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Full Length Research Paper

Influence of polymorphism of adenosine triphosphate binding cassette on oral drug absorption

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Administration of drugs via oral route is the most common and convenient. Three major factors can affect drug absorption from this route: physiologic, physicochemical and formulation. Drug absorption mainly takes place in small intestine after oral administration. Within the small intestine, there are 2 major families of proteins involved in transport that have significant roles on drugs absorption: Solute-carrier and adenosine triphosphate-binding cassette (ABC) superfamilies. There are 48 ABC transporters in humans as well as seven subfamilies: ABCA to ABCG. ABCB1, ABCC1/2, and ABCG2 are widely described for their impacts in the absorption of drugs. Polymorphism on ABC transporters can significantly affect the absorption of drugs from the gastro intestinal (GI) tract. Some polymorphisms can result in over expression of the transporter in the small intestine and hence reduce absorption of drugs and some result in reduction in expression and increase in absorption. These polymorphisms are an important cause of adverse drug reactions and therapeutic failures.

Key words: Polymorphism, adenosine triphosphate binding cassette, oral drug absorption, expression, ABC transporters.

INTRODUCTION

Administering drugs utilizing the oral route is strongly preferred because of its convenience, and relative reduced cost (Engman, 2003). The small intestine is a major area of absorption for most orally administered drugs and so is the end for pharmaco-therapeutic approaches to regulate the oral absorption of drugs and the pharmacokinetics and pharmaco-dynamics parameters (Nakamura et al., 2008). The main mechanisms by which absorption occurs include

transcellular or intracellular transport, paracellular or intercellular transport, active transport and vesicular transport or endocytosis (Salama et al., 2006).

Drugs which are administered orally must cross via the intestinal mucosa prior to arriving at the capillaries that drain to the portal vein causing first pass effect. The barrier of mucosa comprises polarized enterocytes that are intimately connected by way of firm junctions. Many drug transporters are detected in the

cells (Estudante et al., 2013). Drug transporters are proteins that hold xenobiotics or endogenous compounds across membranes. They are divided as either uptake proteins or efflux, based on the way of transport. The two superfamilies of proteins involved in transport and that have significant roles on major pharmacokinetic profiles are the SLC and adenosine triphosphate-binding cassette (ABC) superfamilies (Regmi and Bharati, 2012).

This literature review focused on the ABC family members of transporters which are from the most widely investigated transporters involved in drug absorption and elimination and responses (Evans and McLeod, 2003). This superfamily members exploit adenosine triphosphate (ATP) as a source of energy, permitting to move molecules against a concentration grade. Drugs can be all together substrates and/or inhibitors of more than one efflux transporter, signifying that ABC transporters apply a dual role in detoxification at the intestine (Linton, 2007).

Genetic polymorphism (variation) is a difference in deoxyribonucleic acid (DNA) sequence among individuals, groups, or populations that contribute for a difference in drug absorption probably by affecting the transporter expression (Meletiadis et al., 2006). So many genetic polymorphisms in the transporters are investigated; some of them fascinated considerations as genetic factors linked with expression level and role in small intestine (Nakamura et al., 2008). The two important sites influencing the extent of drug reaching the systemic circulation followed by an administration are the intestine and/or liver, so transporter expression in these sites proposes that factors dictating their role will be important factors of oral drug pharmacokinetics. Factors influencing protein levels, genetic polymorphisms leading to improved or decreased purpose and co-administration with inhibitors are all significant opportunities where a transporter's capacity to transport molecules is changed (Estudante et al., 2013).

BASICS OF HUMAN GENETICS

Major types of genetic polymorphisms

Polymorphism refers to the occurrence, in a proportion of people in a population, of several sequence variations at a particular position in a gene. It is the inherited differences found among the individuals in 68, more than 1% of normal population (National Health and Medical Research Council, 2000). Polymorphism occurs due to mutation of genetics. The various kinds of polymorphism are characteristically indicated by the nature of the mutation that formed them. The kind of polymorphism that forms from a single base mutation which replaces with another nucleotide is called 'single nucleotide polymorphism' (SNP). The first systematic investigations

of single point variants were practiced across the recognition of controlled enzyme locations, where a single base pair alteration might cause the loss or gain of a controlled position. Disintegration of a piece of DNA having the appropriate position with a suitable limitation enzyme might then differentiate alleles or variants based on consequential fragment sizes via electrophoresis, and such a kind of polymorphism is known as 'restriction fragment length polymorphism' (Brandenberg et al., 2011; Schork et al., 2000). Other forms of genetic polymorphism are caused from the addition or removal of DNA part. The most frequent form of such 'addition: removal' polymorphism is the presence of changeable figures of frequent base or nucleotide pattern in a genetic region. Repetitive base patterns range in size from a number of hundreds of base pairs, called 'variable number of tandem repeats', to the more general 'microsatellites' comprising two, three or four nucleotides in repetition with some variable number of times. Microsatellites are often known as 'simple tandem repeats' (Schork et al., 2000).

POLYMORPHISM OF ATP BINDING CASSETTE ON ORAL DRUG ABSORPTION

Introduction to ABC transporters

Structure

The superfamily of ABC proteins (Figure 1) are among the major protein families (Sharom, 2008). There are 48 ABC transporters in humans (Linton, 2007) which are categorized into 7 subfamilies: ABCA to ABCG according to the series homology. Among these, MDR1 drug-transporting P-glycoprotein (P-gp; ABCB1), multidrug resistance-associated 91 protein (MRP2; ABCC2), and breast cancer resistance protein (BCRP; ABCG2) have been well studied for the roles in drug disposition as well as response (Li and Bluth, 2011). The fundamental unit of an ABC transporter comprises 4 interior domains (Higgins, 2001), organized as either full transporters containing two transmembrane domain (TMD) and two nucleotide binding domain (NBD) or as half transporters having one of each domain. The half transporters come together as either homodimers or heterodimers to produce a useful transporter (Dean et al., 2001). The TMD, entrenched in the bilayer of membrane, differentiates a variety of substrates and goes through conformational modification to transport the substrate transversely across membrane (Yang, 2013) and provide specificity, whereas the NBDs attached as well undertake hydrolysis of ATP to force the movement of attached ligand (Linton, 2007).

The TMD possess numerous alpha helices, which cross the lipid bilayer. Typically, there are six predicted
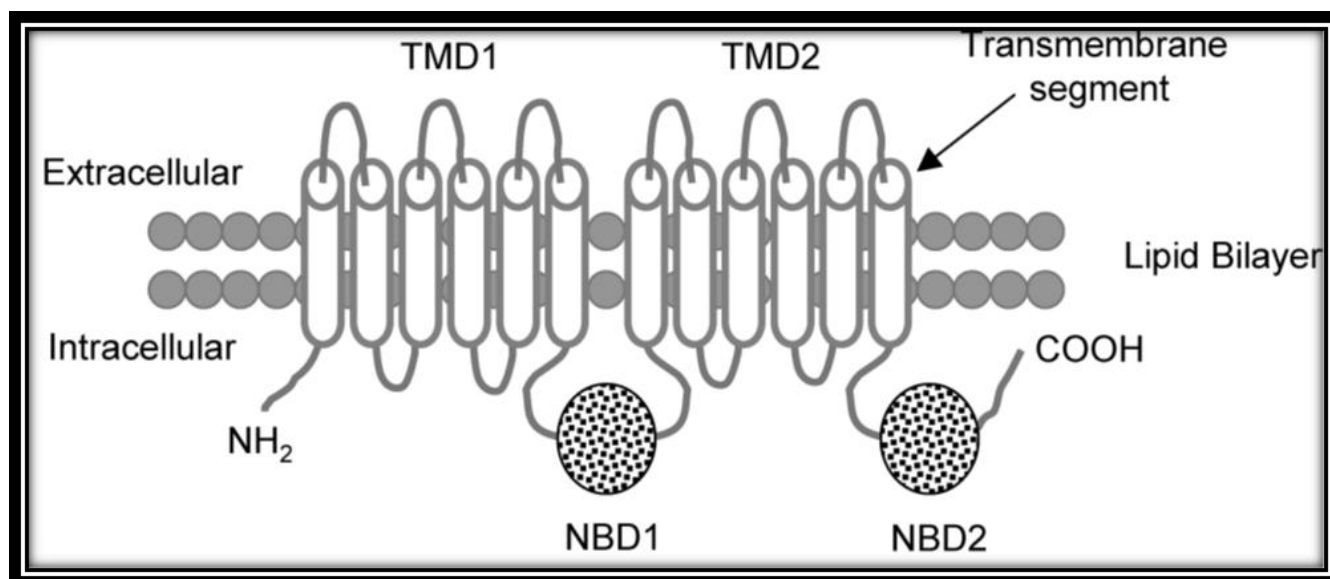
Figure 1. Structure of adenosine triphosphate binding cassette transporter protein (Choudhuri and Klaassen, 2006).

membrane- spanning α-helices per domain (Higgins, 2001). The figure of alpha helices in a TMD varies based on the family. The attaching sites of ATP are situated on the membrane cytoplasmic (Cox, 2010).

The ABCB1 gene, the primary ABC transporter recognized, maps to chromosome 7q21.1 and comprise 28 translated exons and 27 introns. Previously called MDR1 or PGY1, ABCB1 was the initial human ABC transporter gene cloned and distinguished across its capacity to present a multi-drug resistant (MDR) phenotype to cancer cells which caused resistance to some therapy (Franke et al., 2010). The human MDR1 gene encodes a protein of 1280 amino acids which comprises two highly homologous halves. The molecular weight of P-gp is 170e180 kDa. This molecule holds 12 TMD and two putative ATP binding positions (Dallas et al., 2006).

MRP or ABCC family is the other member of ABC transporter and MRP2 is the main transporter of MRP family. MRP2 encodes a 190e200 kDapolytopic transmembrane protein consisting of 1545 amino acids and links to subfamily C of ABC transporter (Dallas et al., 2006). Twelve full transporters have been recognized so far. ABCC subfamily in human comprises ABCC1 through ABCC12, and nine of them belong to MRP transporters (Yang, 2013).

The ABCG2 (called BCRP, ABCP, or MXR) protein is an ABC half-transporter (Li and Bluth, 2011) which is composed of one transmembrane and one nucleotide linking fold section, called an NBF-TM. It is composed of 16 exons and 15 introns and is situated on chromosome 4q22 (Franke et al., 2010).

Expression and function

MDR1 drug-transporting P-glycoprotein: MDR1 is among the common efflux transporters uttered in MDR cancer cells and in numerous organs like intestine, liver, kidney and BBB. In the intestine of humans, P-gp is articulated in enterocytes apical membrane and the mRNA intensity is maximum in the jejunum, then in ileum and colon (Yang, 2013). It has a significant function by eliminating toxic materials/metabolites from cells. For example, the protein is extremely manifested in cells comprising BBB and most likely has a part in transport of toxic materials out of the brain, effectively stopping uptake (Franke et al., 2010). In the intestine, P-gp extrudes various drugs into the lumen, minimizing the rate as well as extent of absorption (Sharom, 2008). Poorly hydrophilic drugs with a polyaromatic skeleton and a positive or neutral charge are often the substrates for P-gp (Cox, 2010).

