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Benzoxazine derivatives of phytophenols show anti-plasmodial activity via sodium homeostasis disruption

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ABSTRACT

Development of new class of anti-malarial drugs is an essential requirement for the elimination of malaria. Bioactive components present in medicinal plants and their chemically modified derivatives could be a way forward towards the discovery of effective anti-malarial drugs. Herein, we describe a new class of compounds, 1,3-benzoxazine derivatives of pharmacologically active phytophenols eugenol (compound **3**) and isoeugenol (compound **4**) synthesised on the principles of green chemistry, as anti-malarials. Compound **4**, showed highest anti-malarial activity with no cytotoxicity towards mammalian cells. Compound **4** induced alterations in the intracellular Na^+ levels and mitochondrial depolarisation in intraerythrocytic *Plasmodium falciparum* leading to cell death. Knowing P-type cation ATPase PfATP4 is a regulator for sodium homeostasis, binding of compound **3**, compound **4** and eugenol to PfATP4 was analysed by molecular docking studies. Compounds showed binding to the catalytic pocket of PfATP4, however compound **4** showed stronger binding due to the presence of propylene functionality, which corroborates its higher anti-malarial activity. Furthermore, anti-malarial half maximal effective concentration of compound **4** was reduced to 490 nM from 17.54 μM with nanomaterial graphene oxide. Altogether, this study presents anti-plasmodial potential of benzoxazine derivatives of phytophenols and establishes disruption of parasite sodium homeostasis as their mechanism of action.

Malaria still remains a major health challenge affecting millions of people worldwide. Prevalence of drug resistance in malaria parasites towards the existing chemotherapy has concrete propositions towards the development of new class of anti-malarial compounds for the eradication of this disease.[1–3]

In Ayurveda, the medicinal plants and their bioactive natural components have been used to cure various diseases including malaria.[4–6] Traditionally, ancient herbal remedies used for the treatment of malaria are of plant-origin such as artemisinin and quinine.[7] A major phenolic phytochemical present in clove oil (45–90%), eugenol, is well-known for its bioactive properties. It is an essential component of several medicinal plants, namely *Eugenia caryophyllata* (clove), *Ocimum gratissimum* (african basil), *Cinnamomum verum* (cinnamon), *Ocimum tenuiflorum* (holy basil), *Myristica fragrans* (nutmeg) and can be easily extracted.[8–10] Even today, it is widely used in dentistry as an antiseptic, and as a flavouring agent in food products and cosmetics.[11] In addition, eugenol also exhibits analgesic, antibacterial, antiviral, antileishmanial, anti-inflammatory, and anti-cancer properties. Eugenol is approved as a anti-mutagenic and non-carcinogenic chemical by the US Food and Drug Administration (FDA).[12–18] The structural isomer of eugenol i.e., isoeugenol also belongs to the phenylpropanoids class of chemical compounds. They are also produced by plants as defense compounds against animals and microorganisms and as floral attractants to pollinators.[19]

We have synthesised 1,3-benzoxazine derivatives of the phytophenols, based on the previous reports advocating the antimalarial, antimicrobial, antifungal, antibacterial impact of amino allylphenol derivatives and benzoxazine derivatives. It is reported that benzoxazine derivatives specifically at 1,3-position exhibit potential antimicrobial, antifungal, antiproliferative and antibacterial activities.[20–26] Earlier, amino allylphenol derivative (SN6771)

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showed a good anti-malarial activity which possess the intramolecular H-bonding between amino and phenolic group (O and N). Accordingly, we have also synthesised 1,3-benzoxazine derivatives of eugenol and isoeugenol with the green chemistry approach where H-bonding is replaced by a methylene linkage (oxazine ring) between the heteroatoms (O and N). This synthesis demonstrates the greener aspect due to the facts: (i) raw materials namely, eugenol, isoeugenol and furfurylamine are bio-sourced from clove oil and corncobs respectively; (ii) solventless synthesis; (iii) one-pot one-step synthesis; and (iv) by-product of the reaction is only water.

In the present work, we describe the synthesis of new class of compounds based on phytophenols, eugenol and isoeugenol and their 1,3-benzoxazine derivatives. Compounds were analyzed for their activity against intraerythrocytic *Plasmodium falciparum*. This contributes to the development of novel class of anti-malarials derived from natural products. Further investigations revealed disruption of sodium homeostasis in intraerythrocytic parasite upon treatment with the lead compounds. This is further supported by *in silico* binding of compounds to the druggable parasite target P-type cation ATPase i.e. PfATP4, a key transporter responsible for maintenance of intraerythrocytic parasite sodium homeostasis.

