*In vivo* Antimalarial Evaluation of Embelin and Some Semi-Synthetic Aromatic Amine Substituted Embelin Derivatives from the Fruit of *Embelia schimperia*

By

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A Thesis Submitted to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medicinal Chemistry.

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<th>Description</th>
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<tbody>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ITNs</td>
<td>Insecticide-treated Nets</td>
</tr>
<tr>
<td>ACTs</td>
<td>Artemisinin combination therapies</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>TS</td>
<td>thymidylate synthase</td>
</tr>
<tr>
<td>CQ</td>
<td>chloroquine</td>
</tr>
<tr>
<td>AQ</td>
<td>amodiaquine</td>
</tr>
<tr>
<td>Fe (II) PPIX</td>
<td>ferroheme ferrous-protoporphyrin</td>
</tr>
<tr>
<td>PPM</td>
<td>parasite plasma membrane</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylincholine</td>
</tr>
<tr>
<td>NPPs</td>
<td>new permeability pathways</td>
</tr>
<tr>
<td>PRQ</td>
<td>primaquine</td>
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<tr>
<td>MQ</td>
<td>mefloquine</td>
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<tr>
<td>PQ</td>
<td>piperaquine</td>
</tr>
<tr>
<td>LUM</td>
<td>lumefantrine</td>
</tr>
<tr>
<td>PYR</td>
<td>pyronaridine</td>
</tr>
<tr>
<td>EHNRI</td>
<td>Ethiopian Health and Nutritional Research Institute</td>
</tr>
<tr>
<td>IR</td>
<td>Infra-red</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>Tetramethylsilane</td>
</tr>
<tr>
<td>$^1$H NMR</td>
<td>Proton Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>MP</td>
<td>melting point</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>Mmol</td>
<td>Mill mole</td>
</tr>
<tr>
<td>Ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One Way Analysis of Variance</td>
</tr>
<tr>
<td>DEPT</td>
<td>Distortional Enhancement Polarization Transfer</td>
</tr>
<tr>
<td>$^{13}$C NMR</td>
<td>Carbon thirteen Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical package for social science</td>
</tr>
<tr>
<td>OECD</td>
<td>Organization for Economic Co-operation and Development</td>
</tr>
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</table>
Malaria is a major public health problem mainly due to the development of drug resistance by the most lethal causative parasitic species. New drugs with unique structures and mechanism of action are urgently required to treat sensitive and drug-resistant strains of malaria. Historically, compounds containing novel structure from natural origin represent a major source for the discovery and development of new drugs for several diseases. The present study aimed at isolating embelin from the fruit of *Embelia schimperia*, preparing some semi-synthesized compounds starting from embelin and evaluating of their *in vivo* antimalarial activities against mice infected with *Plasmodium berghei* using a 4-days suppressive test. Three novel aromatic substituted embelin derivatives were semi-synthesized in good yields (>98%) by using one-step condensation reaction. Structures for the synthesized compounds were determined using IR, $^1$H NMR, $^{13}$C NMR and MS. An attempt has been made in this study to provide some insight on recent semisynthetic approaches to antimalarial drug discovery from natural sources with some structural modification.

The target compounds, at a dose of 100-400 mg/kg, suppressed *P. berghei* parasites by 49-75% which was significantly different from the negative control group ($P<0.05$). The study further showed that 5-(o-toluidino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione(1A) possesses maximum antimalarial activity (75% suppression) at a dose of 400mg/kg. The synthesized compounds possess considerable antiplasmodial activities which justify the recent semisynthetic approaches to antimalarial drugs discovered from natural sources. In conclusion, some of the semi-synthesized compounds from embelin could be good candidates for treatment of malaria.

**Key words:** Antimalarial activities, aromatic substituted embelin, *Embelia schimperia*
1. Introduction

1.1 Background on malaria

1.1.1. Malaria

Malaria is a parasitic disease caused by *Plasmodium* species transmitted from the blood of an infected person and passed to a healthy human by a female Anopheles mosquito. It is a complex disease that varies widely in epidemiology and clinical manifestation in different parts of the world (Riehle *et al*., 2006). This variability is the result of factors such as the species of malaria parasites that occur in a given area. In particular, young children, pregnant women, and non-immune visitors to malarious areas are at greatest risk of severe or fatal illness. Many malaria control strategies exist, but none are appropriate and affordable in all contexts (Beroa *et al*., 2009).

In humans, malaria infection is caused by one or more of four species of intracellular protozoan parasite such as *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, and Plasmodium malariae* and transmitted by the female anopheles mosquitoes. These species differ in geographical distribution, microscopic appearance clinical features, ability to cause relapse and potential for development of resistance to antimalarial drugs (WHO 2010).

Malaria is a mosquito borne parasitic disease that is common in the world poorest countries. It is preventable and treatable, yet it still kills some 881,000 people every year, 90% of whom are in Africa and 85% of whom are children under five. Malaria was eliminated in most western countries more than 50 years ago; today, more than half of all estimated malaria cases occur in just five African countries: Nigeria, Democratic Republic of Congo, Ethiopia, United Republic of Tanzania and Kenya (WHO 2010).
Malaria predominantly affects rural and poor populations that have little or no access to current prevention and treatment tools. It is estimated that malaria costs Africa more than US$12 billion every year in lost Gross domestic product (GDP), due to the heavy toll it inflicts on families in rural areas (Kayser et al., 2001). In areas where malaria occurs, however, there is considerable variation in the intensity of transmission and risk of malaria infection. Women are four times more likely to get sick, and twice as likely to die from malaria if they are pregnant (Li and Weina, 2010).

Ethiopia’s fight against Malaria started more than half century ago. “Initially malaria control began as pilot control project in the 1950’s and then it was launched as a national eradication campaign in the 60’s followed by a control strategy in the 70’s.” The effort has seen alternating periods of success and failures (FMOH, 2007). “In 1976 the vertical organization known as the National Organization for the Control of Malaria and Other Vector-borne Diseases (NOCMVD) evolved from the Malaria Eradication Service (MES)” (Kassahun, 2004). As the case everywhere else where malaria is endemic, the disease is far from being conquered. The *plasmodium* species have developed resistance to a number of drugs while the vector mosquito has learned to fend off the chemical onslaught launched by humans (Guthmann et al., 2007).

It is estimated that three-fourths of the land below 2000 meters is malarious with two-thirds of the country’s population at risk. This makes malaria the number one health problem in Ethiopia with an average of 9.5 million cases per year between 2001-and 2005. The disease causes 70,000 deaths each year and accountant for 17% of outpatient visits to health institutions. It also accounts for “15% of admissions and 29% of inpatient deaths. The burden of malaria has been increasing due to combination of large population movements, increasing large-scale epidemics, mixed infections of *Plasmodium vivax* and *Plasmodium falciparum* increasing parasite resistance to malaria drugs, vector resistance to insecticides, low coverage of malaria prevention services, and general poverty.
In Ethiopia, *Plasmodium falciparum* and *Plasmodium vivax* account for 60-70% and 30-40% of malaria cases, respectively. *Plasmodium falciparum* has been the major cause of epidemics, and of most malaria deaths (Woyessa et al., 2012).

### 1.1.2. *Plasmodium* life-cycle

The *Plasmodium* parasite exhibits a complex life cycle involving an insect and a vertebrate host (figure 1). All four species exhibit a similar life cycle with only minor variations (Matteelli et al., 2000). The comprehensive life-cycle of the species of *Plasmodium* occurring in man is starts when the sporozoites are injected into the blood stream during a blood meal by an infectious mosquito. Although it is assumed that one single sporozoite is capable of initiating the infection in men, the number of sporozoites injected by a mosquito bite is supposed to vary from dozens to thousands. It is likely that this number strongly affects the clinical picture: the greatest the sporozoite load, the shortest the incubation period and the most serious the symptoms.

The sporozoites remain into the circulation for a short period before they actively enter the liver of the host (Derisi, 2009). The intracellular parasite undergoes an asexual replication known asexoerythrocytic schizogonic within the hepatocytes. The liver cycle ends when the mature schizont ruptures and releases the merozoites into the sinusoids of the liver (Srinivasa et al., 2012). Merozoites invade erythrocytes and undergo a trophic period in which the parasite enlarges. The blood stage is responsible for the pathology associated with malaria. The intermittent fever paroxysm is due to the synchronous lysis of the infected erythrocytes. *Plasmodium malariae* exhibits 72 hours, whereas the other three species exhibit 48 hour cycles (Li and Weina, 2010).

Two species of human malaria determine a relapsing infection including *Plasmodium vivax* and *Plasmodium ovale*. In these two species some of the liver trophozoites immediately start the exo-erythrocytic schizogonic cycle which has been described above, while others remain into the liver
in dormant stage for varying periods of time and are termed hypnozoites (Castelli, 2000; Matteelli, 2005).

Figure 1: Life cycle of *Plasmodium* (Batista *et al.*, 2010)

1.1.3. Treatment, prevention and drug targets for antimalarial

Current practice in treating cases of malaria is based on the concept of combination therapy, since this offers several advantages, including reduced risk of treatment failure, reduced risk of developing resistance, enhanced convenience, and reduced side-effects (Cragg *et al.*, 2005). Most of the antimalarial drugs available currently have been in use for decades, but their use is now severely limited by the emergence and spread of drug resistance, primarily in *Plasmodium falciparum*, the malaria parasite that causes severe forms of disease and most of the disease burden. These know more about sites of parasite vulnerability and the basic mechanisms through which drugs act and resistance is generated in order not only to generate new drugs with novel mechanisms of action, but also to make better use of available drugs (Tren *et al.*, 2008; Boulton *et al.*, 2010).
1.1.3.1. Antifolate combination drugs

Antifolate drugs include various combinations of dihydrofolate reductase enzyme (DHFR) inhibitors, such as pyrimethamine, proguanil, and chlorproguanil, also a bifunctional enzyme in plasmodia coupled with thymidylate synthase (TS), thus preventing the NADPH-dependent reduction of dihydrofolate (DHF) to tetra hydro folate (THF) by DHFR. THF is a necessary cofactor for the biosynthesis of thymidylate, purine nucleotides, and certain amino acids. DHFR inhibitors mimic the pteridine ring of the natural substrate DHF, and compete with it for the active site of the enzyme. Pyrimethamine and cycloguanil have a phenyl pyrimidine and a dihydrodiazine group, respectively, that fits the hydrophobic active site pocket of DHFR and that forms H-bonds with the carboxyl oxygen’s of Asp54 (in helix B), as DHF does (Snehasis et al., 2001).