Multidrug resistance-associated protein: The protein is the most investigated ABCC family member (Cox, 2010) contained in the polarized cells apical membrane from range of human and rat tissues together with enterocytes of the small intestine, hepatocytes and renal proximal tubules (Dallas et al., 2006) where it can mediate the efflux of glucuronides, bilirubin and other organic anions, playing a function in the detoxification for various xenobiotic and endogenous compounds (Glavinas et al., 2004). The substrates for ABCC are several organic anions, especially conjugated compounds. In addition to conjugates, MRP2 moves
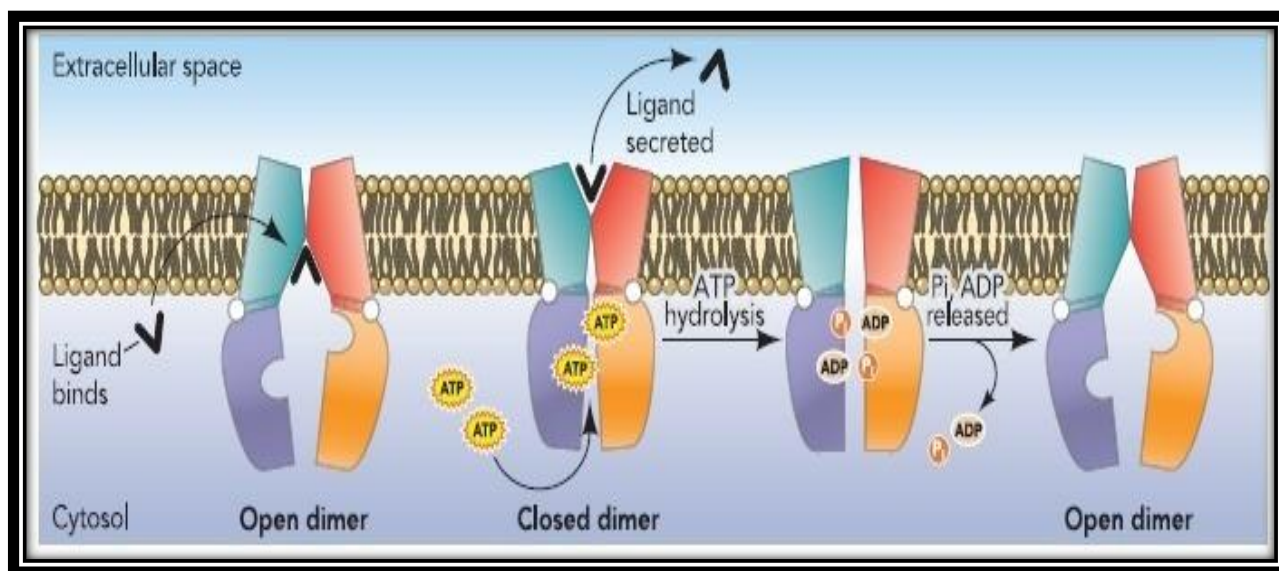
Figure 2. Mechanism of a typical Adenosine triphosphate binding cassette transporter (Linton, 2007).

amphipathic unionized compounds. Different investigations indicate that MRP2 mediated transport of unionized/positively charged substrates is motivated by the existence of low glutathione (Dietrich et al., 2003).

BCRP: ABCG2 was initially revealed, as indicated by the name, in cells of breast cancer. It was also named mitoxantrone resistance protein (MXR) due to one of its substrates (Dietrich et al., 2003). ABCG2 is usually manifested in the hepatocytes canalicular membrane, in the small intestinal, colon, placenta, lung, kidney, adrenal and sweat glands epithelia, as well as in the central nervous system (CNS) endothelia vasculature. It is very important for detoxification of host as well as defense against poisonous xenobiotics (Li and Bluth, 2011).

MECHANISM OF TRANSPORT

ABC transporter protein family, in spite of their great useful diversity, at their center they all contribute to the same field design and are thought to share a basically related to alternating access transport mechanism (Procko et al., 2009). The mechanism of a typical adenosine triphosphate binding cassette transporter is shown in Figure 2. The transport cycle is induced by the contact of substrate with the TMDs from the membrane intracellular face. The number of substrate-linking positions on TMDs is vague, though it is likely to be 2 (Higgins, 2001). Ligand attaches to the TMDs in the high-affinity open NBD dimer modification, stimulating higher affinity for ATP (Linton, 2007). Without nucleotide, NBDs are not together or open, but when

ATP attaches to both NBDs they approach to form a tight dimer with two ATPase active locations at the interface (Procko et al., 2009), which on the other hand stimulates a high conformational alteration in TMDs adequate to move ligand. ATP hydrolysis induces dissolution of the stopped NBD dimer (Linton, 2007) and the transporter returns to the beginning of conformation (Procko et al., 2009).

Polymorphism of ABC drug transporters and influence on drug absorption from GI tract

MDR1 drug-transporting P-gp

P-gp is substrate for many drugs together with bilirubin, several anticancer drugs, cardiac glycosides, immunosuppressive agents, glucocorticoids, and HIV-1 PIs (Evans and McLeod, 2003). ABCB1 genetic polymorphisms were indicated to alter the appearance of mRNA, protein expression and use of P-gp and change in substrate specificity (Sharom, 2008). Greater than 50 SNPs were recognized in the coding section of ABCB1 (Li and Bluth, 2011). C1236T in exon 12, G2677T/A in exon 21 and C3435T in exon 26 are three major SNPs in the ABCB1 gene as shown in Figure 3. The SNPs might influence the pharmacodynamics and pharmacokinetics of drugs that are P-gp substrates (Estudante et al., 2013).

C3435T: A SNP in exon 26 position 3435 (C3435T) was linked with the P-gp expression and function (Ekhart et al., 2009). Variation in the sequence of nucleotide from C to T at site 3435 did not cause an alteration of amino
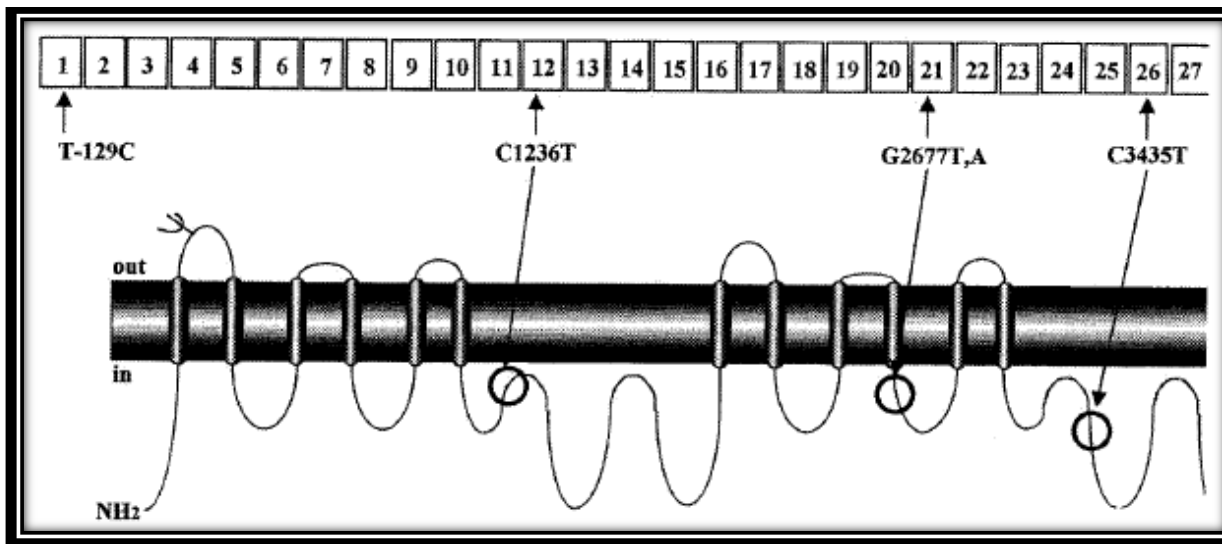
Figure 3. The three most frequent single nucleotide polymorphisms in the multidrug resistance transporter gene (Anglicheau et al., 2003).

acid; however it is a quiet mutation situated in the wobble site of the codon. Although there is no alteration in induced protein, together the intensity of its function and expression may be changeable. For example, a double fall of intestinal P-gp had been seen in individuals who were homozygous for 3435T (Cox, 2010).

Effect on digoxin absorption

Digoxin is not affected by alteration of metabolism; it serves as a model substrate for phenotype-genotype interactions of MDR1 polymorphs. The digoxin uptake, a known P-gp substrate, is affected by intestinal P-gp induction. Consequently, digoxin plasma levels have been established to be significantly elevated in volunteers with a C3435 T nucleotide exchange in exon 26 (C3435T) of the MDR1 gene (Brinkmann and Eichelbaum, 2001). A sizable Dutch study (195 elderly patients) concerning chronic dosing of digoxin rather than single dose kinetics investigated the influence of MDR1 genotype on digoxin concentrations. The 3435C>T subjects were related with concentration of serum digoxin of 0.18 to 0.21 mcg/L per supplementary T allele. The findings of this study are in consistence with other study in healthy Japanese individuals. Individuals porting a T allele at 3435 had considerably lesser area under the curve than those homozygous for C at this site (Cox, 2010). Other study conducted on 32 healthy persons also found that the synonymous polymorphism in point 3435 affects the absorption kinetics of oral digoxin, the homozygous wild-type (CC) genotype eliminating digoxin

more efficiently than the individuals by TT or CT alleles (Li and Bluth, 2011).

Effect on absorption of anticancer drugs

The substrates of P-gp comprises anticancer drugs like vincristine, doxorubicin, vinblastine, etoposide, daunorubicin, and paclitaxel (Novoa et al., 2005). Reports on individual SNPs at 3435 had significantly showed lower exposure to SN-38 (7-ethyl-10-hydroxycamptothecan), irinote can activate metabolite by carboxylesterase enzymes (Cox, 2010), signifying that the variant haplotype is linked by high efflux action (Franke et al., 2010).

Effect on absorption of antiretroviral agents

Most protease inhibitors of HIV-1 are substrates of the P-gp system, which affects their uptake as well as body distribution (Novoa et al., 2005). The MDR1 C3435T genotype influences the absorption constant of indinavir suggesting that P-gp might be concerned in its variability of pharmacokinetics profile. Result of study conducted by Solas et al. (2007) indicated that patients having the MDR1 C3435T genotype had an extensively elevated ka (two-fold increase) compared with patients having the C3435C genotype Solas et al. (2007). Studies conducted on other antiretroviral drug, nelfinavir, also show that T allele at position 3435 in exon 26 is linked with a superior level of P-gp purpose. Individuals with a homozygous T

genotype at exon 26 had a lower level of nelfinavir in plasma in comparison to those individuals with the homozygous C genotype. This result suggested the T allele is linked by a superior concentration of P-gp purpose (Sankatsing et al., 2004).

Effect on other drugs

As a result of hydrophobic structure, antiepileptic drugs might be substrates for P-gp transporter (Buzoianu et al., 2009). Investigations of 108 patients for phenytoin concentrations of serum along with MDR1 C3435T gene polymorphism by Ponnala et al. (2012), indicated steady raise in phenytoin concentrations of serum from homozygous C allele, heterozygous CT, and homozygous T allele. Genotype and serum phenytoin concentration examination as indicated by the MDR1 C3435T gene polymorphism influences serum phenytoin concentrations. The mean serum phenytoin concentrations in seizure re-occurrence genotype groups were 8.65, 9.51, and 17.54 mg/ml for CC, CT, and TT genotypes, respectively (Sailaja et al., 2010).

