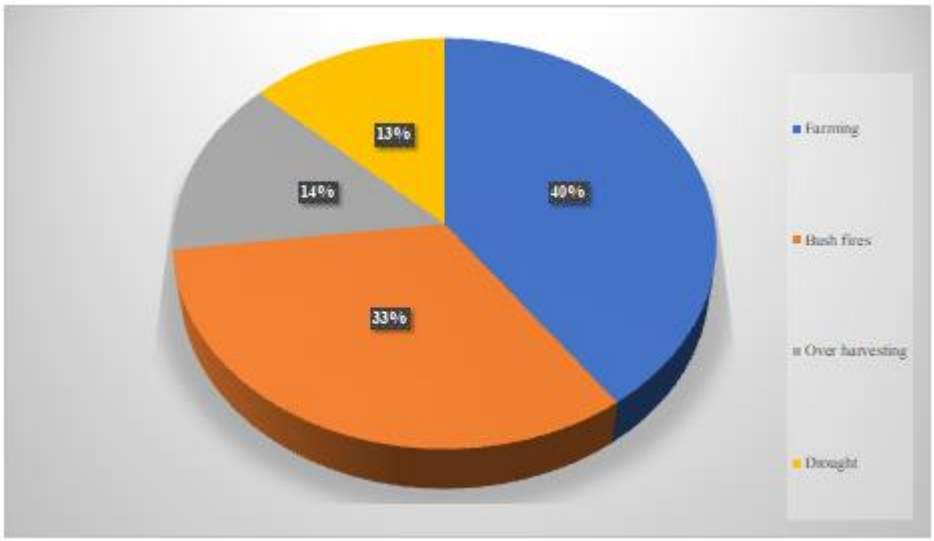

Figure 5. Proportions of different threats to medicinal plants being used by Ghanaian herbalists for the treatment of malaria.

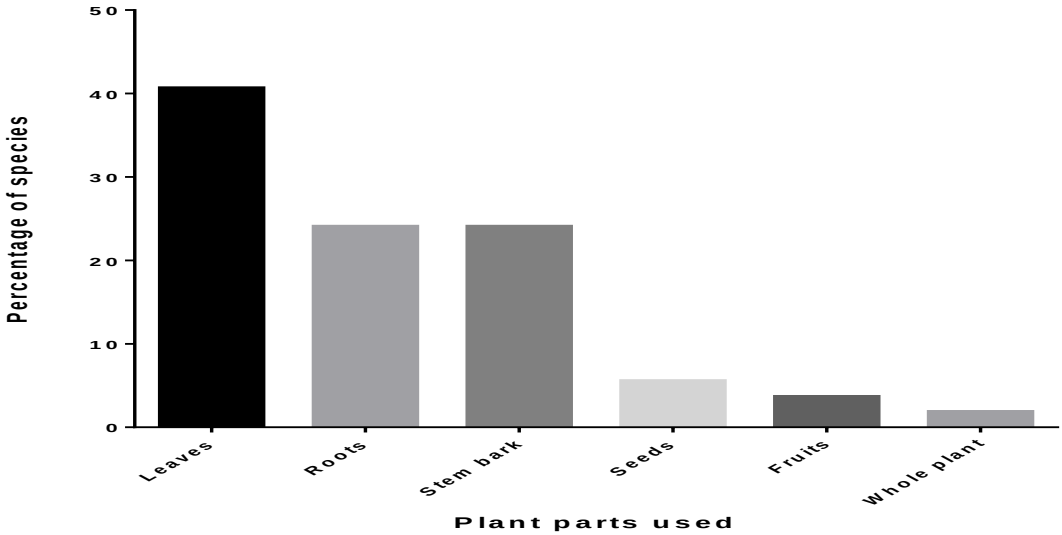

Figure 6. Distribution of plant parts used in the treatment of malaria and their percentages.

medicinal plant for the treatment of malaria itself. Again, C. sinensis may be used together with other plants as combination therapy because the antimalarial characteristics of medicines are contingent on synergy of the plant components. This renders identifying and characterizing the measure of bioactive compounds in herbal preparations very complex and poses immense challenges in coming up with quality control methods. However, WHO provides basic standards for quality of herbal preparations (WHO, 2007, 2011).

Antiplasmodial activity have been shown in some of the species used as sources of antimalarial preparations (Sendagire et al., 2005). For instance, in Ivory Coast, *Alchornea cordifolia* leaves are being used for the treatment of malaria, and an alcoholic concentrate of the leaves has clearly shown antiplasmodial activity at IC50 = 9.2 µg/ml (Okpekon et al., 2004). The leaves of *Alstonia boonei* are used for malaria treatment in Ghana and in Ivory Coast and Okpekon et al. (2004) accounted that the alkaloids extracted from this species possessed antiplasmodial activity at 8.4 µg/ml. In addition, methanol and methylenechloride extracts from root and stem bark

of *Morinda lucida* indicated antiplasmodial activity that may be linked to anthraquinones (Tona et al., 1999). It is worth noting that, the extraction solvent, locality of collection and time of harvesting influence the presence of phytochemical expression levels and the efficacy of extracts from plants (Prance, 1994).

CONCLUSIONS AND RECOMMENDATION

This study has emphasized the significance of plants in the treatment of malaria in Ghana. It has also contributed to the establishment of a complete database of traditional knowledge on medicinal plants used for the treatment of malaria in Ghana. In addition, this study provides useful information for new drug discovery, since it provides a list of plants amongst which an alternative phytochemical, that is more efficacious against the *Plasmodium* parasite could be discovered. More research should be conducted to find out the activities in the leaves of medicinal plants used for the treatment of malaria and other common diseases so that leaves can be used more than other plant parts such as roots and stem barks since harvesting of the roots and stem barks are more destructive and poses a greater threat to local plant populations, especially when whole plants are removed in some instances. The sourcing of plant materials from the wild by majority of the herbalists in this study, emphasizes the need to train them on sustainable cultivation and harvesting methods. Additional work can be done on the plants reported in this study to corroborate their antimalarial claim as well.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Chemical composition, *in vitro* antioxidant and antiparasitic properties of the essential oils of three plants used in traditional medicine in Benin.

Didier Kpadonou[1], Salomé Kpoviessi[1,2,3,*], Joanne Bero[1,2], Pierre Agbani[4], Fernand Gbaguidi[1,3], Bénédicta Kpadonou-Kpoviessi[1], Brice Sinsin[4], Michel Frédérich[5] and Joëlle Quetin-Leclercq[2]

[1]Laboratory of Physic and Synthesis Organic Chemistry (LaCOPS), Faculty of Sciences and Technics (FAST), University of Abomey-Calavi (UAC), BP: 4521 Cotonou, Benin.
[2]Pharmacognosy Research Group, Louvain Drug Research Institute, Université Catholique de Louvain, B1 7203 Av. E. Mounier 72, B-1200 Bruxelles, Belgium.
[3]Laboratory of Pharmacognosy and Essential oils (LAPHE), Faculty of Health Sciences (FSS), Faculty of Sciences and Technics (FAST) University of Abomey-Calavi (UAC), 01BP: 188 Cotonou, Benin
[4]Laboratory of Applied Ecology (LEA), Faculty of Agronomic Sciences (FSA), University of Abomey-Calavi (UAC), 03 BP: 1974 Cotonou, Benin.
[5]Université de Liège, Drug Research Center, Laboratoire de Pharmacognosie, Av. de l'Hôpital 1, B36, B-4000 Liège, Belgium.