1.1.3.2. Blood schizonticides

These drugs act on the blood forms of the parasite and thereby terminate clinical attacks of malaria. These are the most important drugs in antimalarial chemotherapy. These may be subgrouped as: (a) fast-acting high-efficacy blood schizonticides such as chloroquine, quinine, mepacrine, mefloquine, halofantrine, artemisinin and atovaquone. These can be used singly to terminate the attack of malaria promptly; (b) slow acting low-efficacy blood schizonticides such as pyrimethamine, proguanil, sulfonamides and tetracyclines (Andersen et al., 1995).

1.1.3.3. Quinoline containing compounds

Antimalarial agents generally belong to the class of quinoline which acts by interfering with heme metabolism. Quinoline and its related derivative comprise a class of heterocycles, which has been exploited immensely than any other nucleus for the development of potent antimalarial agents. Various chemical modifications of quinoline have been attempted to achieve analogs with potent antimalarial properties against sensitive as well as resistant strains of Plasmodium species together.
with minimal potential undesirable side effects (Bawa et al., 2010). Quinine, extracted from Cinchona bark, has provided the basis for the development of synthetic quinoline-containing drugs such as chloroquine (CQ), amodiaquine (AQ) and mefloquine (Bray et al., 2005).

### 1.1.3.4. Artemisinin-type compounds

Artemisininis the antimalarial principle isolated by Chinese scientists from *Artemisia annua* L. It is a sesquiterpene lactone with a peroxide bridge linkage. Its various derivatives include artemether, arteether, artesunate, dihydroartemisinin and artelinic acid. Artemisinin is poorly soluble in oils or water but the parent compound has yielded dihydroartemisinin, the oil-soluble derivatives artemether and arteether, and the more water-soluble derivatives sodium artesunate.

These derivatives have more potent blood schizonticidal activity than the parent compound and are the most rapidly effective antimalarial drugs known. They are used for the treatment of severe and uncomplicated malaria. They are not hypnozoiticidal but gametocytocidal activity has been observed (Khanna, 2007; Brian et al., 2011).

All members of this drug group have activity throughout the phases of the asexual intra-erythrocytic schizogonicycle, and also act on young gametocytes. The mechanism of action is incompletely understood, but the prevailing hypothesis is that reductive cleavage of the intact peroxide by ferroheme ferrous-protoporphyrin IX (Fe (II) PPIX) generates C-centered radicals, which, in turn, would alkylate biomolecules, leading to the death of the parasite (Khanna, 2007).
1.1.3.5. Novel molecular targets for antimalarial chemotherapy

1.1.3.5.1. Targeting parasite proteases

Proteases have an important role in parasite survival by hydrolyzing a significant proportion of the host erythrocyte proteins. Approximately 80% of the host cell haemoglobin is broken down into individual amino acids, some of which are used for parasite protein synthesis. Several *Plasmodium* proteases that appear to be responsible for vital cleavage of host proteins have been characterized. Although none of the currently marketed antimalarial is targeting plasmodia proteases, The cystein and aspartyl endoproteases involved in the essential pathway of haemoglobin degradation now known as falcipain-2, 2’ and -3 and plasmepsin-I, -II, -III (or HAP, for histo-aspartyl protease) and –IV have first emerged as promising protease targets (Jana *et al.*, 2007).

1.1.3.5.2. Targeting parasite membrane biosynthesis

Intraerythrocytic parasites possess different membranes such as the food vacuolar membrane, the parasite plasma membrane (PPM) and the parasitophorous vacuole membrane. The amount of lipid in infected erythrocytes is therefore significantly higher than that of normal erythrocytes. Growing and dividing malaria parasites require large amounts of phospholipids, which are synthesized from plasma fatty acids. Phosphatidylcholine (PC) is the major parasite phospholipid. Most of the PC (70–80%) is synthesized by de novo synthesis from choline. In infected red blood cells (RBCs), choline mobility increases significantly via a constitutive choline carrier. This occurs from parasite induced over activity of a constitutive host cell transport or parasite-driven synthesis of a new carrier. Molecules are being designed to target the parasite’s supply of PC (Jana *et al.*, 2007).
1.1.3.5.3. Targeting parasite transporters

There is a profound increase in the permeability of the host membrane to a wide range of solutes in malaria-infected erythrocytes. Molecular traffic across the host erythrocyte membrane undergoes dramatic changes with respect to intensity and the nature of permeating solutes. The induced permeability pathways are known as new permeability pathways (NPPs), which are polyspecific, anion selective and confer increased permeability to a wide range of charged and low-molecular-weight solutes. These NPPs are thought to provide the major entry of some essential nutrients (pantothenate) required by the parasites. The properties of the parasite induced transport systems are significantly different from those in normal human cells. Therefore, these transport systems could potentially be exploited as targets for antimalarial chemotherapy. This could be achieved by designing cytotoxic drugs that selectively enter the parasite through these induced transporter routes (Noedl et al., 2008).

1.1.3.5.4. Targeting nucleic acid metabolism

Nucleotides are the precursors of Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) biosynthesis. Nucleic acid metabolism pathways differ between Plasmodium falciparum and the human host. Plasmodia synthesize purines and pyrimidine by salvage and de novo biosynthetic pathways, respectively, whilst mammalian cells synthesize purines de novo and either salvage or synthesize pyrimidine by de novo pathway. The two pathways involve a range of essential enzymes that can be targeted for therapeutic intervention (Noedl et al., 2008).
1.1.3.6. Existing Antimalarial Drugs

1.1.3.6.1. Quinine and Related Drugs

Malaria control has relied upon the traditional quinoline, antifolate and artemisinin compounds. Very few new antimalarial were developed in the last quarter of the 20th century. An alarming increase in drug-resistant strains of the malaria parasite poses a significant problem for effective control (Massaga et al., 2003). Quinine (1), quinidine (2), chloroquine (CQ) (3), primaquine (PRQ) (4), amodiaquine (AQ) (5), mefloquine (MQ) (6), piperaquine (PQ) (7), pyronaridine (PYR) (8), and the amino alcohol lumefantrine (LUM) (9) are structurally related antimalarial drugs shown in (Appendix 1) (Day et al., 2007).

1.1.3.6.2. Antifolates and Antibiotic Antimalarials

Proguanil (10), chlorproguanil (11), pyrimethamine (12), and trimethoprim (13) and sulfa drugs: Dapsones (14), sulfamethoxazole (15), sulfadoxine (16) in (Appendix 2) are drugs which are shown to have antimalarial activity by inhibiting dihydrofolate reductase (DHFR) enzyme as mentioned above (Kalra et al., 2006). These drugs have been used in combination (SP, Sulfamethoxazole/trimethoprim, atovaquone (17)-proguanil) so as to produce synergistic effect, reduce the emergence of drug resistance (Watkins, 1997).

The antibiotic drugs shown in (Appendix 2) tetracycline (18) doxycycline (19) and clindamycin (20) are very potent Antimalarials and are used for both treatment and prophylaxis in combination with other Antimalarials like quinine (Schlitzer, 2008).

1.1.3.6.3. Artemisinins and Artemisinin Combination Therapies (ACTs)

Artemisinin (21) dihydroartemisinin (DHA) (22), artemether (ART) (23), arteether (24) and artesunate (AS) (25) are the most important new class of drugs with excellent safety profiles
(Carrara et al., 2006) as shown (Appendix 3). Intravenous and intramuscular artesunate and artemether have been highly efficacious for the treatment of severe malaria, with less side effects and better activity than quinine (Dondorp et al., 2005).

1.1.4. Drug Candidates for the Treatment of Malaria

Tafenoquine, a new 8-aminoquinoline under Phase III clinical development, is found to be effective in preventing Plasmodium falciparum and Plasmodium vivax malaria as well as relapse of Plasmodium vivax associated with latent hypnozoites in the liver (Crockett et al., 2007). In addition, isoquine, 4-pyridone, AQ 13 and CDRI 97/98 are in Phase I; artemisone, ferroquine, fosmidomycin-clindamycin, SAR 97276, methylene blue-AQ and tinidazole are in Phase II; eurartesim, pyramax, azithromycin-CQ and arterolane-PQ are in Phase III clinical development (Olliaro et al., 2009). More recently, OZ 439 has successfully completed Phase I clinical trials, and Phase Ia trials in patients with malaria are underway (Franklin et al., 2011).

1.1.5. Antimalarial Drug Resistance

Several antimalarial drugs are introduced to reduce the death rates due to malaria, but recently the efficacy of therapy has decreased due to antimalarial drug resistance (Srinivasa et al., 2012). Chloroquine had been the drug of choice for treating Plasmodium falciparum for more than 50 years. However, its use as a prophylactic drug and as a malaria treatment is now limited because of the selection and spread of CQ-resistant Plasmodium falciparum strains throughout malaria endemic areas (Rijken et al., 2011).

In addition, resistant strains evolved to quinine, piperaquine, mefloquine, lumefantrine and atovaquone (Musset et al., 2006; white, 2008). Consequently, the mortality and morbidity from malaria has increased in the past two decades. Thus, Artemisinin and ACTs are recommended as a first-line treatment for Plasmodium falciparum malaria in almost all regions where malaria is endemic (Smithuis et al., 2006).
Although artemisinin are potent and rapidly acting antimalarial drugs, their widespread use for treating patients with *Plasmodium falciparum* malaria raises the question of emerging drug resistance (Dondorp et al., 2005). Research findings revealed that treatment failures to Artesunate/amodiaquine in patients with mixed infections (*Plasmodium falciparum* and *Plasmodium vivax*), observed in Papua, Indonesia. Therefore there is an urgent need for new, more affordable and accessible antimalarial agents possessing original modes of action (Hasugian et al., 2007).
2. Medicinal plants for malaria treatment

Malaria is still the most destructive and dangerous parasitic infection in many tropical and subtropical countries (Koyama, 2006). The burden of this disease is getting worse, mainly due to the increasing resistance of *Plasmodium* species against the widely available antimalarial drugs (Mehlin, 2005). Natural products have played a dominant role in the discovery of leads for the development of drugs to treat human diseases, and this fact anticipates that new antimalarial leads may certainly emerge from tropical plant sources (Sudhanshu *et al*., 2003).

The first antimalarial drug, quinine (26), was extracted from the bark of the *Cinchona* (Rubiaceae) species. In 1967, Chinese scientists isolated a sesquiterpene lactone, artemisinin (27) shown in (Appendix 1) from the leafy portions *Artemisia annua* which is responsible for its reputed medicinal action (Ginsburg and Deharo, 2011). Although artemisinin and its analogues have provided much needed drugs for the treatment of chloroquine-resistant malaria, these are unavailable and/or unaffordable to many people who live in malarious areas. An alternative to manufactured drugs is the use of traditional medicines for the treatment of malaria (David *et al*., 2004; Junko, 2006).

