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Evaluating the Efficacy of Effect of *Eucalyptus camaldulensis* Leaf Extracts Grown in Sandy Habitats on Histological and Gene Expression Changes in the Liver of Mice Infected with *Plasmodium chabaudi*

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ABSTRACT

Background: The plants are considered to be living chemical factories responsible for the production of a wide variety of secondary metabolites (SMs). Geographical and ecological differences result in different chemical compositions even within the same plant. Medicinal plants are promising options at antiparasitic. this study aimed to examine the impact of *Eucalyptus camaldulensis* leaf extracts (ECES) from sandy environments on the histological changes and gene expression caused by *Plasmodium chabaudi* infection in the liver.

Methods: We analyzed *E. camaldulensis* leaf extracts from sandy habitats using GC mass spectrometry, leading to the anticipated identification of 33 compounds. we determined the suitable dose and We divided the mice into five groups before they were infected with *P. chabaudi*: Group 1 represents the non-infected control group without treatment; Group 2 is the uninfected treated group that received 100 mg/kg ECES; Group 3 consists of the infected group that received no treatment; Group 4 includes the infected treated group that received 100 mg/kg ECES; and group 5 includes the infected group treated with chloroquine.

Result: study has demonstrated that the extract of *E. camaldulensis* leaves can mitigate the damage to liver tissue resulting from a *P. chabaudi* infection. Additionally, ECES successfully regulated the expression of liver cytokines, IL-1β, IL-6 and IFN-γ, in mRNA. When the parasite *P. chabaudi* causes liver injury.

Key words: *Eucalyptus camaldulensis*, Gene expression, Habitat, Histological, Malaria, Sandy.

INTRODUCTION

Plants function as living chemical factories, producing a diverse variety of secondary metabolites (SMs) that serve as essential components of many commercial pharmaceutical drugs (Hassan, 2012; Kandpal *et al.,* 2023). Environmental conditions significantly affect the biosynthesis and variations of secondary metabolites in plants (Verma and Shukla, 2015). Different geographical and ecological harvest sites result in different chemical compositions of the same plants (Camara *et al.,* 2021).

An important approach to fight against parasites is to promote the exploration of new antimalarial compounds from different origins, especially from traditional medicinal plants. The focus on research related to medicinal plants is increasing worldwide (Taek *et al.,* 2018; Dakhil *et al.,* 2021; Habibi *et al.,* 2022; Sharma *et al.,* 2021).

Medicinal plants have been used since ancient times to treat malaria and provide a good source for new antimalarial drugs (Dakhil *et al.,* 2021; Al-Jawdah *et al.,* 2022). According to the World Health Organization, malaria is still a serious disease with high morbidity and mortality, affecting 249 million people (World Health Organization, 2022).

According to a recent study by Anigboro *et al.* (2020), *Eucalyptus camaldulensis* can protect *Plasmodium berghei*-infected mice from malaria-induced liver and kidney function abnormalities. Furthermore, in mice infected with *P. Chabaudi*, our team documented the immunomodulatory and antioxidant properties of *E. camaldulensis* (Aljawdah *et al.,* 2022).

Changes in chemical composition can affect the quality and bioactivity of plants (Tian *et al.,* 2016). Several studies have investigated this topic or have recorded changes in the activity and composition of the same species in different regions. This study aims to fully clarify the differences in

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the chemical composition of *E. camaldulensis* collected in two distinct habitats (mud and sand) and to evaluate the antiparasitic activity of *P. chabaudi*.

MATERIALS AND METHODS

Extraction of *E. camaldulensis* (ECE)

The leaves of the plant were collected from sandy habitats in Al-Qassim, Saudi Arabia on 6/15/2022. The plant was identified by a herbalist at King Saud University. Before turning into powder, the leaves were dried in the open air. Then, 70% methanol was used to extract the constituent parts of the leaves (Lubbad *et al.,* 2015). For further studies, the ECE of sand samples (ECES) was diluted with distilled water.