G2677T/A: The non-synonymous G2677T/A SNP is located in exon 21, which represents an amino acid change of alanine by serine (G2677T) or threonine (G2677A) (Ala893Ser/ Thr) (Brambila-Tapia, 2013) in such a manner that the lipophilic residue (Ala) is altered to hydrophilic residue (Ser, Thr) presenting higher resistance to different drugs such as adriamycin and vinblastine (Sailaja et al., 2010). This SNP has shown controversial results in functional effects (Brambila-Tapia, 2013). A Report of study conducted by Lamba et al., 2006 indicated that those who were with the 2677 TT genotype had lesser P-gp messenger RNA expression than those who had 2677 GG genotype (Sailaja et al., 2010). A study conducted by Green et al. (2006) showed that the missense SNP, G2677T/A, correlated with the outcome of paclitaxel treatment. The study compared the wild-type and heterozygous (G/G and G/T) with the homozygously mutated (T/T and T/A) patients and their relation to treatment outcome. A statistically significant association was obtained between homozygously mutated patients as well as successful treatment with paclitaxel. The occurrence of the T and A alleles in the group of patients with a good response was also significantly upper than in poor responders (32 of 56 compared with 18 of 50). One explanation could be that the G2677T/A polymorphism have a functional consequence on P-gp mediated paclitaxel transport. The better response might be due to a lower efflux of paclitaxel from the tumor cells or an increased absorption, giving higher plasma concentrations (Green et al., 2006). On the contrary to the aforementioned

study, G2677T SNP was linked with improved P- gp role *in vitro* and lesser plasma fexofenadine levels in humans (Evans and McLeod, 2003).

C1236T: The synonymous C1236T SNP is located on exon 12 which encodes for the TM6 region, which is essential for substrate binding (Brambila-Tapia, 2013). Variability in MDR1 on exon 12 was linked with variations in plasma drug concentrations as well as response to ART. Bellusci et al. identified genotypes in 100 blood donors and 38 HIV-1-infected patients. Every patient took highly active antiretroviral therapy (HAART) with LPV/r at the moment of lopinavir plasma concentration quantification, prior to and between 1 and 2 h following the next dose of drug administration. CD4 (+) T-cell counts and plasma viral load had been examined before and after the start of LPV/r. The result suggests that the MDR1 C1236T SNP drastically lowers LPV plasma con-centration, influencing the virological response to HAART. Heterozygotes C1236T could have a changed concentration of P-gp expression/activity in enterocytes and CD4 (+) T lymphocytes which confines the absorption of LPV, causing a weakened virological suppression (Bellusci et al., 2013).

Breast cancer resistance protein, BCRP (ABCG2)

Breast cancer resistance protein (BCRP) is one of the newer ABC transport proteins to be examined. The transporter was first identified in drug-resistant breast cancer cells. It actively forces out a broad range of chemically dissimilar hydrophobic compounds from the cells, as well as cytotoxic compounds like topotecan, mitoxantrone, SN-38, flavopiridol, and methotrexate (Li and Bluth, 2011), non-chemotherapy drugs like prazosin, dipyridamole, statins, glyburide, quercetin, temocapril, an ester-type prodrug of temocaprilat, sulfate conjugates, nitrofurantoin, porphyrins, some fluoroquinolones, antivirals such as lamivudine, zidovudine and efavirenz, rifampicin, ciprofloxacin, quercetin, sulfasalazine and nontherapeutic compounds such as dietary flavonoids (Estudante et al., 2013).

Over 80 SNPs, missense and frame shift mutations in the ABCG2 gene were recognized in dissimilar racial group (Sharom, 2008). Functional SNP in exon 5 of the ABCG2 gene, in which a C to A nucleotide transition at site 421 (ABCG2421C.A) exist, causes a nonsynonymous variant protein with a glutamine to lysine amino acid replacement in codon 141. The ABCG2C421A variant was linked with small ABCG2 expression levels and changed substrate specificity, and varied diflomotecan and topotecan pharmacokinetics (Li and Bluth, 2011).

Another study indicated that a higher ratio of patients

existed more than 15 months with docetaxel-based therapy in the occurrence of the ABCG2 C421A polymorphism. The consequence of ABCG2 poly-morphisms on docetaxel disposition is unidentified. The enlarged survival seen in those with an ABCG2 C421A polymorphism can recommend a fewer functional drug efflux pumps, causing improved absorption and concentrations of intracellular docetaxel, as well as enhanced cytotoxic activity (Ekhart et al., 2009).

A study conducted by Zhang et al. (2006) also showed that the pharmacokinetic profiles of rosuvastatin revealed a appreciably high dissimilarity between the two genotyped groups. In the C421A along with A421A mutant group, the area under the curve (0 to 72) and the area under the curve (0 to ∞) of rosuvastatin had been about 80% elevated than that in the C421C wild-type, and the Cmax was about 90% better than that in the C421C wild- type.

The study conducted by Urquhart et al. (2007) shows the influence of general SNPs in BCRP to the disposition of sulfasalazine in healthy individuals. Sulfasalazine is a substrate of BCRP and utilized in the therapy of ulcerative colitis. In the study, individuals with 34GG/421CA genotype, sulfasalazine AUC0- N and Cmax values were considerably better than those obtained from wild-type 34GG/421CC individuals. Fascinatingly, the lone subject transporting variant alleles, 34A and 421A, demonstrated a 4.8-fold raise in the AUC and a 4.4-fold raise in Cmax in comparison to wild-type controls. The data of *in vitro* expression proposed lower cell surface expression of the 421C>A variant as a major influencing factor of lower BCRP mediated transport of sulfasalazine in individuals having an A allele at site 421.

A study conducted Zamboni et al. (2006) also showed that the ABCG2 421C>A genotype radically influenced 9-aminocamptothecin (9AC) pharmacokinetics. The 9-nitrocamptothecin (9NC) is an orally administered camptothecin analogue.

MDR-associated protein family

ABCC2 plays a major part in the transport of drugs like leukotriene, oxidized glutathione, vincristine, cisplatin, methotrexate, daunorubicin, etoposide, glutathione, glucuronide, and sulfate conjugates (Dallas et al., 2006). Most ABCC2 polymorphisms are quite rare in the general population, however, 24C>T, 1249G>A, and 3972C>T are all relatively common in healthy individuals. The functional importance of ABCC2 polymorphisms remains unclear. Few studies assessed results of polymorphisms in this gene and those that have failed to find any functionally significant effects (Sissung et al., 2012). The pharmacokinetics of irinotecan as well as 9-nitrocamptothecin and its metabolite 9-amino-

camptothecin in cancer individuals were not considerably influenced by the polymorphism of ABCC2 –24C>T (Zamboni et al., 2006). ABCC2 gene mutations are linked with the rare autosomal recessive disorder Dubin-Johnson syndrome (DJS). The mutations might form DJS through a diversified system. The common understandable is creation of nonfunctional forms of the protein, that causes the incapability for hepatocytes to produce conjugated bilirubin into the bile (Choudhuri and Klaassen, 2006). Numerous mutations linked with DJS arise on the ATP linking section, which is significant for protein purpose. The intestinal expression and function of ABCC2 in DJS patients are lower. To explain its clinical importance in drug absorption, genotype-haplotype analysis of ABCC2 is necessary (Nakamura et al., 2008).

Application of ABC transporters polymorphism on clinical practice

The vital function by ABC multidrug efflux pumps in carrying tissues from exogenous toxins is broadly documented. These associated transporters determine the drugs uptake as well as release to their tissue target (Sharom, 2008). Polymorphism result in altered drug absorption and then altered drug response. The detection of polymorphisms elucidating separate drug phenotypes transporter proteins put into the acceptance of personal changes (personalization) of drug treatment and enhances drug safety and efficacy (Ma and Lu, 2011).

Personalized medicine is a medical model with molecular profiling technologies for following the correct pharmacologic approach for the appropriate individual at the appropriate time. Accurate prediction about drug response is crucial for individualized treatment, is best made by combining an individual's genetic data with clinical findings and classifying individuals into subpopulations that differ in their response to an exact drug. A pharmacogenomics advancement may permit a precise drug therapy to be applied to genetically distinct subsets of patients and may direct to a new treatment categorization at the molecular stage (Eichelbaum et al., 2006).

The understanding about occurrence of functionally significant SNPs in the ABC gene in the population is a considerable idea for scheming prospect pharmacokinetic and pharmacodynamic investigations conducted with ABC substrates. Pharmacodynamic approaches will permit rising power of the investigations with lower economic expenses and ethical threat for the participating individuals at the right time. The practicing clinicians should be further conscious of the high frequency of functional polymorphisms, particularly when in view of treatment with drugs – substrates with a narrow

therapeutic window. Digoxin is one of the model drugs, where special carefulness is necessary in those with lower activity of transporters to avoid development of severe adverse drug reactions (Pechandova et al., 2006).

Transport activity of ABC is known to influence oral uptake of substrate drugs and polymorphism may sometimes result in treatment failure, for example on HIV, cancer and epilepsy. Identification of these polymorphisms allows improving treatment response through individualization of therapy (Sharom, 2008).

Future perspectives

Drug transporters amend the drugs absorption, distribution, and elimination by regulating the uptake and efflux of drugs in cells. Rising proof shows genetic polymorphisms of transporters can cause intense influence on drug absorption, efficacy, and safety. The vast of investigations had resulted from grim experimental restrictions, such as model variety, sample size, puzzling factors and genotype/phenotype errors, and a complete set of suggestions to circumvent such troubles in the prospect existed. Confirmatory effect of the ABC SNP is desirable. Large-scale genotype-phenotype association trials are required to raise our awareness of the effects of SNP on clinical responses.

A recent review about pharmacogenomics highlights the need for depth analysis of the available whole genome association study data, more advances in technologies and sequencing the complete genome (whole-genome sequencing) to identify low frequency or rare variants that are associated with HIV infection. In addition, data collections should be extended to as many populations as possible to enhance diversity. It is particularly important in African populations due to the high HIV prevalence rate. In the future, the individualized medicine will possibly comprise a mutual approach by means of the knowledge of drug, virus and host factors information to guide the personalized prescription in which the right drug will be given to the right person.

The function of polymorphism in response to drug treatment and disease vulnerability is an increasing area that will visibly be significant in the future as the eventual target of individualized drug is followed.

Conflict of interest

The authors have not declared any conflict of interest

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Full Length Research Paper

Antioxidant and antinociceptive effect of the hydroethanolic extract and fractions of the bark of *Bowdichia virgilioides* in orofacial pain

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Bowdichia virgilioides is used by the population for treating inflammation in general. This study evaluated the antinociceptive activity and possible mechanisms of action of hydroethanolic extract (HEE) and hexane (HXF), chloroform (CLF), ethyl acetate (EAF) and hydromethanol (HMF) fraction of HEE obtained from the plant stem bark against orofacial pain, as well as *in vitro* its antioxidant effect on the scavenging of free radical DPPH•. The antioxidant activity of the extract and fractions was evaluated against DPPH• at concentrations of 5, 15, and 25 µg.mL^{-1} for each sample studied, with gallic acid used as positive control. The absorbance decrease was spectrophotometrically measured at 515 nm up to 60 min to obtain the percentage of inhibition of the free radical. The antinociceptive activity was investigated in Swiss mice treated orally with HEE, EAF and HMF (100, 200, and 400 mg/kg) and morphine (5 mg/kg), using formalin, glutamate and capsaicin orofacial pain models. HEE, EAF and HMF showed the best results regarding the reduction of the DPPH radical (25 µg/mL, 60 min), with percent inhibition of 42.89, 78.52 and 54.96%, respectively. HEE, EAF and HMF significantly (p < 0.001) reduced orofacial nociception in mice in the first (56, 49 and 19%, respectively) and second phases (61, 71 and 69%, respectively) of the formalin pain model, as well as glutamate (82, 69 and 39%, respectively) and capsaicin (49, 68 and 64%, respectively) assays. Animals showed no significant changes in motor performance after treatment with HEE, EAF and HMF in the Rota rod test. In general, the potential of *B. virgilioides* to treat orofacial pain in its central and peripheral components was confirmed, with HEE, EAF and HMF (mainly at 200 and 400 mg/kg) showing antinociceptive effect in three different orofacial pain models related to opioid, glutamatergic and vanilloid receptors. In addition, it is also possible that their antioxidant activity may be related to the observed antinociceptive effect by reducing the biosynthesis of ROS and other inflammatory mediators.