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***Sclerocarya birrea* (*Sb*), *Psidium guajava* (*Pg*) and *Eucalyptus camaldulensis* (*Ec*) are widely used in traditional medicine for the treatment of many diseases, some of which were related to oxidative stress and parasitic diseases. Their essential oils (EO) were analyzed by GC/MS and FID and tested in vitro for their antioxidant activities (DPPH), their anti-trypanosomal and anti-plasmodial activities against *Trypanosoma brucei brucei* (*Tbb*) (strain 427) and *Plasmodium falciparum* (*Pf*) (strain 3D7), respectively. Cytotoxicity was evaluated in vitro against CHO and WI38 cells (MTT) to evaluate the selectivity. They were shown to possess low antioxidant but a strong anti-trypanosomal and a good antiplasmodial activity with a good selectivity, except *Ec* oil whose anti-plasmodial activity was less interesting. Sb oil was the most active against *Tbb*(IC_{50} = 0.46 ± 0.28 µg/ml) and *Pf* (5.21 ± 1.12 µg/ml). All tested oils had low or no cytotoxicity against CHO and WI38 cells. GC/MS and GC/FID analysis revealed that composition of *Sb* (49 compounds) was characterised by the presence as main constituents of 7-epi-α-selinene, α-muurolene and valencene; *Pg* (60 compounds) by β-bisabolene, ar-curcumene and β-bisabolol; *Ec* (43 compounds) by *γ*-terpinene and *p*-cymene. The activity of these oils seems to be the result of a synergistic action of all their constituents, including minor ones. This study shows that essential oils of *Sb* and *Pg* can be good sources of anti-trypanosomal and anti-plasmodial agents.**

Key words: Essential oil, *S. birrea*, *P. guajava, E. camaldulensis*, antimalarial, antitrypanosomal, antioxidant.

INTRODUCTION

The emergence of parasites resistant to current chemotherapies highlights the importance of the search of potential novel anti-parasitic agents which may be used as alternatives or adjuvants to current anti-parasitic therapies (Cheikh-Ali et al., 2011; Nibret and Wink, 2010). Similarly the overproduction of free radicals in

cells induces an oxidative stress inplicated in atherosclerosis, cardiovascular diseases, hypertension, ischemia/reperfusion injury, diabetes mellitus, neuro-degenerative diseases, immuno-inflammatory and malaria (Maloueki et al., 2015; Rashid et al., 2013; Valko et al., 2007; Djordjević et al., 2008; Ayoola et al., 2008). To escape these serious consequences related to oxidative stress and parasitic diseases, the use of aromatic and medicinal plants, and especially their essential oils have been the subject of several studies (Kpoviessi et al., 2014; Safaei-Ghomi et al., 2009).

Sclerocarya birrea (A. Rich.) Hochst (Anacardiaceae), *Psidium guajava* L**.** (Myrtaceae) and *Eucalyptus camaldulensis* Dehnh (Myrtaceae) are aromatic plants used as food for men and cattle, for firewood, wood carving and in traditional medicine for many diseases (Kabiru et al., 2013; Gouwakinnou et al, 2011; Gutiérrez et al., 2008). The stem bark aqueous extract of *S. birrea* has been used to treat malaria in Benin (Gouwakinnou et al, 2011). Bark aqueous and methanolic extracts were shown by Gathirwa et al. (2008) to possess *in vitro* anti-plasmodial and *in vivo* anti-malarial efficacy alone or in combination with other medicinal plant extracts. Maceration, infusion or decoction in water of different parts of *P. guajava* are used in several countries as febrifuge or in skin problems (Gutiérrez et al., 2008; Hermans et al., 2004; Ajaiyeoba et al., 2003). Aqueous decoctions and various extracts from leaves and flowers of *P. guajava*, alone or in combination with other medicinal plant extracts possess *in vitro* anti-plasmodial activities (Kaushik et al., 2015; Tarkang et al. 2014; Rajendran et al., 2014; Chinchilla, et al., 2012). *E. camaldulensis* leaves are used alone and in combination with other plants to treat malaria and typhoid fevers in some Northern parts of Nigeria and ethanolic extracts possess *in vivo* anti-trypanosomal activities (Kabiru et al., 2013).

Essential oils of these plants are known for antimicrobial, antifungal, antioxidant, analgesic, anti-inflammatory, anti-nociceptive, antiradical, larvicidal, and insecticidal properties (Ghalem and Mohamed, 2014; Njume et al., 2011). Furthermore, these oils are used orally (drops) or by inhalation in traditional medicine for the treatment of malaria or its symptoms or sleeping sickness (Knezevic, 2016; Rasoanaivo et al., 1992; Gelfand et al., 1985).The direct activity of these essential oils against *Trypanosoma brucei* and *Plasmodium falciparum* was not very documented except for essential oil of *E. camaldulensis* from Nigeria. This oil was reported to kill in 4 mins *T. brucei brucei* parasites at a concentration of 0.4 g/ml *in vitro* (Habila et al., 2010). So, it seemed interesting to study the anti-plasmodial and anti-trypanosomal activities of these essential oils and their components.

T. brucei is the parasite responsible for human African trypanosomiasis or sleeping sickness, an illness affecting 300,000 African people, while up to 60 million people in 36 countries are at risk of contracting the disease and 6314 cases were recorded in 2013 (WHO, 2015). This parasite is transmitted by the bite of infected Tse-tse flies of the genus *Glossina*. Malaria is also a disease caused by a protozoan parasite of Plasmodium specie and still remains a major public health problem in the world. According to the latest estimates, 219 million cases of this disease occurred globally in 2017 (uncertainty range 203 to 262 million) and the disease led to 435 000 deaths (WHO, 2018).

These two parasitic diseases are the cause of considerable mortality and morbidity throughout the world and parasites develop resistance to most of the drugs used (WHO, 2018). Some of these drugs need a long course parenteral administration, show toxicity and a variable efficacy between strains or species. Free radicals also cause several diseases whose treatments are very expensive for the population. There is a need to search for new anti-trypanosomal, anti-plasmodial and antioxidant lead compounds with new mechanism of action from medicinal plants (Bero et al., 2011).

The present study aims to evaluate *in vitro* anti-trypanosomal, anti-plasmodial and antioxidant activities, along with cytotocycity against chinese hamster ovary cells (CHO) and a human non cancer fibroblast cell line (WI38) for the determination of selectivity, of essential oils from three plants: *S. birrea*, *P. guajava* and *E. camaldulensis* used in traditional medicine in Benin.

MATERIALS AND METHODS

Plant material

Fresh leaves of *S. birrea* (A. Rich.) Hochst (Anacardiaceae), *P. guajava* L. (Myrtaceae) and *E. camaldulensis* Dehnh. (Myrtaceae) were collected in March 2014, from the Botanical Garden of the Abomey-Calavi University. Voucher specimens (n°AA6384, AA6536 and AA6590/HNB respectively) were conserved at the University of Abomey-Calavi Herbarium.

