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# Evaluation of anti-malarial activity and GC–MS finger printing of cannabis: An *in-vivo* and *in silico* approach

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ABSTRACT

The development of an effective and affordable malaria treatment drug is required to reduce the number of deaths caused by this disease around the world. The in-vivo antimalarial activities of Cannabis were investigated, as well as the in-silico antimalarial prediction of cannabis GC–MS compounds. Thirty albino mice were divided into six groups of five animals each at random: A, B, C, D, E and F. The animals in all the groups except the normal control group were infected with Plasmodium berghei NK-65 before the commencement of treatment. Each group was treated differently: A is a positive control; B, mice were administered 0.2 ml 2% DMSO with no infection; C, 10 mg/kg BW of chloroquine was administered; Animals in D, E, and F were given 100, 200, and 400 mg/kg BW of ethanolic cannabis leaf extract, respectively. Blood was drawn from the mice's tail, fixed on a slide, and examined under a microscope to determine the parasitaemia level of the infected mice at various intervals during treatment. The animals were sacrificed and blood collected for hematological studies. There was a significant decrease in percentage parasitemia and an increase in percentage inhibition in the treated group compared to infected group. The red blood cell (RBC), platelet, haematocrit (HCT) and percentage weight gain showed a significant increase ($P \leq 0.05$) in the treatment groups compared to the infected mice. Tetrahydrocannabivarin showed high binding energy to $\alpha/\beta$ tubulin protein of Plasmodium falciparum compared to vinblastine. The decrease in parasitaemia percentage could be attributed to tetrahydrocannabivarin's inhibitory action on microtubules, which eventually leads to the cessation of parasite proliferation in RBC.



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## Introduction

Malaria is a tropical parasitic disease of *plasmodium* notorious for the increased death among children and pregnant women especially in the poor country of the world. The severity of the disease is based on the species of the *plasmodium*. Malaria has always been a health threat that has relentlessly taken life for millennia. Clinical and parasitological data from previously effective antimalarial drugs have revealed widespread parasitic resistance. The use of herbal alternatives was necessitated by the development of an effective drug for the global control of this life-threatening disease. As a result, there is an urgent need to develop antimalarial drugs from chemical, synthetic, and natural sources to mitigate the effects of this tropical disease. However, in the design of antiparasitic drugs, such as in the case of the plasmodium parasite, researchers tend to consider bioactive agents with the potential to alter cellular processes that are harmful to these organisms by binding on strategic target sites. Cannabis extract is renowned for its therapeutic properties due to the presence of a high proportion of antioxidant phytochemicals, particularly coumarins (benzo—pyrone class of natural product) [1]. Coumarin and its synthetic derivatives have been shown to inhibit the proliferation of malarial parasite by targeting microtubule dynamics [2,3]. Tubulin inhibitors, such as coumarin, impede malarial parasite proliferation and growth [4–6]. The vital role of microtubules in cellular processes on the erythrocytic cycle of P. falciparum, include the following: merozoite formation [7], invasion of erythrocytes by daughter merozoites [8], and nuclear division [9]. Furthermore, the protein biosynthesis function of the microtubule [10] has made it a desirable drug target site. The clinical symptoms and manifestations of malarial infection are during the parasite's erythrocytic stage which is also the asexual period of the development of the parasite [11]. The 'silent phase,' also known as the pre-erythrocytic stage of the infection, has no symptoms and affects only a few hepatic cells [12]. One of the aspirations of the African Union's Agenda 2063 is for a prosperous Africa, based on inclusive growth and sustainable development goals, attainable through a high standard of living, quality of life, and well-being for all citizens through a focus on health and nutrition. As a result, one of the identified challenges to this goal is malaria infection, which is endemic in Africa and has been linked to the majority of deaths among pregnant women and children under the age of five. This eventually led to poverty, which is blamed for Africans' low standard of living. This current study could be used to develop a low-cost and effective malaria treatment option for the African continent. This research investigated the anti-malarial properties of cannabis in vivo as well as possible inhibitory effect of a cannabis GC–MS compounds on the *plasmodium* parasite's microtubule target protein in silico.

## Materials and methods

### *Procurement of Cannabis and authentication*

The dried cannabis leaves were obtained from the National Drug Law Enforcement Agency (N.D.L.E.A) Ilorin. The leaf was authenticated at Ahmadu Bello University's Department of Botany in Zaria, Nigeria. It was given the voucher number ABU02438 and placed in the herbarium. All experimental protocols adhered to the ethical guidelines/regulations governing the use of plants.

### *Preparation of Cannabis extract*

The ethanolic leaf extract of cannabis was prepared by soaking 1400 g of pulverized cannabis leaf in 2.8 l of 90 percent ethanol in glassware. The mixture was stirred in an orbital shaker for 2 h and; it is then left to stand for 24 hr. Subsequently, the mixture was filtered; the filtrate was concentrated at 60 °C with a rotary evaporator. The semi-fluid extract was dried at 40 °C using a water bath to dry weight.

### *Parasite strain*

*Plasmodium berghei* NK-65 (a chloroquine-sensitive strain) was obtained from the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Ibadan, Nigeria. The parasite was maintained in a mice host by serial passage of infected mouse to uninfected naïve mouse. The level of parasitemia was monitored by microscopic examination of blood smears.

## Animals procurement

Thirty adult Swiss albino mice (*Mus musculus*) with an average weight of 20 ± 2 g were procured at the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Ibadan, Nigeria. The animals were allowed free access to commercial animal chow (Chikun feed, Ilorin) and tap water *ad libitium*.

### *Ethical clearance*

Ethical approval and license for the handling of experimental animals were obtained from the Ethical Committee of the Landmark University (LUAC/2020/0053B). The approval of the Institutional Ethical Committees was based on the guidelines of the World Health Organization for the care and use of laboratory animals.

*GC–MS analysis*

GC–MS analysis of the ethanolic leaf extract of cannabis was performed using Shimadzu GC–MS - QP2010 PLUS. The equipment was set at the following conditions: Injector temperature - 250ºC, oven temperature - 60ºC, ion source temperature - 200C, interface temperature - 250ºC, pressure - 100.2 KPa, column flow - 1.61 ml/min, purge flow 5.6 ml/min and total flow rate - 39.4 ml/min. Manual injection of the sample was done at a split ratio of 20:0 and the total running time was 11 min. The National Institute of Standard and Technology (NIST) library database was used in the interpretation of the mass spectrum.

*Induction of parasitemia*

Blood (0.1 ml) was obtained by cardiac puncture from mice anaesthetized with diethyl ether. Blood was diluted with 0.1 ml of sterile, non-pyrogenic 0.85% saline to 0.2 ml ($1 \times 10^7$ parasitized red blood cells (PRBC). The diluted blood with parasitized red cell (0.2 ml) was injected into the experimental mice through the intraperitoneal route. Mice in the normal control were inoculated with an equivalent volume of diluted blood containing uninfected red blood cells.

**Experimental design**

Thirty male mice were grouped and kept in animal cages in a well-ventilated animal research laboratory. The animals were allowed access to pellet feed and water *ad libitium*. The animals were grouped into six (6) groups of five (5) animals each as follows;

**Group A**- positive control (infected with no treatment)
**Group B**- normal control (no infection, no treatment)
**Group C**- 10 mg/kg body weight of Chloroquine (infected)
**Group D**- 100 mg/kg body weight of ethanolic extract of Cannabis (infected)
**Group E**- 200 mg/kg body weight of ethanolic extract of Cannabis (infected)
**Group F**- 400 mg/kg body weight of ethanolic extract of Cannabis (infected)

Except for the normal control group, all mice were infected intraperitoneally with *P. berghi* NK-65. The positive control mice received no treatment, while the mice in group C (standard drug) received 10 mg/kg body weight of chloroquine. The extract was given to the mice in groups D, E, and F at doses of 100, 200, and 400 mg/kg body weight, respectively.