Key words: *Bowdichia virgilioides*; black sucupira; antioxidant activity; orofacial pain; antinociception; free radical.

INTRODUCTION

Bowdichia virgilioides, popularly known as "black sucupira", is a leguminous tree belonging to the family Fabaceae and occurs mainly in the North and Northeast, as well as in the Central Plateau of Brazil (Albuquerque and Guimarães, 2007). In folk medicine, *B. virgilioides* is used for treating diarrhea, rheumatism, headache and aneurysm (Arriaga et al., 2000; Gomes et al., 2008), malaria (Mariath et al., 2009) and general inflammation (Smiderle and Sousa, 2003). Previous studies have shown antimalarial activity (Deharo et al., 2001), antimicrobial (Almeida et al., 2006), anxiolytic (Vieira et al., 2013), antioxidant, anti-inflammatory and analgesic activities, which showed the plant can be used for treating diseases in which the patient is affected by pain (Thomazzi et al., 2010).

Among painful disorders is the orofacial pain, which is characterized by painful conditions in the hard and soft tissues of the head, face, neck and all intraoral structures (Macfarlane et al., 2002; Fan et al., 2012). Pain signal is carried by the trigeminal system, which is a dense nerve network that involves the fifth cranial nerve or trigeminal responsible for pain perception in the orofacial region (Viggiano et al., 2005). Anticonvulsants, antidepressants and opioids are used for treating orofacial pain. However, this therapy causes various side effects, which often prevent their use as well as prevent the patient to adhere to the treatment (Rang et al., 2003; Antonialli et al., 2012).

It is also necessary a better understanding of the physiological and pathophysiological pathways in the trigeminal system to found drugs able to reduce pain. Studies have demonstrated that reactive oxygen species (ROS) are produced during persistent facial pain. It was found that these substances are necessary for the transmission of pain signals (Meotti, 2006; Guimarães et al., 2010), thus showing that a pathway to minimize pain propagation is the reduction of ROS production. Therefore, there is a correlation with antioxidant systems (Viggiano et al., 2005; Lewis et al., 2007).

The antinociceptive and antioxidant activities of the aqueous extract of *B. virgilioides* were demonstrated by Silva et al. (2010) and Thomazzi et al. (2010). However, the effect of this species was not reported for orofacial pain.

This study aimed to improve the understanding of the antioxidant mechanisms based on the evaluation of the scavenging activity against free radical DPPH• as well as to evaluate the orofacial antinociceptive effect in the models of orofacial pain induced by formalin, capsaicin and glutamate, using the hydroalcoholic extract and fractions obtained from the bark of *B. virgilioides*.

MATERIALS AND METHODS

Collection and identification of plant

The bark of *B. virgilioides* was collected in March 2011 in the village Fazenda Riachão, city of Japaratuba, state of Sergipe, Brazil (10°32'4.49" S and 36°53'57" W). The plant was identified by Dr. Ana Paula Silvera, botanist of the Department of Biology, Federal University of Sergipe, Brazil (DB-FUS). A voucher specimen was deposited in the DB-FUS herbarium under the registration number ASE 23107.

Preparation of the hydroethanolic extract and its fractions

A total of 1.2 kg of *B. virgilioides* bark were dried at room temperature, reduced to powder and subjected to maceration with 90% ethanol for 5 days. Afterwards, the material was filtered and concentrated in a rotatory evaporator under reduced pressure at 45°C to give 114.35 g of the hydroethanolic extract (HEE, yield of 9.53%). A portion of HEE (78.0 g) was dissolved in methanol: water (2:3) and subjected to liquid-liquid extraction with organic solvents to obtain hexane (HXF, 6.020 g, yield of 7.71%), chloroform (CLF, 1.590 g, yield of 2.03%), ethyl acetate (EAF, 2.010 g, yield of 2.57%) and hydromethanol (HMF, 57.920 g, yield of 74.26%) fractions.

Phytochemical screening

Extracts and fractions were qualitatively analyzed by precipitation and colorimetric methods as described by Matos (2009) to detect phenols and tannins (red and blue precipitate, respectively, after treatment with 1 mol/L alcoholic ferric chloride), flavonoids (red-orange precipitate at pH 11 with no color change at pH 3 and 8.5 after treatment with 3 mol/L sodium hydroxide or 1 mol/L hydrochloric acid), catechins (yellow precipitate after heating at pH 2 with no color change at pH 11 after treatment with 1 mol/L hydrochloric acid or 3 mol/L sodium hydroxide), steroids and triperpenoids (blue or red coloration, respectively, after a Lieberman-Buchard reaction), saponnins (Lieberman-Buchard reaction and foam formation) and alkaloids (orange color in presence of the Hager, Mayer and Dragendorff reagent). Ethanol solutions (500 µg/ml) of the extract and fractions (5 ml) were treated with the different reagents.

Quantification of total phenolics

Total phenol content (TFC) was determined according to the methodology of Sousa et al. (2007) with modifications. HEE and its fractions (10 mg) were dissolved in 10 ml of methanol and an aliquot (100 µl) of the resulting solution was transferred to a Falcon conical tube with 6 ml of distilled water and 500 µl of 1N Folin-Ciocalteu reagent. The solution was stirred for 1 min. Afterwards, 2 ml of 15% sodium carbonate were added to each solution, which was again stirred for 30 s. Distilled water was then added to each Falcon tube to give a final volume of 10 ml and they were incubated for 120 min at 23°C. The absorbance of each solution was spectro-photometrically determined at 750 nm against the blank using

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Table 1. Phytochemical screening of the hydroethanolic extract (HEE) and its hexane (HXF), chloroform (CLF), ethyl acetate (EAF) and hydromethanol (HMF) fractions obtained from the bark of *B. virgilioides.*

Compound	HEE	HXF	CLF	EAF	HMF
Phenol	+	-	+	+	+
Tannins	+	-	+	+	+
Flavonoids	+	-	+	+	+
Xanthones	+	-	-	+	-
Catechins	+	-	+	+	+
Pentacyclic triterpenes and free steroid	+	+	+	-	-
Saponins	-	-	-	+	-
Alkaloids	+	-	+	-	+

quartz cuvettes. TFC was determined by interpolating each solution absorbance against a calibration curve built using gallic acid (GA) as the standard. Results were expressed as mg GA per g of extract or fraction.

DPPH• free radical scavenging activity

Aliquots of HEE and its fractions solutions (0.5 mg/ml in methanol) were added to 40 µg/ml DPPH• to give final concentrations of 5, 15, and 25 µg/ml in a final reaction volume of 3 ml. The blank was a mixture of HEE/fraction with methanol, while gallic acid was used as positive control.

Absorbance values for each sample were spectrophotmetrically obtained at 515 nm after 1, 5, 10, 20, 30, 40, 50, and 60 min (Sousa et al., 2007). The percentage of remaining DPPH ($DPPH_{REM}$%) was calculated according to Brand-Willams et al. (1995) from the equation: $DPPH_{REM}\% = [DPPH]_T/[DPPH]_{T0} \times 100$, where $[DPPH]_T$ is the radical concentration in the reaction medium after the reaction with HEE/fractions, and $[DPPH]_{T0}$ the initial concentration of DPPH. Inhibition percentage (IP) after 60 min of reaction was obtained from the $DPPH_{REM}$% at this same time.

Activity antinociceptive

Swiss mice (*Mus musculus*) males, 60 to 90 days of age, weighing 28 to 32 g each, were obtained from the Central Animal Facility of FUS. They were randomly kept in cages under controlled temperature ($22 \pm 3°C$) with light/dark cycle of 12 h. The animals had free access to food (Purina™) and water. The Ethics Committee on Animal Research of FUS approved the experimental protocols and procedures under registration number 65/11.

Groups of mice (n=6) were systematically pretreated with vehicle (tween 80 in phosphate buffered saline), HEE, EAF and HMF (100, 200 and 400 mg/kg; p.o.) 1 h prior to administration of the pain agent. Morphine (MOR, 5 mg/kg; i.p.) was used as positive control and administered 0.5 h before the injection of the orofacial pain inducer. Nociception was quantified by measuring the time (s) that the animals remained rubbing the area of the face where the pain inducer was administered with the front or hind legs.

Nociceptive orofacial pain was induced in mice by injection of 2% (20 µl, subcutaneously, s.c) formalin on the right upper lip (perinasal area). The behavioral response characteristic of the biphasic pain-related high intensity periods was observed from 0 to 5 min (first phase) and 15 to 40 min (second phase) (Luccarini et al., 2006). The glutamate nociceptive assay was performed as described by Beirith et al. (2002) with some adaptations, wherein the glutamate (40 µl, 25 mM, s.c.) was injected in the perinasal area and the animals were individually observed for 15 min after the

administration. Regarding the capsaicin nociceptive pain model, capsaicin (20 µl, 2.5 µg, s.c.) was dissolved in ethanol, dimethyl sulfoxide and distilled water (1:1:8) and injected in the perinasal area, followed by observation of the behavior of experimental animals during 42 min.

To investigate whether the treatment could influence the motor activity of animals and consequently affect the assessment of the nociceptive behavior, motor activity was evaluated in a rotarod apparatus. Initially, the mice that were able to remain on the rotarod up to 180 s were selected 24 h before the test. Then, the selected animals were divided into five groups and treated with vehicle (tween 80 in phosphate buffered saline), HEE, EAF and HMF (400 mg/kg; p.o.) and diazepam (1.5 mg/kg; i.p.). After 30, 60 and 120 min of administration, each animal was tested in the apparatus and recorded over time (s) remaining in the bar up to 180 s (Dunham and Miya, 1957).

Statistical analyses

Data were evaluated by One-Way Analysis of Variance (ANOVA) followed by Dunnett's post hoc test. Mean differences were significant for $p<0.05$.

RESULTS

The phytochemical screening revealed the presence of several classes of phenolic compounds (Table 1) as well as triterpenes and alkaloids, especially in HEE and EAF, which showed the greatest diversity of chemical compounds. Tannins and flavonoids appeared in HEE and all fractions, except for HXF. Triterpenes were only found in the apolar fractions HXF and CLF, while saponins were only present in EAF.

Table 2 shows the TPC of HEE and its fractions, with EAF providing the highest concentration of phenolic compounds (198.17 ± 9.06 GA mg/g fraction), while HXF was shown to have the lowest TPC (37.17 ± 8.41 GA mg/g fraction). EAF had 54.8% more TPC than HEE. Regarding the DPPH free radical scavenging activity, Table 3 shows that DPPH exposition to HEE, EAF and HMF caused the highest inhibition of the radical by 42.9, 78.5 and 54.7%, respectively.

Figure 1 shows that at the lowest dose (100 mg/kg), HEE did not reduce significantly ($p > 0.05$) the formalin-

Table 2. Total phenol content of the hydroethanolic extract (HEE) and its hexane (HXF), chloroform (CLF), ethyl acetate (EAF) and hydromethanol (HMF) fractions obtained from the bark of *B. virgilioides*.