Chemical analysis of plant extract (ECES)

In continuation of our previous studies (Dkhil *et al.,* 2023 and Aljawdah *et al.,* 2022) on the chemical analysis of plant extract (ECE), we used an optical spectrometer with infrared spectroscopy of transformation of Fourier (FT-IR) Nicolet 6700 to analyze the extract of plants that inhabit sand and mud habitats. In addition, antioxidant activity and phenolic and flavonoid compounds were evaluated. In this study, we used gas chromatography mass spectrometry to analyze the ECE of sand habitats (ECES) to verify the most significant differences between the chemical compounds in the *E. camaldulensis* extract. in different habitats (muddy and sandy) and evaluation of its antimalarial activity In the laboratories of the College of Science, King Saud University, during the period from 09/04/2023 to 01/01/2024.

Analysis for ECES use gas chromatography-mass spectrometry (GC-MS)

Kanthal *et al.* (2014) developed the protocol for the phytochemical analysis of ECES. The analysis was conducted using a GC-MS Triple Quad 7000D system from Agilent Technologies, USA, for gas chromatography coupled with mass spectrometry (GC-MS).

Infection and animals

Female C57BL/6 mice, aged 9–11 weeks, were provided with normal feed and water during the breeding period. The Research Ethics Committee for the Care of Laboratory Animals of King Saud University gave approval for the procedures (approval number: KSU-Se-21-77).

Mice infection and treatment

Introduction of *P. chabaudi* parasites into experimental mice was performed as reported in a previous study (Maksoud *et al.,* 2019). The injection dose (106 parasitized erythrocytes) was determined using a Neubauer chamber and *P. chabaudi* parasitized erythrocytes were administered intraperitoneally to mice (Wunderlich *et al.,* 1982). Blood smears were made from the mouse tails using Giemsa staining to assess the parasitemia that *P. chabaudi* induced.

Based on our recently published study (Aljawdah *et al.,* 2022), we determined the suitable dose and divided the mice into the following groups.

Group 1 represents the non-infected control group without treatment; Group 2 is the uninfected treated group that received 100 mg/kg ECES; Group 3 consists of the infected group that received no treatment; Group 4 includes the infected treated group that received 100 mg/kg ECES; and group 5 includes the infected group treated with chloroquine. Over the course of four days, Group 5 received 10 mg/kg of chloroquine phosphate (CQ) (Sigma-Aldrich, St. Louis, USA) (Abay *et al.,* 2015).

Sample collection

Mice were sacrificed on day 7 postinfection and the livers were removed and minced. Some sections were stored at “80°C for RNA extraction for subsequent gene expression studies and other sections were stored in neutral buffered formalin for histological examination.

Histopathology study

Liver samples were fixed in 10% neutral formalin and embedded in paraffin. Samples were cut into 5 µm thick sections and stained with hematoxylin and eosin (Drury and Wallington, 1980). Liver histology was performed according to the guidelines of Ishak *et al.* (1995). In summary, the quantitative measurements were based on the evaluation of liver sections at different magnifications. The quantity measure assigns scores from 1-3, 4-8, 9-12 and 13-18 for minimal, mild, moderate and severe liver damage, respectively, according to the severity of the injuries.

Gene expression

TRIzol (QIAGEN, Hilden, Germany) was used to extract hepatic RNA for quantitative real-time polymerase chain reaction (RT-qPCR). DNase (Applied Biosystems, Darmstadt, Germany) prepared the RNA and a reverse transcription kit (QIAGEN, Hilden, Germany) converted the samples to cDNA. The ABI Prism 7500HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany) and the SYBR Green PCR Master Mix (QIAGEN, Hilden, Germany) were used for the PCR analysis. The levels of interleukin-IL-1β, IL-6 and IFN-γ were measured using primers from Macrogen Inc. (Seoul, South Korea). We followed the guidelines set out by Dkhil *et al.* (2019) for doing PCR. We calculated the fold change in mRNA expression using the 2-DDCT technique (Livak and Schmittgen, 2001).