*In vivo antimalarial test*

The method was carried out according to the method described by [13]. The mice were infected by intraperitoneal injection of parasitized blood. The treatment of the infected mice was commenced 72 hour after the infection.

*Light microscopy*

Parasitized erythrocytes were extracted from the tails of experimental mice and thin blood smears were made on glass slides. Slides were fixed in methanol, air dried, and stained with Giemsa before being examined under a light microscope at magnification of 100.

**Evaluation of inhibition of parasitemia**

Parasitemia was calculated as a percentage of infected red blood cells (RBC) relative to total number of cells in a microscopic field at x100 magnification as given below.

$$\text{Parasitemia } (\%) = \frac{\text{Total number of parasitised RBCs}}{\text{Total number of RBCs}} \text{X } 100$$

Mice were infected by intraperitoneal injection of parasitized blood. Treatment of the respective groups commenced 3 h after infection. Treatments lasted 4 days. Percentage suppression of parasitemia was calculated as:

$$\text{Percent Inhibition } = \frac{\text{mean \% parasitemia of untreated group} - \text{mean \% parasitemia in treated group}}{\text{mean \% parasitemia in untreated}} \text{X } 100$$

*Bioinformatics experiments*

Protein sequences for *Plasmodium falciparum* $\alpha$-tubulin (P14642) and $\beta$-tubulin (P14140) were retrieved from UniProt [14,15]. The sequences were utilised for the homology modeling of $\alpha$ and $\beta$ chains of *Plasmodium falciparum* tubulin using the Swiss-Prot [16] server the crystal structure (2.30 Å) of *Drosophila melanogaster* tubulin (PDB ID: 6TIS, $\alpha$-tubulin: P06603, $\beta$-tubulin: Q24560). The *Drosophila melanogaster* templates for homology modeling of $\alpha$ and $\beta$ chains of tubulin have sequence identity of 84.28% and 87.18% respectively compared to *Plasmodium falciparum* sequences. Structures of ligands were

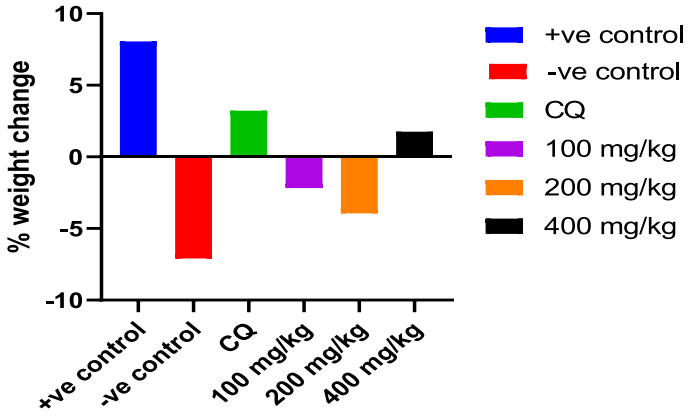


**Fig. 1.** The weight change in mice infected with *Plasmodium berghei* NK65 and the treated with ethanolic leaf extract of Cannabis. CQ; Chloroquine +ve: positive control; -ve; negative control.

downloaded from PubChem database and converted into their 3D configuration using the Marvin Sketch Software 19.20 and were assigned Gasteiger charges. The homology model of *Plasmodium falciparum* tubulin dimer was refined using AutoDock tools 4.2. Docking of ligands to the receptor was performed using AutoDock vina using a grid box size of 40 × 40 × 40 in the x,y,z dimensions and center values of 23.963, 4.552 and 79.579 in the x,y and z dimensions respectively. Visualization of docked poses was done using Pymol, Ligplus and Discovery studio softwares. Drug likeness properties of the ligands were analyzed using SWISSADME server (http://www.swissadme.ch/) [17].

*Statistical analysis*

The data presented were expressed as mean ± SEM, two way analysis of variance (ANOVA) was used followed by Dunnet and Tukey post hoc mean as comparison to access for significant differences among the variables at $P \leq 0.05$. All statistical evaluations were carried out using the Statistical Package for Social Science version 22 (SPSS Inc. Illinois, Chicago, USA) and Graph Pad Prism version 8

## Result and discussion

*Weight, parasitaemia level and growth inhibition*

The anti-plasmodial function of medicinal plants is due to the presence of bioactive ingredients in the plants, which have been reported to inhibit growth or cell replication [18]. These bioactive ingredients exhibit this therapeutic property by binding to protein receptors or by blocking the binding site of an enzyme in a critical pathway for the survival of the organism. Symptoms of *plasmodium* infection in mice include anemia, hypoglycemia, weight loss, and a drop in body temperature [19].

The positive control group showed an increase in weight because the mice in this group were not infected but given food and water only (Fig. 1). The negative control group showed a decrease in weight compared to the standard drug which showed an increase in weight (Fig. 1). Also, the mice treated with 100 mg/kg BW and 200 mg/kg BW respectively showed a decrease in weight and the mice treated with 400 mg/kg BW showed an increase in weight (Fig. 1). There was a significant decrease in the percentage parasitaemia level in the mice treated with the extract on the 10th and 12th day post infection treatment compared to the infected untreated mice (Figs. 2D & E). The standard drug, chloroquine (CQ) treated mice however showed a significant decrease in the percentage parasitaemia level from the 4th day post infection treatment compared to the mice in the infected untreated mice (Fig. 2A). On the 10th and 12th days of post-infection, mice treated with extract had between 80 and 96 percent inhibition in *Plasmodium berghei* NK-65 in infected mice compared to mice infected and treated with chloroquine (standard antimalarial drug) which had 100 percent inhibition at the same time (Figs. 3D & E). Certain criteria, however, are required to track the therapeutic progress of drugs or medicinal plants. The increase in percentage weight change could be attributed to the therapeutic effect of cannabis extract [20]. Also the therapeutic efficacy of antimalarial drugs is determined by their ability to rid the host organisms of parasites in the blood or the tissue. Furthermore, Plasmodium parasite merozoites invade red blood cells, where they feed on hemoglobin and multiply, thereby increasing the parasite's population [21]. If no antimalarial agent is applied, the parasite multiplies rapidly in the blood, increasing the parasite count by up to 20 times in 48 h [22]. The parasitaemia level in the blood is an important criterion for determining the severity of malaria infection or an indicator of antimalarial drug therapeutic efficacy [23]. The lower percentage of parasitaemia reported in this study could be due to the extract's antiplasmodial activity. The percentage inhibition of plasmodium parasites is a measure of how well an antimalarial drug prevents them from multiplying and