2.1. Ethiopian medicinal plants used for malaria treatment

In Ethiopia it is estimated that about 80% of the Ethiopian population is still dependent on traditional medicine, which essentially involves the use of plants. Despite their wide use in the traditional health care, the work that has been done to evaluate the safety, efficacy and semi-synthesis of Ethiopian traditional medicinal plants is not extensive (Kiprono *et al*., 2004). Previous studies have shown the antimalarial activities of *Vernonia amygdalina* and *Withania somnifera* in *vitro* against *Plasmodium falciparum* and activities of these plants against *Plasmodium berghei* in mice (Kassaye *et al*., 2006).
Dikasso et al. (2007) reported that in vivo, hydroalcoholic extracts of Asparagus africanus displayed a very good activity against the Plasmodium berghei malaria parasite. Bogale and Petros. (1996), also reported that among nine Ethiopian medicinal plants that are used to treat malaria the in vitro antimalarial activities of Withnia somnifera and Vernonia amygdalina against Plasmodium falciparum are substantial. Asres and Balcha (1998) reported that the acetone and methanol extracts of the stem bark of Comberatum molle inhibit schizont maturation of Plasmodium falciparum (Dikasso et al., 2006).

2.1.1. Myrsinaceae family

The family myrsinaceae consists of nearly 1000 species of trees and shrubs spread over 33 genera including four genera namely Myrsine, Maesa, Rapanea and Embelia, which are widely used in herbal medicines. In traditional medicine, plant preparations have always been used to treat infectious disease such as malaria and skin infection with varying degree of success. Use of Embelia schimperia and Embelia ribes plant extract uses dewormers and a wound cleaner have been reported in many part of African country (WHO, 2007).

2.1.1.1. Biological activity of Myrsinaceae

Different bioactive benzoquinone were isolated from the fruits of Maesa lanceolata (Myrsinaceae). The ability of this compound to inhibit the growth of bacteria, fungi, protozoa and parasites, contraceptive and pro-apoptotic properties of are described. For instant Maesanin, 2-hydroxy-5-methoxy-3-(10¢-pentadecenyl) - 1, 4-benzoquinone are a natural p-benzoquinone isolated from the fruits of Ardisia japonica (Myrsinaceae).

A number of Myrsinaceae plants were found to exhibit antimalarial activity. For example, the methanol extracts of the stem part of the Myrsine africana L (Myrsinaceae) have been reported to have significant antiplasmodic activity on chloroquine resistant Plasmodium falciparum strain (Nanyingi et al., 2004).
Various biological activities have been attributed to diverse secondary metabolites, among them Benzoquinone derivatives (1) 2,5-dihydroxy-3- (nonade- 14- enyl)- benzoquinone (2) lanciaquinone and novel phenolic compounds such as maesol were also isolated from the genus Maesa(Appendix 4) and evaluated for in vivo cytotoxic , antioxidant and antimalarial activity (Mohammad et al., 2010).

2.1.1.2. The genus Embelia

Embelia a genus of climbing shrubs in the family Myrsinaceae There are about 130 species which occur in tropical and subtropical areas across a wide range including Africa and Madagascar and from eastern Asia to the Pacific Islands as well as Australia.

Figure 2: Image Embelia schimperia Vatke
2.3. Ethnobotonical use of *Embelia schimperia*

In India Embelia root bark is acrid, astringent anthelmintic, antifertility, *anti-oestrogenic* carminative, digestive laxative, soothing, stimulant, stomachic, and thermo genic (Rochfort *et al.*., 2008). It is effective against intentional parasites and intestinal worms. It is used in abdominal disorders, skin fungal infections, flatulence, constipation indigestion, headache, hemorrhoids, lung diseases, obesity, piles, Pneumonia, mouth ulcers, toothache and sore throat. Its decoction is useful in insanity and heart diseases (Midiwo *et al.*, 2009).

In Kenya *Embelia schimperia* is traditionally used for the treatment of malaria, microbial and helmintics (Nanying *et al.*, 2004; Muthaura *et al.*, 2007)). In Pakistan powder of dried fruit is mixed with milk and taken orally for the treatment of helmintics (Kumar *et al.*, 2011). In Ethiopia the fruit of *Embelia schimperia* crush and drink liquid to cure tapeworm. Women use the leaves to roll the dough in before putting it in the oven so that it does not burin. The seeds also crushed and the oil is used to grease the baking plate before baking (Kiprono *et al.*, 2004). In India the fruit of the *Embelia ribes* has been used to treat fever, inflammatory diseases, and a variety of gastrointestinal diseases (kuruvilla *et al.*, 2010).

2.3.1. Pharmacological activity

2.3.1.1. Analgesic and Antioxidant activity

*Para* quinones are derived from *Embelia ribes* Burm *Embelia schimperia*. The analgesic effect of potassium embelate has been studied in rats and mice. The test drug was found to be effective by oral, intramuscular and intravenous routes and the results compared well with morphine (Singh *et al.*, 2009). The Antioxidant activity ethanolic extract of *Embelia ribes* was examined and the study concluded that *Embelia ribes* enhances the antioxidant defense against reactive oxygen species produced under hyperglycemic condition and this protects beta-cells against loss, and exhibit ant diabetic property (Wan *et al.*, 1974; Lushof *et al.*, 1990).
2.3.1.2. Anthelmintic activity

Because of increasing anthelmintic resistance and the impact of conventional anthelmintic on the environment, it is important to look for alternative strategies against gastrointestinal nematodes. Phytotherapy could be one of the major options to control these pathologies. For instance, extracts of the fruit of *Embelia ribes* (Myrsinaceae) showed an anthelmintic efficacy of up to 93%, relative to pyrantel tartrate (Srinivas et al., 2010).

2.3.1.3. Antimicrobial and Antimalarial activity

Antimicrobial activities of the ethyl acetate, chloroform and hexane extracts of the fruits of *Embelia schimperia* and *Embelia ribes* were tested on several microorganisms. These extracts showed antimicrobial activity against Gram-positive strains including *Enterococcus fecalis*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* with the ethyl acetate extract showing the highest activity. Two species from this genus were found to exhibit antimalarial activity. The methanolic extract of the fruits of *Embelia schimperia* and *Embelia ribes* were found to be active *in vitro* with good IC₅₀ value of, in chloroquine resistant *Plasmodium falciparum* (Srinivas et al., 2010).

2.4. Antimalarial potential of Some Natural compounds

2.4.1. Flavonol glycoside

Fractionation of the methanolic extract of *Embelia schimperia* leaves has led to the isolation of two novel flavonol glycosides (28 and 29) shown in (Appendix 5) also being reported are eight known ones identified as isorhamnetin 3-O-β-glucoside (3), quercetin 3-O-a-rhamnoside (Lawrence et al., 2004).
2.4.2. Triterpenes


2.4.3. Pentacyclic triterpenoids

Five oleanane-type pentacyclic triterpenoids were isolated by chromatographic separation of a chloroform extract of the stem bark of *Embelia schimperia*. Three of these compounds have a methylenedioxy bridge. Two compounds, embelinone and schimperinone in (Appendix 7) are reported here for the first time from a natural source (they have been synthesized previously during chemical transformations). Three of the triterpenoids exhibited mild antibacterial properties against the gram-positive bacterial strain (Connolly *et al.*, 2003).

2.5. Semi-synthetic approaches to antimalarial drugs discovery from natural sources

2.5.1. Semi-synthetic artemisinin derivatives

Medicinal plants have provided valuable and clinically used antimalarial like quinine and artemisinin. Several compounds containing unique structural composition have been isolated and characterized from natural sources (Butle, 2009). These natural products have exhibited promising antimalarial activities *in vitro* and *in vivo* (David, 2001). However, limitations such as toxicity, low bioavailability and/or poor solubility have restricted the scope of use for several natural products in humans. There are some compounds which could be modulated to obtain antimalarial active without limitations. In this direction, semisynthetic approaches to newer and modified
antimalarial have provided useful insights into their applicability in antimalarial drug discovery (Kaur et al., 2009).

Artemisinin and its derivatives artemether and arteether were isolated from Artemisia annua (Cragg et al., 2005). The biological activity and the challenging structure of artemisinin have prompted extensive synthetic efforts to disclose more potent analogs. Artemisinin analogs modified at C-3 and C-13 were prepared by Han et al. (2004) from artemisinic acid, artemisinic acid was modified through allylic oxidation at C-3 or conjugate addition at C-13 to afford methyl artemisinates, which upon photo oxidation yielded artemisinin analogs. Among these analogs, 13-nitromethylartemisinin produced activity comparable to artemisinin. 13-(1-Nitroethyl) artemisinin was 20-fold less active, indicating that the activity was sensitive to the bulkiness of the side chain shown in (Appendix8) (Kaur et al., 2009).

2.5.2. Semi-synthetic alkaloids

Alkaloids, especially quinine, were used in the treatment of malaria for ages. With changes in human lifestyle and environment, Cinchona alkaloids are no more effective, because of resistance developed by the parasite (Ejebe et al., 2008). A series of mono-and dimeric natural and structurally modified carbazoles has been tested for activity against Plasmodium falciparum. One of the monomers, the synthetic compound 1, 4-diacetoxy-3-methylcarbazole , displays the highest activity. It is distinctly more active than the as yet best natural compound, 1-hydroxy-3-methylcarbazole. In the series, suggesting that a free phenolic hydroxyl function as in 268 is not required for antiplasmodial activity (Bringmann, 1998).

Cryptolepine is a major benzo-d-carboline alkaloid from shrub C. sanguinolenta. Cryptolepine and its hydrochloride, display potent in vitro antiplasmodial activity but were cytotoxic that precluded their clinical use. The cytotoxicity can be ascribed to the ability of Cryptolepine to intercalate into
DNA and inhibit topoisomerase II as well as DNA synthesis. Several synthetic cryptolepine analogs have been reported (Kaur et al., 2009).

Miert et al. (2005), synthesized dimethylatedindoloquinoline derivative, N-methyl-isocryptolepinium iodide and isoneocryptolepine, to compare their biological activities with the naturally occurring cryptolepine. The quaternary alkaloid was more active and selective than Isoneocryptolepine. Compounds were also evaluated in vivo in mice infected with Plasmodium berghei. The carbon (indenoquinoline) and oxygen (benzofuroquinoline) isosteres were significantly less potent than the parent nitrogen (indoloquinolines) isostere. Miert et al. (2005), designed cryptolepine analogs like compound 272 by incorporating an alkyldiamino side-chain at C-11, aiming to increase accumulation in the parasite food vacuole in (Appendix 9) (Kaur et al., 2009).