Chemicals and drugs

Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 culture media were purchased from Life technologies corporation (Grand Island, NY 14072, USA); Dulbecco's Phosphate Buffered Saline (DPBS 1X) from Invitrogen (Grand Island, NY 14072, USA); tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) (MTT), DPPH (2,2-diphenyl-1-picrylhydrazyl), (S) - (+), ascorbic acid, (S)-(+)-camptothecin,

*Corresponding author. E-mail: salome.kpoviessi@fast.uac.bj.

suramine, chloroquine, artemisinin, dimethyl sulfoxide (DMSO) and *n*-alkanes "C_7-C_{28}" were obtained from Sigma-Aldrich (Steinhein, Germany), Acros Organics (New jersey, USA), and Fluka Chemie (Buchs, Switzerland). All compounds were of analytical standard grade. Ter-Butyl methyl ether (TBME) was an analytical grade solvent purchased from Fluka Chemie, and anhydrous Na_2SO_4 was of analytical reagent grade from UCB (Brussels, Belgium).

Isolation of essential oils

Five hundred grams (500 g) of fresh leaves were steam distillated for 3 h in a modified Clevenger-type apparatus (Bruneton, 2009). The extraction was carried out in triplicate. The oils were preserved in a sealed vial at 4°C. The essential oil yields were calculated based on the fresh plant material (Kpoviessi et al., 2014).

Chemical analysis of essential oils

GC/MS analysis

GC/MS analysis was carried out using a TRACE GC 2000 series (Thermo-Quest, Rodano, Italy), equipped with an autosampler AS2000 Thermo-Quest. The GC system was interfaced to a Trace MS mass spectrometer (ThermoQuest) operating in the electronic impact mode at 70 eV. HP 5MS column (30 m × 0.25 mm, film thickness: 0.25 μm) was used; injection mode: splitless; injection volume: 1 µl (TBME solution); split flow: 10 ml/min; splitless time: 0.80 min; injector temperature: 260°C; oven temperature was programmed as following: 50 to 250°C at 6°C/min and held at 250°C for 5 min; the carrier gas was helium with a constant flow of 1.2 ml/min. The coupling temperature of the GC was 260°C and the temperature of the source of the electrons was 260°C. The data were recorded and analyzed with the Xcalibur 1.1 software (ThermoQuest) (Kpoviessi et al., 2014).

Identification of oil components

Individual components of the volatile oils were identified by comparison with computer matching of their retention times against those of commercial EI-MS spectra library (NIST/EPA/NIH, 1998; Adams, 2007), home-made mass spectra library made from pure substances and components of known oils (Kpoviessi et al., 2011). Mass spectrometry literature data were also used for the identification, which was confirmed by comparison of the GC retention indices (RI) on a non-polar column (determined from the retention times of a series of *n*-alkanes "C_7 - C_{28}" mixture) (VanDenDool and Kratz, 1963). The minimum Relative Strength Index (RSI) for MS analysis was 937. The Kovats indices (KI) calculated were in agreement with those reported by Adams (Adams, 2007). Quantification (expressed as percentages) was carried out by the normalization procedure using peak areas obtained by FID. Values are expressed as mean ± standard deviation (n = 3).

In vitro test for antioxidant activity

The DPPH method was used to evaluate the antioxidant activity of oils. In a 96-well microplate, a series of 10 successive dilutions (at 1/2) of each oil, was prepared from sample solutions at 150 µL/ml in methanol. For each concentration, three (03) tests were carried out by adding 100 µl of DPPH at 100 µg/ml in methanol at all dilutions in cascade. Thus, the DPPH was tested at a single concentration of 50 µg/ml. The plate was incubated in the dark for 20 min and the absorbance at 517 nm using a spectrophotometer. The negative control consists of 1 ml of methanolic solution and 1 ml of DPPH solution (100 µ/ml). Positive control was the solution of Ascorbic acid (1 mg/ml) (Otohinoyi et al., 2014; Brand-Williams et al., 1995)

The antiradical activity was estimated according to the following equation:

$$\%\ \text{antiradical activity} = \frac{Absorbed\ \ (negative\ \ control) - Absorbed\ \ (oil)}{Absorbed\ \ (negative\ control)} * 100$$

The extract concentration that reduces the absorbance of DPPH by 50% (EC_{50}) was obtained with the GraphPadPrism 4.0 software.

Parasites, cell lines and media

T. brucei brucei strain 427 (Molteno Institute in Cambridge, UK) bloodstream forms were cultured *in vitro* in HMI9 medium containing 10% heat-inactivated foetal bovine serum (Hirumi and Hirumi, 1994**)**. *P. falciparum* chloroquine-sensitive strain 3D7 (from Prof. Grellier of Museum d'Histoire Naturelle, Paris-France) asexual erythrocytic stages were cultivated continuously *in vitro* according to the procedure described by Trager and Jensen (1976) at 37°C and under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The host cells were human red blood cells (A or O Rh+). The culture medium was RPMI 1640 (Gibco) containing 32 mM $NaHCO_3$, 25 mM HEPES and 2.05 mM L-glutamine. The medium was supplemented with 1.76 g/L glucose (Sigma–Aldrich), 44 mg/mL hypoxanthin (Sigma–Aldrich), 100 mg/L gentamycin (Gibco) and 10% human pooled serum (A or O Rh+). Parasites were subcultured every 3 to 4 days with initial conditions of 0.5% parasitaemia and 1% haematocrit.

The macrophage-like cell line, CHO Chinese Hamster Ovary cells (ATCC N° CCL-61, batch 4765275), were cultivated *in vitro* in Ham's F12 Nutrient Mixture 21765 medium (Gibco) containing 2 mM L-glutamine supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and penicillin–streptomycin (100 UI/mL to 100 μg/mL). The human non cancer fibroblast cell line, WI38 (ATCC N° CCL - 75 from LGC Standards) was cultivated *in vitro* in DMEM medium (Gibco) containing 4 mM L-glutamine, 1 mM sodium pyruvate supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and penicillin–streptomycin (100 UI/mL to 100 μg/mL).

***In vitro* test for antiplasmodial activity**

Parasite viability was measured using parasite lactate dehydrogenase (pLDH) activity according to the method described by Makler et al. (1993). The *in vitro* test was performed as described by Murebwayire et al. (2008). Chloroquine (Sigma) or artemisinin (Sigma) were used as positive controls in all experiments with an initial concentration of 100 ng/mL. First stock solutions of essential oils and pure compounds were prepared in DMSO at 20 mg/mL. The solutions were further diluted in medium to give 2 mg/mL stock solutions. The highest concentration of solvent to which the parasites were exposed was 1%, which was shown to have no measurable effect on parasite viability. Essential oils were tested in eight serial threefold dilutions (final concentration rang: 200 to 0.09 μg/mL, two wells/concentration) in 96-well microtiter plates. The parasitaemia and the haematocrit were 2 and 1%, respectively. All tests were performed in triplicate.

In vitro test for anti-trypanosomal activity

The *in vitro* test was performed as described by Hoet et al. (2004). Suramine (a commercial antitrypanosomal drug, MP Biomedicals, Eschwege, Germany) was used as positive control in all experiments with an initial concentration of 1 μg/mL. First stock

solutions of essential oils and compounds were prepared in DMSO at 20 mg/mL. The solutions were further diluted in medium to give 0.2 mg/mL stock solutions. Essential oils and compounds were tested in eight serial threefold dilutions (final concentration range: 100 to 0.05 μg/mL, two wells/concentration) in 96-well microtiter plates. All tests were performed in triplicate.