Sample	Total phenol content (GA mg/g of extract/fraction)
HEE	128.00 ± 26.10
HXF	37.17 ± 8.41
CLF	169.31 ± 13.36
EAF	198.17 ± 9.06
HMF	154.21 ± 20.09

Table 3. Antioxidant activity (%) of the hydroethanolic extract (HEE) and its hexane (HXF), chloroform (CLF), ethyl acetate EAF) and hydromethanol (HMF) fractions obtained from the bark of *B. virgilioides* against the DPPH$^{\bullet}$ free radical.

Concentration (µg/ml)	HEE IP (%)	HXF IP (%)	CLF IP (%)	EAF IP (%)	HMF IP (%)	GA IP (%)
5	8.34±0.59	1.81±2.22	3.21±1.14	21.72±2.68	5.79±2.45	92.17±0.06
15	23.97±1.80	4.85±1.13	13.85±1.77	68.58±3.00	32.56±2.21	92.22±0.06
25	42.89±1.74	7.31±1.15	23.14±1.19	78.51±1.96	54.96±2.37	92.32±0.10

Results are expressed as inhibition percent (mean ± SD, n = 3). Gallic acid (GA) was used as positive control.

induced nociceptive response in both neurogenic (Figure 1A) and inflammatory phase (Figure 1B). However, at the highest does (200 and 400 mg/kg), HEE significantly ($\rho < 0.05$) reduced the nociception in both phases by 40 to 61% compared with the control group vehicle, whereas the group treated with MOR reduced more than 80% of the nociception in both phases.

Although the highest inhibitions of the nociceptive response were observed in the inflammatory phase (Figure 1D) in a dose-dependent manner, EAF produced antinociception in both phases of the formalin test (Figure 1C and 1D). The highest inhibition of the inflammatory response (Figure 1D) at the highest dose (400 mg/kg) was similar to the inhibitory result exhibited for MOR treatment (71 and 75%, respectively, compared to the vehicle group).

HMF also showed antinociception in the two phases of the formalin test (Figure 1E and 1F). However, the reduction was significant only at highest dose for the neurogenic phase (Figure 1E), while this fraction showed an antinociceptive effect in all doses used in the inflammatory phase (Figure 1F), reducing the nociceptive behavior by 55 to 69% against 75% for MOR.

To further analyze the antinociceptive effect of the bark of *B. virgilioides*, the glutamate and capsaicin pain models were used. As shown in Figure 2, HEE, EAF and HMF showed significant ($\rho<0.05$) antinociception when glutamate was used to cause orofacial pain, with HEE inhibiting up to 82% of the nociceptive response at 400 mg/kg. This is higher than observed for MOR, which inhibited only 67% of the gluatamate induced-pain.

HEE, EAF and HMF showed antinociceptive effect against pain induced by capsaicin as well (Figure 3A, 3B and 3C, respectively). However, only HEE and EAF were active at 100 mg/kg (Figure 3A and 3B), while HMF was not able to reduce significantly ($\rho>0.05$) the nociceptive effect of capsain (Figure 3C) compared to the vehicle group. The extract and both fractions showed similar results to MOR, with EAF and MOR reducing 71 and 68% of the nociceptive response time at 400 and 5 mg/kg, respectively, compared with the control group vehicle.

The effect of HEE, EAF and HMF on mice motor activity was investigated to evaluate its influence in the nociceptive behavior using a rotarod apparatus. Pre-treatment with HEE, EAF and HMF at the highest concentration used in the present study (400 mg/kg) or vehicle did not induce alterations in motor performance, since the animals remained on the rotating rod for 180 s at intervals of 30, 60 and 120 min after the dose administration. As expected, diazepam (1.5 mg/kg), a CNS depressant, reduced the residence time of the animal on the rotary shaft at all tested times.

DISCUSSION

The phytochemical prospecting (Table 1) confirms results previously seen in other studies with *B. virgilioides* (Smiderle and Sousa, 2003; Almeida et al., 2006). The presence of tannins, terpenoids, alkaloids, flavonoids and glycosides has been reported by Smith et al. (2007), with triterpenes and isoflavones being isolated in their study. Condensed tannins were also isolated in the aqueous and hydroethanolic extracts of *B. virgilioides* bark (Trugilho et al., 1997). Leite et al. (2014) isolated gallic, chlorogenic and caffeic acids and flavonoids quercetin,
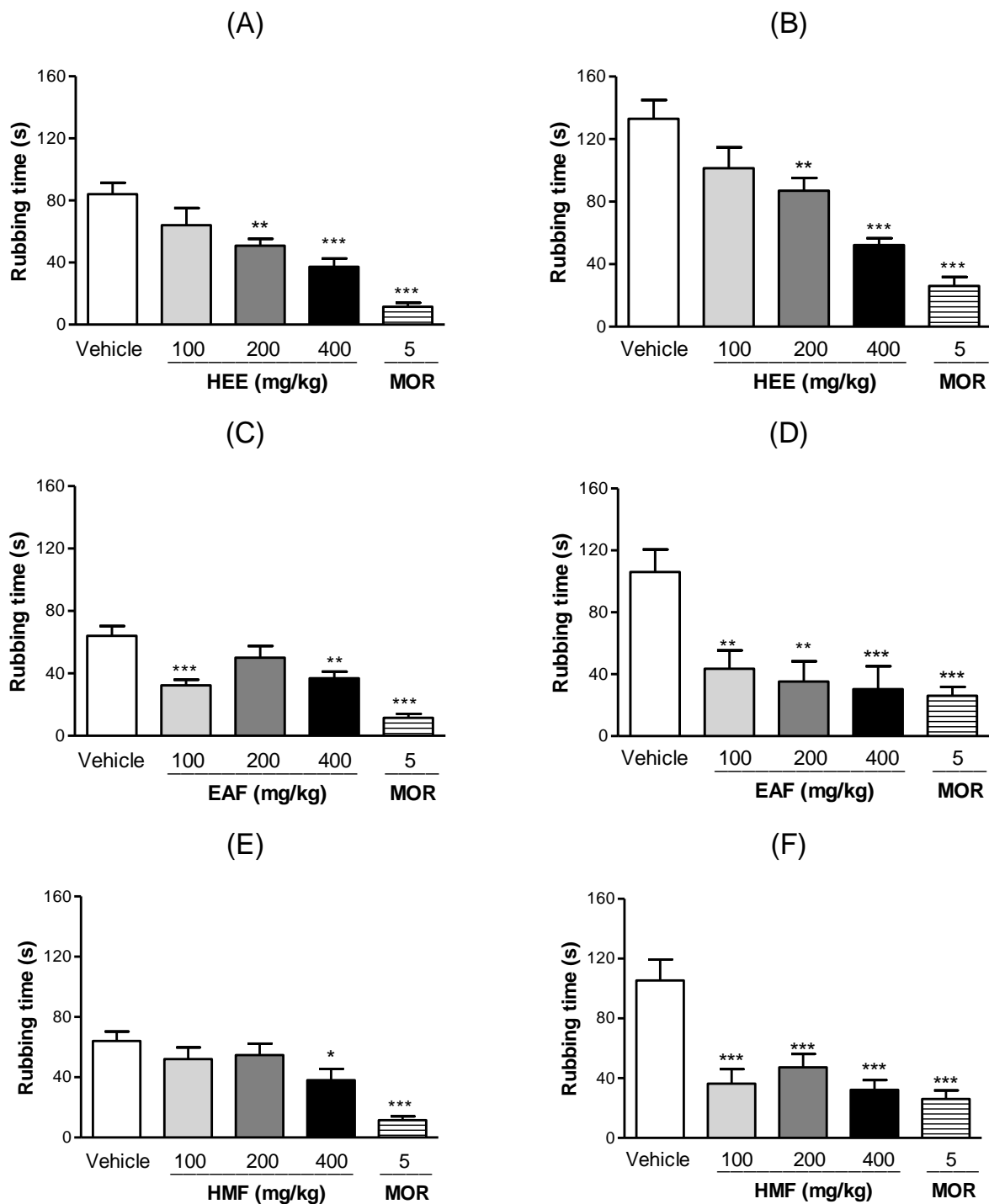
(A)

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(E)

(F)

Figure 1. Antinociceptive effect of the hydroethanolic extract (HEE, A and B), ethyl acetate (EAF, C and D) and hydromethanol fraction (E and F) of *B. virgilioides* in the orofacial model of pain induced by formalin. First (A, C and E) and second phase (B, D and F). Results are shown as mean ± SE (n= 6) of the time (s) that mice spent rubbing the orofacial region treated with formalin. Statistical differences were determined by ANOVA followed by Dunnett's post hoc test. (*) Denotes significant differences compared to the vehicle control group. **$p<0.01$, ***$p<0.001$.

rutin and kaemferol of extracts from the stem and heartwood of the *B. virgilioides*, while b-elemene (6.9%), β-caryophyllene (44.1%), germacrene D (7.9%), bicyclogermacrene (6.4%) and caryophyllene oxide (8.9%) were found as the main components of the plant seed (Rodrigues et al., 2009). All of these compounds
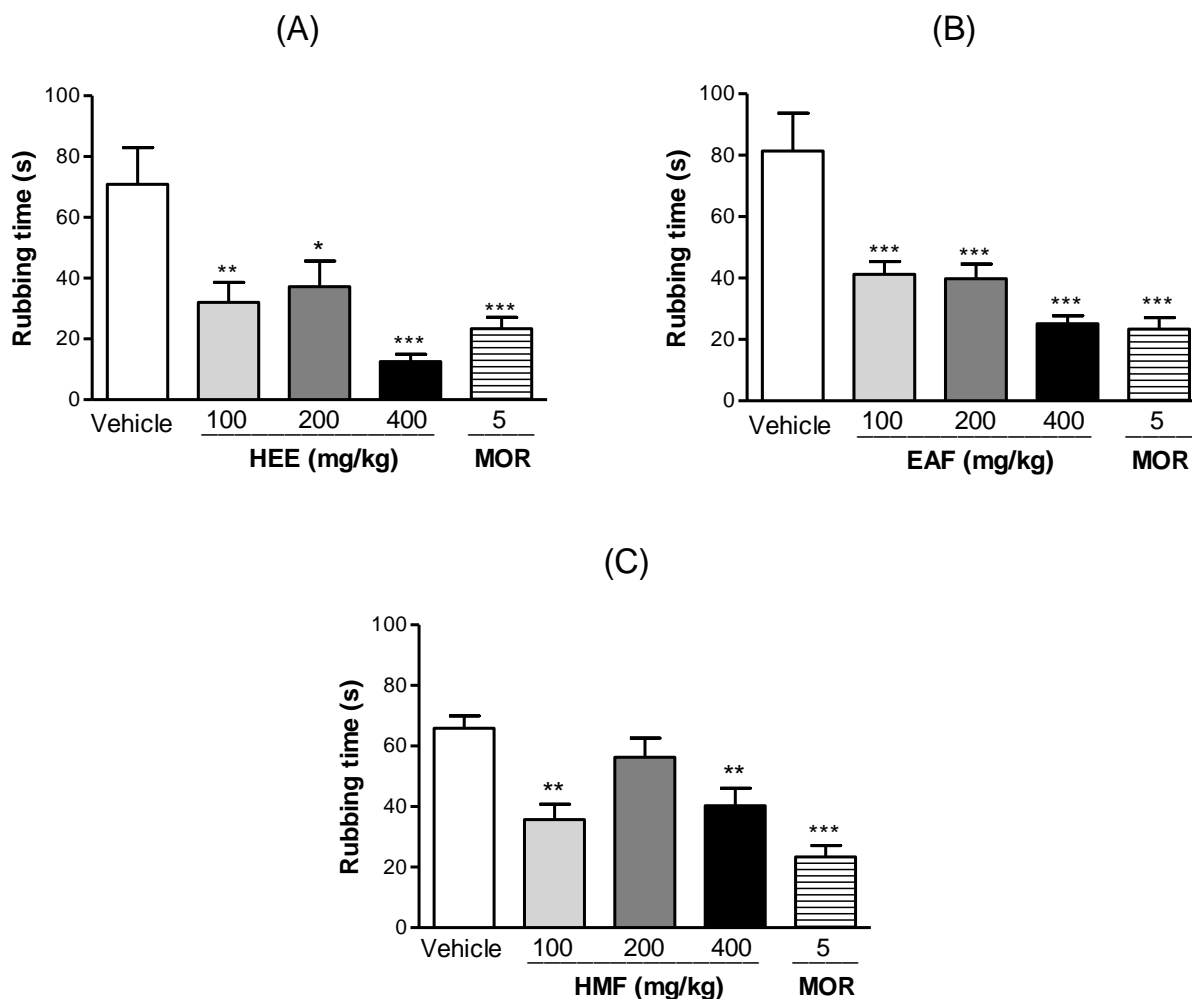
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Figure 2. Antinociceptive effect of hydroethanolic extract (HEE, A), and ethyl acetate (EAF, B) and hydromethanol (HMF, C) fractions of *B. virgilioides* against the orofacial pain induced by glutamate. Results are shown as mean ± SE (n= 6) of the time (sec) that mice spent rubbing the orofacial region treated with glutamate. Statistical differences were detected by ANOVA followed by Dunnett's post hoc test. (*) Denotes significant differences compared to the vehicle control group. *p<0.05, **p<0.01, **vfgw*p<0.001.

