

# A Study on Toxicity and Antimalarial Efficacy of *Ocimum Gratissimum*, Influence on Lung Antioxidant Defense and Nrf2 Expression in *Plasmodium Berghei* (Nk65) Infected Mice

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<https://dx.doi.org/10.13005/bpj/3271>

(Received: 07 June 2025; accepted: 27 August 2025)

Malaria chemotherapy, the mainstay of treatment is currently failing due to the emergence and spread of resistance of the causative parasites to conventional drugs endangering public health. The most severe complication of malaria is pulmonary involvement which occurs in 3-10% of the infection with about 70% mortality. So, discovery of antimalarial therapeutic agent with the capacity to combat associated lung complications is imperative. In Nigeria, herbs, like *Ocimum gratissimum* are used to treat malaria and associated fevers. Hence, this study evaluated the qualitative phytochemical constituents, toxicity, and antimalarial properties of *O. gratissimum* with its capacity to resolve the malarial-induced disturbances in lungs. The curative murine models and standard methods were adopted for the study. Results indicates that *Plasmodium berghei* (NK65) malarial parasite induced biochemical derangement in lungs of experimental adult male BALB/c mice, traceable to free radical-elicited lipid peroxidation of lung's membrane, occasioned by the down regulation of the tissue's Nuclear Factor Erythroid 2 Related Factor (Nrf2) as evidenced by its immunohistochemical expression. However, the ethanol extract of *O. gratissimum* (EEOg) was observed to be non-toxic with rich medicinal antimalarial phytochemicals capable of curing malaria and combating the associated lung complications by stimulating the expression of Nrf2 in lung tissue which enhanced the level of reduced glutathione (GSH) and activities of superoxide dismutase (SOD) and catalase antioxidants in the lungs of malarial infected mice treated with EEOg (125, 250, 500 mg/kg) and standard drug Artemether-lumefantrine (LDS). Our computational study identified eugenol as the most active compound with drug-likeness property which strongly inhibited *Plasmodium falciparum* lactate dehydrogenase (PfLDH), and modulated Keap1-Nrf2/p62 pathway. These properties indicate that eugenol is a potential antimalarial and antioxidant compound which could be optimized for enhanced performance as potent antimalarial agent, capable of resolving the associated lung impairments and oxidative stress.

**Keywords:** Antimalaria; Antioxidant; Eugenol; Lungs; *Ocimum gratissimum*.

The escalating worldwide prevalence of drug-resistant *Plasmodium* strains, particularly in endemic areas, is a critical concern to global health.<sup>1</sup> Malaria infection induces oxidative stress, which is characterized by an imbalance in the host's

redox system as a result of excessive formation of reactive nitrogen and oxygen species (RNS-OS).<sup>2</sup> RNS-OS play dual effect in the human system, serving as signaling molecules under physiological conditions, but inducing oxidative stress when

produced uncontrollably.<sup>3,4</sup> The source of these oxidizing compounds includes the degradation of haemoglobin by the parasite and the host's immune response, leading to respiratory burst and ischemia-reperfusion syndrome.<sup>5</sup> This oxidative stress contributes to systemic and tissue damage, particularly in organs like the lungs and brain, with severe manifestations which include acute respiratory distress syndrome acute lung injury.<sup>6</sup> Pulmonary alterations occur in 3-10% of the cases of *P. falciparum* malaria and represents the most severe complications of malaria with 70% lethality.<sup>6</sup>

Consequently, the Nrf2 emerges as a crucial moderator of resistance to oxidative stress, with studies highlighting its role in cellular protection.<sup>7</sup> In humans, Nrf2 is encoded by nfe2l2 gene and it belongs to the cap 'n' collar family due to the basic leucine zipper in its structural composition.<sup>8</sup> Initially identified as a homolog of the nuclear factor-erythroid 2 p45 (NF-E2) associated with  $\alpha$ -globin gene expression.<sup>7, 9</sup> Nrf2 was later found to bind to the antioxidant responsive element (ARE) in deoxyribonucleic acid, activating the expression of antioxidant enzymes and detoxifying enzymes (Phase II) which include cysteine ligase, glutamate NAD(P)H dehydrogenase (quinone 1) and haem oxygenase-1 (HO-1).<sup>10, 11</sup>

The ARE, also known as the electrophile response element, is a crucial cis-acting enhancer sequence located in the promoter section of most genes coding for phase II and antioxidant enzymes. It is involved in the expression of inducible genes which respond to oxidative stress and associated with low-level basal gene expression to antagonize RNS-OS formed during cellular respiratory processes.<sup>2, 13</sup>

Under basal conditions, Nrf2 associates with the Kelch-like ECH-associated protein 1 (Keap1), leading to Nrf2 ubiquitination and subsequent degradation through the proteasome.<sup>14</sup> However, under cellular stress, this association is disrupted, preventing Nrf2 degradation and allowing its accumulation within the cell. This results in elevated translocation of Nrf2 into the nucleus, heterodimerization occurs with nuclear factors and then binds to AREs, thereby activating antioxidant gene transcription. The complex transcriptional machinery involved in this process

includes co-activators like receptor-associated co-activator (RAC3) and co-regulators such as CREB binding protein.<sup>15, 16</sup> The activation of antioxidant genes, then, lowers oxidative stress.

In murine study, *Plasmodium berghei*-mediated Nrf2 activation in infected hepatocytes enhanced parasite survival.<sup>17</sup> However, it has been demonstrated that Nrf2 protects against cerebral malaria (CM) by inducing HO-1,<sup>18</sup> but protects the spleen by inducing p62 expression which binds to Keap1 and increases free Nrf2 level.<sup>17</sup> It has been observed that respiratory symptoms are common among African children with uncomplicated malaria (ECDC, 2017), and in adults, a frequency of 4-18% has been reported.<sup>18</sup> Therefore, the role of Nrf2 in protecting the lungs during malarial infection is not clear. Hence, the need to search for inexpensive, more effective, easily accessible and safer bioactive compounds from natural products, particularly, herbal plants used in traditional treatment of malaria has become urgent and imperative.

*Ocimum gratissimum*, commonly known as clove basil or African basil is one of such medicinal plants.<sup>19</sup> *Ocimum gratissimum* has been recognized for its diverse therapeutic properties, and its leaves have been traditionally employed for various medicinal purposes. Notably, plants extract exhibit antioxidant potential, attributed to their phytochemical constituents.<sup>19</sup> Hence, this research aims to explore the *in vivo* antimalarial activity of *Ocimum gratissimum* and its relationship to the expression of lung's Nrf2 and associated oxidative damage. The overall objective is to contribute valuable insights into the potential of *Ocimum gratissimum* as a natural resource for combating malaria and understanding its impact on oxidative stress and parasite survival in the lungs.

## MATERIALS AND METHODS

### Plant collection and authentication

The plant (*Ocimum gratissimum*) was harvested fresh from their natural habitat in Abraka, Ethiope East Local Government Area of Delta State, Nigeria, in the month of May, 2023. It was identified and authenticated by Mr. O.E. Michael, Delta State University Herbarium, Abraka, Nigeria (Voucher Number, DELSUH 196).

### Preparation of Ethanol Extract

The plucked fresh leaves were washed, air-dried at room temperature, pulverized, macerated in ethanol (1:8 w/v) for 72 h and filtered. The obtained filtrate was concentrated 45 °C with the aid of rotary evaporator and water bath, labelled and, refrigerated at 4°C.