Cytotoxicity assay

The cytotoxicity of the oils against CHO and WI38 cells was evaluated as described by Stevigny et al. (2002), using the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (Sigma)) colorimetric method based on the cleavage of the reagent by dehydrogenases in viable cells. Camptothecin (Sigma) was used as positive cytotoxic reference compound. Stock solutions of compounds and essential oils were prepared in DMSO at 10 mg/mL. The solutions were further diluted in medium with final concentrations of 200 to 6.25 μg/mL. The highest concentration of solvent to which the cells were exposed was 1%, which was shown to be non-toxic. Each oil was tested in six serial fourfold dilutions in 96-well microtitre plates. All experiments were made at least in duplicate.

Statistical analysis

Student's t-test was used to test the significance of differences between sets of results for different samples, and between results for samples and controls (GraphPad Prism 4.0; GraphPad Software Inc., San Diego, USA). Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of the essential oils

Yields (w/w) of oils extracted from fresh leaves of *Sb*, *Pg* and *Ec* (0.24, 0.78 and 1.38%, respectively) collected in the same place at the same time are given in Table 1. The yield (1.38%) of *Ec* leaves oil obtained in the present study confirms the work of Moudachirou et al. (1999) who reported the highest rate (1.30%) for this plant in Benin at Calavi in the period of February and March or at Kétou between April and May 1996. However, this yield was higher than that obtained in Morocco (0.84%) (Farah et al., 2002) and between the values 0.75 and 1.42% obtained from Tunisia (Haouel et al., 2010). For *Pg*, the yield (0.78%) was closer to that reported for this plant at Tchaada in Benin (0.82%) (Noudogbessi et al., 2013) and in Nigeria (0.75%) (Ogunwande et al., 2003) but different from the one described by Noudogbessi et al. (2013) in Missérété (0.25%) and Adjarra (0.30%) in outhern Benin. The leaves of *Sb* gave an oil yield (0.24%) in accordance with that indicated by Kpoviessi et al. (2011) for the same plant in the same area during the rainy season. These authors had also showed that this yield varies depending on the season (Kpoviessi et al., 2011). The difference between essential oil yields or chemical composition of the same plant could be explained by the influence of the location, season, and time of harvest in the day or the vegetative stage of the plant (Noudogbessi et al., 2013; Kpadonou-Kpoviessi et al., 2012; Kpoviessi et al., 2011; Moudachirou et al., 1999).

A total of 49 (*Sb*), 43 (*Ec*) and 60 (*Pg*) compounds, representing respectively 97.96% (*Sb*), 98.50% (*Ec*) and 96.10% (*Pg*) of hydrodistillate, were identified (Table 1). These oils contained more hydrocarbon compounds (60.60 to 92.55%) than oxygenated ones. Sesquiterpenes were the major terpenoids in *Sb* and *Pg* oils (95.12 and 93.81%, respectively) while *Ec* oil was characterized by the predominance of monoterpenes (96.95%) (Table 2).

The essential oil of *Sb* was characterized by the presence of 7-epi-α-selinene (37.86 ± 0.03%) of α-muurolene (25.03 ± 0.03%), and valencene (17.12 ± 0.06%) as major constituents followed by β-selinene (4.32 ± 0.01%), β-caryophyllene (3.24 ± 0.02%), epoxy-allo aromadendrene (1.54 ± 0.03%), 14-hydroxy-α-humulene (1.51 ± 0.03%) and α-copaene (1.20 ± 0.04%). The study of this oil was not very documented. Its chemical composition was close to that described by Kpoviessi et al. (2011) by GC/FID and GC/MS analysis methods.

In the *Ec* oil, γ-terpinene (57.24 ± 0.04%) predominated followed in decreasing order of rate by *p*-cymene (18.22 ± 0.02%), terpinen-4-ol (7.50 ± 0.07%), 1,8-cineole (7.49 ± 0.07%), limonene (1.82 ± 0.02%) and terpinolene (1.02 ± 0.01%). This composition was similar to that described at Calavi (Moudachirou et al., 1999) but different from those studied in Spain (Verdeguer et al., 2009), Jerusalem (Chalchat et al., 2001), Tunisia (Haouel et al., 2010), Australia, Morocco and Ivory Coast (Kanko et al., 2012), which were richer in *p*-cymene, spathulenol, cryptonne or 1, 8-cineole.

No component of the *Pg* oil exceeded a rate of 15%. Over twenty compounds exhibit a percentage higher than 1% with β-bisabolene (14.38 ± 0.03%), *ar*-curcumene (12.39 ± 0.02%), β-bisabolol (11.40 ± 0.08%) and β-caryophyllene (8.04 ± 0.03%) as major compounds. These results were more similar to those obtained in the locality of Banigbe (Benin) by Noudogbessi et al. (2013) than those obtained in other parts of the country by the same authors. Furthermore, the content of 1,8-cineole (0.44 ± 0.01%) in Benin oil was lower than that in Brazil (21.40%; with GC/MS method), Taiwan (12.40%, with GC/FID and GC/MS methods) and China (18.90% with GC/MS method) ones (Chen et al., 2007; Da Silva et al., 2003).

Anti-trypanosomal, anti-plasmodial activities and cytotoxicity

All studied oils were tested *in vitro* for their anti-trypanosomal and anti-plasmodial activities respectively on *T. brucei brucei* and *P. falciparum* 3D7 and their cytotoxicity against WI38 and CHO cells. The results are

Table 1. Chemical composition and yield of essential oils from *Sclerocarya birrea* (*Sb*), *Eucalyptus camaldulensis* (*Ec*) and *Psidium guajava* (*Pg*) (mean ± sd, n = 3).