2.6. Benzoquinones

Quinones are wide spread in nature and many are of major industrial importance such as dyes, pigments and plant protection chemicals. Some quinones are related to more complicated aromatic systems which have been isolated from biological sources. In many cases, they seem to take part in oxidation reduction cycles as they have a vital role in electron transport in respiratory and photosynthetic elements (Pink et al., 2000; Ross et al., 2000). Since quinones are alpha- beta – unsaturated cyclic diketones with both the oxygen atoms in simple or fused conjugated ring system, they are capable of forming 1,4- addition products. Compounds containing the thiol (SH) and amino (NH₂) groups react readily with quinones (Song and Jeon, 2003).

Para-quinones were known to possess antitumor activity (Long and Jaiswal, 2000), antimicrobial activity (Haraguchi, 1998) and antimalarial activity. Thiophene ring containing quinones were reported to possess antiprotozoal activity against Leishmania and Trypansomacruize(Valderramaet et al., 1999). Alkylated hydroxy -1,4- Naphthoquinone were inhibitors of succinoxidase and NADH -
oxidase (Porter et al. 1978). Hydroxynaphthoquinones inhibits parasite respiratory systems with outstanding efficacy against *Plasmodium* species.

The antimalarial activities of *Cassia occidentalis* and *Garsinia kola* have been reported by (Tona et al., 2001). It is likely that these activities could be attributed to the presence of quinones in these plants (Liu et al., 2009). A growing interest is granted to these compounds from the description of the antimalarial activity of Atovaquone (Basco et al., 1995) and three quinones isolated from *Salacia krausii* presented very high activity on *Plasmodium falciparum* (Figueiredo et al., 1998). Quinones could be used as starting point for the synthesis of molecules much more active than the natural extract (Joanne et al., 2009; Kayembe et al., 2010).

2.6.1. Synthesis of 1, 4- benzoquinone derivatives

Moreover, it is essential to mention that some of 1, 4- benzoquinone derivatives were synthesized and have shown promising antimalarial activities. It has been reported that some N-, S-, O- substitututed naphtho- and benzoquinone compounds were synthesized from 2,3-dichloro-1,4-naphthoquinone or 1,4 benzoquinone .Saeed et al. (2009), synthesized some of simple p-benzoquinone derivatives. The basic ring was designed to be a 1, 4- benzoquinone with additional derivatives as halogens. Selected amino compounds with intrinsic biological features were allowed to react with 2,3,5,6-tertabromo- 1,4-benzoquinones to furnish the target 2,5-diarylamino- 3,6-dibromo-1,4- benzoquinones as shown (Appendix 10) (Ibis and Deniz, 2012).

Embelin is the major chemical constituent of *Embelia vibes* Burn and *Embelia schimperia* (Balawant et al., 1995). It has a long alkyl chain (undecyl) as a substituent, which confers solubility in the non-polar phase and cell permeability. The adjacent benzoquinone and hydroxyl groups on embelin form intramolecular hydrogen bonds. Due to the presence of this structure embelin shows a wide spectrum of biological activities, Such as antibacterial, antitumor, anti-
inflammatory, anti-malaria and analgesic activity of embelin has been reported (Mahesh et al., 2008).

These antecedents justify the interest in the construction of newer embelin derivatives by using some aromatic amino-derivatives which have better activity than the parent compound (Kiprono et al., 2011). This study initiated to semi-synthesize some new 1, 4-benzoquinone derivatives starting from Embelin and study their antimalarial activities and antibacterial activity with minimal toxicity rapid efficacy, and low cost (Rosanne et al., 2011).
2.7. Significance of the study

Medicinal plants play an important role in the treatment of malaria especially in developing countries where resources are limited. They have in the past been the source of some of the most successful antimalarial agents such as the quinolines and artemisinin compounds. The spread of multi drug-resistant *Plasmodium falciparum* has highlighted the urgent need to develop new antimalarial drugs, preferably inexpensive drugs that are affordable for developing countries, where malaria is prevalent.

In Ethiopia, the use of indigenous plants still plays an important role in malaria treatment and these plants might be interesting sources for the detection of novel antiplasmodial compounds. The recently developed new isolation and characterization techniques together with development of new pharmacological testing have led to interest in plants as a source of new drugs. However, a promising approach is needed to use these agents as templates for designing new derivatives with improved properties.

The emergence and spread of this antimalarial drug resistance has been a major obstacle to efforts to reduce malaria-related morbidity and mortality throughout all parts of the world. So there is an urgent need to discover new compounds with an original mode of action from plants community which used in traditional medicine are a source of active new compounds.

Therefore, traditionally used medicinal plants modification may need to improve their activity before they are recommended for treatment of malaria and need for scientific validation, safety evaluation of traditionally used medicinal plants and designing new derivatives with improved antimalarial activities.
3. Objectives

3.1. General objective

- Isolation of embelin from *Embelia schimperia*, preparation of some new semisynthesized products from embelin and evaluation of their *in vitro* antimalarial activity against mice infected with *Plasmodium berghei*

3.2. Specific objectives

- To extract and isolate embelin from the fruit of *Embelia schimperia*;
- To semi-synthesis some embelin derivatives using condensation reactions of embelin with some aromatic amines;
- To confirm the chemical structure of the synthesized compounds using some analytical and spectroscopic techniques;
- To evaluate *in vivo* antimalarial activity of the synthesized compounds against mice infected with *Plasmodium berghei*;
- To perform acute toxicity test for the most active compounds; and
- To propose structure activity relationships of the synthesized compounds for antimalarial activities.
4. Materials and Methods

4.1. Materials

4.1.1. Plant material

The fruit of *Embelia schimperia* were purchased from local market in March 2012 in Addis Ababa, Central Ethiopia. The authenticity of the plant material was confirmed by Ato Melaku Wondafrash, the National Herbarium, Department of Biology, and Addis Ababa University.

4.1.2. Chemicals, reagents and drugs

The following chemicals and reagents were used for the experiments. Giemsa, trisodium citrate, n-hexane, diluted hydrochloric acid, Tween 80, Petroleum ether, glacial acetic acid, n-hexane, diethyl ether, benzene, Acetic anhydride, Tertiary ammonia, Aniline, Ethanol, sodium hydroxide, potassium hydroxide, chloroform, absolute methanol (Reagent Chemical Limited, UK), ethyl acetate (Research-Lab-Fine, India), ortho-toluidine, Para-toluidine, n-butanol, n-propanol and sodium carbonate. All the chemicals were analytical grade and most of them were purchased from Pharmaceutical Fund and Supply Agency, Addis Ababa-Ethiopia, while the rest were obtained from Department of Pharmaceutical Chemistry and Pharmacognosy, School of Pharmacy, Addis Ababa University.

4.1.3. Equipment and instruments

Conical flask, Graduating cylinder, Beakers, Erlenmeyer flask, Round bottom flask, water bath, Condenser, Buchner funnel, filter paper etc, TLC plates, Thermometer, Ice bath, thin layer chromatography jar and Digital balance were used. $^1$H and $^{13}$C NMR spectra were recorded on Bruker Avance DMX400 FT-NMR spectrometer using TMS as internal standard, at the Department of Chemistry, Collage of Natural Sciences Addis Ababa University.
The NMR data were measured using deuterated chloroform as a solvent and the chemical shifts are reported in (ppm). Melting points (MP) were determined in open capillaries using electro-thermal 9100 melting point apparatus at the Department of inorganic Chemistry, Collage of Natural Sciences Addis Ababa University and are uncorrected. IR spectra were recorded on a SHIMADZU 8400SP FT-IR spectrophotometer at Department of Chemistry, Collage of Natural Sciences Addis Ababa University.

4.1.4. Experimental animals and Test organisms

Swiss albino mice of both sex weighing 20-30 g and age 6-8 weeks were obtained from Department of Biology Addis Ababa University animal house, all animals were housed in an air-conditioned room and were allowed to acclimatize for one week before the study. The animals were kept at room temperature and were exposed to a 12 h light/dark cycle. All the experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline (ILAR, 1996). Before and during the experiment, the mice were allowed free access to standard pellets and water ad libitum.

Plasmodium berghei ANKA strain (CQ sensitive), was obtained from Department of Biology Addis Ababa University. The parasite was subsequently maintained in the laboratory by serial blood passage from mouse to mouse on weekly bases. Chloroquine phosphate (EPHARM) was used as a reference drug in determination of the antimalarial activity of the semi-synthesized compounds.
4.2. Methods

4.2.1. Preparation of plant material and Isolation of embelin

The fruits were cleaned, air-dried at room temperature and crushed into fine powder. A half (1/2) kg of the material was macerated in ethyl acetate at room temperature for 72 hour. The mixture was filtered and the solvent evaporated under reduced pressure using a rotary evaporator (Buchi Rota Vapor R-200, Switzerland) to afford 25 g of dark brown solid. A quantity of 10 g of the crude extract was subjected to column chromatography using a column packed under \( n \)-hexane with dry de-activated silica gel. The column was first eluted with pure \( n \)-hexane followed by a mixture of \( n \)-hexane/ethyl acetate with increasing polarity. Elution of the column with an \( n \)-hexane/ethyl acetate mixture (1:10 v/v) led to isolation of bright orange crystalline embelin. Then Embelin was characterized on the basis of physical and spectroscopic data (MS, \(^1\)H NMR, and \(^{13}\)C NMR) and identified as 2, 5 dihydroxy-3-undecyl 1, 4 benzoquinone.

4.2.2.1. Condensation of embelin with some aromatic amines

The synthesis of target compounds (5-aromatic amine substituted- 2- dihydroxy-3-undecyl 1, 4 benzoquinone) was achieved using one step condensation reactions. It involved the synthesis of 5- (\( p \)-toluidino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (1A), 5-(\( o \)-toluidino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione(2A) and (\( E \))-3, 6-dihydroxy-4-(phenylimino)-2-undecylcyclohexa-2,5-dienone (3A) The details of each reactions and reaction conditions are mentioned in the following sections.
4.2.2.1.2. Preparation of 5-(p-toluidino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione

Compounds (1A) was synthesized by condensation of 2,5-dihydroxy-3-undecylcyclohexa 2,5-diene-1,4-dione (Embelin; 320mg, 1 mmol) with p-toluidine (450mg, 1 mmol) in glacial acetic acid (30ml) were boiled under reflux on a water bath at 100 °C for 4 hour, Cooled and poured into ice-cooled diluted HCl and the deep violet precipitate formed was filtered, washed with ethanol, dried and then recrystallized from chloroform/ethanol mixture (4:1). Yielded the products 5-(p-Tolylamino)-2-hydroxy-3-undecyl-1,4-benzoquinone (1A) Obtained as deep violet prisms, MP 149-151 °C; yield 408mg, 98.3%.