### Phytochemical Profiling

The phytochemical composition of the prepared EEOg was investigated using standard procedures and documented methods. Tannins, phlobatannins, saponins, steriods and terpenoids were screened following the procedures outlined by Ezeonu and Ejikeme *et al.*<sup>20</sup> Furthermore, flavonoids, alkaloids and glycosides were estimated using the methods described by Sofowara,<sup>21</sup> while, anthraquinones, phenols, and triterpenoids were identified according to the techniques stated by Sharma *et al.*,<sup>22</sup> Shah and Yadav,<sup>23</sup> and Hasmi *et al.*,<sup>24</sup> respectively.

### Ethical Approval

All procedures were carried out in accordance with the approved guidelines for animal use by the Research, Ethics and Grants Committee, Faculty of Basic Medical Sciences, Delta State University (RBC/FBMC/DELSU/25/794).

### Oral Acute Toxicity

Oral acute toxicity was assessed *in vivo* through a single oral administration of the EEOg at a dose of 5,000 mg/kg to five (5) mice. Thereafter, the mice were monitored hourly for 3 hours and then, observed for 14 days in line with the protocol described by Pouaha.<sup>25</sup>

### Malarial Curative Test

Thirty (30), adult male BALB/cmice were maintained under standard housing condition, fed with chow and water *ad libitum* and acclimatized for a week before the study was commenced.

### Study Design

Twenty-five (25) mice were peritoneally inoculated with *P. berghei* (NK 65) malarial parasite infected blood containing about  $1 \times 10^7$  parasites/mL. Inoculation success was confirmed 72 hours after, and the mice were grouped (n= 5) as follows:

Group 1 Mice (Naïve); were neither infected nor treated (NINT). Group 2 Mice (Sham); were infected, but not treated (INT). Group 3 Mice (Standard treatment); were infected and treated

with 10 mg/kg b.wt of the standard ACT drug used for the treatment of malaria, containing; Artemether (80mg) and Lumefantrine (480mg). Groups 4-6 Mice; were infected and treated with 125, 250 and 500 mg/kg body weight of the ethanol extract of *Ocimum gratissimum*.

### Animal euthanization and sample collection

Blood samples were collected from the cut tail tip at Day 0 (end of acclimatization and inoculation), Day 3 (confirmation of parasitaemia and commencement of the 4-day treatment), Day 6 (end of treatment), Day 9 (third day post treatment) and Day 12 (sixth day post treatment), for determination of malarial parasite.

After the Day 12, the mice were euthanized by cervical decapitation and blood samples were collected by ocular puncture using capillary tube and then, placed in plain, sterile tubes and centrifuged (10,000 xg for 10 mins at room temperature) to obtain serum for lung function (bicarbonate, glucose and lactate) assays. Laparotomy was carried out to expose the internal organs and lungs were excised and prepared for parasite count, tissue antioxidant and immunohistochemical evaluations.

### Parasitemia Assessment

Thin smears were made on a microscope slide from the collected blood samples, smears were fixed with methanol, stained with field stain A and B, and viewed microscopically using a X 100 objective lens to determine the parasite density.<sup>26</sup>

### Collection of bronchoalveolar lavage fluid (BALF)

Tracheostomy was performed, cannula was inserted into the trachea, 2 mL of sterile saline was instilled into the lungs and the BALF was collected and centrifuged (500 x g for 10 min) to remove debris and cells.<sup>27</sup>

### Estimation of lung function markers

Markers of lung function (bicarbonate, glucose, and lactate) in both serum and BALF were determined using kits (Sigma-Aldrich) according to the manual instructions.

### Determination of lung tissue antioxidants and marker of oxidative stress

The collected lungs were washed in an ice cold 1.15% potassium chloride solution, homogenized with 0.1M phosphate buffer (pH 7.2), centrifuged at 2500 rpm for 15 min. and

the supernatant was decanted. The resultant was analysed for antioxidants and oxidative stress marker.

#### Lung Antioxidant Assay

The activities of superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) in the lung tissue homogenate were estimated spectrophotometrically in line with the method of Misra and Fredorich,<sup>28</sup> Cohen *et al*<sup>29</sup> and Ellman,<sup>30</sup> respectively. The breakdown product of lipid peroxidation, malondialdehyde (MDA) was evaluated in line with the method of Gutteridge and Wilkins.<sup>31</sup>

#### Immunohistochemical staining of lung tissue for Nrf2 expression

Method of Kansanen *et al*<sup>32</sup> was adopted for the staining of formalin-fixed and paraffin-embedded (FFPE) lung tissue sections (4-6  $\mu$ m thickness). The stained sections were developed and counterstained with haematoxylin and mounted with permount mounting medium. Nrf2 staining intensity and distribution in the lung tissue sections were evaluated using light microscope.

#### Data Analysis

Results were presented as Mean  $\pm$  Standard Error of Mean (SEM) for n=5 mice per group. Data were subjected to ANOVA using GraphPad prism (v. 10.4.0; 2024), and  $p < 0.05$  was taken as statistically significant.

## RESULTS

### Qualitative Phytochemistry of *Ocimum gratissimum* Ethanol Leaf Extract

The findings from the qualitative phytochemical evaluation of *Ocimum gratissimum* ethanol extract (EEOg) are shown in Table 1.

The results (Table 1) indicate high amounts of cardiac glycosides, triterpenoids, phenols and alkaloids, but moderate level of flavonoids with low saponin content.

#### Toxicity Assessment

The oral acute toxicity of EEOg was evaluated to determine the safety limits for selection of appropriate dosing regimens for the murine study. During the assessment, no mortality was recorded within the first day and the tracked duration of 14 days following the administration of 5,000 mg/kg body weight of the mice. Also, the animals did not display any outward sign of

abnormalities like posture, diarrhoea, lacrymation, movement, salivation, tremors, loss of appetite, and depression. These observations showed that the LD<sub>50</sub> of EEOg is  $> 5,000$  mg/kg; indicating non-toxicity. Therefore, 2.5% (125 mg/kg), 5% (250 mg/kg), and 10% (500 mg/kg) doses of EEOg were selected for the curative study using murine model.

#### Parasite levels, suppression and mean survival time of infected mice treatment with EEOg

Changes in parasite levels (Figure 1a) and parasite suppression (Figure 1b) in both blood and lungs induced by the treatment with EEOg, and associated mean survival time, MST, (Figure 1c) as shown as indicated.

#### Expression of Nrf2 in lungs of infected mice

Next, we examined the impact of parasite survival in the lung on the immunohistochemical expression of Nrf2 (Figure 2) and histological grading (Table 2) of the infected lungs.

Figure 2 shows the staining pattern in lungs of malarial infected mice treated with *Ocimum gratissimum* varying doses (125, 250, 500 mg/kg) and controls.

The INT group showed strong staining indicating consistent expressions of Nrf2 but those of the other groups were low. This expression induced inflammation in infected lungs (Table 2) and modified parasite sequestration receptors (Figure 3 a-d) which altogether promoted parasite survival, growth, and infectivity (Table 1).