N°	[a]Compounds	[b]IK	%*Sb*	%*Ec*	%*Pg*
1	4-hydroxy-4-methyl-pentan-2-one[&o]	835	0.19 ± 0.06	0.10 ± 0.00	-
2	α-thujene[*h]	931	0.10 ± 0.05	0.19 ± 0.00	-
3	α-pinene[*h]	939	0.09 ± 0.05	0.36 ± 0.00	-
4	camphene[*h]	953	-	tr	-
5	benzaldehyde[&o]	961			0.26 ± 0.00
6	sabinene[*h]	976	0.21 ± 0.08	0.14 ± 0.00	-
7	β-pinene[*h]	980	0.19 ± 0.10	0.31 ± 0.00	-
8	6-methylhept-5-en-2-one[&o]	985	-	-	0.17 ± 0.00
9	myrcene[*h]	991	0.10 ± 0.02	0.24 ± 0.00	
10	α-terpinene[*h]	1018	-	0.19 ± 0.00	-
11	*p*-cymene[*h]	1026	0.52 ± 0.13	18.22 ± 0.02	0.28 ± 0.00
12	limonene[*h]	1031	0.10 ± 0.01	1.82 ± 0.02	0.13 ± 0.00
13	1.8-cineole[*o]	1033	-	7.49 ± 0.07	0.44 ± 0.01
14	(*Z*)-β-ocimene[*h]	1040	-	tr	0.21 ± 0.00
15	(*E*)-β-ocimene[*h]	1050	0.20 ± 0.04	0.06 ± 0.00	0.22 ± 0.00
16	γ-terpinene[*h]	1062	tr	57.24 ± 0.04	-
17	terpinolene[*h]	1088	-	1.02 ± 0.01	-
18	*p*-cymenene[*h]	1089	-	0.10 ± 0.00	-
19	linalol[*o]	1096	0.37 ± 0.05	0.09 ± 0.00	0.13 ± 0.00
20	valerate d'isoamyle[&o]	1107	-	0.10 ± 0.00	-
21	1-methyl-4-(1-methyl propyl)-benzene[&h]	1113		-	0.17 ± 0.00
22	(*E*)-4.8-dimethyl-1.3.7-nonatriene[*h]	1113	0.10 ± 0.03	-	-
23	citronellal[*o]	1153	-	0.12 ± 0.00	-
24	verbenol[*o]	1164	-	0.06 ± 0.00	-
25	boneol[*o]	1175	-	tr	-
26	terpinene-4-ol[*o]	1182	-	7.50 ± 0.07	-
27	*p*-cymene-8-ol[*o]	1183	-	0.09 ± 0.00	-
28	α-terpineol[*o]	1196	tr	0.54 ± 0.01	-
29	(*Z*)- sabinol[*o]	1214	-	0.19 ± 0.00	-
30	isovalerate de n-hexyle[&o]	1243	-	0.10 ± 0.00	-
31	piperitone[*o]	1252	-	0.28 ± 0.00	-
32	*p*-cymene-7-ol[*o]	1287	-	0.29 ± 0.00	-
33	thymol[*o]	1298	0.13 ± 0.01	0.25 ± 0.00	0.18 ± 0.00
34	carvacrol[*o]	1298	-	0.16 ± 0.00	-
35	cyclosativene[**h]	1378	0.28 ± 0.03	-	-
36	α-copaene[**h]	1379	1.20 ± 0.04	-	1.00 ±0.02
37	β-bourbonene[**h]	1388	0.20 ± 0.01	-	-
38	β-elemene[**h]	1391	tr	-	-
39	7-epi-α-cedrene[**h]	1404	-	-	0.38 ± 0.01
40	helifolene[**h]	1406	-	-	1.13 ± 0.02
41	α-gurjunene[**h]	1409	-	0.13 ± 0.00	-
42	(*Z*)-α-bergamotene[**h]	1411	-	-	0.59 ± 0.01
43	α-cedrene[**h]	1418	-	-	1.01 ± 0.02
44	β-caryophyllene[**h]	1418	3.24 ± 0.02	-	8.04 ± 0.03
45	β-cedrene[**h]	1424	-	-	0.45 ± 0.01
46	β-copaene[**h]	1430	0.11 ± 0.04	-	-
47	β-gurjunene[**h]	1432	-	0.07 ± 0.00	-
48	(*E*)-α-bergamotene[**h]	1434	-	-	0.41 ± 0.01
49	aromadendrene[**h]	1441	0.10 ± 0.05	0.24 ± 0.00	-
50	selina-5.11-diene[**h]	1444	0.10 ± 0.05	-	-

Table 1. Contd.

51	epi-β-santalene[**h]	1446	-	-	0.19 ± 0.00
52	α-humulene[**h]	1454	0.10 ± 0.01	-	1.32 ± 0.02
53	(*E*)-β-farnesene[**h]	1458	-	0.12 ± 0.00	0.90 ± 0.01
54	β-santalene[**h]	1460	-	-	1.08 ± 0.02
55	allo-aromadendrene epoxyde[**0]	1461	-	tr	-
56	α-acoradiene[**h]	1464	-	-	2.89 ± 0.05
57	β-acoradiene[**h]	1465	-	-	0.73 ± 0.01
58	4.5-di-epi-aristochene[**h]	1470	0.21 ± 0.05	-	-
59	α-neocallitropsene[**h]	1475	-	-	1.66 ± 0.01
60	selina-4.11-diene[**h]	1475	0.44 ± 0.06	-	-
61	germacrene-D[**h]	1480	tr	-	-
62	*ar*-curcumene[**h]	1483	-	-	12.39 ± 0.02
63	β-selinene[**h]	1485	4.32 ± 0.01	-	1.23 ± 0.00
64	ledene[**h]	1491	-	0.07 ± 0.00	-
65	(*Z*)-α-bisabolene[**h]	1494	-	-	1.28 ± 0.02
66	α-selinene[**h]	1494	0.36 ± 0.20	-	1.22 ± 0.02
67	valencene[**h]	1494	17.12 ± 0.06	0.07 ± 0.00	-
68	α-zingiberene[**h]	1495	-	-	0.31 ± 0.00
69	α-muurolene[**h]	1496	25.03 ± 0.03	-	-
70	β-curcumene[**h]	1503	-	-	0.22 ± 0.00
71	β-bisabolene[**h]	1509	-	-	14.38 ± 0.03
72	γ-cadinene[**h]	1510	-	tr	0.45 ± 0.00
73	β-sesquiphellandrene[**h]	1516	-	-	3.02 ± 0.05
74	δ-cadinene[**h]	1520	-	tr	0.60 ± 0.01
75	(*E*)-γ-bisabolene[**h]	1521	-	-	2.07 ± 0.03
76	7-epi-α-selinene[**h]	1522	37.86 ± 0.03	-	-
77	(*E*)-α-bisabolene[**h]	1530	-	-	0.64 ± 0.01
78	Selina-3.7(11)-diene[**h]	1557	0.27 ± 0.40	-	-
79	(*E*)-nerolidol[**O]	1564	-	-	2.38 ± 0.04
80	viridiflorol[**O]	1564	-	0.06 ± 0.00	-
81	ar-tumerol[**O]	1578	-	-	0.70 ± 0.01
82	caryophyllene oxyde [**O]	1581	0.06 ± 0.04	-	2.20 ± 0.03
83	β-copaen-4-α-ol[**O]	1587	-	-	0.11±0.00
84	globulol[**O]	1595	-	0.25± 0.01	-
85	guaïol[**O]	1607	-	-	0.75±0.01
86	humulene-1.2-epoxyde[**O]	1608	-	-	-
87	epi-globulol[**O]	1612	-	-	0.82±0.01
88	humulene epoxyde-D[**O]	1616	-	-	0.25±0.00
89	1.10-diepi-cubenol[**O]	1619	-	-	0.22±0.00
90	epi-cubenol[**O]	1627	0.07 ± 0.10	-	1.07±0.02
91	α-acorenol[**O]	1629	-	-	0.21±0.00
92	γ-eudesmol[**O]	1632	0.13 ± 0.10	0.05 ± 0.00	-
93	β-acorenol[**O]	1634	-	-	2.21 ± 0.03
94	gossonorol[**O]	1638	-	-	1.50 ± 0.02
95	allo-aromadendrene epoxyde[**O]	1640	1.54 ± 0.03	-	-
96	epi-α-muurolol[**O]	1641	0.10 ± 0.01	-	0.30 ± 0.00
97	α-muurolol[**O]	1646	0.08 ± 0.10	-	0.80 ± 0.01
98	α-eudesmol[**O]	1652	-	0.14 ± 0.00	0.50±0.01
99	α-cadinol[**O]	1654	0.16 ± 0.10	-	2.20±0.03
100	selin-11-en-4-α-ol[**O]	1660	0.23 ± 0.03	-	2.00±0.03
101	intermedeol[**O]	1667	0.22 ± 0.01	-	-
102	β-bisabolol[**O]	1671	-	-	11.40 ± 0.08

Table 1. Contd.