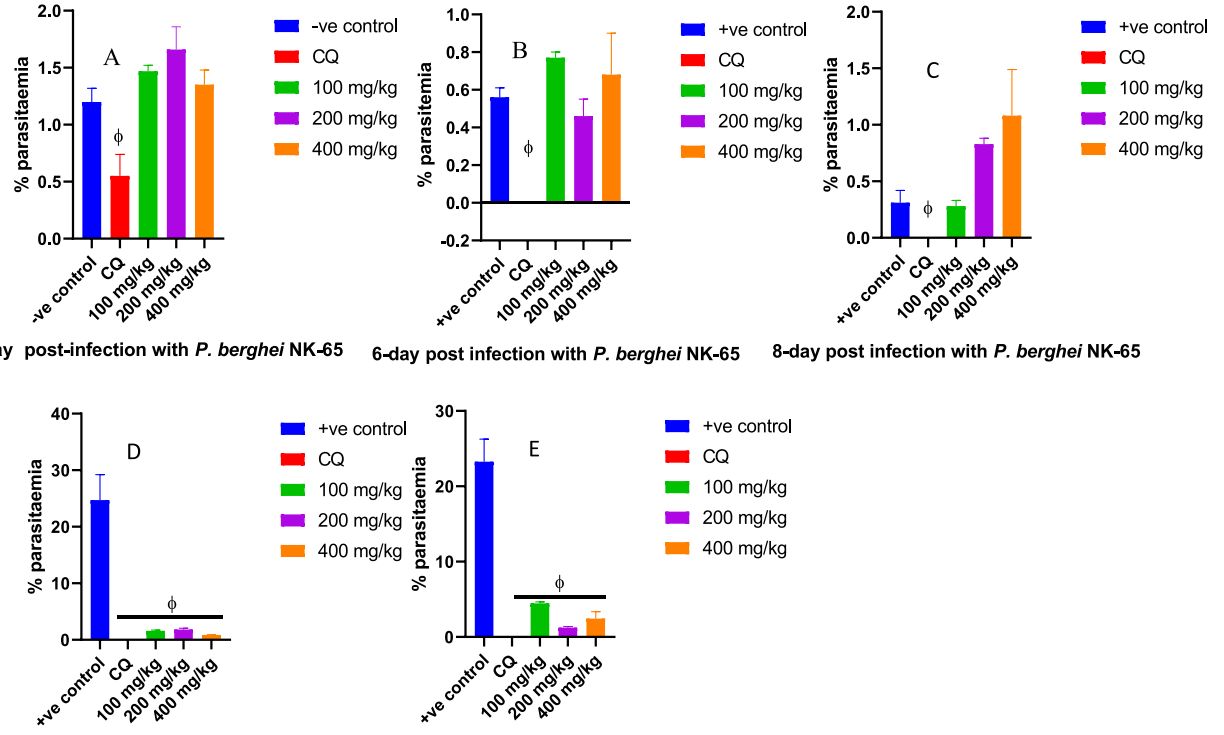


**Fig. 2.** Parasitemia level in *Plasmodium berghei* NK-65 in infected mice treated with ethanolic leaf extract of Cannabis (Curative test). Values are means of three replicates. CQ; Chloroquine +ve: positive. The values are expressed as means of three replicates ± SEM: $^{\phi}$ $p \leq 0.05$ vs normal control.

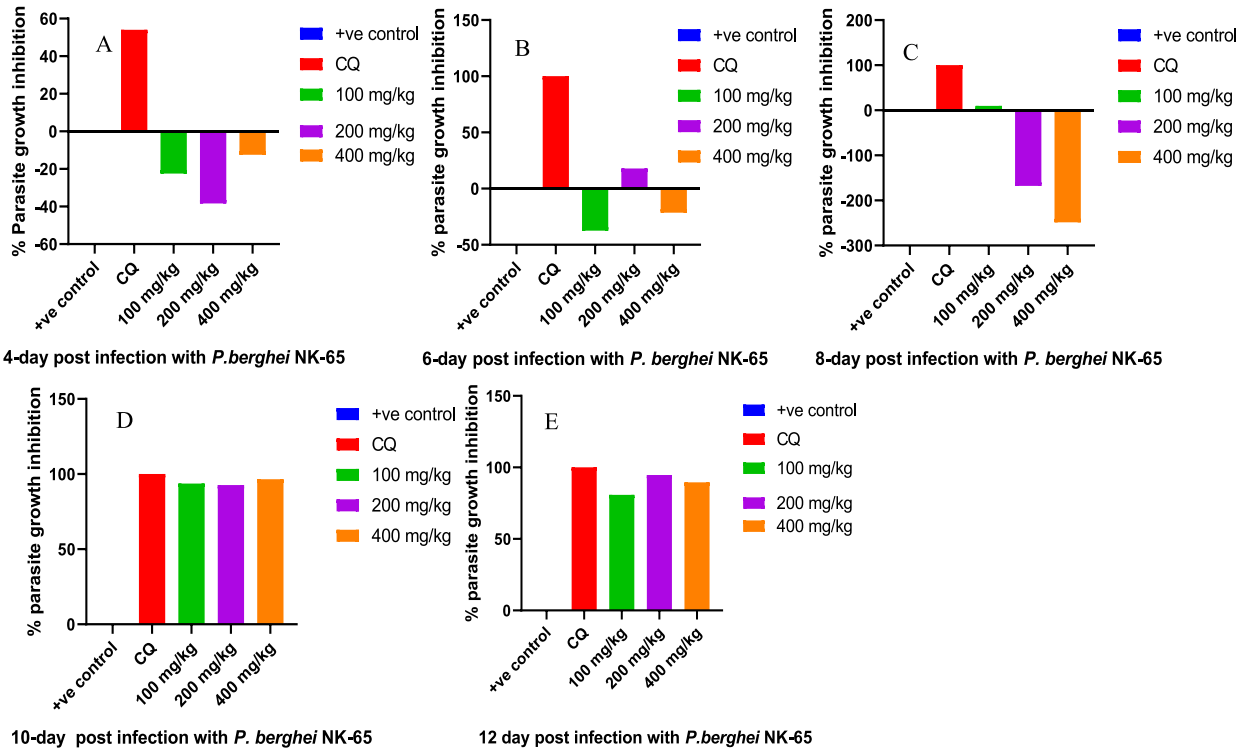


**Fig. 3.** Parasite growth inhibition in *Plasmodium berghei* NK-65 -infected mice treated with ethanolic leaf extract of Cannabis (Curative test). CQ; Chloroquine +ve: positive.

**Table 1.**
Hematological indices of *Plasmodium berghei NK-65* Infected mice and treated with ethanolic leaf extract of Cannabis.