#### Histological grading

The histological grading of the lung tissue obtained from the malarial parasite infected mice, with and without treatment, including the

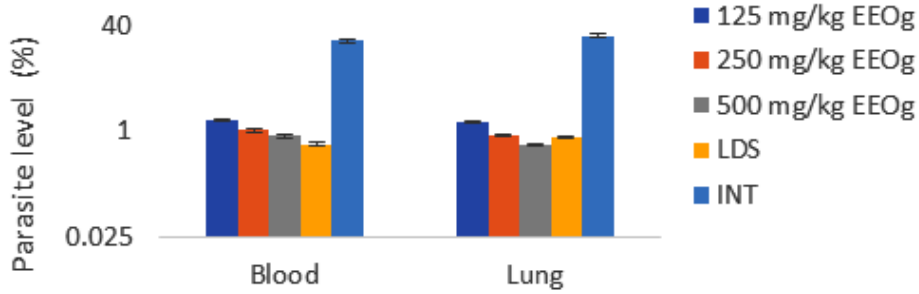
**Table 1.** Qualitative phytochemical contents of *Ocimum gratissimum* ethanol leaf extract

Phytochemical	Inference
Saponins	+
Cardiac glycoside	+++
Flavonoids	++
Tannins	+
Terpenoids	+++
Alkaloids	+++
Phenols	+++

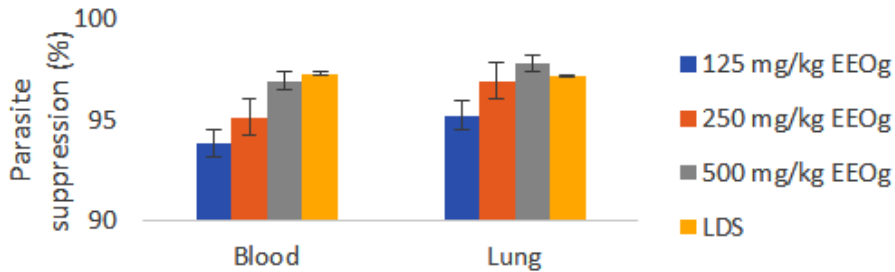
+ Low, ++ Moderate, +++ High

controls are shown in Table 2. Also, data for the sequestration receptors are indicated in Figure 3a-d  
 Peribronchial inflammation for NINT reveals no detectable inflammation, LDS shows occasional cuffing with inflammatory cells.

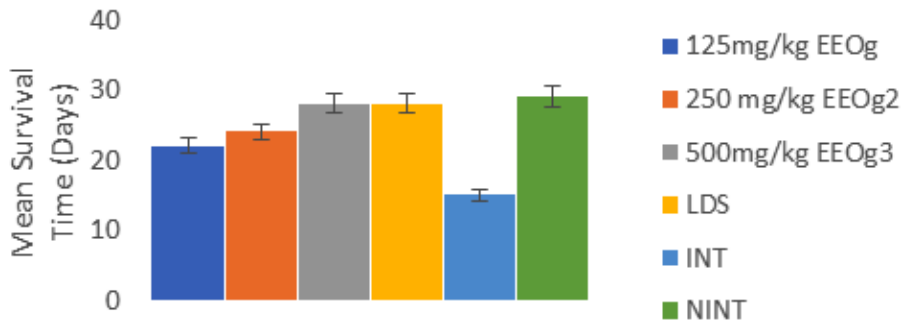
However, INT indicates that most bronchi or vessels were surrounded by a thin layer (1-5 cells thick) of inflammatory cells, but 125 mg/kg like the LDS showed occasional cuffing with inflammatory cells, and 250 mg/kg like the NINT,



**Fig. 1a.** Changes in parasite levels in blood and lung of malarial-infected mice treated with EEOg (LDS = Artemether-lumefantrine, INT = Infected, not treated)



**Fig. 1b.** Changes in parasite suppression in blood and lung of malarial-infected mice treated with EEOg (LDS = Artemether-lumefantrine, INT = Infected, not treated)



**Fig. 1c.** Mean survival time of *Plasmodium berghei* malarial parasite infected mice treated with EEOg and tracked for 30 days (LDS = Artemether-lumefantrine, INT = Infected, not treated, NINT = Not infected, not treated)

had no detectable inflammation. So all so, with the 500 mg/kg group.

**Sequestration receptors**

Levels of parasite sequestration receptors (eosinophils, lymphocytes, macrophages, and neutrophils) in lungs and BALF of malarial-infected mice, treated with EEOg are shown in Figure 3 a-d.

Malarial parasite infection in experimental mice induced sequestration of parasites via increase in receptors in lung tissue, hence, the increased level in parasitized mice without treatment (INT), indicating favourable reception, evasion of host immune surveillance and remodelling of the host environment for survival in the lungs (Figure 1a), and this significantly reduced MST (Figure 1c).

Infection of experimental mice with *P. berghei* (NK 65) caused increase amounts of

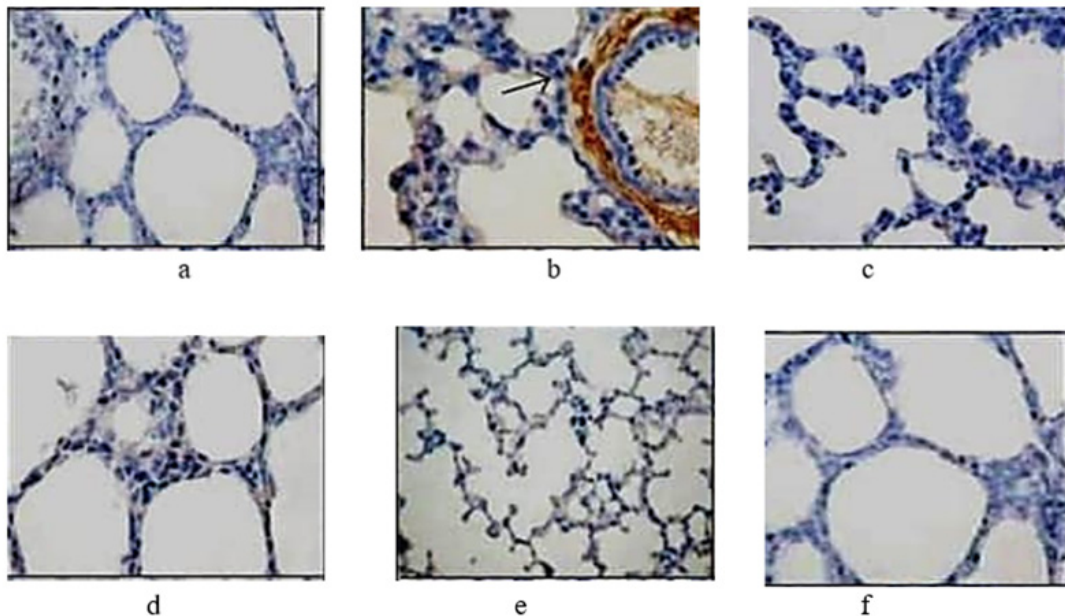
parasite sequestration receptor in infected–lungs and BALF (Figure 3 a-d) hence the elevated load of parasites in the tissue (Figure 1). Such sequestration induced inflammation (Table 2) and oxidative stress (Figure 4 a-d). Changes in lung and BALF oxidative stress marker induced by the treatment of malarial infected mice with ethanol leaf extract of *Ocimum gratissimum*

The changes in lung and BALF oxidative stress marker induced by the treatment of malarial infected mice with ethanol leaf extract of *Ocimum gratissimum* are shown in Figure 4a-d.