Statistical analysis

Groups were statistically compared using SPSS version 17.0 and Duncan’s test. Significance was assessed using univariate analysis of variance. Standard deviations and means are presented for all values. All p values were two-sided and statistical analyses were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

Phytochemical analysis performed by GC-mass revealed that ECES consisted of 33 phytochemicals (Table 1), each

Table 1: identifies phytochemicals in ECES using GC-Mass.

Component RT	Compound name	Molecular weight	[M-H]- (m/z) molecular weight^{-1}	Formula	Peak %
1.1045	1,2-Ethanediol	62.0678	61.0678	$C_2H_6O_2$	7.368962
1.7978	Ethane, fluoro-	48.0595	47.0595	C_2H_5F	1.665879
2.3075	Benzyl mandelate	242.27	241.27	$C_{15}H_{14}O_3$	1.749506
2.6749	Arsenous acid, trimethyl ester	168.0234	167.0234	$C_3H_9AsO_3$	0.152004
3.1731	4-(1-Benzyl-1H-tetrazol-5-ylsulfanylmethyl) -6- morpholin-4-yl-[1,3,5]triazin-2-ylamine	242.27	241.27	$C_{16}H_{19}N_9OS$	0.259789
3.9132	Benzene, 1,2,4,5-tetramethyl-	134.2182	133.2182	$C_{10}H_{14}$	44.59885
4.5150	1H-Indene, 2,3-dihydro-4,7-dimethyl-	146.2289	145.2289	$C_{11}H_{14}$	0.737307
5.0353	Phenol, 2-methoxy-4-(1-propenyl)-	164.2011	163.2011	$C_{10}H_{12}O_2$	0.897163
5.0353	Eugenol	164.2011	163.2011	$C_{10}H_{12}O_2$	0.94772
5.9352	2,4-Di-tert-butylphenol	206.3239	205.3239	$C_{14}H_{22}O$	1.820367
6.6847	5-Azulenemethanol, 1,2,3,4,5,6,7,8 -octahydro- .alpha.,.alpha.,3,8 -tetramethyl-, acetate,	264.4030	263.403	$C_{17}H_{28}O_2$	0.119677
7.6655	Methyl tetradecanoate	242.3975	241.3975	$C_{15}H_{30}O_2$	0.37017
8.2885	1,4-Benzenedicarboxylic acid, bis(2- hydroxyethyl) ester	254.24	253.24	$C_{12}H_{14}O_6$	0.095972
8.6555	1,3-dithiole-4,5-dicarbonitrile, 2-thioxo-	164.2011	163.2011	$C_5N_2S_3$	0.018827
9.0983	N-{4-[4-(4-Acetamido-1,2,5-oxadiazol -3-yl)- 1,2,5-oxadiazol-3-yl]-1,2,5 -oxadiazol-3- yl}acetamide	206.3239	205.3239	$C_{10}H_8N_8O_5$	0.004067
9.6861	Hexadecanoic acid, methyl ester (Palmitic acid)	270.4507	269.4507	$C_{17}H_{34}O2$	17.82442
9.9441	Benzenepropanoic acid, 3, 5-bis(1,1- dimethylethyl)-	292.4131	291.4131	$C_{18}H_{28}O_3$	0.179299
10.9771	4,4'-(Hexafluoroisopropylidene)diphenol	336.23	335.23	$C_{15}H_{10}F_6O_2$	0.048641
11.8079	Methyl stearate	298.5038	297.5038	$C_{19}H_{38}O_2$	8.264177
12.0765	Piperidine, 1-ethyl-	113.2007	112.2007	$C_7H_{15}N$	0.023494
12.8110	4H-Indazol-4-one, 6-(2-furanyl) -1,5,6,7- tetrahydro-	202.21	201.21	$C_{11}H_{10}N_2O_2$	0.006351
14.1018	Methyl 3-(3,4-dihydroxyphenyl) propanoate, 2TMS derivative	340.56	339.56	$C_{16}H_{28}O_4Si_2$	0.013985
14.5140	Butanamide, N-(2-iodo-4-methylphenyl	336.23	335.23	$C_{11}H_7F_7INO$	0.042817
14.8665	Hexadecanoic acid, 2-hydroxy -1- (hydroxymethyl)ethyl ester	330.5026	329.5026	$C_{19}H_{38}O_4$	7.751457
15.1025	Phthalic acid, octyl 2-propylpentyl ester	390.6	389.6	$C_{24}H_{38}O_4$	0.745948
15.6988	Thiophen-2-methylamine, N-(2-fluorophenyl)-	207.27	206.27	$C_{11}H_{10}FNS$	0.050374
16.0905	Octadecanoic acid, 2, 3-dihydroxypropyl ester	358.5558	357.5558	$C_{21}H_{42}O_4$	2.335814
16.5630	Phthalic acid, cyclobutyl nonyl ester	346.5	345.5	$C_{21}H_{30}O_4$	0.016676
16.9439	Methanol, [4-(1,1-dimethylethyl) phenoxy]-, acetate	222.28	221.28	$C_{13}H_{18}O_3$	0.175841
17.3641	propanoic acid, 2-[[4-(acetyloxy) -1- naphthalenyl]oxy]-	274.27	273.27	$C_{15}H_{14}O_5$	0.009405
17.8650	2-Methyl-3-phenyl-pyrrolo(2,3-b)pyrazine	209.25	208.25	$C_{13}H_{11}N_3$	1.615099
18.7018	3-(4-Methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazole	341.4	340.4	$C_{19}H_{19}NOs$	0.048772
19.2664	2,2'-Thiodipyridine	188.249	187.249	$C_{10}H_8N_2S$	0.041171