| Treatment | N-control | P-control | 100 mg/kg | 200 mg/kg | 400 mg/kg | Chloroquine |
|---|---|---|---|---|---|---|
| **WBC** | 9.50 ± 0.15[a] | 8.60 ± 0.15[b] | 11.10 ± 0.46[c] | 10.53 ± 0.48[c] | 7.87 ± 0.08[d] | 5.87.0.43[e] |
| **RBC** | 6.13 ± 0.06[a] | 4.71 ± 0.65[b] | 8.90 ± 0.16[c] | 8.60 ± 0.28[c] | 8.85 ± 0.16[c] | 7.97 ± 0.48[c] |
| **HGB** | 11.40 ± 0.35[a] | 12.07 ± 0.34[a] | 11.17 ± 0.12[a] | 12.17 ± 0.97[a] | 11.80 ± 0.29[a] | 10.70 ± 0.26[a] |
| **HCT** | 38.80 ± 1.36[a] | 36.47 ± 1.13[a] | 40.10 ± 1.21[b] | 36.00 ± 0.35[a] | 40.23 ± 0.96[b] | 34.56 ± 1.68[c] |
| **MCV** | 44.20 ± 1.25[a] | 44.37 ± 1.06[a] | 44.93 ± 0.52[a] | 41.53 ± 0.58[a] | 44.57 ± 0.24[a] | 43.84 ± 0.57[a] |
| **MCH** | 12.13 ± 0.49[a] | 13.50 ± 0.20[a] | 12.87 ± 0.13[a] | 12.73 ± 0.39[a] | 13.16 ± 0.29[a] | 12.76 ± 0.17[a] |
| **MCHC** | 26.43 ± 1.42[b] | 29.00 ± 1.97[a] | 28.27 ± 0.59[a] | 27.17 ± 2.24[a] | 30.24 ± 1.37[a] | 31.03 ± 0.88[a] |
| **PLT** | 741.33 ± 30.01[c] | 392.67 ± 15.98[a] | 396.00 ± 14.00[b] | 334.33 ± 7.45[a] | 441.67 ± 14.05[b] | 381.67 ± 14.88[a] |
| **LYM** | 77.86 ± 0.58[a] | 77.73 ± 3.41[a] | 73.03 ± 0.49[a] | 77.53 ± 0.37[a] | 76.58 ± 4.62[a] | 75.33 ± 1.37[a] |
| **MXD%** | 9.18 ± 0.25[a] | 10.42 ± 0.43[c] | 9.94 ± 0.36[b] | 8.51 ± 0.24[a] | 10.46 ± 0.13[c] | 8.57 ± 0.25[a] |
| **NEUT%** | 3505.67 ± 147.20[a] | 1180.02 ± 49.31[a] | 1793.01 ± 104.71[b] | 1550.43 ± 125.91[a] | 2292.82 ± 111.11[c] | 1295.70 ± 28.63[a] |
| **LYM#** | 275.62 ± 0.80[d] | 263.23 ± 0.50[c] | 253.77 ± 4.61[b] | 235.87 ± 2.13[a] | 232.43 ± 7.46[a] | 266.93 ± 4.03[c] |
| **MXD#** | 4.89 ± 0.49[a] | 4.40 ± 0.49[a] | 4.29 ± 0.38[a] | 3.93 ± 0.43[a] | 3.53 ± 0.19[a] | 4.95 ± 0.13[a] |
| **NEUT#** | 916.88 ± 78.48[a] | 442.64 ± 16.32[b] | 450.37 ± 8.87[a] | 888.63 ± 63.76[a] | 888.63 ± 63.76[a] | 562.06 ± 26.90[b] |
| **RDW-SD** | 42.87 ± 1.94[a] | 53.50 ± 4.09[b] | 33.57 ± 0.76[a] | 42.50 ± 2.84[a] | 39.13 ± 0.62[a] | 54.13 ± 0.92[c] |
| **RDW-CV** | 14.13 ± 0.97[b] | 12.13 ± 1.18[a] | 11.33 ± 1.22[a] | 11.60 ± 0.25[b] | 9.70 ± 0.42[a] | 14.67 ± 0.99[b] |
| **PDW** | 12.47 ± 0.62[a] | 12.47 ± 0.70[a] | 12.67 ± 0.93[a] | 12.57 ± 0.88[a] | 13.70 ± 0.17[b] | 13.50 ± 0.15[b] |
| **MPV** | 8.97 ± 0.30[a] | 9.43 ± 0.03[a] | 8.67 ± 0.60[a] | 9.40 ± 0.10[a] | 9.30 ± 0.06[a] | 9.20 ± 0.06[a] |
| **P-LCR** | 22.20 ± 2.05[b] | 26.27 ± 0.30[a] | 25.37 ± 0.78[a] | 25.03 ± 0.44[a] | 25.57 ± 0.45[a] | 25.40 ± 0.21[a] |
| **PCT** | 0.65 ± 0.20[b] | 0.68 ± 0.02[b] | 0.64 ± 0.02[b] | 0.65 ± 0.17[b] | 0.65 ± 0.00[b] | 0.67 ± 0.00[b] |

WBC- White Blood Cells, RBC- Red Blood Cells, HGB- Haemoglobin, HCT- Haematocrit, MCV- Mean Corpuscular Volume, MCH- Mean Corpuscular Hemoglobin, MCHC- Mean Corpuscular Hemoglobin Concentration, PLT- Platelets, LYM- Lymphocytes, MXD%- Mixed Cell Count (percentage), NEUT%- Neutrophil (percentage), LYM#- Lymphocytes, MXD#- Mixed Cell Count, NEUT#- Neutrophil, RDW-SD- Red Blood Cell Distribution Width (standard deviation)., RDW-CV- Red Blood Cell Distribution Width (coefficient variation), PDW- Platelet Distribution Width, MPV- Mean Platelet Volume, P-LCR- Platelet- Large Cell Ratio, PCT- Platelet Crit Circulation.

growing. This study found that a high concentration of the antimalarial bioactive compound inhibited parasite growth in a dose-dependent manner, implying that a higher dose of the extract contained a higher concentration of the antimalarial bioactive compound. Furthermore, isolated cannabis compounds were found to have mild antimalarial activity in vitro with 4-acetoxycannabichromene, 5-acetyl-4-hydroxycannabigerol, and −1′S hydroxycannabinol [24,25]. In another study, Akinlola et al. reported mild antimalarial activity in infected mice fed cannabis compounded feed [26]. The use of crude extract in this study, on the other hand, results in a high antimalarial activity.
[24–26].

*Hematological parameters*

Moreover previous researches have linked disease condition and therapeutic effect to changes on some hematological parameter.

There was a significant decrease in the WBC volume in the positive control, the groups administered 400 mg/kg of the extract and CQ treated groups compared to the normal control while groups administered 200 and 400 mg/kg body weight of the extract showed a significant increase (Table 1). There was a significant decrease in the RBC level in the mice in the positive control and significant increase in the treatment groups and standard drugs compared to the normal control (Table 1). There was no significant change in the hemoglobin (HGB) concentration in all the groups compared to the normal control (Table 1). There was a significant increase in the HCT level in the groups treated with 100 and 400 mg/kg body weight of the extract compared to the normal control (Table 1). There was no significant change in the treatment groups, positive control and standard drug compared to the normal control (Table 1). Mean Corpuscular Volume, (MCV) and Mean Corpuscular Hemoglobin (MCH) did not show significant change across groups compared to the normal control (Table 1). MCHC showed significant increase across the groups compared to the normal group (Table 1). There was a significant decrease in platelet (PLT) across the groups compared to the normal control (Table 4). No significant change was observed in the level of lymphocyte (LYM) in all the groups compared to the normal control (Table 1). Mixed Cell Count (MXD) showed a significant increase in the positive control. There was a significant increase in level of plasma Platelet Crit Circulation (PCT) in the infected and treatment groups compared to the normal control (Table 1). Because the parasite's primary target is RBC, the decrease in RBC can be attributed to either the invaded parasite destroying RBC or the spleen rapidly removing parasitized RBC from circulation [27,28]. The normal RBC volume in the treated groups supported the antiplasmodial activity by the cannabis extract. Red cell volume is measured by the haematocrit (HCT). The parasite's invasion of the RBC and subsequent destruction of the RBC will eventually overwhelm the RBC production rate, resulting in a lower haematocrit. The HCT volume in the treated group was normal because there was no or a low level of parasitemia, which could destroy the RBC. The infection did not affect the levels of HGB, MCV, MCH, or MCHC, which may be attributed to some physiological compensation by the variant red blood cell [29,30]. Platelet reduction is linked to malaria, and thrombocytopenia may be a sign of plasmodium infection [31–33].