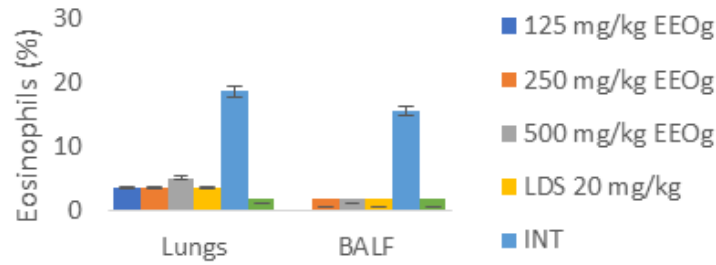
MDA level for the treated groups decreased significantly ( $p<0.05$ ) when compared with INT. GSH, SOD and catalase for the treated groups increased significantly ( $p<0.05$ ) when compared with INT. This indicate that EEOg treatment enhanced antioxidant molecules and

**Table 2.** Histological grading of lung tissue after staining with haematoxylin

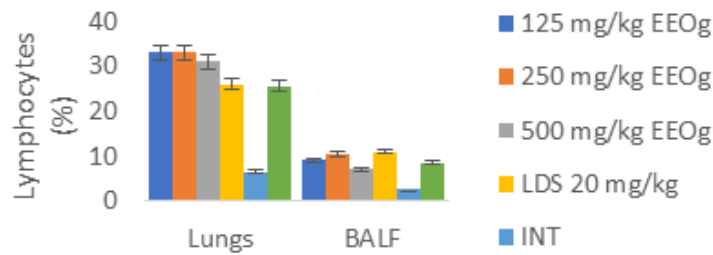
Grading	NINT	LDS	INT	EEOg (mg/kg)		
				125	250	500
Peribronchial inflammation	0	1	2	1	0	0
Perivascular inflammation	0	1	3	1	1	1
Total lung inflammation	0/6	2/6	5/6	2/6	1/6	1/6



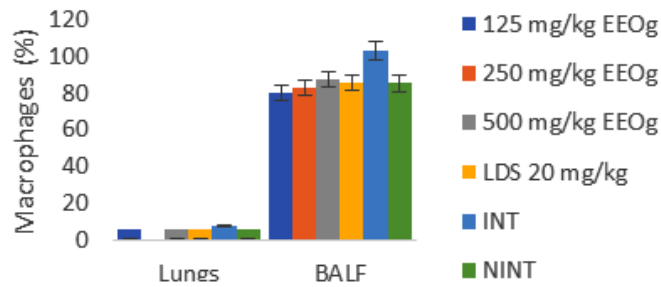
**Fig. 2.** (a-f) indicating Positive control NINT (a), Negative control, INT (b), Standard Control, LDS (c), EEOg 125mg/kg (d), EEOg 250mg/kg (e) and EEOg (500mg/kg (f), INT= infected, but not treated, NINT: No infected, not treated, LDS = Artemether-lumefantrine (Standard drug)



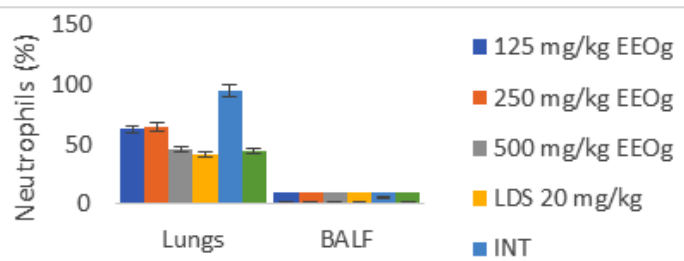
3a. Levels of eosinophils in lungs and BALF of malarial-infected mice treated with ethanol extract of *Ocimum gratissimum* (EEOg)



3b. Amounts of lymphocytes in lungs and BALF of malarial-infected mice treated with EEOg



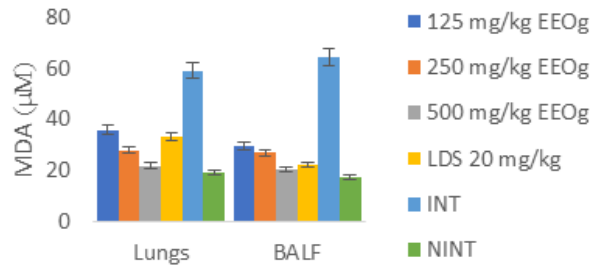
3c. Levels of macrophages in lungs and BALF of malaria infected mice treated with EEOg



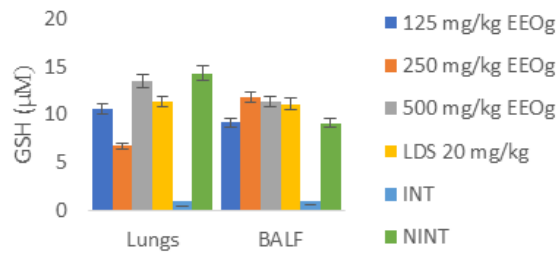
3d. Levels of neutrophils in lungs and BALF of malarial-infected mice treated with (EEOg)

**Fig. 3a-d.** Levels of parasite sequestration receptors (eosinophils, lymphocytes, macrophages and neutrophils) in lungs and BALF of malarial parasite infected mice, with or without treatment by EEOg

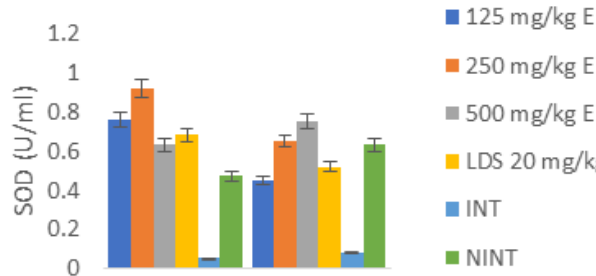
(INT= infected, but not treated, LDS= Lonart®DS (Standard drug; Artemether, 80 mg, Lumefantrine 480 mg), NINT: No infected, not treated)



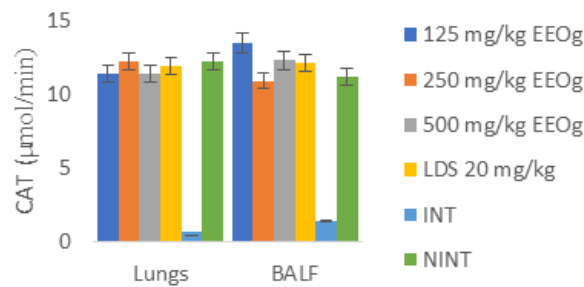
4a. Levels of malonaldehyde (MDA) in lungs and BALF of malarial-infected mice treated with EEOg



4b. Levels of reduced glutathione (GSH) in lungs and BALF of malarial-infected mice treated with EEOg