were linked to the plant biological activities.

In the present study, most of these compounds found in the literature as present in *B. virgilioides* were detected in HEE and EAF, suggesting the extract and its ethyl acetate fraction are the most biologically active samples of the plant. It should be noted that saponins were detected in EAF, but not in HEE. This may be linked to the interactions of the different secondary metabolites present in the extract. Compounds may be masked by others due to coupling, polymerization, glycosides formation and simple competition for reagents (Ainsworth and Gillespie, 2007). However, these interactions can be broken due to the fractioning of the original extract in solvents with different polarities so substances that were not present in the extract are found in the fractions (Muller et al., 2006; Dias et al., 2012).

To further analyze the biological potential of the plant

extract and fractions, the total phenol content was also evaluated. Phenolic compound amount in extracts or fractions is frenquently linked to the biological properties besides the classes of secondary metabolites found in them. Phenolic compounds such as phenols, flavonoids and tannins are characterized by having one or more hydroxyl groups attached to an aromatic ring, which will be used to stabilize free radicals. Therefore, the higher the amount of phenolic compounds in an extract or fraction, the higher the amount of hydroxyls groups available for their antioxidant activity (Angelo and George, 2007; Simões et al., 2010), which is attributed to electron donation from hydroxyl to free radical (Sousa et al., 2007). In the present study, EAF showed a total phenol content higher than HEE, from which it was obtained (Table 2). As previously said, this can be explained by synergistic and antagonistic interactions in
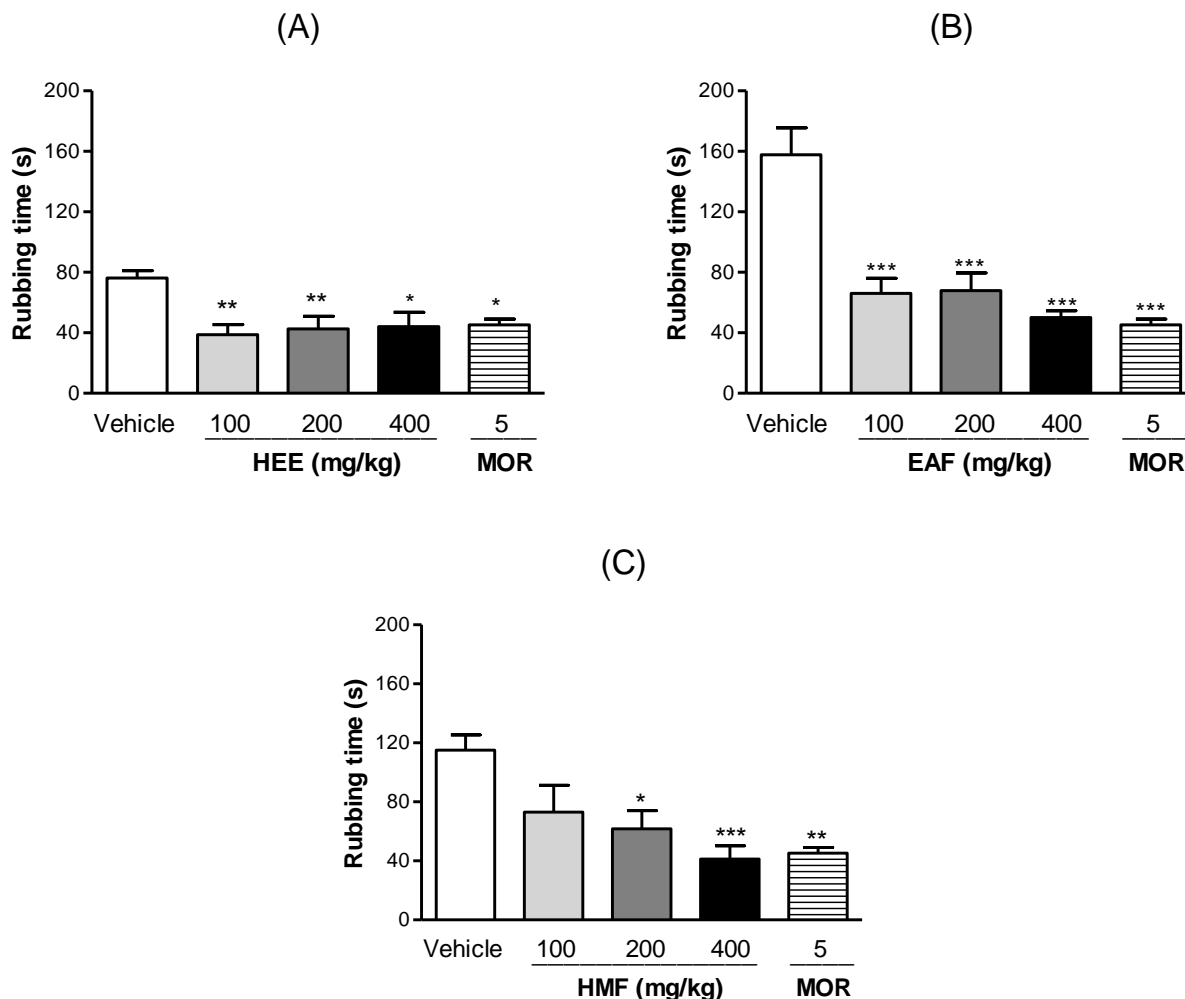
(A)

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Figure 3. Antinociceptive effect of hydroethanolic extract (HEE, A), and ethyl acetate (EAF, B) and hydromethanol (HMF, C) fractions of *B. virgilioides* against the orofacial pain induced by capsaicin. Results are shown as mean ± SE (n= 6) of the time (sec) that mice spent rubbing the orofacial region treated with capsaicin. Statistical differences were detected by ANOVA followed by Dunnett's post hoc test. (*) Denotes significant differences compared to the vehicle control group. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

HEE and their competition for the Folin-Ciocalteau reagent (Ainsworth and Gillespie, 2007). As of general, the order for TPC was EAF > CLF > HMF > HEE > HXF.

Considering that ROS are involved in pain propagation (Viggiano et al., 2005), the antioxidant potential of HEE and its fractions was evaluated using the DPPH• free radical method (Pérez-Jiménez et al., 2008; Scherer and Godoy, 2009). This method is used worldwide because the radical is stable and its reaction with the antioxidant can be easily followed using a UV/VIS spectrophotometer as the radical changes its color from purple to yellow due to its neutralization by the donation of electrons from the antioxidant to its structure (Scherer and Godoy, 2009). In the present study, HEE and its two polar fractions, EAF and HMF, were able to remove 42 to 79% of the DPPH at 40 µmol/L (Table 3). As of general, the order for the DPPH scavenging potential was EAF > HMF > HEE >

CLF > HXF. Regarding HEE and its polar fractions, there is a correlation between TPC and DPPH removal: EAF > HMF > HEE. The correlation between a higher TPC and DPPH free radical removal was previously reported in other studies involving medicinal plants (Djeridaneet al., 2006; Li et al., 2008; Melo et al., 2010; Vaher et al., 2010; Moura et al., 2011; Silva et al., 2011).

As previously stated, other studies have reported the antinociceptive activity of *B. virgilioides*. Silva et al. (2010) and Thomazzi et al. (2010) described the peripheral antinociceptive effect of the aqueous extract of the inner bark of *B. virgilioides* using the writhing, hot-plate and formalin tests, while the anti-inflammatory activity was evidenced by the paw oedema and peritonitis methods assays. Thus, considering those results and the findings regarding phenol content and *in vitro* antioxidant activity against DPPH in the present study, as well as the

use of the plant in the folk medicine to threat pain, the effect of the plant ethanol extract and fractions with the higher antioxidant activity (HEE, EAF and HMF) was also investigated against orofacial pain.

HEE, EAF and HMF showed antinociceptive responses in the inflammatory phase (second phase) of the formalin test (Figure 1B, 1D and 1F), confirming the studies of Silva et al. (2010) and Thomazziet al. (2010) with the plant aqueous extract. However, they also caused antinociceptive responses in the neurogenic phase (first phase) of the same test (Figure 1A, 1C and 1E). These results indicate that *B. virgilioides* HEE, EAF and HMF have central and peripheral actions regarding the orofacial pain that may be associated with opioid receptors, which are the same used by morphine (Roseland et al., 1990; Stein et al. 2001). In their studies, Silva et al. (2010) and Thomazzi et al. (2010) did not found any central effect of the aqueous extract from *B. virgilioides* bark. This difference in results may be explained by the different extraction methods of the same part of plant, since more substances can be extract with ethanol, which is an organic solvent, than with water, a polar solvent that can only extract polar water soluble substances. In addition, although HMF was active in the first phase of the formalin test, this was only observed at 400 mg/kg. This may be explained by the observation of the chemical composition of HEE, EAF and HMF. The phytochemical screening showed that HEE and EAF have a similar pattern of secondary metabolites, differing only in their triterpenes, steroids and alkaloid content, while HMF lacks xanthones and saponins besides triterpenes, steroids and alkaloids, which can explain its lower antinociceptive activity (Ayres et al., 2009).

When the orofacial antinociceptive properties were further evaluated by the glutamate test, HEE, EAF and HMF showed the highest nociceptive effect at 400 mg/kg, with HEE reducing up to 82% in this concentration (Figure 2). Results were similar or even higher than morphine, suggesting there is antagonistic activity in the glutamatergic system, which is basically characterized by the action on ionotropic NMDA (N-methyl-D-aspartate) and non-NMDA receptors located in peripheral, spinal and supra spinal structures. Glutamate is involved in the nociceptive transmission in the spinal cord and trigeminal subnucleus caudalis through primary afferent fibers that are excited by the release of nitric acid, inflammatory cytokines and other pain mediators (Bonjardim et al., 2011). Endogenous glutamate released peripherically activates NMDA receptors to stimulate the axon to release more glutamate in the periphery, creating a vicious circle that culminates in a central sensitization characterized by chronic pain in the orofacial pain. Glutamatergic antagonist prevents activation of these peripheral receptors as well as the consequent central sensitization. Chen et al. (2010) and Cardoso et al. (2006) found that ROS interfere with glutamatergic transmission, possibly by helping the release and/or

uptake of glutamate as well as the activation of its receptors. The antinociceptive activity observed for HEE, EAF and HMF may also be related to the antioxidant activity of the phenolic compounds in the extract and its fractions, which may be inhibiting ROS action in the signaling pathway in the glutamatergic system besides their direct action in the nociceptive fibers and receptors, corroborating the studies of Meotti (2006), Valerio et al. (2009) and Guimarães et al. (2012).