**Table 2.**
Compounds of GC–MS Analysis of ethanolic leaf extract of Cannabis.

| Ligand label | Peak# | Retention time | Percentage area (a) | Compounds | Chemical formula |
|---|---|---|---|---|---|
| A | 1 | 10.186 | 1.69 | Caryophyllene Oxide | $C_{15}H_{24}O$ |
| B | 3 | 10.628 | 1.39 | Isoaromadendrene Epoxide | $C_{15}H_{24}O$ |
| C | 4 | 12.239 | 4.05 | Hexadecanoic acid | $C_{16}H_{24}O_2$ |
| D | 5 | 13.175 | 1.27 | Phytol | $C_{20}H_{40}O$ |
| E | 6 | 13.339 | 7.98 | 9,12-Octadecadienoic Acid | $C_{18}H_{32}O_2$ |
| F | 7 | 13.504 | 1.92 | 9,12-Octadecadienoic Acid, Ethyl Ester | $C_{20}H_{36}O_2$ |
| G | 8 | 14.974 | 3.52 | Tetrahydrocannabivarin | $C_{19}H_{26}O_2$ |
| H | 9 | 15.174 | 1.36 | 6-Hydroxymelatonin | $C_{13}H_{16}ON_2O_3$ |
| i | 10 | 15.563 | 1.51 | Cannabispiran | $C_{15}H_{18}O_3$ |
| j | 11 | 15.627 | 1.66 | Cannabivarin | $C_{17}H_{22}O_2$ |
| k | 12 | 16.115 | 4.49 | Cannabichrome | $C_{21}H_{30}O_2$ |
| l | 13 | 17.551 | 24.99 | Delta9-Tetrahydrocannabinol | $C_{21}H_{30}O_2$ |
| m | 14 | 18.351 | 3.02 | Cannabigerol (Cbg) | $C_{21}H_{32}O_2$ |
| n | 15 | 18.715 | 27.37 | Cannabinol | $C_{21}H_{26}O_2$ |
| o | 18 | 22.256 | 1.93 | 11 **α**-Hydroxycephalotaxine | $C_{14}H_{21}NO_{14}$ |

In response to increased parasitized red cells, platelets protect the erythrocyte by releasing platelet factor 4 (PF4) molecules, as was demonstrated in previous studies. The plasmodial activity of platelet factor 4 (PF4) molecules has been documented [34,35]. The platelet's antiplasmodial activity could kill the platelet, which could result in a decrease in PCT. The PCT was not affected in this study, which was in contrast to the findings of Asare et al., who reported a decrease in PCT [36]. Platelet distribution width (PDW) is a measure of platelet volume variability, and it increases when platelet anisocytosis is present. It is used to explicitly quantify platelet size variability, changes with platelet activation, and represents platelet morphology heterogeneity. In contrast to Yusuf et al. [37] who observed increased PDW due to increased development of larger platelets as mitigation for excess platelet destruction in malarial conditions, this study found no increase in PDW in infected mice as compared to non-infected or treated infected mice [37]. The neutrophil is abundant in the peripheral blood, where it performs phagocytic action against antigens or microorganisms in the same environment as parasitized red blood cells. The decrease in neutrophil levels recorded in this study backs up a previous study that found a decrease in leukocyte (neutrophil) levels in the blood stage of plasmodium infection and an increase in leukocyte levels in the liver stage of infection [38]. The presence of blood parasitaemia is indicated by a decrease in the number of leukocytes in the blood. The most widely studied markers of platelet activation are mean platelet volume (MPV). The infection by plasmodium parasite in the present study did alter the level of MPV; this may imply that the infection did not induce increased development of new megakaryocytes [39,40].

*GCMS compounds and molecular docking*

Drug design involves developing a chemical agent with a therapeutic effect. The mechanisms of the therapeutic function depend on the type of ailment. An anti-parasitic agent primarily targets and blocks certain receptor vital for the physiological or biochemical function necessary for the survival of the organism. Fifteen compounds were identified in the GCMS analysis of the crude extract of cannabis (table 2) while the GC–MS chromatogram identified Delta9-Tetrahydrocannabinol to be the most abundance compound (Fig. 4).

As of the time of writing this report, no structure of tubulin of *Plasmodium falciparum* origin had been deposited in the Protein Data Bank (PDB). Therefore, the $\alpha\beta$ dimer was modeled using the Swis-Prot server (Fig. 5). The primary sequences of the modeled $\alpha$ and $\beta$ subunits shares 84.28% and 87.18% identity with the template 6TIS. In order to pry into the molecular interactions between compounds from Cannabis and $\alpha\beta$ dimer, the vinblastine binding site of tubulin located at the interphase of the $\alpha$ and $\beta$ subunits (as highlighted in the homology model) was utilised for molecular docking. All compounds showed favourable molecular interactions via hydrogen bonding and hydrophobic interactions (Table 4) with binding energies ranging from 4.7- 7.9 kcal/mol. Vinblastine used as positive control for the docking experiment exhibited binding energy of 7.8 kcal/mol. Tetrahydrocannabivarin, 6⟨13:glyph 0:name="sbnd"⟩⟨/13:glyph⟩Hydroxymelatonin and Delta9-Tetrahydrocannabinol possessed the lowest binding energy compared to other docked compounds. Tetrahydrocannabivarin had the best (lowest) binding energy forming interactions with A: ALA247, B:ALA97, B:ASN99 (Fig. 6). 6⟨13:glyph 0: name="sbnd"⟩⟨/13:glyph⟩Hydroxymelatonin docked with binding energy of −7.7 kcal/mol forming molecular interactions with A: ALA247, B: GLN11, B: THR72, B: ASN99. Delta9-Tetrahydrocannabinol on the other hand had binding energy of 7.6 kcal/mol and interacted with A: ALA247, B: GLN11, B: ASN99. Analysis of the nature of interactions revealed the involvement of electrostatic interactions (HB) and hydrophobic between the compounds and amino acids with Plasmodium tubulin but the interaction was predominantly with the $\beta$-subunit (Table 4). Detail of molecular interaction between the $\alpha\beta$-tubulin and compounds of the cannabinoid family is shown in Fig. 7. The results of the drug-likeness prediction of the docked compounds identified are presented in Table 3. The essence of this analysis was to predict the likelihood of the compounds been utilized as a drug based on their physic-chemical properties based on the Lipinski's rule of 5, bioavailability score (BS) and Topology Polar Surface Area (TSPA). The parameters for the prediction was set at $<$ 500 g/mol(Da) molecular