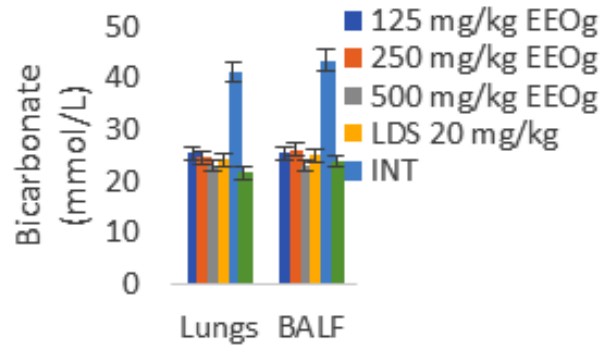
4c. Levels of superoxide dismutase (SOD) in lungs and BALF of malaria infected mice treated with EEOg



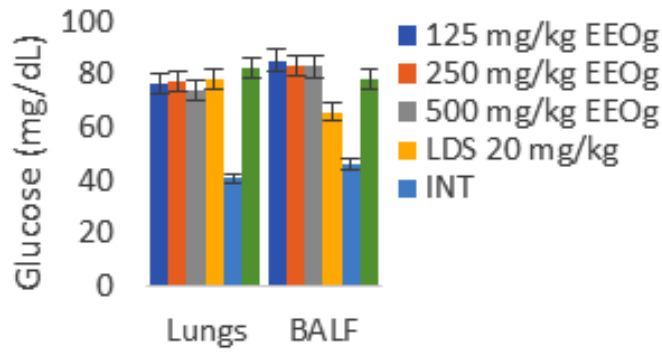
4d. Levels of catalase (CAT) in lungs and BALF of malarial-infected mice treated with (EEOg)

**Fig. 4 a-d.** Changes in oxidative stress biomarkers in lungs and BALF of malarial infected mice, treated with EEOg

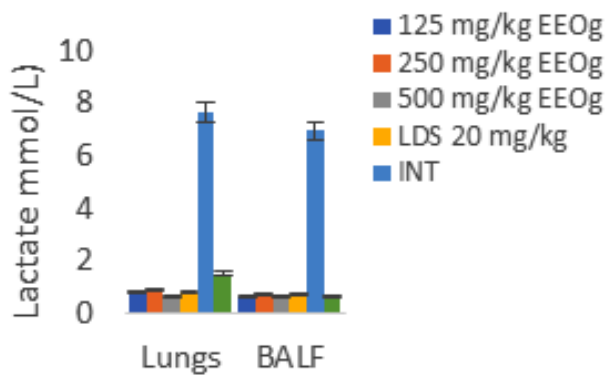
BALF= Bronchoalveolar Lavage Fluid, INT= Infected, not treated, NINT= Non infected, Not treated, LDS= Lonart ®DS, GSH= Glutathione, CAT= catalase, SOD= Superoxide Dismutase, MDA= malonaldehyde.



5a. Levels of bicarbonate in lungs and BALF of malarial-infected mice treated with EEOg



5b. Levels of glucose in lungs and BALF of malarial-infected mice treated with EEOg



5c. Levels of lactate in lungs and BALF of malarial-infected mice treated with EEOg

**Fig. 5a-c.** Changes in markers of lung function and BALF balance in malarial-infected mice treated with or without EEOg

thus, reduce oxidative stress in lungs and BALF of malarial infected mice.

Parasite sequestration in lungs induced inflammation and oxidative stress which affected lung functions (Figure 5 a-c).

Evaluation of biochemical function in lung and BALF of malarial infected mice treated with or without EEOg

Malarial infection in experimental mice induced oxidative stress in lungs and BALF by hampering the synthesis of antioxidant molecules, and the effect of such stress on biochemical markers (bicarbonate, glucose and lactate) of lung function were estimated and results are presented in Figure 5a-c

In the BALF, malarial infection significantly ( $p<0.05$ ) reduced glucose content, but

**Table 3.** Drug likeness scores for the two prioritized compounds

Scores	Prioritized compounds	
	Eugenol	Carvacrol
Lipinski's Rule		
• Molecular weight (g/mol)	164.21	150.22
• LogP	2.73	3.38
• HBD	1	1
• HBA	2	1
• TPSA	2	1
• Rotable bonds	4	3
• Molar refractivity	55.11	48.41
• LogD	2.53 (pH 7.4)	3.18 (pH 7.4)
Pharmacokinetic properties		
• Solubility (water) (mg/mL)	1.9	0.12
• Permeability (HIA) (%)	85.14	92.11
• Bioavailability (%)	65-80	70-90
• Volume of distribution Vd (L/kg)	1.23	1.54
• Clearance (ml/min/kg)	1.15	0.81
• Half-life (hours)	2.5	3.5
Pharmacodynamic properties		
• Target	COX-2, Receptor (TRPV1)	COX-2, Receptor (TRPV1)
• Mechanism	Antioxidant, anti-inflammatory, analgesic	Antimicrobial, antioxidant, anti-inflammatory
• Binding affinity, Ki (μM)	10-50	5-20
Toxicity and Safety		
• LD <sub>50</sub> (oral) (g/kg) rat	2.65	3.4
• LD <sub>50</sub> (dermal) (g/kg) rabbit	>2	>2
• Genotoxicity	Non-genotoxic	Non-genotoxic
• Carcinogenicity	Non-carcinogenic	Non-carcinogenic
Drug-likeness scores		
• DL-Score	0.43 (moderate)	0.53 (good)
• QED	0.61 (good)	0.73 (excellent)
• Drug-likeness probability	0.71	0.85

**Table 4.** Dock scores for eugenol against important parasite (*P. falciparum*) targets (*Pf* LDH)

Targets	PDB ID	Docking score (kCal/mol)	XP Gscore (kCal/mol)	MMGBSA dG Bind (kCal/mol)
<i>Pf</i> LDH	1TQJ	-3.962	-3.963	-38.900

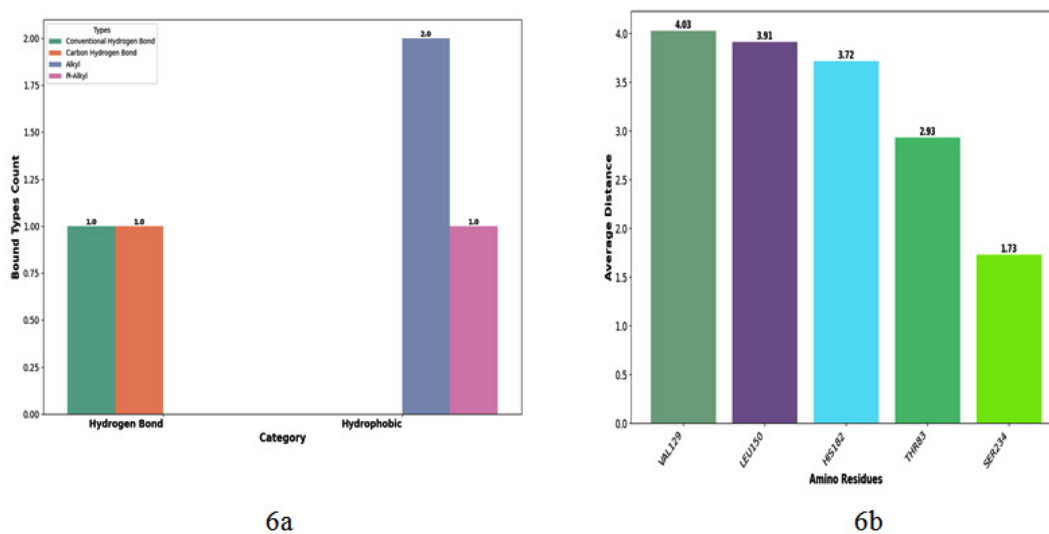


Fig. 6. Post docking analysis of eugenol binding to *Plasmodium falciparum* lactate dehydrogenase