Till date, there is no report on utility of either isoeugenol and eugenol or their benzoxazines derivatives for anti-malarial activity in literature. The main focus of our study is to highlight the effect of substituents namely phenolic-OH and position of the double bond on the propylene chain attached to benzene ring in phytophenolic compound, eugenol **1** that may play an important role in the biological activity. Isoeugenol **2** is a structural isomer of **1** where the double bond is present in conjugation with the benzene ring in the former, while it is an isolated double bond in the latter (Fig. 1A). Isoeugenol **2** also occurs naturally and inter-conversion of **1** to **2** is well known in literature.[27,28] The benzoxazine derivatives of naturally occurring eugenol **1** and isoeugenol **2** were synthesised and the structures of the studied compounds are illustrated in Fig. 1A. The 1,3-benzoxazine derivatives of **1** and **2** were synthesised via Mannich-like condensation reaction with furfurylamine (**ffa**) and paraformaldehyde to form the corresponding benzoxazine derivatives **E-ffa** (compound **3**) and **I-ffa** (compound **4**) respectively. It must be noted that **ffa** is also derived from agricultural byproducts such as corn corbs and wheat bran. The structure of the synthesised compounds **3** and **4** was confirmed by FT-IR, ^{1}H NMR, ^{13}C NMR spectroscopy and mass spectrometry. The FT-IR spectrum (Fig. S1(A)) of compounds **3** and **4** showed the characteristic absorption peaks due to asymmetric C–O–C stretching of benzoxazine ring at 1225 and 1221 while symmetric C—O—C stretching observed at 1092 and 1093 cm^{-1} respectively. Fig. S1 (B)(i), (ii) shows the ^{1}H NMR spectra of compounds **3** and **4**. The characteristic NMR resonances attributed to the benzoxazine structure, Ar–CH_2–N– and –O–CH_2–N– for compounds **3** and **4** are observed at 3.98 (3.98) and 4.95 (4.97) ppm respectively. The benzoxazine structure formation in compounds **3** and **4** was further verified by ^{13}C NMR spectroscopy [Fig. S1(B)(iii), (iv)]. The appearance of resonances at 55.85 (55.84) and 82.37 (82.55) ppm due to Ar–CH_2–N– and –O–CH_2–N– respectively indicate condensation of phenolic-OH in **1** and **2** with **ffa** and paraformaldehyde to form benzoxazine structure. All compounds **1**–**4** appear as attractive candidates towards the medicinal chemistry studies due to the natural origin, non-toxicity, and have potential to degrade due to bio-origin accounting for minimal side effects. We systematically investigated whether the presence of phenolic-OH group is essential, and the effect of extensive conjugation in the structure has any effect on the initial studies towards drug's utility.

In vitro growth inhibitory activities of eugenol and isoeugenol derivatives against *Plasmodium falciparum* (*P. falciparum*) were explored. Eugenol **1**, isoeugenol **2** and their respective derivatives (**3**–**4**) were initially screened for their biological activity. Screening was done at 10 μM and 50 μM conc. each of compounds **1**–**4** (as described in supplementary information) for inhibition of intraerythrocytic growth of *P. falciparum* strain 3D7. Compounds were tested over one intraerythrocytic cycle of parasite growth where untreated parasites served as control. The percentage of growth inhibition was measured by flow cytometry. Out of four compounds, compounds **3** and **4** exhibited the best growth inhibition. Compound **3** showed inhibition of 4.7% and 58.5% at concentrations 10 μM and 50 μM, whereas compound **4** showed inhibition of 14.9 and 73% at concentrations 10 μM and 50 μM respectively [Fig. 1B(i)]. Comparatively, eugenol and isoeugenol showed negligible growth inhibition against *P. falciparum*. This lead supports that the modified eugenol and isoeugenol compounds are potent bioactive compounds and showed a better inhibitory effect on malaria parasite growth.

The most potent compounds **3** and **4** were further investigated for the determination of half maximal effective concentration (EC_{50}). To determine the EC_{50} value, 36–40 h of sorbitol synchronized *P. falciparum* strain 3D7 at a late trophozoite-stage were treated with the increasing concentrations (1, 10, 20, 30, 40, and 50 μM) of compounds **3** and **4**. A dose-response curve was plotted using Graph Pad Prism 6. Compounds **3** and **4** exhibited EC_{50} of 22 μM & 17.54 μM respectively in the dose-dependent manner [Fig. 1B(ii)]. The effect of compounds **1**–**4** was also checked for their effect on mammalian cell viability. Upon screening, compounds (**1**–**4**) showed no cytotoxicity towards Human liver hepatocellular carcinoma cells (HepG2 cells) as an effect of compounds at 50 μM of concentration for 24 h [Fig. 1C].

The regulation of low sodium [$Na^{\pm}$] levels is the fundamental characteristic of almost all types of cells. A low cytoplasmic [$Na^{\pm}$] is maintained in the human erythrocytes through the action of sodium transporters.[29] There occurs an alteration in the ionic homeostasis upon infection with *P. falciparum*. The intraerythrocytic malaria parasite is enclosed in the parasitophorous vacuole membrane, which, at mature trophozoite stage, is freely permeable to low molecular weight solutes and inorganic ions such as [$Na^{\pm}$] etc. *P. falciparum* P-type cation ATPase PfATP4 is reported previously to be involved in maintaining a low cytoplasmic [$Na^{\pm}$].[30,31] To confer [$Na^{\pm}$] status, the levels of intracellular [$Na^{\pm}$] were determined by using sodium green dye (as described in supplementary information). An increase in [$Na^{\pm}$] levels was observed in compound treated parasites as compared to solvent treated controls (Fig. 2A). Swelling of the intraerythrocytic parasites *P. falciparum* was observed after the treatment with compound **4** which might be a consequence of increase in intracellular sodium levels. To further ascertain this, live cell images of trophozoite stage parasites were taken after 4 h of treatment with compound **4** by Nikon eclipse Ti microscope (Fig. 2B). The average diameter of parasitised red blood cells (pRBCs) and parasites in the untreated control and compound **4** treated parasites were measured. The diameter of compound treated pRBCs was found to increase to 6.3 μM from 5.03 μM diameter of the untreated pRBCs. In addition, an increase in the diameter of parasites to 4.4 μM was observed in compound treated parasites as compared to 3.5 μM in case of untreated parasites. Diameter of around fifty parasites was measured using NIS element analysis software. Increase in the diameter is consistent with the swelling of parasite after the treatment with compound. The increase in [$Na^{\pm}$] levels was most significant in compound **4** treated parasites as to compound **3** treated parasites. It was reported earlier that increase in cytoplasmic [$Na^{\pm}$] levels due to the inhibition of PfATP4 results in osmotic imbalance leading to parasite death. This indicates that PfATP4 might be the possible target to above synthesised class of compounds.