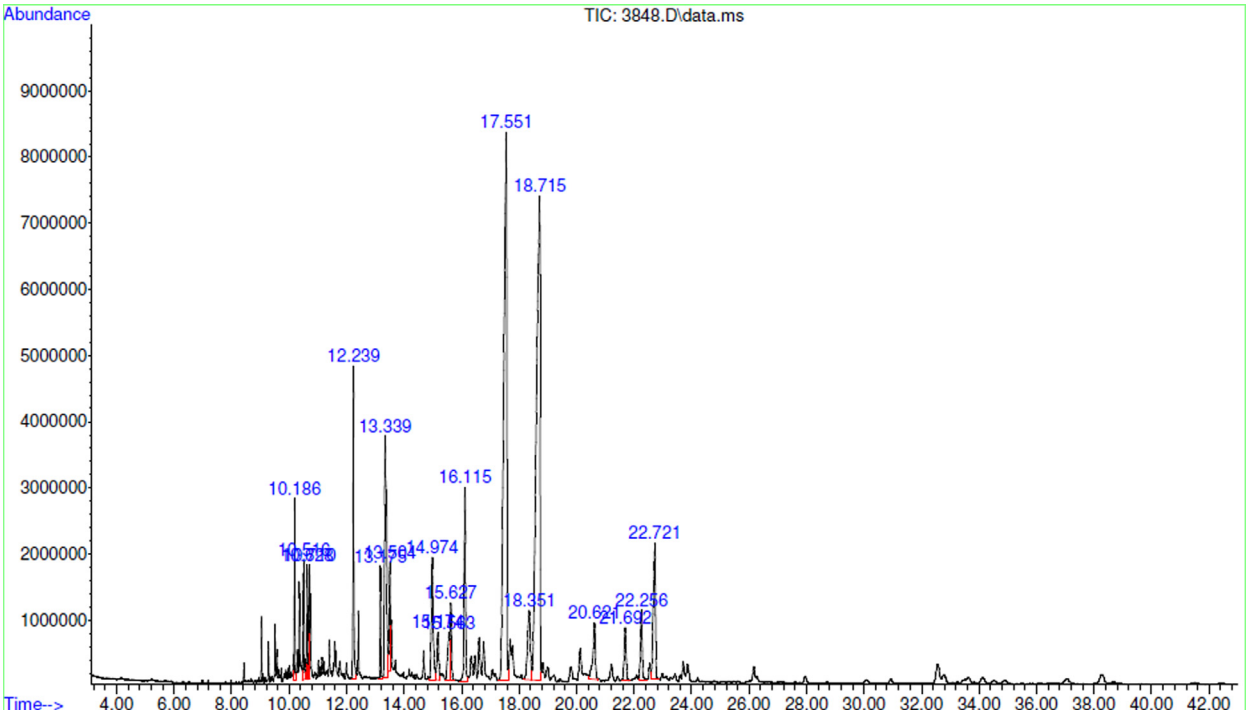


**Fig. 4.** GC–MS Chromatogram of ethanolic leaf extract of Cannabis.

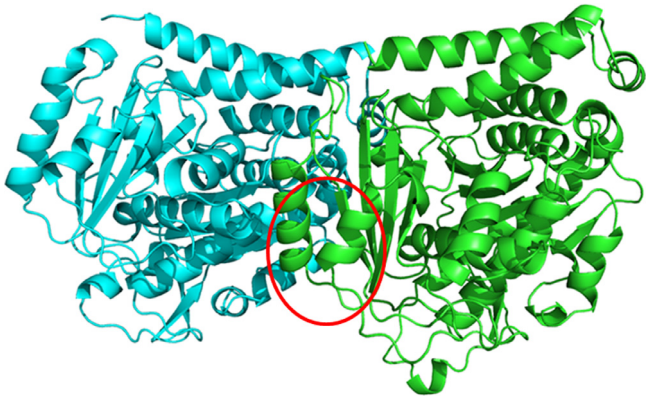

**Fig. 5.** Homology model of $\alpha/\beta$ tubulin of *Plasmodium falciparum*, A chain is coloured green; $\beta$ chain is coloured cyan. Red sphere indicates the vinblastine binding site.

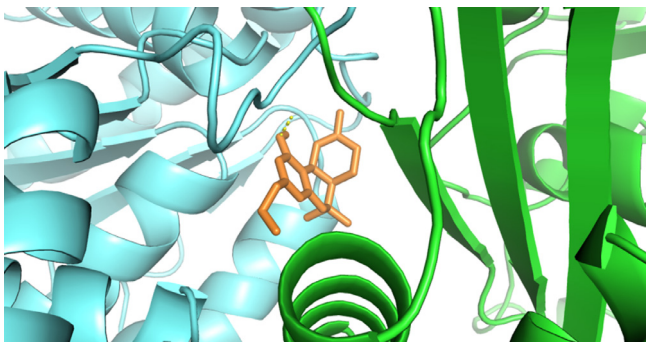

**Fig. 6.** Three dimensional (3D) docking pose of Tetrahydrocannabivarin with homology model of $\alpha/\beta$ tubulin of *Plasmodium falciparum*, A chain is coloured green; $\beta$ chain is coloured cyan. Tetrahydrocannabivarin is coloured as orange stick.

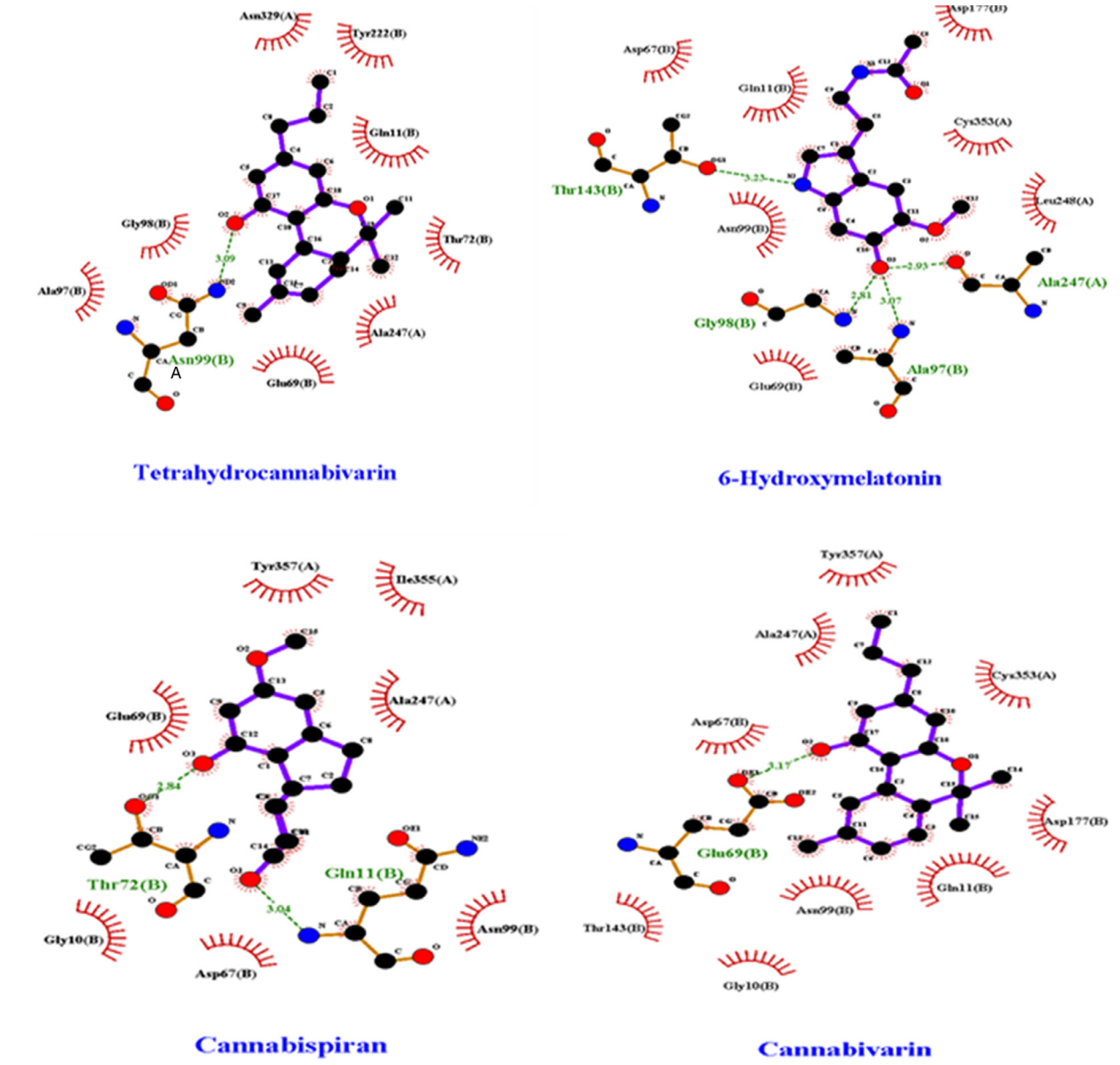


**Fig. 7.** Intermolecular interactions of selected compounds of *Cannabis sativus* with $\alpha/\beta$ tubulin of *Plasmodium falciparum.* Interacting amino acids are presented as brown sticks while purple sticks represent the ligand. Green dotted lines show hydrogen bonds while dashed half-moons represent hydrophobic interactions with amino acids of the protein.