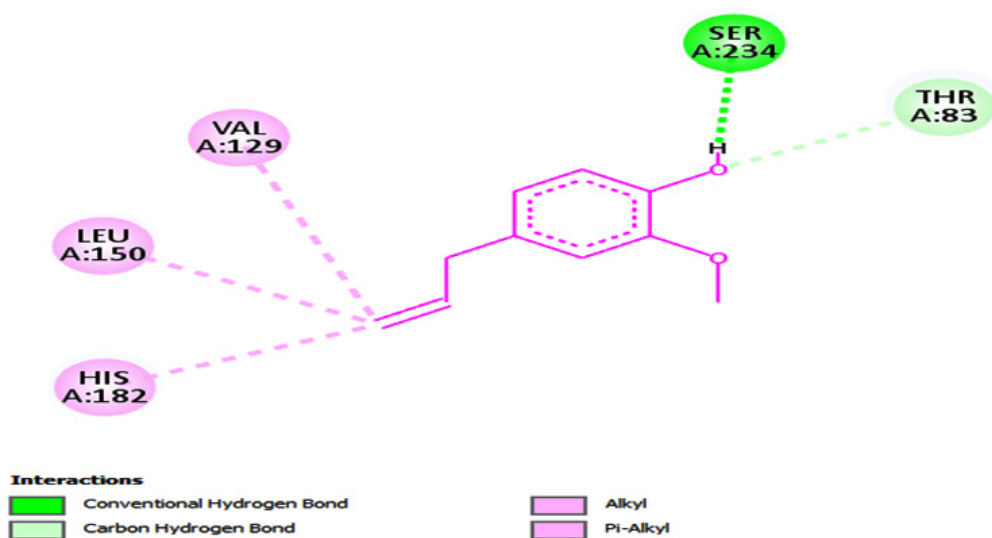


Fig. 7. Bond interaction of eugenol with *PfLDH*

Table 5. Molecular docking scores for eugenol against Keap1

Targets	PDB 1D	Docking score (kCal/mol)	XP Gscore (kCal/mol)	MMGBSA dg Bind (kCal/mol)
Keap1	4CX1	-7.784	-7.784	-35.220

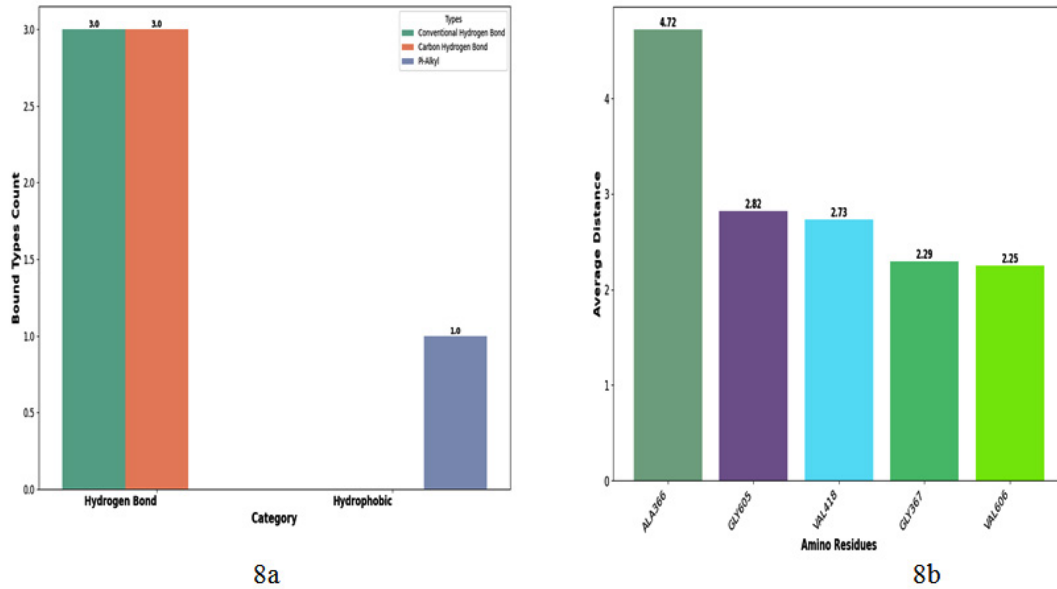
Keap1= Kelch-like ECH-associated protein 1, inhibition of Keap1-p62 by eugenol =75.2%, Nrf2 activation by eugenol=82.1%, Inhibition of Keap1-Nrf2 interactions by eugenol =85.6%

increased ( $p < 0.05$ ) bicarbonate and lactate levels. Similar pattern was observed in the lung. Malarial-infection disturbs biochemical function, possibly due to the increased oxidative stress (Figure 4) and inflammation (Table 2).

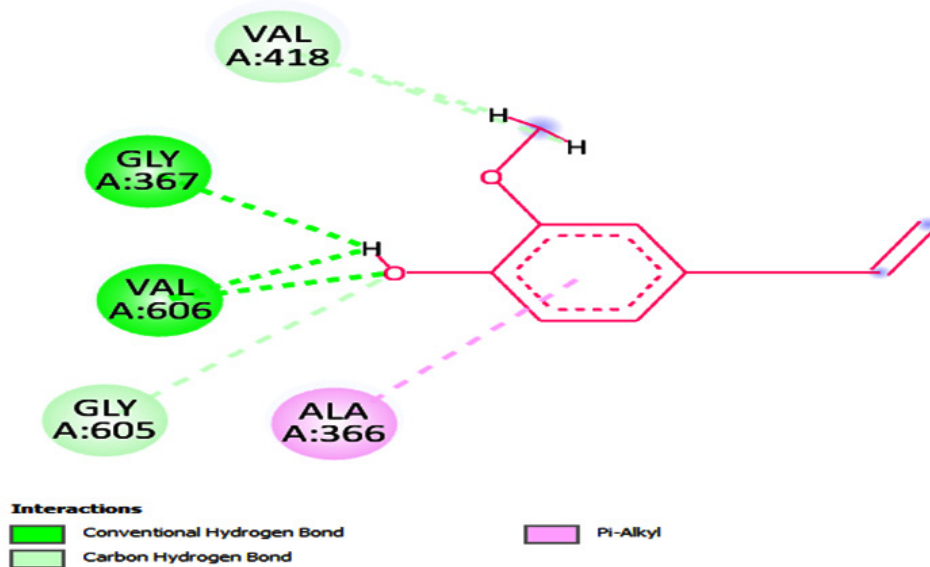
**Effect of treatment of malarial infection with EEOg in mice**

The treatment of *P. berghei* (NK65) parasite induced malaria in experimental mice

showed good anti-malarial activity (Figure 1b) with the additional capacity to significantly ( $p < 0.05$ ) decrease parasite sequestration receptors in lungs (Figure 3), which in turn, reduced ( $p < 0.05$ ) parasite load in the tissue (Figure 1a). The significant reduction in lungs's parasite level halted tissue (lung) inflammation (Table 2), improved antioxidant capacity (Figure 4) and restored cellular



**Fig. 8.** Post docking analysis of eugenol binding to Keap1 [Kelch-like ECH-associated protein 1 [AF-Q14145]



**Fig. 9.** Eugenol binding to Keap1 [Kelch-like ECH-associated protein 1 [AF-Q14145]

biochemical function (Figure 5) which enhanced the mean survival time (MST) of the treated when tracked for 30 days (Figure 1c). These observations compared well with the standard ACT treatment (Lonart®DS).