characterized by different peak areas and retention times. The compounds benzene, 1,2,4,5-tetramethyl-,hexadecanoic acid, ethyl ester (palmitic acid), methyl stearate, hexadecanoic acid and 2-hydroxy-1-(hydroxymethyl) ethyl ester are shown in Fig (1) with pointed peaks.

The results of the study showed tissue changes in the liver of mice. Significant liver damage characterized by the presence of inflammatory cells. Mice infected with *P. chabaudi* showed changes, while healthy controls and ECES-administered groups maintained normal liver architecture. Hepatic injury was attenuated and liver score decreased in mice treated with ECES and CQ, which correlated with reduced infiltration of inflammatory cells (Fig 2). The Ishak score revealed that the liver activity index of the infected group varied between 13 and 15. The administration of ECES to mice resulted in a reduction of the liver index to 5-8, while the chloroquine treatment gave a score of 6-8 (Fig 3).

The levels of pro-inflammatory cytokines IL-1β, IL-6 and IFN-γ increased significantly ($p<0.05$) after *P. chabaudi* infection, suggesting the inflammation of the hepatic tissue (Fig 4). The preventive effect of ECES and CQ on the inflammatory events associated with the development of malaria is proven by the significant reduction of this inflammatory response after treatment with ECES or CQ. *E. camaldulensis* contains phenolic compounds and flavonoids, which are bioactive. These compounds may contribute to the antimalarial effect and the reduction of parasitemia that occurs during the treatment of infected animals. Anigboro *et al.* (2020) recently reported that the aqueous extract of *E. camaldulensis* leaves may be effective against *P. berghei* significantly reduces the metabolic defects caused by malaria in the liver and kidney, as well as serum electrolytes, thanks to the active phytochemical components in the extract.