weight, <5 LogP, <5 H-bond donor and <10 H-bond acceptor. Our results reveal that vinblastine which was used as the reference compound violated the molecular weight and H-bond acceptor properties of the Lipinski's rule. LogP which is a measure of partition coefficient between hydrophilic and hydrophobic face was >5 for compounds c (Hexadecanoic acid), d (Phytol), e (9,12-Octadecadienoic Acid), f (9,12-Octadecadienoic Acid, Ethyl Ester), k (Cannabichrome), and m (Cannabigerol) indicating that they will likely accumulate in fat deposits in vivo. Over all compounds a (Caryophyllene Oxide), b (Isoaromadendrene Epoxide), g (Tetrahydrocannabivarin), h (6-Hydroxymelatonin), i (Cannabispiran), j (Cannabivarin) and l (Delta9-Tetrahydrocannabino) satisfied all the properties of the Lipinski's rule and therefore good drug candidates. More noteworthy are compounds Tetrahydrocannabivarin, Cannabivarin and Delta9-Tetrahydrocannabinol that displayed complementarily good docking scores.

In this present study, the compounds in cannabis were explored for anti-tubulin function through molecular docking. Anti-tubulin agents have been reported to be specific in their inhibitory action on the parasite microtubule against that of the host microtubule [41]. Previous studies have reported two alpha ($\alpha$I and $\alpha$II) and one beta ($\beta$) tubulin gene for *Plasmodium falciparum* [42–44]. Tublins are present in both the sexual and sexual developmental stages (erythrocytic phase)

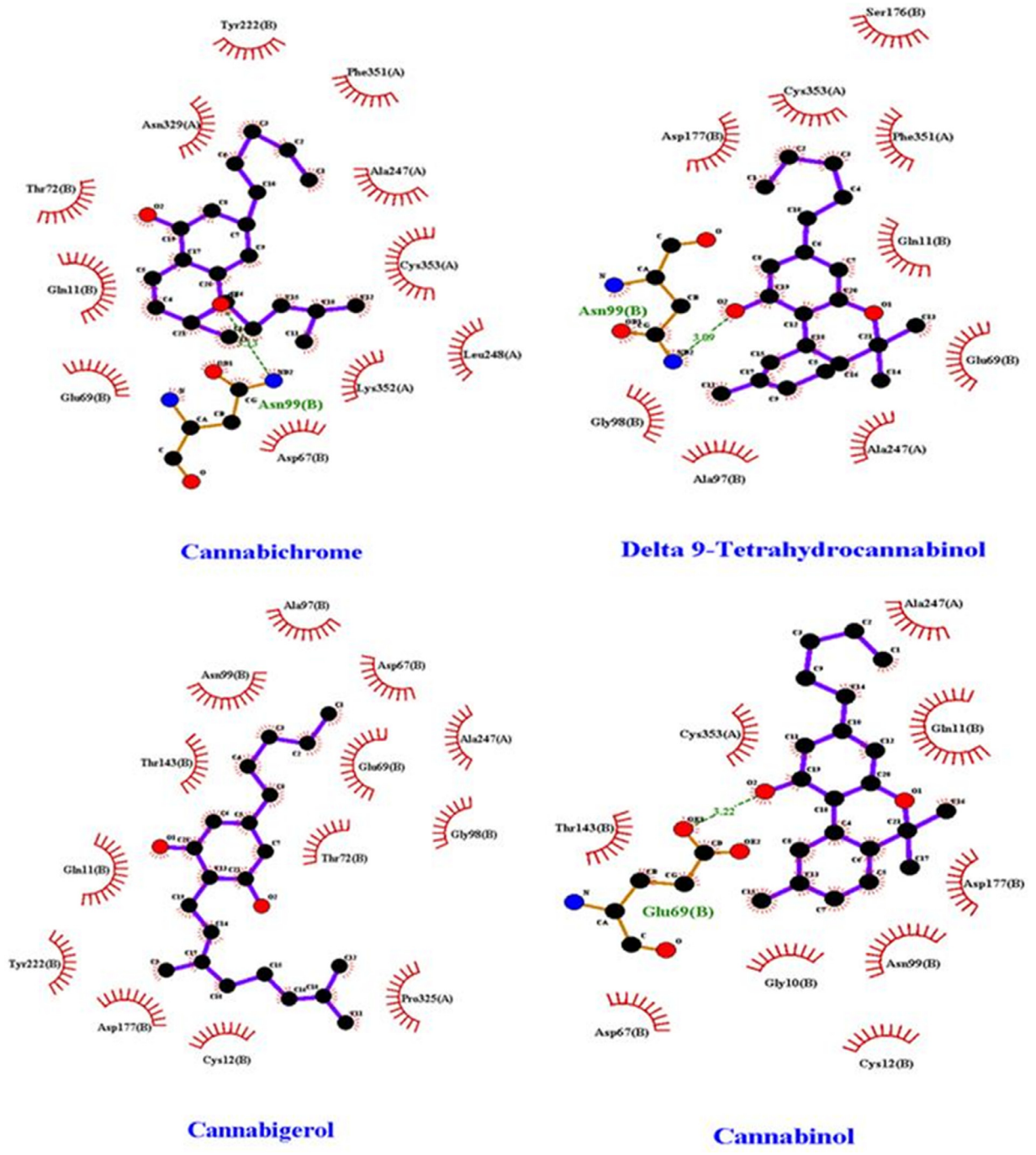


**Fig. 7.** Continued

of the plasmodium parasite but at different transcription levels. While the high rate of transcription is common in $\alpha$I and $\beta$ in both the sexual and asexual blood stages, stage $\alpha$II is expressed in male gametocytes, gametes, and newly formed zygotes during the sexual blood, and it was later discovered to have a promiscuous expression in both male and female gametocytes [45]. Tubulin levels were reported to increase sequentially from the ring stage, trophozoite stage, and schizont stage of the parasite, with the highest level found at the segment stage [46]. These evidences formed the basis for selection of tubulin protein for the computational analysis of cannabinoids in this study.

Due to the dearth of evidence to justify a binding site for any of the approved anti-malarial compounds, this work utilised vinblastine as a reference for bioinformatics analysis. Vinblastine is a proven inhibitor of tubulin of *Plasmodium fal-*