### Drug Property Test

To evaluate the drug properties of the already identified eighteen (18) compounds in EEOg<sup>[33]</sup>, their drug likeness scores were computationally determined in order to select the compounds with likelihood of being developed into drugs. The drug properties obtained for two prioritized compounds (eugenol and carvacrol) are presented in Table 3.

Following the drug properties analysis, two compounds (eugenol and carvacrol) were prioritized and so, selected based on their profile ranking (Table 3). Eugenol possessed moderate solubility, clearance, lipophilicity and polar surface area, but good oral bioavailability with analgesic, antioxidant and anti-inflammatory properties, and low toxicity.

Carvacrol has moderate lipophilicity, polar surface area, and clearance, but good oral bioavailability and poor solubility. It possesses antimicrobial, antioxidant and anti-inflammatory characteristics with low toxicity. Eugenol and carvacrol, obtained from EEOg demonstrated promising drug-likeness properties, and analysis indicate potential as therapeutic agents in the treatment of especially inflammatory and infectious diseases.

EEOg has been shown to possess good antimalarial activity and two compounds (eugenol and carvacrol) have been identified with drug-likeness properties with potential for combating infectious diseases and associated inflammatory and oxidative stress complications. Following, therefore, the two compounds were docked against *Plasmodium falciparum* protein targets, important for parasite survival and perpetuation of the malarial infection and results (Table 4) showed that eugenol is topmost and inhibited *Pf* LDH (*Plasmodium falciparum* lactate dehydrogenase) the more.

Results (Table 4) indicate that eugenol showed high binding affinities for *Pf*LDH; suggesting interruptions of *P. faciparium* energy metabolism

In all, *Pf*LDH seems to be the primary

target for eugenol. So, the post dock binding analysis, and bond interactions between eugenol and *Pf*LDH are shown in Figures 6 and 7, respectively.

This analysis presents insights into the binding interactions between eugenol and *Plasmodium falciparum* lactate dehydrogenase (*Pf*LDH). The first bar chart (6a) categorizes the types of bonds involved in the binding interaction, showing both hydrogen bonds and hydrophobic interactions. Two hydrogen bonds were identified: one conventional hydrogen bond and one carbon hydrogen bond, each with a count of 1. These hydrogen bonds contribute to the stability and specificity of eugenol's binding with *Pf*LDH. Hydrophobic interactions are also significant, with one alkyl and one  $\delta$ -alkyl interaction, indicating the role of nonpolar interactions in stabilizing eugenol within the enzyme's binding pocket.

The second bar chart (6b) displays the average distances between eugenol and specific amino acid residues in the *Pf*LDH binding site. The residues VAL129 and LEU150 show the longest interaction distances (4.03 Å and 3.91 Å, respectively), while SER234 has the shortest interaction distance at 1.73 Å, suggesting it may play a key role in the binding affinity due to closer proximity to eugenol. Other notable residues include HIS182 and THR83, with distances of 3.72 Å and 2.93 Å, respectively. These distances indicate that various amino acids contribute differently to the binding, with closer residues potentially having stronger interactions with eugenol.

In summary, both hydrogen bonding and hydrophobic interactions are essential in the binding of eugenol to *Pf*LDH, with specific amino acid residues at varying distances playing crucial roles in stabilizing the complex. The presence of close hydrogen bond interactions (e.g., with SER234) and multiple hydrophobic contacts supports the potential effectiveness of eugenol as an inhibitor of *Pf*LDH.

These bond interactions reduce *Pf*LDH activity which disturbs glycolysis and decrease the ability of *P. faciparium* to produce energy and defend oxidative stress.

Alongside malaria, *P. faciparium* induces oxidative stress in the host. To investigate the ability of eugenol to combat this malarial associated complications, molecular docking was

conducted with host's vital targets (Keap1-Nrf2 pathway) and results are shown in Table 5.

The analysis identifies several key interactions between the ligand and specific amino acid residues, revealing a mix of hydrogen bonding and hydrophobic interactions (Figure 8a). ALA536 forms a conventional hydrogen bond with the ligand, although with a relatively large average distance of 4.72 Å, indicating a weaker or spatially distant interaction. GLY605 and VAL418 both engage in carbon hydrogen bonds, with average distances of 2.82 Å and 2.73 Å, respectively, suggesting stronger, closer interactions than ALA536. Also, GLY367 and VAL606 exhibit close contact with the ligand, with distances of 2.29 Å and 2.25 Å, respectively, further stabilizing the interaction network. Lastly, a single R-Alkyl hydrophobic interaction is observed, adding stability to the overall binding complex. Together, these interactions underscore a binding profile dominated by hydrogen bonds, supported by hydrophobic contacts, which collectively enhance the ligand's binding affinity (Figure 8b and 9).

## DISCUSSION

Malaria is a hemoparasitic infection, caused by the *Plasmodium* sp and transmitted by an infected female *Anopheles* mosquito, leading to severe life-threatening disease, ranging from mild to severe clinical forms depending on host's infection history, immune status, parasite virulence, and genetic variations in both host and parasite.<sup>1, 3, 32,</sup>

Respiratory disturbances have been observed in uncomplicated malaria, and are common among African children (ECDC, 2019). However, in adults, respiratory involvement has also been reported in uncomplicated *P. falciparum* malaria with a frequency of 4-18%, and such pulmonary alterations include: impaired ventilation, increased pulmonary phagocytic activity, obstruction of airflow and reduced gas transfer<sup>18</sup>. But, in severe and complicated malaria, multi-organ dysfunctions occur and these include: acute lung injury, ALI, and acute respiratory distress syndrome (ARDS) which contribute to patient's high mortality.

In our present experimental murine study, we observed that *P. berghei* malarial parasite

infection in mice increased the expression of Nrf2 in lungs of the infected mice and this resulted in parasite sequestration and induction of oxidative stress and inflammation in mice (host) pulmonary tissues. Malaria parasite reside within a vacuole created during invasion which act as the main interface between the host and the parasites. These parasites coordinate several activities (uptake of host cell cytosol, nutrient import, effector protein export, and waste efflux) to subvert host cell and support their own metabolism, survival and virulence.<sup>5</sup> Evidence for the involvement of Nrf2-signalling pathway leading to *Plasmodium* parasite survival has been provided.<sup>36</sup>