Capsaicin activates nociceptors by direct stimulation of C fibers through their vanilloid receptor (TRPV1), which causes and influx of positive ions into neurones that depolarize with consequent release of substance P, glutamate, nitric oxide and pro-inflammatory mediators (Pelissier et al., 2002). HEE, EAF and HMF at 400 mg/kg (Figure 3) reduced the time mice remained rubbing the area where capsaicin was administered similarly to morphine, suggesting the antinociceptive activity of *B. virgilioides* extract and fractions is also associated with the inhibition of capsaicin action on TRPV1 or mediator production. As for glutamate, the observed effect can also be mediated by their antioxidant properties because the pro-inflammatory signaling of the TRPV1 receptor is also ROS-dependent (Akada et al. 2006).

Due to the concern for human motor impairment associated with drug administration, the Rota rod test was used to evaluate the depressive and muscle relaxant effect of HEE, EAF and HMF in mice. Results showed *B. virgilioides* extract and fractions did not interfere with the motor coordination of the experimental animals, which significantly differed from diazepam.

It should be noted that it is well known that flavonoids (phenolic compounds) are effective for treating pain because they inhibit enzymes related to pain mediator production such as phospholipase A2, COX, LOX and nitric oxide synthase. In this context, the production of arachidonic acid, prostaglandins, leukotrienes and nitric oxide, important mediators of inflammation mechanism, is minimized (Havsteen, 2002; Rathee et al., 2009; Valério et al., 2009; Miyashiro et al., 2010). However, the action of other metabolite secondary found in the present study cannot be discarded because the literature also report that alkaloids (Bonjardim et al., 2011) and terpenes (Guimarães et al., 2010) have antinociceptive effect.

Conclusion

The potential of *B. virgilioides* to treat orofacial pain in its central and peripheral components was confirmed. HEE, EAF and HMF (mainly at 200 and 400 mg/kg) showed antinociceptive effect in three different orofacial pain models, formalin, capsaicin and glutamate, suggesting the active metabolites extracted from the inner bark of *B. virgilioides* produce a peripheral and central anti-nociceptive response related to opioid, glutamatergic and vanilloid receptors. In addition, it is also possible that

their antioxidant activity may be related to the observed antinociceptive effect by reducing the biosynthesis of ROS and other inflammatory mediators.

Conflicts of interest

ACKNOWLEDGEMENTS

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Full Length Research Paper

Phytochemistry and antibacterial activity of extracts from medicinal plant *Olea africana*

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Increase in prevalence of resistant microorganisms especially to synthetic drugs, has necessitated the need to search for new bioactive compounds having natural origin. Phytochemical investigation of *Olea africana* extracts afforded two triterpenoids namely erythrodiol and uvaol which were obtained through repeated column chromatography. The compounds were characterized using Nuclear Magnetic Resonance (NMR) spectroscopy and by comparison with literature values. The isolated triterpenoids exhibited moderate antibacterial activity whereas crude extracts exhibited relatively high antibacterial activity against Gram positive bacterial strains; methanol showed 12.4 mm zone of inhibition against *Staphylococcus aureus,* Erythrodiol exhibited higher antibacterial activity than uvaol against Gram positive bacteria *S. aureus* with zones of inhibition of 5.2 mm and 5.0 mm respectively. None of the pure compounds showed significant activity against Gram negative bacteria *Escherichia coli.* The results give a scientific validity and credence to the ethno-medicinal use of this medicinal plant as a chewing stick.

Key words: *Olea africana*, triterpenoids, antibacterial activity.

INTRODUCTION

Medicinal plants have been identified and used throughout human history because they have the ability to synthesize a wide variety of chemical compounds (Bulunas et al., 2005). These compounds are used to perform important biological functions, and to defend the medicinal plants against attack from predators such as insects, fungus and herbivorous mammals (Fabricant et al., 2001). At least 12,000 such compounds have been isolated so far, a number estimated to be less than 10% of the total (Tapsell et al., 2006). According to WHO report, 80% of the world's population, mostly from developing countries still rely on traditional medicine for the treatment of common ailments (Shagal et al., 2012).

Oral diseases, like most diseases, are unevenly distributed, with the greatest burden falling on needy and poor populations (Ogunbodede et al.*,* 2015). Thus, lower socioeconomic status populations have higher rates of caries and periodontal diseases than their higher SES counterparts (Chidzonga et al.*,* 2015). Elsewhere, there are also disparities in oral health between those from rural and urban areas of Africa (Ogunbodede et al.*,* 2015). According to World Health Organization (2008),

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the main determinants of health in general are the social, economic, and environmental conditions. These factors influence the oral health of individuals and populations. Therefore proper oral hygiene for all implies use of cheaper and readily available alternatives.

Dental carries and periodontal diseases are among the most pursued global oral health problems. They are caused by plaque forming bacteria such as *Myces, Actinobacillus, Streptococcus* and *Candida* species which reside in the oral cavity (More et al*.,* 2008). These bacteria have been reported to be unsusceptibility to antimicrobial agents compared with cultures grown in suspension (Sonkos and Gondson, 2011). It is therefore not surprising that bacteria growing in dental plaque, a natural occurring biofilm show increased resistance to antimicrobial agents.

They generate acids that eat away at a tooth or form dental plaque or biofilm. Due to the structure of these biofilms, physical removal is the most effective means of control. Regular and thorough removal of food deposits from teeth also plays a vital role in reducing dental and periodontal diseases especially if accompanied by the use of chewing sticks.

Chemical investigations on chewing sticks and other medicinal plants reported to have medicinal properties has also generated numerous purified compounds which have proven to be essential in the practice of modern medicine (Ncube et al*.,* 2012). The use of teeth cleaning agents like tooth brush and tooth pastes or chewing sticks varies from country to country, from urban to rural areas and culture to culture. Its use has been documented in parts of Asia, Africa, the Middle East and South America (Verenne et al*.,* 2006). Chewing stick has remained a common and acceptable teeth cleaning agent in different parts of the world especially in developing countries despite the widespread use of tooth brushes and tooth pastes (Al-Otaibi et al*.,* 2004). Its regular use is based on factors such as availability, cost, therapeutic and social-cultural reasons.

Religious reasons have also contributed to the increasing popularity in the use of this form of teeth cleaning. It is often mentioned that the Islamic Prophet Mohammed recommended its use as he is quoted in various Hadith advising the use of siwak (*Salvadora persica*) (Almas, 2001; Hyson, 2003). Olive plant (*Olea europea*), Lime tree (*Citrus aurantafolia*), Neem (*Azadirachta indica*) and Orange tree (*Citrus sineusia*) are also used as a chewing stick.

In response to emerging trends of bacterial resistance to antibiotics there is continuous and urgent need to discover new antimicrobial compounds from natural products like chewing sticks with diverse chemical structures and novel mechanisms of the new emerging oral infectious diseases. The current research focused on the medicinal plant *Olea africana* that is used traditionally by local inhabitants of Kabianga in Kericho, Kenya, as a chewing stick, so as to isolate and test the antibacterial activity of the compounds extracted.

MATERIALS AND METHODS

General experimental procedure

The samples were prepared for analysis by dissolving in 2 mL DCM in 5 mm NMR tubes. NMR spectra was recorded at room temperature on a 400 MHz variant UNITY INOVA spectrometer. ^{1}H NMR spectra was referenced against the CHCl$_3$ signal at δ$_H$ 7.24 and ^{13}C NMR spectra against the corresponding signal at δ$_C$ 77.0. Coupling constants were given in Hz. solvent against an air background.

Plant material

The leaves and twigs of *O. africana* were collected in January, 2015 from University of Kabianga Botanical garden, Kericho County in Kenya. The plant was identified by a taxonomist, Mr. D. Maritim, University of Kabianga, Kenya and a voucher specimen (kemboi 01) was deposited in the Herbarium, University of Kabianga, Kericho County, Kenya.

Extraction and isolation

The air dried and ground plant materials of *O. africana* (1000 g leaves and 1000 g twigs) was sequentially extracted with organic solvents in the order of increasing polarities including; hexane, dichloromethane, ethyl acetate and methanol, using cold extraction for 24 h in each case. The extracts were then concentrated under a reduced pressure to a minimum volume using a Rotavapor. The yields obtained were hexane 30.0 g (leaves), 23.0 g (twigs); dichloromethane 24.1 g (leaves), 15.3 g (twigs); ethyl acetate 6.24 g (leaves), 5.22 g (twigs) and methanol 5.35 g (leaves), 4.35 g (twigs).

Phytochemical analysis

Qualitative tests for terpenoids, tannins, flavanoids, flavones, carotenoids and alkaloids was carried out by standard method as described by Edeoga et al. (2005). The tests were based on the visual observations of color change or formation of a precipitate after addition of specific reagent.

Isolation and purification of compounds 1 and 2

The DCM extracts of the leaves was separated by column chromatography using a step gradient of hexane: dichloromethane: ethyl acetate, starting with 100 % hexane stepped to 10, 20, 30, 50, 80 and 100% dichloromethane, followed by 20 and 30% ethyl acetate in dichloromethane. Twenty fractions of 100 mL each were collected in each step. Fractions 1 to 19 were combined and purified using 80% dichloromethane to produce erythrodiol (18 mg), fractions 34 to 36 were combined and separated with 20 and 30% ethyl acetate in DCM to afford Uvaol (12 mg).

Erythrodiol (1)

White amorphous solid, The ^{1}H-NMR spectral data δ$_H$ 5.17 (H-12), δ$_H$ 3.54 (H-3), δ$_H$ 3.20 (H-28), δ$_H$ 1.22, 1.13, 0.96, 0.91, 0.89, 0.85, 0.76 (3H each). The ^{13}C-NMR spectral data; δ$_C$ 144.2 (C-13), δ$_C$ 122.4 (C-12), δ$_C$ 79.02 (C-3) and δ$_C$ 69.65 (C-28).
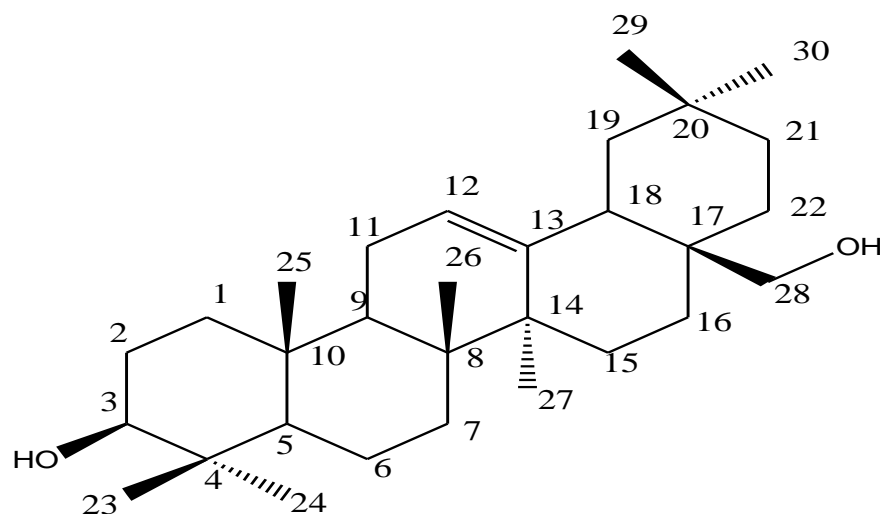
Uvaol (2)

White amorphous solid, The ^{1}H-NMR spectral data δ$_H$ 5.17 (H-12),

Table 2. ^{13}C data of compounds 1 and 2.