**Table 3.**
Druglikeness prediction of compound identified in GCMS.

| Compound | Pubchem ID | Chemical formular | Molecular wt (g/mol) | Log P | H -bond donor | H- bond acceptor | BS | TSPA |
|---|---|---|---|---|---|---|---|---|
| A | 1,742,210 | $C_{15}H_{24}O$ | 220.35 | 3.68 | 0 | 1 | 0.55 | 12.53 |
| B | 534,398 | $C_{15}H_{24}O$ | 220.35 | 3.53 | 0 | 1 | 0.55 | 12.53 |
| C | 985 | $C_{16}H_{24}O_2$ | 256.42 | 5.20 | 1 | 2 | 0.85 | 37.30 |
| D | 5,280,435 | $C_{20}H_{40}O$ | 296.53 | 6.22 | 1 | 1 | 0.55 | 20.23 |
| E | 3931 | $C_{18}H_{32}O_2$ | 280.45 | 5.45 | 1 | 2 | 0.85 | 37.30 |
| F | 5,365,672 | $C_{20}H_{36}O_2$ | 308.50 | 6.09 | 0 | 2 | 0.55 | 26.30 |
| G | 93,147 | $C_{19}H_{26}O_2$ | 286.41 | 4.57 | 1 | 2 | 0.55 | 29.46 |
| H | 1864 | $C_{13}H_{16}ON_2O_3$ | 248.28 | 1.51 | 3 | 3 | 0.55 | 74.35 |
| I | 162,936 | $C_{15}H_{18}O_3$ | 246.30 | 2.56 | 1 | 3 | 0.55 | 46.53 |
| J | 622,545 | $C_{17}H_{22}O_2$ | 282.38 | 4.50 | 1 | 2 | 0.55 | 29.46 |
| K | 30,219 | $C_{21}H_{30}O_2$ | 314.30 | 5.45 | 1 | 2 | 0.55 | 29.46 |
| L | 16,078 | $C_{21}H_{30}O_2$ | 314.46 | 2.28 | 1 | 2 | 0.55 | 29.46 |
| M | 5,315,659 | $C_{21}H_{32}O_2$ | 316.48 | 5.74 | 2 | 2 | 0.55 | 40.46 |
| N | 2543 | $C_{21}H_{26}O_2$ | 310.43 | 5.21 | 1 | 2 | 0.55 | 29.46 |
| O | 285,343 | $C_{14}H_{21}NO_{14}$ | 331.36 | 1.05 | 2 | 6 | 0.55 | 71.39 |
| Vinblastine | 13,342 | $C_{46}H_{58}N_4O_9$ | 810.97 | 3.77 | 3 | 11 | 0.17 | 154.10 |

**a** (Caryophyllene Oxide), **b** (Isoaromadendrene Epoxide), **c** (Hexadecanoic acid), **d** (Phytol), **e** (9,12–Octadecadienoic Acid), **f** ( 9,12-Octadecadienoic Acid, Ethyl Ester ), **g** (Tetrahydrocannabivarin), **h** (6-Hydroxymelatonin), **i** (Cannabispiran), **j** (Cannabivarin), **k** (Cannabichrome), **l** (Delta9-Tetrahydrocannabinol), **m** (Cannabigerol), **n** (Cannabinol), **o** (11 $\alpha$-Hydroxycephalotaxine).

**Table 4.**
Binding scores of the ligands from cannabis *with* $\alpha/\beta$ tubulin of *Plasmodium falciparum* considered in the molecular docking simulation and their interacting residues.

| Compound | Binding energy (kcal/mol) | Interacting residues |
|---|---|---|
| A | −6.9 | A:ALA247, B:ALA97, B:GLY98,B:ASN99 |
| B | −6.8 | A:CYS353, B:THR72 |
| C | −7.4 | A:CYS353 |
| D | −5.3 | B:ALA97, B:THR222 |
| E | −4.8 | B:ALA97, B:GLY98 |
| F | −4.7 | A:PRO325, A:CYS353, A:TYR357 |
| G | −7.9 | A:ALA247, B:ALA97, B:ASN99 |
| H | −7.2 | A:ALA247, A:CYS353, B:ASP67, B:ALA97, B:GLY98, B:ASN99 |
| I | −7.1 | A:ALA247, A:ILE355, B:GLN11, B:GLU69, B:THR72 |
| J | −7.7 | A:ALA247, B:GLN11, B:THR72, B:ASN99 |
| K | −7.0 | A:ALA247, A:LEU248, A:LYS352, B:ASN99 |
| l | −7.7 | A:ALA247, B:ALA97, B:ASN99 |
| m | −6.5 | A:PRO325, B:CYS12, B:ASN99, B:ASP177, B:TYR222 |
| n | −7.6 | A:ALA247, B:GLN11, B:ASN99 |
| o | −7.4 | A:PRO325, A:ILE355, B:CYS12, B:ASN99 |
| VLB | −7.8 | A:LYS19, A:ARG276, B:GLN11, B:PRO325, B:LYS326, B:VAL324 |

**a** (Caryophyllene Oxide), **b** (Isoaromadendrene Epoxide), **c** (Hexadecanoic acid), **d** (Phytol), **e** (9,12-Octadecadienoic Acid), **f** ( 9,12-Octadecadienoic Acid, Ethyl Ester ), **g** (Tetrahydrocannabivarin), **h** (6-Hydroxymelatonin), **i** (Cannabispiran), **j** (Cannabivarin), **k** (Cannabichrome), **l** ( Delta9-Tetrahydrocannabinol), **m** (Cannabigerol), **n** (Cannabinol), **o** (11 $\alpha$-Hydroxycephalotaxine).

*ciparum* with $IC_{50}$ 0.10 – 1.70 µM *in vitro* [47]. Furthermore, the antiplasmodial effect observed in this study demonstrated the extract's therapeutic efficacy against plasmodium infection. This suggests that the extract's compounds are acting synergistically to inhibit *plasmodium* parasite growth and proliferation in infected mice, possibly through molecular interactions between these compounds and some target macromolecules such as proteins. Also Bioinformatics techniques such as molecular docking and drug-likeness prediction are commonly used to predict and rank the potentiality of a compound in drug discovery. The *in silico* methods are less time consuming and effective at screening a large number of compounds. Literature abounds to justify the use of *in silico* methods for the screening of plant compounds targeted at the management of several human diseases. Such studies often probe into the interactions between plant compounds and protein targets to identify those with favourable binding characteristics. In this study, we combined molecular docking of compounds from Cannabis crude extract with Plasmodium tubulin to predict drug-likeness of the compounds. Tetrahydrocannabivarin, 6-Hydroxymelatonin, and Delta9-Tetrahydrocannabinol had the highest binding scores, indicating that they could inhibit *Plasmodium* tubulin and thus interfere with malaria cycle progression. This study could not find a significant correlation between the physicochemical properties of the compounds and the binding affinities of the compounds to *Plasmodium* tubulin. However, 6-hydroxymelatonin and delta9-Tetrahydrocannabinol were found to have favourable drug description properties thereby suggesting a role of these compounds as drug leads. This claim is supported by the fact that tetrahydrocannabinol has a higher binding affinity than other cannabinoids, as demonstrated in the current study. However, to the best of our knowledge, there is no literature to support the potential anti-malarial activity of tetrahydrocannabivarin. Judging by the

available computational data and evidence from literature, tetrahydrocannabinol and tetrahydrocannabivarin could be recommended as possible anti-malarial drug leads targeting $\alpha\beta$ tubulin. The amino acids interaction of the cannabinoids and vinblastine with the tubulin protein differed significantly with each compound. This reflects the diversity of physicochemical properties of the compounds. However, certain amino acids ASN99, ALA97, ALA247 and CYS353 were found to participate in the interaction for more than one ligand tested.

## Conclusion

The antimalarial therapeutic efficacy of drugs or medicinal plants is measured by a reduction in the percentage parasitaemia load and an increase in the percentage inhibition of parasite proliferation, as demonstrated in this study by an ethanolic extract of Cannabis in *Plasmodium berghei* NK-65 infected mice. Furthermore, Tetrahydrocannabivarin, 6-Hydroxymelatonin, and Delta9-Tetrahydrocannabinol, with binding energies of −7.9, −7.7, and 7.7 respectively, which inhibited *plasmodium* microtubules predictably in an *in-silico* study, could be responsible for Cannabis' antimalarial action. These compounds may have inhibited the parasite's multiplication and growth in red blood cells by interfering with cellular processes required for parasite multiplication and growth. The new information in this study should pique the interest of the larger research community, as it identifies and characterizes the antimalarial active ingredient of cannabis. Similarly, there may be interest in screening for additional chemical agents with tubulin inhibition, as reported in this study, and redirecting them to the *plasmodium* tubulin inhibitor.

## Declaration of Competing Interest

All authors declared that there is no conflict of interest

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