Scheme 1: synthesis of embelin derivative by using p toluidine (1A)

4.2.2.1.3. Preparation 5-(o-toluidino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione

Compound (2A), which is also an amine derivative was synthesized by condensation of 2,5-dihydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (Embelin) (320mg, 1 mmol) with the o-toluidine (409.2mg, 1 mmol) in glacial acetic acid (40ml) were boiled under reflux on water bath at 100 °C for 4 hour), Cooled and poured into ice-cooled diluted HCl The solid products formed (2A) were filtered, dried and recrystallized from chloroform/ethanol (4:1) yielded the products 5-(o-toluidino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione obtained as reddish brown prisms (2A) MP 119-120°C; yield 410mg, 98.8%.
**Scheme 2: synthesis of embelin derivative by using o-toluidine (2A)**

4.2.2.1.4. Preparation \((E)-3,6\text{-dihydroxy-4-(phenylimino)-2-undecylcyclohexa-2,5-dienone}\)

Compound (3A) was synthesized by condensation of 2,5-dihydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (Embelin) (320mg, 1 mmol) with aniline (409.20mg, 1 mmol) in glacial acetic acid (40ml) were boiled under reflux on a water bath at 100 °C for 5 hour, Cooled and poured into ice-cooled diluted HCl The solid products formed (3A) were filtered, dried and recrystallized from chloroform/ethanol (2:1) yielded compound (3A). Obtained as violet amorphous, MP 157-159 °C; yield 0.40 g, 96.1%

**Scheme 3: synthesis of embelin derivative by using o-toluidine (3A)**
4.3. Determination of physical constants for synthesized compounds

Some physical constants like percentage yield, melting point and $R_f$ values were determined. Precoated silica gel of 0.25 mm thickness plates and ultra-Violate-light and iodine vapor were used to visualize the spots. Petroleum ether and chloroform (1: 1) was used as a mobile phase to develop chromatogram.

4.3.1. Spectroscopic analysis of synthesized compounds

IR spectra of the synthesized compounds were recorded in the range of 4000-500 cm$^{-1}$ in nujol and $^1$H NMR spectra were recorded with a Bruker Avance DMX400 FT-NMR spectrometer operating at 400 MHz. All the compounds were dissolved in CDCl$_3$ for the analysis. The chemical shift values are reported in $\delta$, ppm using TMS as an internal standard.

4.4. Biological activity tests

4.4.1. Acute oral toxicity test

Female Swiss albino mice were used for acute oral toxicity study. Oral toxicity study was conducted as per the internationally accepted protocol drawn under Organisation for Economic Co-operation and Development guidelines 423 (OECD, 2001). Fifteen mice were randomly divided into 5 groups of 3 mice per cage. The animals were physically active and were consuming food and water in a regular way. Before oral administration of a single dose of the test samples, the mice were deprived from food for 2 h. Then the mice in the first group were given distilled water while the second groups were given embelin (2 g/kg) dissolved in 7% Tween 80 and 3% ethanol orally, and the mice in the third, fourth and fifth group were provided with the synthesized compound(1A-3A) 2g/kg dissolved in 7% Tween 80 and 3% ethanol, respectively. The mice were observed continuously for one h after administration of the compounds; intermittently for 4 hours, over a period of 24 hours and for 14 days. Gross behavioral changes such as loss of appetite, hair
erection, lacrimation, tremors, convulsions, salivation, diarrhea, mortality and other signs of toxicity manifestation were observed (OECD, 2001).

4.4.2. Antimalarial activity test

The standard 4-day suppressive method was used to evaluate *in vivo* antimalarial activities of the newly semisynthesized products against mice infected with *Plasmodium berghei* (Fidock *et al*., 2004). Blood was taken from a donor mouse with approximately 30% parasitemia and diluted in physiological saline to $5 \times 10^7$ parasitized erythrocytes per ml. Swiss albino mice weighing 22-30 g were infected with 0.2 ml ($1 \times 10^7$ parasitized erythrocytes) *Plasmodium berghei* intraperitoneally (i.p.) and randomly divided into five groups of five mice per cage with three test groups and two control groups (each for chloroquine as standard drug and distilled water as a negative control).

The embelin and synthesized compounds were prepared in three different doses (100 mg/kg, 200 mg/kg, and 400 mg/kg of body weight) and chloroquine at 25 mg/kg in a volume of 0.2 ml. the embelin and synthesized compounds or the standard was administered as a single dose per day. The compounds and the drug were given through oral route by using standard oral gavages. Treatment was started 3 h after infection on day 0 and was then continued daily for four days (i.e. from day 0 to day 3).

On the fifth day (D4) thin smears of blood films were obtained from the peripheral blood on the tail from each mouse. The smears were placed on microscopic slides (Westtmed Praxis, Germany), fixed with methanol and stained with 10% Gemsa at pH 7.2 for 20 min. The parasitemia level was determined by counting the number of parasitized erythrocytes out of three random fields of the microscope (Reichert-jung microscope Neovar Germany). Average percent parasitaemia and suppression were calculated by using the following formula (Fidock *et al*., 2004; Kalra *et al*., 2006).
4.5. Coding system

A stands for the isolated embelin, 1A, 2A and 3A were codes given for the three semi-synthetic embelin derivatives.

4.6. Statistical Analysis

Results of the study were expressed as mean ± standard deviation and statistical significance for suppressive test was determined by One Way Analysis of Variance (ANOVA) using Windows SPSS Version 16. software. Data on survival time, % parasitemia and % suppression was analyzed using Microsoft office excel 2007. All data was analyzed at 95% confidence interval (P=0.05). The % parasitemia and % suppression of the synthesized compounds were calculated using the following formulae (Gessler et al., 1995).

\[
\text{% Parasitemia} = \frac{\text{Number of infected RBC}}{\text{Number of total RBC}} \times 100
\]

\[
\text{% Suppression} = \frac{\text{Parasitemia in untreated group} - \text{Parasitemia in treated group}}{\text{Parasitemia in untreated group}} \times 100
\]
5. Results and Discussion

5.1. Structural elucidation of isolated embelin and its semi-synthetic derivatives

**Compound (A)**

Compound A (embelin) was isolated as a yellow crystal with Rf value of 0.3. The negative-mode of electrospray ionization gave a pseudomeolecular ion at \( m/z = 292.17 \) ([M-H]), which indicating a molecular formula of \( \text{C}_{17}\text{H}_{26}\text{O}_{4} \). \(^1\)H-NMR spectrum of embelin showed a singlet at 5.80 ppm, which was assignable to H-6. The signal at 0.87 ppm (3H, 11'), the broad singlet at 1.56 ppm (18H, H-2'-H-10') and a signal at 2.46 ppm (2H, H-1') showed the presence of the undecyl chain at C-3 position of embelin. \(^{13}\)C-NMR of 2,5-dihydroxy-3-alkyl-1,4-benzoquinones do not show the ring carbon peaks particularly those attached to oxygen atoms due to fluxional effect caused by intramolecular hydrogen bonding. The result of this is long spin relaxation time which leads to saturation of oxygen–carbon signals.
Table 1: $^1$H NMR and $^{13}$C NMR spectral signals of embelin

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<th>Assignment</th>
<th>$^1$H NMR (ppm)</th>
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<td>A(Embelin)</td>
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<td>_</td>
</tr>
<tr>
<td>5</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>6</td>
<td>6.12</td>
<td>5.99.</td>
</tr>
<tr>
<td>1’</td>
<td>2.46</td>
<td>2.46</td>
</tr>
<tr>
<td>2’</td>
<td>1.56</td>
<td>1.60</td>
</tr>
<tr>
<td>3’</td>
<td>1.56</td>
<td>1.56</td>
</tr>
<tr>
<td>4’</td>
<td>1.56</td>
<td>1.55</td>
</tr>
<tr>
<td>5’</td>
<td>1.56</td>
<td>1.57</td>
</tr>
<tr>
<td>6’</td>
<td>1.56</td>
<td>1.56</td>
</tr>
<tr>
<td>7’</td>
<td>1.56</td>
<td>1.56</td>
</tr>
<tr>
<td>8’</td>
<td>1.56</td>
<td>1.56</td>
</tr>
<tr>
<td>9’</td>
<td>1.56</td>
<td>1.49</td>
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<tr>
<td>10’</td>
<td>1.56</td>
<td>1.57</td>
</tr>
<tr>
<td>11’</td>
<td>0.87</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Therefore, the isolated compound was finally identified as compound A (2,5-dihydroxy-3-alkyl-1,4-benzoquinone), by comparing its spectroscopic data with those reported in the literature (Appendix 11-14) (Brhmeshwari, 2009).
Compound (1A)

Compound (1A) is the first synthesized compound obtained as deep violet prisms, with Rf value of 0.48. The negative-mode of electrospray ionization gave a pseudomolecular ion at $m/z = 382.23$ ([M-H]), which indicating a molecular formula of $C_{24}H_{33}NO_3$. The IR spectra of 1A showed absorptions at 3310 cm$^{-1}$ due to $-OH$ and the absorption bands at 2921 cm$^{-1}$ is due to the presence of long aliphatic chain. The compounds also exhibited absorptions at 1572 cm$^{-1}$ due to the presence of $\delta, \beta$-unsaturated C=O and at 1443 cm$^{-1}$ due to the presence of aromatic C=C and 1221 cm$^{-1}$ due to NH groups and The carbonyl stretching band was observed at a low frequency because the conjugation of the carbonyl with a double bond and resulting resonance.

The $^1$H NMR spectrum of compound 1A is presented in (Appendix17). The up field triplet peak observed at 0.87 ppm and integrated for 3 protons is attributed to the methyl group. The multiplet peaks observed at 1.1-2.14 were due to the presence of aliphatic chain. Singlet at 5.86 ppm, integrated to one proton is attributed to the Para benzoquinone proton. It is deshielded due to the electron withdrawing effect of the carbonyl group. The multiplet from 7.15-7.28 ppm and integrated for 5 protons is attributed to thearomatic protons and C-NH proton. The two doublets between 7.15-7.25 ppm each integrated for two protons are attributed to $p$- tolyl-C$_{3,5}$ H and $p$- tolyl-C$_{2,6}$ H respectively. The peak for $p$-tolyl-C$_{2,6}$ H is more deshielded because of its proximity to the electron withdrawing N of amine group. The expected singlet for C-NH might has
overlaped with the multiplet peak of aromatic protons. These are the characteristic peaks of the
target compounds formed, due to the condensation reaction between embelin and \( p \)-toluidine.