During quiescent conditions, Nrf2 is negatively regulated by Kelch-like ECH associated protein 1 (Keap 1), a redox-sensitive E3 ubiquitin ligase substrate adaptor.<sup>37</sup> Keap 1 mediates the ubiquitination of Nrf2 via E3 ubiquitin ligase Cullin3 (CUL3) by binding to it, and this elicits the degradation of Nrf2 by the ubiquitin proteasome pathway.<sup>38, 39</sup> However, in response to oxidative stress, the oxidation of cysteine residues in Keap1 causes a change in conformation and that releases Nrf2 from Keap1 which protects it from degradation.<sup>40, 41</sup> Consequently, the free Nrf2 is translocated to the nucleus where it heterodimerizes with the Maf protein family, inducing transcription of its target genes. Besides, the stability of Nrf2 can be determined by p62/sequestosome-1 (p62) which functions in Nrf2 regulation via interaction with Nrf2 binding site of Keap1 and hence, competitive inhibition of the Keap1-Nrf2 binding which leads to Nrf2 stability and then, nuclear translocation.<sup>42, 43</sup> Phosphorylation of p62 at serine 349 increases its binding with Keap1, freeing Nrf2 whose activation supports *Plasmodium falciparum* survival during its liver stage.<sup>44</sup>

Nrf2 inhibits ferroptosis (accumulation of lipid peroxidation products and ROS from iron metabolism) by regulating the expression of SLC7a11.<sup>45</sup> This controls intracellular oxidation homeostasis of the host cell which favours parasite survival.<sup>43, 46, 47</sup> It has been demonstrated that the high oxidative stress in brain of experimental cerebral malaria induced Nrf2 activation<sup>47</sup> which in turn stimulated heme oxygenase-1 (Hmox1) that enhanced the breakdown of heme and reduction of ROS, leading to parasite survival and perpetuation of infection in both brain<sup>[48]</sup>

and liver.<sup>49</sup> Upregulation of genes controlled by Nrf2; including p62, was seen in all infected cells; indicating the vital role of this transcription factor for parasite persistence and survival.<sup>50</sup>

Essentially, Nrf2 activation that promotes parasite survival and infectivity seems not be restricted to the liver and brain cells, our study shows that sequestered pulmonary parasites survived and induced Nrf2 expression, high oxidative stress and inflammation with accompanying increase in sequestration receptors. It seems that the Keap1-Nrf2 pathway plays critical role in protecting against parasites' oxidative stress and promoting their survival in lungs of *P. berghei* infected mice. Activation of the pathway appears to enable the parasites to neutralize harmful ROS and maintain cellular homeostasis.<sup>35</sup> Also Nrf2, a key transcription factor, regulates the expression of antioxidants and detoxification genes that provide protection for parasite survival. The Keap1-Nrf2 pathway, in addition, influences host immune response, modulating pro-inflammatory cytokines and chemokines productions which creates a favourable environment for parasite growth and survival, enabling parasites' evasion of host immune system.<sup>35</sup> Furthermore, the pathway regulates biochemical processes (glucose metabolism, amino acid biosynthesis, and lipid synthesis) important for parasite survival and growth in the host. These speculations have nonetheless provided valuable insights regarding the role of Keap1-Nrf2 pathway in *P. faciparium* survival in lungs of malarial infected mice.

Albeit, treatment of the malarial infection with *EEOG*, a non-toxic extract ( $LD_{50}$  e<sup>27</sup> 5,000 mg/kg) containing important medicinal phytochemicals (Table 1) reduced parasitemia (Figure 1), lungs' parasite load (Figure 1), oxidative stress (Figure 4), inflammation (Table 2), sequestration receptors (Figure 3) and Nrf2 expression (Figure 2), but improved lung function (Figure 5) in manners that compared well with the standard treatment and the naïve mice.

The *in silico* evaluation of compounds in *EEOG* identified eugenol and carvacrol as the topmost bioactive compounds with drug properties (Table 3). However, docking analysis with protein targets (*Pf*LDH, *Pf*DHFR, *Pf*SUB1, and *Pf*ASL) important for parasite growth,

survival and infectivity (Table 4) indicated that eugenol interacted the more with *Pf*LDH with both hydrogen bonding and hydrophobic interaction (Figure 6). It bonded closely and strongly with SER234 of *Pf*LDH via hydrogen bonding, and the other multiple bond interactions contribute to the stability of the binding complex (Figure 7), supporting the potential effectiveness of eugenol as a strong inhibitor of *Pf*LDH which is expressed in all stages of the parasite life cycle. *Pf*LDH aids the parasites production of lactate from pyruvate, and this generates energy for the parasite required for multiplication, growth and survival. Also, it yields NAD<sup>+</sup> from NADH which maintains parasites' redox balance. Therefore, the eugenol in *EEOG* may have inhibited *Pf*LDH, affecting parasites' energy supply and ability to maintain redox homeostasis which hamper survival, hence, the reduction of parasitaemia and sequestration in pulmonary tissues. Other natural compounds (quercetin, apigenin, curcumin, and berberine) that inhibit *Pf*LDH have been demonstrated to possess antiplasmodial activity and our study aligns with theirs.<sup>51</sup>

In order to investigate the roles of eugenol on the Keap1-Nrf2 pathway, docking was performed and we discovered that eugenol strongly inhibited Keap1 via a mix of hydrogen bonding and hydrophobic interactions (Figure 8) and such binding particularly with Gly 367 and Val 606 inhibited Keap1's association with Nrf2 (85.6%) and p62 (75.2%) (Table 5). This frees Nrf2 which is then activated to increase the production of antioxidants (Figure 8) improved oxidative stress defence in lungs of infected mice (Figure 4). So, eugenol binds to Keap1, distorting its association with p62 known to favour parasites' survival. Eugenol's binding with Keap1 prevents the association with p62, the parasites' machinery employed to maintain redox balance and evade host's immune system,<sup>36</sup> and stimulation of the released (free) Nrf2 to favour host's redox maintenance and hence, parasites' inability to survive due to oxidative stress and immune capture. The study of Annina *et al.*<sup>36</sup> has also shown that Nrf2 activation is driven by p62 recruitment of Keap1 into PVM (parasitophorous vacuole membrane) of *P. berghei* parasites, and this activation was demonstrated to be essential for

optimal parasite development, suggesting it to be another example of how parasites evade signalling for survival.

### CONCLUSION

EE $O_g$  is a non-toxic, culinary plant, containing several important classes of phytochemicals, and our study has identified its eugenol compound as the most active antiplasmodial agent which strongly inhibits *Pf*LDH and modulates the Keap1-Nrf2/p62 pathway. These properties regulate host's redox system and induce oxidative stress in invading parasites which hampers lung sequestration. Therefore, eugenol can be optimized for further study as an antiplasmodial and antioxidant compounds, capable of resolving malarial associated lung symptoms, the commonest complication of malaria.

### ACKNOWLEDGMENT

We wish to acknowledge the contributions of the laboratory staff members of the Department of Medical Biochemistry, Delta state University, Abraka, and the technical assistance of the CEO and Management of Affemefune Biomedical Consultt, Abraka, Delta State, Nigeria.

### Funding Sources

The author(s) received no financial support for the research, authorship, and/or publication of this article.

### Conflict of Interest

The author(s) do not have any conflict of interest.

### Data Availability Statement

This statement does not apply to this article.

### Ethics Statement

An Ethical approval with the number RBC/FBMC/DELSU/25/794 was obtained from the Research, Ethics and Grant Committee, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria. The study was conducted in line with guidelines provided.

### Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

### Clinical Trial Registration

This research does not involve any clinical trials.

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Not Applicable.

### Authors' Contribution

Onyesom I: Conceptualization and supervision; Onobrudu DA: Writing, review and editing; Acha JO: Data collection; Orororo OC: Methodology and data analysis; Enudinisu GN: Laboratory investigations and data collection

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