103	nerolidyl acetate**O	1675	-	-	0.80±0.01
104	α-bisabolol**O	1683	-	-	3.40 ± 0.06
105	(2*Z*.6*Z*)-farnesol**O	1694	-	-	0.10±0.00
106	(2*Z*.6*E*)-farnesol**O	1712	-	-	0.20±0.00
107	14-hydroxy-α-humulene**O	1714	1.51 ± 0.03	-	-
108	(2*E*.6*E*)-farnesol**O	1753	-	-	0.10±0.00
109	benzyl benzoate &o	1777	-	-	0.10±0.00
110	nootkatone**O	1800	0.08 ± 0.01	-	-
111	phtalates&o	1852	0.12 ± 0.02	-	-
112	acide hexadecanoïque&o	1951	0.09 ± 0.01	-	-
113	phytol***O	2097	0.33 ± 0.01	0.05 ± 0.00	-
	Total		97.96±0.06	98.50±0.03	96.10±0.02
	γYield (%)		0.24±0.01(a)	1.38±0.02(c)	0.78±0.02(b)

[a] Compounds listed in order of elution from HP-5 MS column; [b]= Kovats indices (KI) on HP-5 MS column; * = monoterpenes; *Sb* = Essential oil from *S. birrea*; *Ec* = Essential oil from *E. camaldulensis*; *Pg* = Essential oil from *P. guajava*; ** = sesquiterpenes; *** = diterpene; & = non terpenes; h = hydrocarbons; o = oxygenated; t = traces (inferior or equal to 0.05%); (-) = absence or not detected; γYield calculated based on the fresh plant material; Values are means±standard deviation of three separate experiments.

Table 2. Chemical groups of essential oils from *Sclerocarya birrea* (*Sb*). *Eucalyptus camaldulensis* (*Ec*) and *Psidium guajava* (*Pg*) (mean ± sd. n = 3).

N°	Chemical groups	%*Sb*	%*Ec*	%*Pg*
1	Hydrocarbon compounds	92.55 ±1.60	80.59 ±0.09	60.60 ±0.44
2	Oxygenated compounds	5.41 ±0.71	17.91 ±0.16	35.50 ±0.41
3	Hydrocarbon monoterpenes	1.61 ±0.51	79.89 ±0.09	0.84 ±0.00
4	Oxygenated monoterpenes	0.50 ±0.06	17.06 ±0.15	0.75±0.01
5	Monoterpenes	2.11 ±0.57	96.95 ±0.24	1.59 ±0.01
6	Hydrocarbon sesquiterpenes	90.94 ±1.09	0.70 ±0.00	59.59 ±0.44
7	Oxygenated sesquiterpenes	4.18 ±0.56	0.50 ±0.01	34.22 ±0.40
8	Sesquiterpenes	95.12 ±1.65	1.20 ±0.01	93.81 ±0.84
9	Diterpenes	0.33 ±0.01	0.05 ±0.00	-
10	Others	0.40 ±0.09	0.30 ±0.00	0.70±0.00

Sb = Essential oil from *S. birrea*; *Ec* = Essential oil from *E. camaldulensis*; *Pg* = Essential oil from *P. guajava*; (-) = absence or not detected; Values are means±standard deviation of three separate experiments, calculated from the individual percentages of the components.

summarized in Table 3.

These oils show an interesting anti-trypanosomal activity, the most interesting being *Pg* (IC_{50} = 1.16 ± 0.16 μg/ml) and *Sb* (IC_{50} = 0.46 ± 0.28 μg/ml). According to Bero et al. (2014), Ec oil has a moderate anti-trypanosomal activity (2 ≤ IC_{50} ≤ 20 μg/ml). While the other oils exhibited good activities (IC_{50} ≤ 2 μg/ml) and could be of interest for future development (Bero et al., 2014). The activity of *Sb* oil was not significantly different (P value = 0.1628> 0.1) than that of suramin (IC_{50} = 0.11 ± 0.02 μg/ml), the standard compound used against this parasite. The selectivity index of the three tested oils (*Sb* = 79; *Ec* > 19 and *Pg* = 33) showed that *Sb* was also the most selective. *In vivo* studies should be performed to assess its efficacy on sleeping sickness and determine if the essential oil from *Sb* already consumed by livestock and extensively used in traditional medicine, can be recommended for the treatment of this illness. It will be necessary to search for adequate formulation as LBDDS (lipid based drug delivery systems) (Mu et al., 2013) and to verify the absence of toxicity. To our knowledge, this is the first report of the activity of the essential oil of these three plants from Benin on *T. brucei brucei* except Habila et al. (2010) who showed that a concentration of 0.4 g/ml of Ec oil from Nigeria killed *T. brucei brucei* parasites in 4 min. Essential oils of plants from the same family (Myrtaceae) as *Leptospermum scoparium* Forst., *Melaleuca alternifolia*, *Syzygium aromaticum* (L.) Merr

Table 3. *In vitro* antitrypanosomal, antiplasmodial and antioxidant activity, cytotoxicity and selectivity index of essential oils from *S. birrea* (*Sb*), *E. camaldulensis* (*Ec*) and *P. guajava* (*Pg*) (mean ± sd. n = 3) and some of their major components.

Sample	Antioxydant activity (IC_{50}, µg/ml) Average ± standard deviation	Cytotoxicity (IC_{50}, µg/ml) Average ± standard deviation		Antitrypanosomal activity *Tbb* (IC_{50}, µg/ml) average±standard deviation	Antiplasmodial activity *Pf* (IC_{50}, µg/ml) average±standard deviation	[T]Selectivity indices		
		CHO	WI38			WI38/Tbb	WI38/3D7	3D7/Tbb
Sb	5106	31.19±1.80	36.17±3.31	0.46±0.28[a]	5.21±1.12[b]	78.6	6.9	11.3
Ec	9510	>50	>50	2.65±0.48b[b]	51.30±4.35[d]	>18.9	> 1.0	19.4
Pg	19290	39.00±0.80	38.00±2.00	1.16±0.16[b]	12.02±2.99[c]	32.8	3.2	10.4
myrcene[€]	-	>50	>50	2.24±0.27[b]	nd	>22.3	-	-
R(+)-limonene[€]	-	>50	>50	4.24±2.27[c]	nd	>11.8	-	-
citronellal[€]	-	>50	>50	2.76±1.55[b]	nd	>18.1	-	-
β-pinene[€]	-	>50	>50	47.37±15.65[e]	nd	>1.1	-	-
p-cymene[€]	-	>50	>50	76.32±13.27[f]	-	-	-	-
Campthotecin	-	0.74±0.09	0.44±0.12	nd	nd	-	-	-
Ascorbic acid	20	nd	nd	nd	nd	-	-	-
Suramine	-	nd	nd	0.11±0.02[a]	nd	-	-	-
Chloroquine	-	nd	nd	nd	0.02±0.01[a]	-	-	-
Artemisinin	-	nd	nd	nd	0.01±0.001[a]	-	-	-

Sb = Essential oil from *S. birrea*; *Ec* = Essential oil from *E. camaldulensis*; *Pg* = Essential oil from *P. guajava*; WI38 = human normal fibroblast cells; CHO = Chinese Hamster Ovary cells; nd = not determined; *Tbb* = *Trypanosoma brucei brucei*; 3D7 = Chloroquine-sensitive strain of *Plasmodium falciparum*; IC_{50} = sample concentration providing 50% death of cells or parasites; [T]Selectivity index = IC_{50} (WI38) / IC_{50} (*Tbb* or 3D7); [€]IC_{50} values from Kpoviessi et al., 2014; Data in the same column followed by different letters ($^{a,b,c,\cdots}$) are statistically different by Student's t-test ($P < 0.05$).

and L. M. Perry Cheel and *Kunzea ericoides* (A. Rich) Joy Thomps, showed anti-trypanosomal activities, respectively with IC_{50} values of 16.90, 0.50, 1.90 and 13.60 µg/ml (Bero et al., 2014). It was also reported that the ethanolic extract of *Pg* leaf was able to produce alterations in the biochemical parameters in the kidney and liver of rats experimentally infected with *T. brucei brucei* (Adeyemi and Akanji, 2011).