In 13C-NMR spectrum of compound 1A in (Appendix 18) aromatic carbons appeared between
115-150 ppm, C-6 appeared at 93.62 and C-5 appeared at 116.00 to 136.48. Spectrums of ten
methylene groups were observed between 14.14-29.80, in this compound nitrogen is bonded to
C-5. These data confirms that the reaction has taken place at C-5 position. The reaction has
occurred in only one position at C-5, but not in the C-2 position and this may be due to the steric
factor.
Table 2: $^1$H NMR and $^{13}$C NMR spectral signals of the synthesised compound (1A-3A)

<table>
<thead>
<tr>
<th>Assignment</th>
<th>$^1$H NMR (ppm)</th>
<th>$^{13}$C NMR (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbons</td>
<td>Comp 1A</td>
<td>Comp 2A</td>
</tr>
<tr>
<td>1</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>2</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>3</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>4</td>
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<td>_</td>
</tr>
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<td>5</td>
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<td>6</td>
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<td>_</td>
</tr>
<tr>
<td>1'</td>
<td>2.14</td>
<td>2.14</td>
</tr>
<tr>
<td>2'-10'</td>
<td>1.12</td>
<td>1.12</td>
</tr>
<tr>
<td>11'</td>
<td>0.87</td>
<td>0.87</td>
</tr>
<tr>
<td>1''</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>2'',6''</td>
<td>7.28</td>
<td>_</td>
</tr>
<tr>
<td>3'',5''</td>
<td>7.21</td>
<td>6.82</td>
</tr>
<tr>
<td>4''</td>
<td>7.15</td>
<td>6.50</td>
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<tr>
<td>2''-CH$_3$</td>
<td>_</td>
<td>2.35</td>
</tr>
<tr>
<td>4''-CH$_3$</td>
<td>2.47</td>
<td>_</td>
</tr>
<tr>
<td>1''-NH</td>
<td>7.8</td>
<td>7.91</td>
</tr>
<tr>
<td>1''N=C</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>2'''-6'''</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>3'''-5'''</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>1'-N-C</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>4'''</td>
<td>_</td>
<td>7.31</td>
</tr>
</tbody>
</table>
Compound (2A)

Compound (2A) was obtained as violet prisms, with Rf value of 0.488. The negative-mode of electrospray ionization gave a pseudomolecular ion at \( m/z = 382.23 \) ([M-H]), which indicating a molecular formula of \( \text{C}_{24}\text{H}_{33}\text{NO}_3 \). The IR spectrum of compound 2A (Appendix21), showed a Band for O-H stretch was observed at 3260 cm\(^{-1}\). The band around 2922 cm\(^{-1}\) belonged to the aliphatic chain (C-C). Strong characteristic band at 1567 cm\(^{-1}\) due to the carbonyl group of the benzoquinone moiety and Band for C-N stretch was observed at 1213 cm\(^{-1}\). The bands at 765-581 cm\(^{-1}\) were attributed to the presence of ortho substituent aromatic ring. The \(^1\)H NMR spectrum of compound 2A is presented in (Appendix22). The up-field triplet peak observed at 0.87 ppm and integrated for 3 protons is attributed to the methyl group. The multiplet peaks observed at 1.1-2.14 were due to the presence of aliphatic chain. Singlet at 5.86 ppm, integrated to one proton is attributed to the P-benzoquinone proton. It is deshielded due to the electron withdrawing effect of the carbonyl group. The multiplet from 7.15-7.28 ppm and integrated for 5 protons is attributed to the aromatic protons and singlet at 7.91 ppm attributed to C-NH proton. The two doublets between 7.15-7.25 ppm each integrated for two protons are attributed to o-tolyl-C\(_{3,5}\) H and p-tolyl-C\(_{2,6}\) H respectively. The peak for p-tolyl-C\(_{2,6}\) H is more deshielded because of its proximity to the electron withdrawing N of amine group. The expected singlet for C-NH might has overlaped with the multiplet peak of aromatic protons. In 13C-NMR spectrum of compound 1A, aromatic carbons appeared between 115-150 ppm, C-6 appeared at 93.62 and
C-5 appeared at 1116.00 to 136.48. Spectrums of ten methylene groups were observed between 14.14-29.80, in this compound nitrogen is bonded to C-5. These data confirms that the reaction has taken place at C-5 position. The reaction has occurred in only one position at C-5, but not in the C-2 position and this may be due to the steric factor.

![Chemical structure](image)

**Compound (3A)**

Compound (3A) was obtained as reddish brown prisms, with MP 157-159°C; yield 0.40 g, 96.10%; and Rf value of 0.53. The negative-mode of electrospray ionization gave a pseudomeolecular ion at $m/z = 269.23$ ([M-H]), which indicating a molecular formula of C$_{23}$H$_{31}$NO$_3$. The IR spectrum of compound 3A (Appendix 26), showed a Band for O-H stretch was observed at 3260 cm$^{-1}$. The band around 2922 cm$^{-1}$ belonged to the aliphatic chain (C-C). Strong characteristic band at 1567 cm$^{-1}$ due to the carbonyl group of the benzoquinone moiety and Band for C=N stretch was observed at 1443 cm$^{-1}$. The bands at 1221 cm$^{-1}$ were attributed to the presence of aromatic ring.

The up-field triplet peak observed at 0.87 ppm and integrated for 3 protons is attributed to the methyl group. The multiplet peaks observed at 1.1-2.14 were due to the presence of aliphatic chain. Singlet at 5.86 ppm, integrated to one proton is attributed to the *Para* benzoquinone
proton. It is deshielded due to the electron withdrawing effect of the carbonyl group. The multiplet from 7.15-7.28 ppm and integrated for 5 protons is attributed to the aromatic protons. The two doublets between 7.15-7.25 ppm each integrated for two protons are attributed to phenyl-C\textsubscript{3,5} H and phenyl-C\textsubscript{2,6} H respectively. The peak for phenyl-C\textsubscript{2,6} H is more deshielded because of its proximity to the electron withdrawing N of imine group. In 13C-NMR spectrum of compound 3A, aromatic carbons appeared between 122-129.09ppm, C-6 appeared at 93.62 and C-5 appeared at 154.00 to 136.48. Spectrums of ten methylene groups were observed between 14.14-29.80, in this compound nitrogen is bonded to C-1. These data confirms that the reaction has taken place at C-1 position.

The chemical structure of the synthesized compounds and embelin were further verified based on the data obtained from UV Spectrophotometric. Embelin and synthesized compounds were dissolved in methanol and these solutions were scanned in the UV range 200-800 nm. Embelin and synthesized compounds (1A-3A) shows maximum absorbance at 290 nm, 341.4nm, 317.4nm 201.9nm respectively. Di-substituted aromatic group that both groups are electron donating and they are para- to one another, the magnitude of the shift is similar to the effect of the stronger of the two groups as if it were mono-substituted ring effects (1A). The two electronically similar groups are ortho- to one another, the effect is usually the sum of the two individual effects (ortho-steric hind, 2A).The attachment of substituent groups (other than H) can shift the energy of the transition that increase the intensity and often wavelength of absorption are called Auxochromes. Auxochromes include alkyl, hydroxyl, alkoxy and amino groups and the halogens. Methyl groups also cause a bathochromic shift, even though they are devoid of p- or n-electrons. This effect is thought to be through what is termed “hyperconjugation” or sigma bond resonance (Sudani et al., 2011).
5.2. Synthesis of Target Compounds

Synthesis of all target compounds 1A-3A was accomplished starting from embelin which was initially isolated from *Embelia schimperia*, and commercially available primary amines. The synthesis was accomplished using nucleophilic reaction and condensation reaction (1A, 2A, and 3A). The proposed mechanism of reactions for the target compounds is discussed below:

5.2.1. Proposed Mechanism of Synthesis of (1A-2A)

The proposed mechanism for synthesis of 5-(p-toluidino) 2-hydroxy-3-undecylcyclohexa-2, 5-diene-1, 4-dione involved nucleophilic attack of the strong electron-donating group (NH$_2$) of the para toluidine with the electron deficient carbonyl group of the embelin (benzoquinone), as shown in scheme 1. Glacial acetic acid was used as a catalyst to protonation of oxygen to make hydroxyl group as a good leaving group. In the first stage the more nucleophilic amino group of para toluidine attack the carbonyl carbon of embelin at C-1 later dehydration and ketoenole tautomerism would bring about the formation of compound (1A-2A).
Scheme 4: Proposed mechanism of synthesis of synthesis of (1A-2A)

\[ \text{R} = \text{H}_3\text{C}-\text{C}_6\text{H}_4\text{NH}_2 \quad \text{and} \quad \text{H}_3\text{C}-\text{C}_6\text{H}_4\text{NH}_2 \]

The less hindered 5-hydroxy-group of embelin appears to be replaced first by aromatic amine and gave compound (1A, 2A). Nucleophilic displacement of halogeno, alkoxy-, or alkyl substituents from benzoquinones by alkyl amines has been reported. However, some alkoxy-hydroxytoluquin ones do not undergo replacement of the hydroxyl-substituent when treated with aromatic amine (Joshi, B. S. et al., 1975). The formation of (1A, 2A) from embelin therefore could be rationalized in terms of a 1, 2 addition of aromatic amine, followed by elimination of water (Scheme 1) (Brahmeshwari, 2009). It can be concluded that the \textit{para} position of aromatic amine may be a better place for introducing substituents than \textit{Meta} and \textit{ortho} positions since para substituted aromatic amine has resonance structure with positive charge on the methyl substituent which releasing electrons and thus destabilizing the amine substituent((Saeed, et al., 2009).
5.2.2. Proposed Mechanism of Synthesis of (3A)

Compound 3A was synthesized by applying condensation reaction involves nucleophilic addition of a phenyl amine to embelin. Proton transfer occurs between cat-ion and an ion group, a weak acid, glacial acetic acid was used as a catalyst to protonation of oxygen to make hydroxyl group as a good leaving group. Finally the lone pair of electrons expels water to produce imine as shown below (Balawant et al., 1975).

Scheme 5: Proposed mechanism of synthesis of 3A
5.2.3. Physical properties and percentage yield of the synthesized compounds

TLC was used to monitor the progress and confirm the completion of the chemical reactions. The purity of the synthesized compounds was also inferred on TLC from one spot for each target compound in three different developing solvents. Percentage yield, $R_f$ values and melting point of the synthesized compounds were determined as shown in table(1). All synthesized compounds were produced in the highest yield (98.60%) while the least percentage yield was observed for compound (3A) (96.1%). All the synthesized compounds were completely soluble in chloroform.

Table 3: Physical constants and percent yield of the synthesized compounds.