GC mass analysis revealed that ECES contains several active compounds, including hexadecenoic acid and its methyl ester (palmitic acid), known for their antiseptic and anti-inflammatory intestinal properties (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2002). Additionally, methyl stearate is employed in the management of neurological and cardiac disorders (Chen *et al.,* 2020). Aissaoui *et al.* (2019) reported the antibacterial, antifungal, antioxidant and anticancer properties of 2,4-di-tert-butylphenol. Abbaszadeh *et al.* (2014) reported the

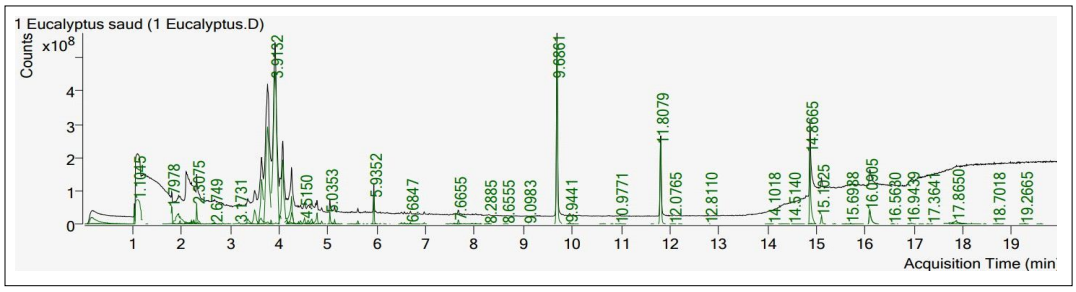
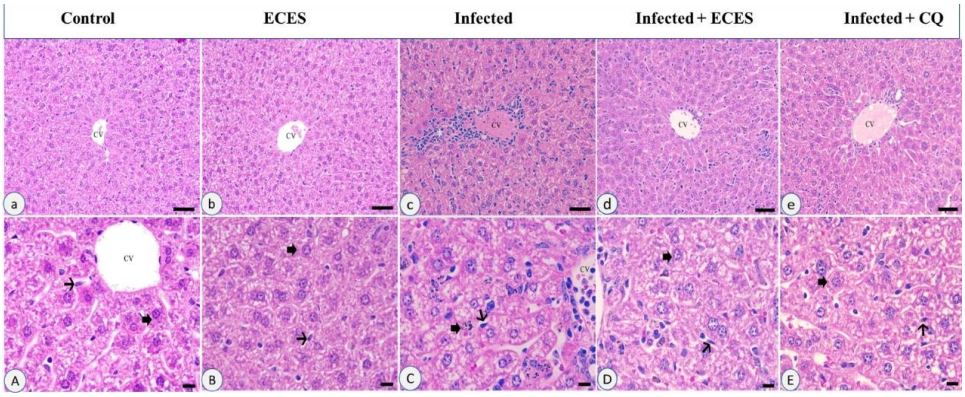


Fig 1: GC-MS chromatogram of aqueous ECES.



(Capital letters) Use the liver at higher magnification than the figure (small letters). Hematoxylin and eosin were used to stain the sections. 100 µm is the scale bar. {CV, central vein; Kupffer cells (arrow); hepatocytes (arrow head)}. the group of the control and group that was not infected and received ECES treatment, the liver showed up with its typical form. the group infected and non-treated appeared Liver with *P. chabaudi*. the group infected and with (ECES)-treated. Liver showed up alongside *P. chabaudi*. the group infected and chloroquine (CQ)-treated. Liver showed up alongside *P. chabaudi*.

Fig 2: Photomicrograph of the liver histology of the five groups.

potential activity of eugenol as antifungal, antibacterial, antiviral and antiparasitic. Phthalic acid-cyclobutyl nonyl ester (Kok *et al.,* 2008), methyl tetradecanoate (Mallu *et al.,* 2019), epizonarene (Mohammed *et al.,* 2020), benzenamine, N,N,3-trimethyl- (Yang *et al.,* 2021) and piperidine (Guo *et al.,* 2022) have been documented for their therapeutic activity as antiviral and anti-cancer agents.