Concerning the anti-plasmodial activity against the chloroquine-sensitive 3D7 *P. falciparum* strain, *Sb* (IC_{50} = 5.21 ± 1.12 µg/ml) and *Pg* (IC_{50} = 12.02 ± 2.99 µg/ml) essential oils showed moderate activity with $2 \leq IC_{50} \leq 20$ µg/ml. The *Ec* (IC_{50} = 51.30 ± 4.35 µg/ml) oil was less interesting against this parasite. *Sb* aqueous extract in combination with three other medicinal plants was reported to exhibit high malaria parasite suppression (chemo-suppression >90%) *in vivo* with a doses of 100 mg/kg/d at different ratios tested interperitoneally or per os (Gathirwa et al., 2008). Recently, promising *in vitro* antiplasmodial activity against 3D7 ($IC_{50} \leq 20$ µg/ml), was seen in leaves ethyl acetate extracts and methanol extracts of *Pg* (Kaushik et al., 2015) and synergistic activities of combination with ethanol and water macerations of *Mangifera indica*, *Carica papaya*, *Cymbopogon citratus*, *Citrus sinensis*, and *Ocimum gratissimum* were reported against *P. falciparum* 3D7 and Dd2 strains (Tarkang et al., 2014). Aqueous decoctions of *Pg* also showed anti-plasmodial activity against chloroquine resistant *P. berghei* (Rajendran et al., 2014).

With a selectivity index > 6 and 3, respectively, the essential oils of *Sb* and *Pg* can also be good candidates for bio-guided fractionation to yield a more active and less toxic fraction against *P. falciparum*. These results may explain, at least in part the use of these plants in the treatment of malaria in Benin (Gouwakinnou et al., 2011; Hermans et al., 2004). Moreover, these results indicate the selectivity of the activity of the studied oils on *T. brucei brucei* as compared to *P. falciparum* (SI > 10 for all studied oils). The cytotoxicity tests against the Chinese Hamster

Table 4. Correlation between activity and chemical components of the essential oils.

Compound	Concentration (%) in essential oils			Antitrypanosomal activity (IC_{50}. µg/ml)	Reference
	Sb	*Ec*	*Pg*		
Myrcene	0.10 ±0.02	0.24 ±0.00	-	2.24 ±0.27	Kpoviessi et al. (2014)[&]
β-pinene	0.19 ±0.10	0.31 ±0.00	-	47.37 ±15.65	Kpoviessi et al. (2014)[&]
p-cymene	0.52 ±0.13	18.22 ±0.02	0.28 ±0.00	76.32 ±13.27	Kpoviessi et al. (2014)[&]
Citronellal	-	0.12 ±0.00	-	2.76 ±1.55	Kpoviessi et al. (2014)[&]
Limonene	0.10 ±0.01	1.82 ±0.02	0.13 ±0.00	4.24 ±2.27	Kpoviessi et al. (2014)[&]
α-pinene	0.09 ±0.05	0.36 ±0.00	-	4.09	Bero et al. (2014)
Sabinene	0.21 ±0.08	0.14 ±0.00	-	17.67	Bero et al. (2014)
1.8-cineole	-	7.49 ±0.07	0.44 ±0.01	83.02	Bero et al. (2014)
γ-terpinene	tr	57.24±0.04		136.91	Bero et al. (2014)
Linalol	0.37±0.05	0.09±0.00	0.13±0.00	39.26	Bero et al. (2014)
Terpinen-4-ol	-	7.50±0.07	-	39.51	Nibret and Wink (2010)
Piperitone	-	0.28±0.00	-	41.06	Nibret and Wink (2010)
Thymol	0.13±0.01	0.25±0.00	0.18±0.00	22.83	Bero et al. (2014)
Carvacrol	-	0.16±0.00	-	11.23	Bero et al. (2014)
α-cedrene	-	-	1.01±0.02	4.06	Nibret and Wink, (2010)
Aromadendrene	0.10±0.05	0.24±0.00	-	18.77	Bero et al. (2014)
β-caryophylene	3.24±0.02	-	8.04±0.03	13.76	Bero et al. (2014)
(*E*)-nerolidol	-	-	2.38±0.04	1.70	Bero et al. (2014)
caryophyllene oxyde	0.06±0.04	-	2.20±0.03	17.67	Nibret and Wink (2010)

Sb = Essential oil from *S. birrea*, *Ec* = Essential oil from *E. camaldulensis*, *Pg* = Essential oil from *P. guajava*, [&]values were previously published (Kpoviessi et al., 2014)

Ovary (CHO) cells and the human non cancer fibroblast cell line (WI38) showed that all tested oils and components had a low cytotoxicity (IC_{50} > 31 µg/ml) (Table 3).

Correlation of activity and chemical composition of essential oils

The antitrypanosomal activity of available major compounds of these studied oils was also evaluated or obtained from literature (Table 4).

The essential oil of *Sb* contains active compounds as myrcene (IC_{50} = 2.24 µg/ml), limonene (IC_{50} = 4.24 µg/ml), α-pinene (IC_{50} = 4.09 µg/ml), while sabinene (IC_{50} = 17.67 µg/ml), aromadendrene (IC_{50} = 18.77 µg/ml), β-caryophyllene (IC_{50} = 13.76 µg/ml) and caryophyllene oxide (IC_{50} = 17.67 µg/ml) had moderate activity. Other compounds as β-pinene (IC_{50} = 47.37 µg/ml), *p*-cymene (IC_{50} = 76.32 µg/ml), linalool (IC_{50} = 39.26 µg/ml) and thymol (IC_{50} = 22.83 µg/ml) had low activities (Bero et al., 2014). All these compounds do not explain the interesting activity (IC_{50} = 0.46 µg/ml) of this essential oil. The absence of described activity for not available and major compounds as 7-epi-α-selinene (37.86 ± 0.03%), α-muurolene (25.03 ± 0.03%), and valencene (17.12 ± 0.06%) could not help to explain this activity.