<table>
<thead>
<tr>
<th>Test compounds</th>
<th>Molecular formula</th>
<th>Molecular weight (gram/mol)</th>
<th>% yield</th>
<th>Melting point (°C)</th>
<th>$R_f$ values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>C$<em>{24}$H$</em>{33}$NO$_3$</td>
<td>383.23</td>
<td>98.3</td>
<td>150-151</td>
<td>0.48</td>
</tr>
<tr>
<td>2A</td>
<td>C$<em>{24}$H$</em>{33}$NO$_3$</td>
<td>383.23</td>
<td>98.6</td>
<td>120-121</td>
<td>0.488</td>
</tr>
<tr>
<td>3A</td>
<td>C$<em>{23}$H$</em>{31}$NO$_3$</td>
<td>269.23</td>
<td>96.1</td>
<td>157-159</td>
<td>0.53</td>
</tr>
</tbody>
</table>
5.3. Biological Activity Testing Results

5.3.1. Acute toxicity

No sign of toxicity or mortality was observed in mice after oral administration of the embelin as well as the synthesized compounds, even at doses as high as 5000 mg/kg, signifying that the oral LD$_{50}$ was greater than 5000 mg/kg. Generally, toxicity is the main concern of indigenous therapeutic preparations. Sign of toxicity such as change in animal behavior, lacrimation, weight loss, hair erection and mortality were not recorded in acute toxicity testing. This fulfills the criteria set by OECD (2001), lack of acute toxicity by the plant extract. Therefore, it can be said that the embelin and its derivatives with various substituted aromatic primary amines are relatively safe for mice when given orally.

5.3.2 In vivo Antimalarial activity of embelin

Malaria parasite degrades up to 80% of the haemoglobin in the host cell and releases heme which is toxic to parasite and their host (Junko, 2006). Neutralization of heme occurs mainly by haemozoin formation by the parasites. Inhibition of hemozoin formation is important drug target to kill the parasite cell (David et al., 2004). Inhibition of hemozoin formation took place through different routes via drug binding to the heme or inhibition of Glutathione (GSH) dependent heme degradation. According to Huang et al. (1982), embelin acts as an antimalarial with a mechanism of action similar to that of the well-known 4-aminoquinoline chloroquine, in inhibiting hemozoin formation. It was reported that the presence of hydroxyl groups in chloroquine bind iron of heme and lead to the formation of $\pi-\pi$ adducts, which inhibited hemozoin formation. Similarly
hydroxyl groups present in embelin may bind iron of heme and inhibit the formation of the hemozoin which is essential for survival of parasites (Basilico et al., 1997).

According to Ginsburg et al. (1998), the major degradation pathway of heme is not the hemozoin formation, since around 70% of non-crystallized heme exits in the food vacuole and is subsequently catabolized by GSH, leading to the formation of oxidized glutathione. Hence, a drug that can inhibit the interaction between GSH and heme could have potential antimalarial properties. Embelin may form a heme-embelin complex and inhibit glutathione-dependent degradation of heme. Since trapping of a drug in the acidic vacuole is enhanced if the base character which can accept more than one proton at the lysosomal pH. Embelin may act on two different targets of the parasite as mentioned above therefore chances of development of resistance may less in such cases.

The results of this study indicated that the isolate (embelin) possesses activity against Plasmodium berghei malaria parasite in vivo. The result also revealed that embelin has dose dependent activity. Thus, at the doses of 100, 200 and 400 mg/kg/day, caused 48.10%, 50.2% and 55.00% suppression respectively. The chemosuppression was significant \( P<0.001 \) when compared with the negative control. In the same assay, on day 4, chloroquine had a chemosuppression of 100% at the dose level of 25 mg/kg/day and showed significant \( P<0.05 \) suppression when compared to embelin treated groups (Table 4).

From the result shown in (Table 4) it is evident that embelin possesses blood schizontocidal activity in the early infection of mice by Plasmodium berghei parasite. The average percent of parasitemia observed at a higher dose (400 mg/kg/day) was found to be 21.3±3.8 with 55% of chemosuppression. A dose of 200mg/kg/day of the embelin showed 23.22±2. Parasitemia
with 50% chemosuppression, while the lowest dose (100 mg/kg/day) lowered the average percent parasitemia to 24.6±1.73 with chemosuppression of 48.1%.

**Table 4:** Percentage suppression of the embelin against *Plasmodium berghei* in mice.

<table>
<thead>
<tr>
<th>Drug/Embelin</th>
<th>Dose (concentration) mg/kg/day</th>
<th>% Parasitaemia ±SEM</th>
<th>% Suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.2 ml</td>
<td>47.12±3.21</td>
<td>–</td>
</tr>
<tr>
<td><em>Embelin</em></td>
<td>100</td>
<td>24.6±1.73</td>
<td>48.1</td>
</tr>
<tr>
<td><em>Embelin</em></td>
<td>200</td>
<td>23.220±2.64</td>
<td>50.72</td>
</tr>
<tr>
<td><em>Embelin</em></td>
<td>400</td>
<td>21.32±3.82</td>
<td>55.00</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>25</td>
<td>0.00*</td>
<td>100</td>
</tr>
</tbody>
</table>

*The mean value is significant (*P*<0.001) when compared with the negative control; data are expressed as means ± SEM for five mice per group

The embelin prolonged the mean survival time of the study mice indicating that the embelin suppressed *Plasmodium berghei* and reduced the overall pathologic effect of the parasite on the study mice. The mean survival time of mice of the given embelin was shown to be 7.80±0.41, 8.00±0.71, 9.00±0.00 days, for doses of 100, 200 and 400 mg/kg/day, respectively, which was statistically significant (*P*<0.002), when compared to vehicle treated mice (5.6±.55 days). All animals treated with the standard drug, chloroquine 25 mg/kg/day, survived more than 28 days.
Table 5: Mean survival time of *Plasmodium berghei* infected mice after treatment with Embelin

<table>
<thead>
<tr>
<th>Drug/Isolate</th>
<th>Dose (concentration) mg/kg/day</th>
<th>Survival time ± SEM (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.2 ml</td>
<td>5.6±0.55</td>
</tr>
<tr>
<td>Embelin</td>
<td>100</td>
<td>7.8±0.44</td>
</tr>
<tr>
<td>Embelin</td>
<td>200</td>
<td>8.00±0.71</td>
</tr>
<tr>
<td>Embelin</td>
<td>400</td>
<td>9.00±0.00**</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>25</td>
<td>28.00±0.00*</td>
</tr>
</tbody>
</table>

The body weight of each mouse in all groups was taken before infection (day 0) and on day 4. There was a significant (p<0.05) loss of body weight between days 0 and 4 in both negative control and embelin treated groups. Treatment with embelin did not prevent body weight loss due to parasitemia. the loss of body weight in the embelin treated mice was possibly due to appetite suppressant effect of the embelin. This result is in agreement with that of a previous study on other plants (Chinchilla et al., 1998).

Table 6: Body weight of *Plasmodium berghei* infected mice after the administration.

<table>
<thead>
<tr>
<th>Drug/extract</th>
<th>Dose (concentration) mg/kg/day</th>
<th>Weight D0 ±SEM</th>
<th>Weight D4 ±SEM</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.2 ml</td>
<td>24.34±0.34</td>
<td>23.04±1.21</td>
<td>-1.3</td>
</tr>
<tr>
<td>Embelin</td>
<td>100</td>
<td>22.3±0.70</td>
<td>20.94±1.57</td>
<td>-1.34</td>
</tr>
<tr>
<td>Embelin</td>
<td>200</td>
<td>22.94±0.13</td>
<td>21.46±1.61</td>
<td>-1.48</td>
</tr>
<tr>
<td>Embelin</td>
<td>400</td>
<td>24.04±0.20</td>
<td>23.24±1.75</td>
<td>-0.80</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>25</td>
<td>22.34±0.54</td>
<td>23.39±1.73</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM for five mice per group; D0: weight pre-treatment on day zero; W4: weight post-treatment on day five.
5.2.3. In vivo Antimalarial Activity of the Synthesized Compounds

The standard four day suppressive test was used to evaluate the antimalarial activities of the synthesized compounds on \textit{P. berghei} infected mice. Chloroquine phosphate (25 mg/Kg/day was use as a positive control and a vehicle containing 7% tween 80, 3% absolute ethanol in distilled water as a negative control. at the doses of 100, 200 and 400 mg/kg/day of the synthesized compounds were administered through the oral route after dissolving in a vehicle containing 7% tween 80, 3% absolute ethanol in distilled water. The percent suppression, percent parasitemia, and mean survival time of the mice treated with the synthesized compounds were compared against the control groups.

The results of this study indicated that compound (1A-3A) possesses activity against \textit{Plasmodium berghei} malaria parasite \textit{in vivo}. The result also revealed that compounds (1A-3A) have dose dependent activity. The chemosuppression was significant ($P<0.001$) when compared with the negative control as well as the parent compound. In the same assay, on day 4, chloroquine had a chemosuppression of 100% at the dose level of 25 mg/kg/day and showed significant ($P<0.05$) suppression when compared to compounds (1A-3A) treated groups (Table7).

Compound 1A has less hydrophilic than embelin but it more basic character than embelin due to the present of amine substituens which is important to enhance $pK_a$ due to protonation of anime group. This may important to accumulate compounds in the acidic digestive vacuole contents where they undergo protonation. Compound 2A has ortho substituted methyl group which may has steric hindrance to protonation of amine group after accumulation of acidic digestive vacuole finally less antimalarial activity was observed. Compound 3A have enhanced
hydrophilic property due to hydrogen bonding of the hydroxy group with the benzoquinone lowered pKa. The replacement of carbonyl group by imine may affect the benzoquinone structure which acts as electron transporting chain in the parasites. Compound 3A also More acidic than embelin as well as compound 1A and 2A. These may reduce the activity of compounds by affecting accumulation of the compounds in the acidic digestive vacuole contents.

According to Rudrapal et al. (2011), structural activity relationship studies the replacement of 4’-OH group in 4-aminoquinolines structure by several amino substituents, particularly in 5’-position, was provide interesting antimalarial activities. 4-aminoquinolines accumulate at high concentrations into the parasite’s acid food vacuole, which is their site of action. By taking this into account, the stronger basicity of the molecule increases the antimalarial activity due to a better uptake in the vacuole owing to the pH gradient between cytosol and the acidic vacuole.