A limited number of studies have documented changes in species composition and/or activity in different geographic regions. Few studies have examined the antimalarial properties of natural resources in the region, although Saudi Arabia has many different plants and people have long used indigenous ethnic medicinal plants (Mothana *et al.,* 2014; Al-Asmari *et al.,* 2017; Aati *et al.,* 2019). This study explores the antimalarial efficacy of ECE in two geographically distinct environments: sandy and muddy environments.

The difference in chemical constituents, their concentration and antimalarial efficacy between the ECEM and ECES samples can be attributed to the differences in the collection sites. ECEM derived from well-watered soil, while ECES derived from sand, which is less watered and therefore less favorable for optimal plant growth (Traore *et al.,* 2013).

P. chabaudi infection induces an inflammatory response in the liver of mice. This response represents

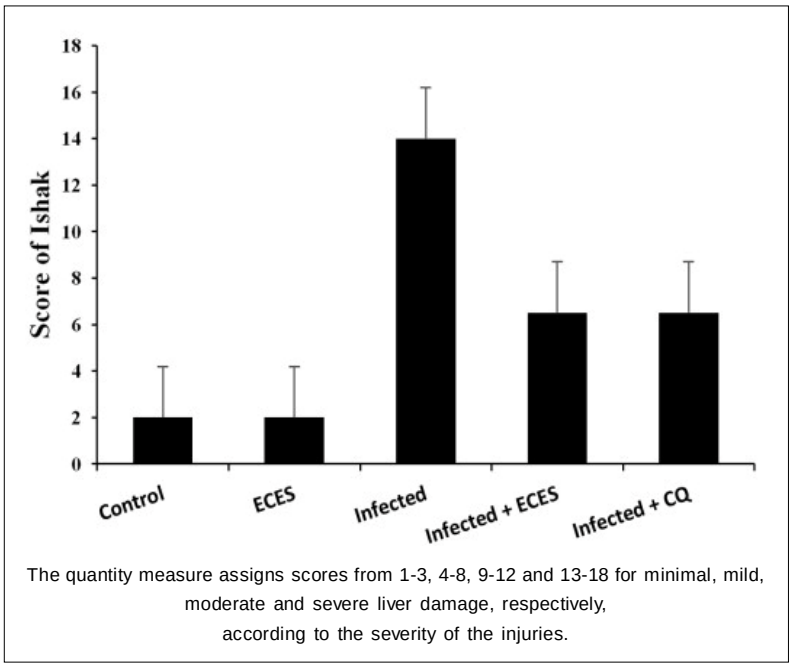


Fig 3: Modified histological score of Ishak, provides the histology score comparing the effects of *E. camaldulensis* and chloroquine (CQ) on erythrocytes parasitized by *P. chabaudi* in the livers of mice.