The first four major constituents of the essential oil of *Ec*: γ-terpinene (57.24%; IC_{50} = 136.91 µg/ml), *p*-cymene (18.22%; IC_{50} = 76.32 µg/ml), 1,8-cineole (7.49%; IC_{50} = 83.02 µg/ml) and terpinen-4-ol (7.50%; IC_{50} = 39.51 µg/ml) have very low anti-trypanosomal activities with IC_{50} values > 20 µg/ml which could not explain the moderate activity (IC_{50} = 2.65 µg/ml) observed for this oil. Furthermore, the oil contains minor compounds showing activity close to the activity of the crude oil with IC_{50} values < 5 µg/ml. Indeed, myrcene, citronellal and α-pinene with a concentration lower than 1%, showed IC_{50} values of 2.24 µg/ml; 2.76 µg/ml and 4.09 µg/ml respectively. Limonene (1.82%) had also a low IC_{50} value (IC_{50} = 4.24 µg/ml). These components seemed to act synergistically in the oil. These results confirm those obtained by Kpoviessi et al. (2014) that described the possibility of synergy effect in the essential oil of *Cymbopogon* spp.

No compound in *Pg* oil does exceed the concentration of 15%. β-caryophyllene (8.04 ± 0.03%), the fourth compound of this oil, showed moderate activity (IC_{50} = 13.76 µg/ml), while caryophyllene oxide (2.20%; IC_{50} = 17.67 µg/ml), α-cedrene (1.01%; IC_{50} = 4.06 µg/ml) and limonene (0.13%; IC_{50} = 4.24 µg/ml) were more effective. (*E*)–nerolidol (2.38 %) showed an interesting activity with an IC_{50} value of 1.70 µg/ml (Bero et al., 2014) similar to that (IC_{50}=1.16 µg/ml) obtained from the oil. Furthermore,

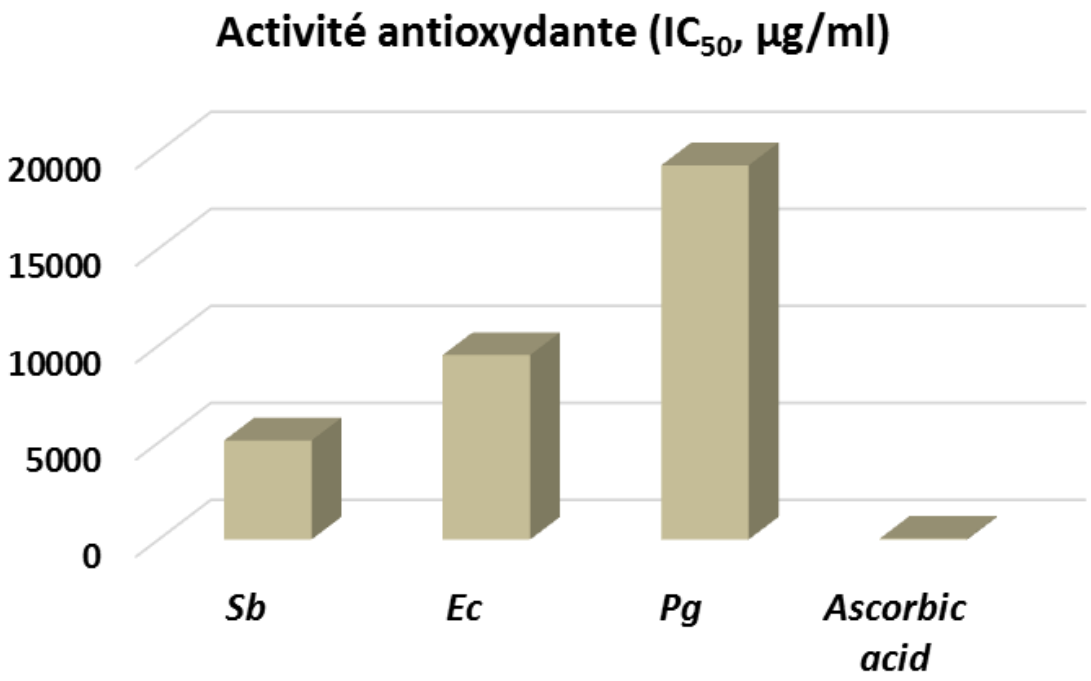

Figure 1. Comparison of the antioxidant activity of essential oils and ascorbic acid.
Sb = Essential oil from *S. birrea*, *Ec* = Essential oil from *E. camaldulensis*, *Pg* = Essential oil from *P. guajava* ,

β-bisabolene (14.38 ± 0.03%), *ar*-curcumene (12.39 ± 0.02%) and β-bisabolol (11.40 ± 0.08%), the three first major constituents of this oil, were not available and could not be tested. Recently, bisabolol oxide derivatives from *Artemisia persica* ethyl acetate extracts, exhibited *in vitro* antimalarial activity against *P. falciparum*, with IC_{50} values ranging from 1.14 to 7.92 µg/ml (Moradi-Afrapoli et al., 2013).

Given the activity observed for pure compounds, these essential oils seem to be the result of a synergistic action of all its constituents, including minor ones.

Antioxidant activity

The antioxidant activity of the studied oils was expressed in IC_{50} values and recorded in Table 3. The essential oil of *Sb* (IC_{50} = 5106 µg/ml) showed the highest activity, followed by that of *Ec* (IC_{50} = 9510 µg/ml) and by that of *Pg* (IC_{50} = 19290 µg/ml). The studied oils were all active, but less than ascorbic acid (IC_{50} = 20 µg/ml), the reference compound used in the test (Figure 1). This activity is quite low and could be explained by the presence in these oils of some high active components as β-caryophyllene (IC_{50} = 3.68 µ/ml; Pujiarti et al., 2012) and some less active components as *β*-pinene (IC_{50} = 20.05± 0.03 µg/ml; Kazemi, 2015); *p*-cymene (IC_{50} = 20.05± 0.4 µg/ml; Kazemi, 2015). Safaei-Ghomi et al. (2009), showed that the antioxidant activity of the major components tested separately gives lower results compared to the activity of the whole components of the essential oil. Furthermore, presence of allylic compounds and / or benzyls (less than 1% in the studied oils; Table 2) could contribute to this activity. Therefore, the antioxidant activity of our oils could be explained by a synergy of action between their different constituents (Safaei-Ghomi et al., 2009; Vardar-Unlu et al., 2003).

Conclusion

Our study shows that the essential oils of *S. birrea*, *E. camaldulensis* and *P. guajava* from Benin were more active on *T. brucei brucei* than on *Plasmodium falciparum* (3D7) and very weakly antioxidants. The essential oils of *S. birrea* and *P. guajava* already used extensively in traditional medicine and consumed by livestock were the most active and could be interesting for the treatment of sleeping sickness but may also have some interest on *Plasmodium*. These plants contain components with low, moderate or very good activities, which appear to act synergistically in their essential oils. These oils had a low cytotoxicity against CHO and WI38 cells. This is the first report on the activities of these essential oils against *T. brucei brucei*, *P. falciparum* and their cytotoxicity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

ABBREVIATIONS

Sb: *Sclerocarya birrea*, **Pg:** *Psidium guajava,* **Ec:** *Eucalyptus camaldulensis.*

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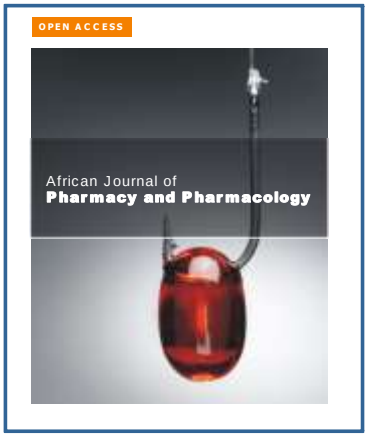

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