Carbon position	Compound 1 (ppm)	Compound 2 (ppm)
1	38.8	39.3
2	27.2	27.2
3	79.0	79.0
4	38.8	38.5
5	55.2	54.0
6	18.3	17.3
7	32.8	32.5
8	39.8	40.0
9	47.6	47.5
10	36.9	36.9
11	23.6	23.4
12	122.3	125.0
13	144.2	138.7
14	41.7	42.0
15	25.9	25.5
16	22.0	23.3
17	36.9	35.2
18	42.3	54.0
19	46.5	39.7
20	31.0	39.4
21	34.1	29.7
22	30.9	35.2
23	28.1	28.1
24	16.7	16.7
25	16.7	16.7
26	16.8	16.8
27	25.9	23.3
28	69.9	69.7
29	33.2	17.3
30	23.6	21.3

by correlation. Single olefinic proton signal (a triplet) was observed at $δ_H$ 5.17 and was assigned to H-12, it also revealed two oxygenated protons resonating at $δ_H$ 3.54 and $δ_H$ 3.20. They were assigned to H-3 and H-28 respectively indicating the presence of hydroxyl moiety. Also present were seven methyl peaks at $δ_H$ 1.22, 1.13, 0.96, 0.91, 0.89, 0.85 and 0.76 each 3H. The ^{13}C-NMR spectra of compound exhibited the presence of 30 carbon signals (Table 2). The DEPT spectrum shows 7 methyl carbons, 11 Methylene, 5 Methine and 7 Quarternary carbon. The Sp^2 signals at $δ_C$ 144.2 and 122.4 were attributed to C-13 and C-12, respectively; characteristic of oleanane triterpenoids. Also present were two oxygenated carbon signals at $δ_C$ 79.02 (C-3) and $δ_C$ 69.65 (C-28) which correlated to the signals at $δ_H$ 3.54 and $δ_H$ 3.20 in the HSQC spectrum. The HMBC spectrum showed correlation of H-23 and H-24 with C-3, H-23 and H-24 with C-5, H-25 with C-9, H-29 with C-19, H-27 with C-8, C-14, H-11 with C-13, H- 18 with C-12 and C-15.The NOESY spectrum exhibited correlations of H-3 and H-28,

indicating that they are both in the alpha position. Based on these spectral data, the compound was identified as a triterpenoid. The structure was proposed by comparison of data with those found in literature and was identified as the known erythrodiol (Marizeth et al., 2002). Erythrodiol has also been isolated from *O. europea* (Hansen et al., 2006).

Compound 2 (Figure 2) was a white amorphous powder from dichlomethane extracts. The ^1H NMR spectrum showed the presence of a single olefinic proton signal (a triplet) at $δ_H$ 5.11 and was assigned to H-12. This proton correlated to methine carbon signal at $δ_C$ 122.3 in the HSQC spectra The ^1H NMR also revealed two oxygenated protons resonating at $δ_H$ 3.50 and $δ_H$ 3.14. They were assigned to H-3 and H-28 respectively indicating the presence of 3β-hydroxyl moiety. Methyl peaks were at $δ_H$ 1.35, 1.34, 1.34, 1.32, 1.31, 1.31 and 1.07 each 3H. The ^{13}C-NMR spectrum of compound 2 (Table 2) exhibited the presence of 30 carbon signals, the DEPT spectrum showed the presence of 6 methyl
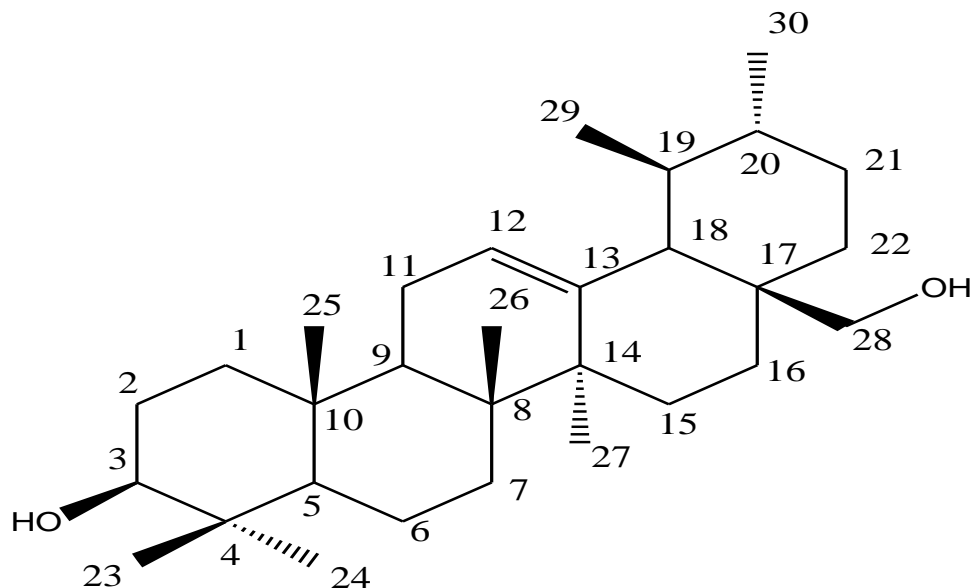
Scheme 2. Uvaol.

Table 3. The minimum inhibition concentration (mgmL^{-1}) of Methanol, Hexane, DCM and ethyl acetate against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*.

Extract	*E. coli*	*S. aureus*	*B. subtilis*
Methanol	1.50	0.30	1.00
Hexane	2.30	1.00	1.00
DCM	2.30	0.50	0.30
Ethyl acetate	2.30	1.00	-

carbons, 7 methine, 10 methylene and 7 quartenary carbon. The *sp^2* signals at δ$_C$ 138.7 and δ$_C$ 122.3 in the ^{13}C NMR spectrum were attributed to C-13 and C-12 respectively, characteristic of ursane triterpenoids. Also present in the ^{13}C NMR spectrum were two oxygenated carbon signals at δ$_C$ 79.0 (C-3) and 69.7 (C-28). The HMBC spectrum showed correlation of H-18 and H-21 with C-29, H-30 and H-11 with C-9, H-9 with C-11. The position of the double bond at C-12, 13 was also evident from the HMBC correlations of olefinic proton with carbons at δ$_c$ 138.87 (C-13), δ$_c$ 125.30 (C-18). The NOESY spectrum exhibited correlations of H-3 and H-28. HSQC spectrum showed correlations between C-5 and H-5, C-9 and H-9, C-16 and H-16. Based on spectral data and literature values it was identified as the known uvaol (2) (Marizeth et al*., 2002).

The MIC values (Table 3) recorded for the crude extracts and pure compounds suggested moderate antibacterial activity. The most active extracts were methanol with MIC value of 0.3 mg/mL against *S. aureus* and 1.0 mg/mL against *B. subtilis.* The least active extracts were from hexane with MIC values of 1.0 mg/ml

against *S. aureus* and *B. subtilis.* This could be because plant-based constituents have also been reported to exhibit different modes of action against bacterial strains which range from interference with the phospholipoidal cell membranes, this has a consequence of increasing the permeability profile and loss of cellular constituents, damage of the enzymes involved in the production of cellular energy and synthesis of structural components and destruction or inactivation of genetic material (Kotzekidou et al*., 2008). The study revealed that none of the pure compounds showed significant activity against Gram negative bacteria *E. coli* (Table 4). This could be due to the fact that *E. coli* is reported to be multiresistant to antibiotics (Cos et al*., 2006). They have frequently been reported to have developed multi-drug resistance to many of the antibiotics currently available in the market. It is therefore not surprising to learn that *E coli* was the least responding bacteria strain to the tested plant extracts. Previous study using hot water extracts of *O. europea* leaves at concentration of 62.5 mg/mL were also found to be inactive against *S. aureus* and *E. coli* (Hansen et al*., 2006). Erythrodiol (1) (Scheme 1)

Table 4. Antibacterial activity of hexane, DCM and ethyl acetate pure compounds.

Compound	Concentration	Zones of inhibition (mm) against			
		E.c	**P.a**	**S.a**	**B.s**
1	10.00	-	5.20	5.20	5.10
	5.00	-	5.00	5.00	5.00
	2.50	-	-	-	-
2	10.00	-	5.30	5.20	5.00
	5.00	-	5.20	5.10	5.20
	2.50	-	-	-	-

E.c: *Escherichia coli*; **P.a**: *Pseudomonas aeruginosa*; **S.a**: *Staphylococcus aureus*; **B.s**: *Bacillus subtilis*. -: no activity: **Conc**; Concentration in mgmL^{-1}.

exhibited the higher antibacterial activity than uvaol (2) (Scheme 2) against Gram positive bacteria *S. aureus.* The results are comparable to findings by Waffo et al*. (*2000) on *O. europea,* a species from the same genus. Higher activity was exhibited by Gram positive bacteria than Gram negative bacteria. The difference in the sensitivity between them can be associated to the variance in morphological constitutes between these microorganisms. Gram negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide components, this makes the cell wall impermeable to antimicrobial agents. The Gram positive bacteria on the other hand are more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier. Therefore, the cell wall of Gram negative organisms which are more complex than the Gram positive organisms act as a diffusional barrier and making them less susceptible to the antimicrobial agents than Gram positive (Nostro et al*.,* 2000). Erythrodiol and uvaol have also been reported to possess unique biological properties such as antibacterial and wound healing effects. This may support the traditional use of this plant in healing open wounds (Marizeth et al*.,* 2002).

Conclusion

O. africana is used in Kenyan traditional medicine as a chewing stick and for treatment of other periodontal diseases. Previous phytochemical studies have found presence of alkaloids, flavanoids, carotenoids and terpenoids among others in *O. europea*. Our study has revealed that the leaves crude extracts contains terpenoids, tannins, flavones and flavanoids while carotenoids were absent. Stem extracts tested positive for terpenoids, tannins, flavones and flavanoids while alkaloids tested negative. Purification of the leaves extracts afforded two triterpenoids, identified as erythrodiol and uvaol which belongs to oleanane and ursane classes respectively. They exhibited relatively good antibacterial activity against the bacterial strains used in this study. The triterpenoids have been reported to be used as antibacterial, antifungal, anti-inflammatory, anti-leishmanial and antimalarial. The plant could therefore be used in traditional medicine to treat the symptoms of inflammation and infections by bacteria and as a chewing stick upon further investigation.

Conflict of interest

The authors have not declared any conflict of interest

Abbreviations: ATCC, American type culture collection; **BCC**, Belgian coordinated collection microorganisms; **COSY,** correlation spectroscopy; **DCM,** dichloromethane; **HMBC,** heteronuclear multiple-bond correlation spectroscopy; **HSQC**, Heteronuclear single quantum correlation spectroscopy; **MH**, Mueller Hinton; **NMR,** nuclear magnetic resonance; **NOESY**, nuclear overhauser effect spectroscopy; **TLC**, thin layer chromatograph; **WHO,** World Health Organization.

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