As the presence of mono substituents amine group seems important to improve basicity of embelin and we designed a new series of 5-amino and 1- imine analogs of embelin which expected to have better activity than the parent compound.
Table 7: Percentage suppression of the synthesized compounds (1A-3A) against *Plasmodium berghei* in mice

<table>
<thead>
<tr>
<th>Synthesized compounds /drug</th>
<th>Dose (concentration) mg/kg/day</th>
<th>% Parasitaemia ±SEM</th>
<th>% Suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.2 ml</td>
<td>51.53±8.32</td>
<td>-</td>
</tr>
<tr>
<td>Compound(1A)</td>
<td>100</td>
<td>22.84±1.49</td>
<td>55.7</td>
</tr>
<tr>
<td>Compound(1A)</td>
<td>200</td>
<td>17.92±5.46</td>
<td>65.2</td>
</tr>
<tr>
<td>Compound(1A)</td>
<td>400</td>
<td>13.06±3.19</td>
<td>74.7</td>
</tr>
<tr>
<td>Compound(2A)</td>
<td>100</td>
<td>26.20±2.79</td>
<td>49.2</td>
</tr>
<tr>
<td>Compound(2A)</td>
<td>200</td>
<td>25.16±2.44</td>
<td>51.22</td>
</tr>
<tr>
<td>Compound(2A)</td>
<td>400</td>
<td>22.31±4.98</td>
<td>58.65</td>
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<tr>
<td>Compound(3A)</td>
<td>100</td>
<td>24.00 ±1.87</td>
<td>53.4</td>
</tr>
<tr>
<td>Compound(3A)</td>
<td>200</td>
<td>23.80±1.78</td>
<td>53.8</td>
</tr>
<tr>
<td>Compound(3A)</td>
<td>400</td>
<td>21.80±2.16</td>
<td>57.7</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>25</td>
<td>0.00±0.0*</td>
<td>100.00</td>
</tr>
</tbody>
</table>

The synthesized compounds significantly inhibited parasitemia (p <0.05) dose-dependently. Compound (1A-3A) increased the survival time of infected mice and none of the compounds, however, prevented body weight loss.
Table 8: Mean survival time of *Plasmodium berghei* infected mice after treatment with compounds obtained from the synthesis

<table>
<thead>
<tr>
<th>Synthesized comp/drug</th>
<th>Dose (concentration)</th>
<th>Survival time ±SEM (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.2 ml</td>
<td>6.20±1.37</td>
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<tr>
<td>Compound(1A)</td>
<td>100</td>
<td>7.80±.44</td>
</tr>
<tr>
<td>Compound(1A)</td>
<td>200</td>
<td>7.90±.54</td>
</tr>
<tr>
<td>Compound(1A)</td>
<td>400</td>
<td>8.00±.00</td>
</tr>
<tr>
<td>Compound(2A)</td>
<td>100</td>
<td>7.00±1.26</td>
</tr>
<tr>
<td>Compound(2A)</td>
<td>200</td>
<td>7.00±1.00</td>
</tr>
<tr>
<td>Compound(2A)</td>
<td>400</td>
<td>7.40±.89</td>
</tr>
<tr>
<td>Compound(3A)</td>
<td>100</td>
<td>6.50± .57</td>
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<td>Compound(3A)</td>
<td>400</td>
<td>7.70±.67</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>25</td>
<td>28.00±00*</td>
</tr>
</tbody>
</table>

***The mean value is significant (*P*<0.05) when compared with vehicle treated group; **the mean value is significant (*P*<0.01) when compared with vehicle treated group; *the mean value is significant (*P*<0.001) when compared with vehicle treated group; data are expressed as means ± SEM for five mice per group.
Table 9: Body weight of *Plasmodium berghei* infected mice after the administration of synthesized compounds.

<table>
<thead>
<tr>
<th>Synthesized comp/drug</th>
<th>Dose (concentration) mg/kg/day</th>
<th>Weight D0 ±SEM</th>
<th>Weight D4 ±SEM</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.2 ml</td>
<td>23.00±.70</td>
<td>22.42±0.54</td>
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<tr>
<td>Compound(1A)</td>
<td>200</td>
<td>23.00±.21</td>
<td>21.66±.59</td>
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<tr>
<td>Compound(1A)</td>
<td>400</td>
<td>23.20±.16</td>
<td>22.52±1.22</td>
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<tr>
<td>Compound(1A)</td>
<td>600</td>
<td>24.12±.16</td>
<td>24.64±1.07</td>
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</tr>
<tr>
<td>Compound(2A)</td>
<td>200</td>
<td>22.24±.23</td>
<td>21.20±1.48</td>
<td></td>
</tr>
<tr>
<td>Compound(2A)</td>
<td>400</td>
<td>22.28±.17</td>
<td>22.62±1.07</td>
<td></td>
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<tr>
<td>Compound(2A)</td>
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<td>22.56±.13</td>
<td>20.98±1.16</td>
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<tr>
<td>Compound(3A)</td>
<td>200</td>
<td>22.06±.89</td>
<td>20.22±.83</td>
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<tr>
<td>Compound(3A)</td>
<td>400</td>
<td>22.10±.17</td>
<td>20.24±.93</td>
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</tr>
<tr>
<td>Compound(3A)</td>
<td>600</td>
<td>23.60±.089</td>
<td>22.40±.89</td>
<td></td>
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<tr>
<td>Chloroquine</td>
<td>25</td>
<td>24.18±0.17</td>
<td>24.28±2.02</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM for five mice per group; Weight D0: weight pre-treatment on day zero; D4: weight post-treatment on day five.

Though the target compounds contain both a long aliphatic substituents (essential for strong hydrophobic interaction with the back bone of the receptor) and hydroxyl group (essential for the formation of hydrogen bonding with the back bone of the receptor) at the 5-position of the embelin moiety, in addition to improved basic characteristic of the compounds. These results offer an invaluable guide to decisions regarding which drugs to combine in the next-generation of antimalarial drugs.
6. Conclusion

In this study, the acute toxicity and antiplasmodial evaluations of embelin and some aromatic amine substituted embelin derivatives were carried out. From the results of this study it can be concluded that embelin and synthesized compounds are relatively safe to mice. All the synthesized compounds displayed potential antimalarial activities as compared to negative control. Evaluation of the antimalarial effect of the synthesized compounds, compound 1A indicated that highly active than the parent compound Thus, 5-(p-toluidino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione containing p-toluidino substitution at 5-position represent a fruitful matrix for the development of antimalarial agents. From the foregoing, it can be concluded that embelin could be good candidates for the semi synthesis of antimalarial agents from natural therapeutic agents giving scientific support for their traditional claim.
7. **Recommendation**

As embelin used as a lead compound for semi synthesis of antimalarial agents, further studies should be carried out on these compound as well as semi synthesized compounds. Therefore, the following additional investigations are proposed:

1. The mechanisms of action of most active compounds observed on antimalarial study need to be determined.
2. *In vitro* studies should be conducted for the tested compounds as they have potent *in vivo* antimalarial activity.
3. Isolation of other minor compounds from the ethyl acetate and methanol fraction and investigate their antimalarial activities.
4. Synthesize other embelin derivatives and evaluate for their antimalarial activity
References


WHO. (2007). Special program for research and training in tropical disease 1-44.


Appendix

Appendix 1: List of quinine and Related Drugs includes Quinine (1), quinidine (2), CQ (3), primaquine (PRQ) (4), amodiaquine (AQ) (5), mefloquine (MQ) (6), piperaquine (PQ) (7), pyronaridine (PYR) (8), and the amino alcohol lumefantrine (LUM) (9).
Appendix 2: List of Antifolates and Antimalarials includes Proguanil (10), chlorproguanil (11), pyrimethamine (12), and trimethoprim (13) and sulfa drugs: Dapsones (14), sulfamethoxazole (15), sulfadoxine (16) atovaquone (17) tetracycline (18) doxycycline (19) and clindamycin (20) (Kalra et al., 2006; Watkins, 1997; Schlitzer, 2008; Hellgren et al., 2010)
Appendix 3: List of Artemisinins and Artemisinin Combination Therapies (ACTs) includes Artemisinin (21) dihydroartemisinin (DHA) (22), artemether (ART) (23), artether (24) and artesunate (AS) (25) (Carrara et al., 2006; Dondorp et al., 2005).

23: $R = \text{CH}_3$; 24: $R = \text{C}_2\text{H}_5$; 25: $R = \text{COCH}_2\text{CH}_2\text{COOH}$

Appendix 4: List of Benzoquinone derivatives extracted from the stem part of the *Myrsine africana* L (Myrsinaceae) includes (1) 2,5-dihydroxy-3- (nonade- 14- enyl)- benzoquinone (2) maesol (3,4).
Appendix 5: List of novel flavonol glycosides extract from *Emelia schimperi* leaves (28 and 29) (Lawrence et al., 2004). Also being reported

(28) R = galactosyl (1® 4)-galactoside, R1 = Me; 2 R = [rhamnosyl (1® 2)][rhamnosyl (29) R= rhamnoside,(1® 4) R1 = H; 3 R = glucose, R1 = Me; 4 R = rhamnose R1 = H (Lawrence et al., 2004).

Appendix 6: List of 5oleanane-type triterpenes extract from *Emelia schimperia* leaves

(1) R=R1= OAc, R2=R3 H (2) R=OAc, R1=R2=O, R3=H (3) R=OAc, R1=OH, R2= R3=H (4) R=OAc, R1= OH, R2=H, R3=O, (5) R= R3=OH, R1=R2 =O
Appendix 7: List of Pentacyclic triterpenoids

Appendix 8: Some of Semi-synthetic Artemisinin Analogs (Kaur et al., 2009)

(269), 1,4-diacetoxy-3-methylcarbazole

(270), 1-hydroxy-3-methylcarbazole

(271)

(272)
Appendix 10: Some of synthesized 1, 4 benzoquinone derivatives
Appendix 11: IR spectrum of compound A (embelin) In Nujol
Appendix 12: $^1$H NMR spectrum of compound A (embelin) in CDCl$_3$

Appendix 13: $^{13}$C NMR spectrum of compound A (embelin) in CDCl$_3$
Appendix 14: DEPT-135 spectrum of compound A (embelin) in CDCl

Appendix 15: HR-ESIMS of Compound 1A
Appendix 16: IR of compound 1A in Nujol.

Appendix 17: $^1$H NMR spectrum of compound 1A in CDCl$_3$
Appendix 18: $^{13}$C NMR spectrum of compound 1A in CDCl$_3$

Appendix 19: DEPT-135 spectrum of compound 1A in CDCl$_3$
Appendix 20: HR-ESIMS of Compound 2A

Appendix 21: IR spectrum of compound 2A in Nujol.
Appendix 22: $^1$H NMR spectrum of compound 2A in CDCl$_3$

Appendix 23: $^{13}$C NMR spectrum of compound 2A in CDCl$_3$
Appendix 24: DEPT-135 spectrum of compound 2A in CDCl₃
Appendix 25: HR-ESIMS of Compound AE 3A

Appendix 26: IR spectrum of compound 3A in Nujol.
Appendix 27: $^1$H NMR spectrum of compound 3A in CDCl$_3$

Appendix 28: DEPT-135 spectrum of compound 3A in CDCl$_3$