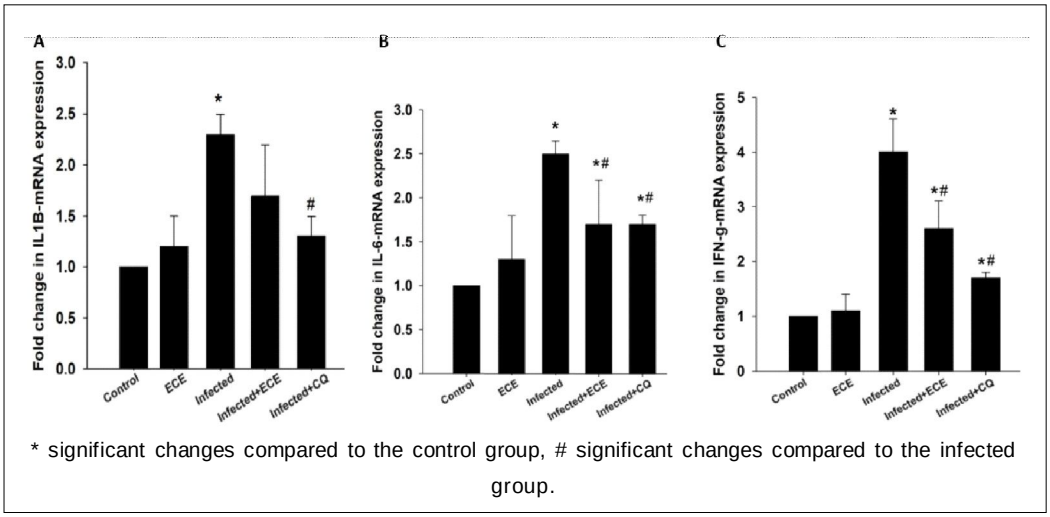


Fig 4: Effect of *E. camaldulensis* extract (ECES) on (A) IL1β, (B) IL-6 and (C) IFN-γ-mRNA expression in liver of mice infected with *P. chabaudi.*

changes in the hepatic architecture resulting from the parasites consuming hemoglobin, which then cause the release of heme. This process leads to oxidative damage and histological changes in the liver (Kumar and Bandyopadhyay, 2005). The histological and biochemical results are consistent with recent studies (Lubbad *et al.,* 2015; Dkhil *et al.,* 2019) that examined the hepatoprotective effects of plant extracts related to *P. chabaudi* infection. The investigation showed that the ECES treatment improved the health of the host mice. Previous studies on other medicinal plants have shown similar results (Dkhil *et al.,* 2021). The results show that the hepatic inflammation caused by *P. chabaudi* is associated with an inflammatory response in the liver. The results show that ECES effectively eradicates Plasmodium parasites in mouse models and has anti-inflammatory properties that maintain liver function.

The improvement in the structure of liver tissue indicates the potential protective effects of *E. camaldulensis* after malaria-induced infection, as shown by our results. In addition, *E. camaldulensis* reduced liver inflammation and restored the balance between antioxidants and antioxidants. The results show that *E. Camaldulensis* can act as a hepatoprotective agent in the context of malaria. Further study is needed to elucidate the mechanisms underlying the response liver of *E. camaldulensis* and *P. chabaudi*.

P. chabaudi infection can result in significant changes in the inflammatory cytokines IFN-, IL-1β and IL-6. There are early and powerful effector mechanisms mediated by cytokines that clear or eliminate parasite-infected cells. These include acquired and innate immune responses (Gowda and Wu, 2018). Furthermore, increased levels of IL-6 and IFN-γ are associated with hyperparasitemia (Angulo and Fresno, 2002). Hepatocytes, activated leukocytes and inflamed macrophages produce more cytokines and proinflammatory enzymes.in case of inflammation, which causes the initiation of the innate immune response (Wunderlich *et al.,* 2014). IL-1 and IL-6 are proinflammatory cytokines that play a crucial role in inflammation and immunity, with elevated levels observed during Plasmodium infection (Dkhil *et al.,* 2021). By decreasing cytokine production and altering oxidative changes in the liver, ECES treatment significantly slowed down the onset of inflammatory responses after *P. chabaudi* infection. Initially, we attributed the reduced inflammatory responses of ECES to the lower number of parasitized erythrocytes. Many studies support our findings that medicinal plants can reduce parasitemia and protect the liver from inflammation (Wunderlich *et al.,* 2014; Dkhil *et al.,* 2019; Dkhil *et al.,* 2021).

CONCLUSION

Extracts of *Eucalyptus camaldulensis* leaves grown in sandy habitats contain several active antiparasitic compounds. It is generally agreed that the *E. camaldulensis* species, which grows in muddy habitats, has a more significant impact than those that grow in sandy environments. Additional studies are needed to evaluate the effectiveness of the extracts in the rat organs and to elucidate the molecular mechanisms of action involved in their activity.

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Disclaimers

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Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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