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Research Article

Paludose™ Herbal Mixture: Antimalarial Activity, Safety and Chemical Composition in a Mouse Model of *Plasmodium berghei*

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Abstract
Paludose™ is a herbal mixture with wide usage in Cotonou, Republic of Benin for the treatment of malaria, but with little information on its beneficial effects. Here we report its antimalarial activity, safety and chemical composition in a mouse model of *Plasmodium berghei.* Seventy Swiss mice, infected intravenously with 1 x 10^6 chloroquine sensitive *P. berghei* NK65 strain were assigned to 5 treatment groups to receive, orally; 1x, 2x and 4x Paludose™ (0.143, 0.29 and 0.57 mL/kg), artemether/lumefantrine (AL; 0.5 mg/3.0 mg/mL) or 2 mL/kg normal saline. Suppression of parasite growth in treatment groups was calculated. Liver and kidney function tests of the experimental mice were determined and mortality monitored till D21 of the experiment. The phytochemical constituents of Paludose™ were identified using Gas Chromatography-Mass Spectrometry. The median lethal dose LD_{50} of Paludose™ herbal mixture is > 7.15 mL/kg. 2x and 4x Paludose™ produced significant suppression of parasite growth of 92 and 87 % respectively on day 4 post treatment, which was comparable to 99.3 % observed in infected mice treated with AL. However, the initial significant suppression of parasite growth in mice treated with Paludose™. was not sustained as parasites recrudesced. Treatment with 4x Paludose™ and AL significantly prolonged survival of *P. berghei* infected mice. As parasites recrudesced in experimental mice, the serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN) increased ($p < 0.05$) in groups treated with Paludose™ and the untreated group compared to AL group on D14 and D21. Five major compounds identified in the GCMS were 3,5-Dimethylpyrazole, Limonene, Borneol, 5-Hydroxymethylfurfural and β-D-Glucopyranose. Paludose™ appears to be a safe but short acting antimalarial herbal mixture.

Key Words: Paludose-herbal, efficacy, safety, *Plasmodium-berghei* malaria, chemical constituents

INTRODUCTION

Malaria remains a major public health problem. Pregnant women and children under 5 years of age are the most vulnerable to malaria infection. An estimated 219 million cases of malaria and 435,000 malaria deaths occurred globally (WHO, 2018). The majority of the estimated cases (92%) and deaths (93%) occur in sub-Saharan Africa. The emergence of resistance to almost all available antimalarial drugs, high cost of efficacious drugs and their non-availability, especially in rural areas (Barine *et al.,* 2017) have further compounded the problems of malaria case management. Antimalarial drug resistance, particularly *Plasmodium falciparum* resistance, has been a major setback in the fight against malaria and its attendant complications (Menard and Dondorp, 2017). Partial resistance has emerged to Artemisinin-based combination therapy (ACT) which is the first-line treatment for uncomplicated malaria globally, necessitating the need to search for new antimalarial agents (Menard and Dondorp, 2017; Ashley *et al*., 2014).

Plants have been the basic source of sophisticated traditional medicine systems for thousands of years and were instrumental to early pharmaceutical drug discovery and industry (Shah and Bhat, 2019). There is huge dependence on traditional medicines; particularly the plant based antimalarial products for the treatment of febrile illnesses in Africa (Mojab, 2012). Paludose™, is an antimalarial herbal mixture sold in Cotonou, Republic du Benin. The dosage ranges from half to one teaspoon full twice daily for three days for children more than 30 months of age to 10 mL (2 teaspoon full) for three days for adults. Preventive treatment is one teaspoon full daily as recommended by the manufacturer. This herbal mixture is used for the treatment of malaria and other febrile illnesses in the Republic du Benin. However, there is no available data to

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proof scientifically, the efficacy and safety of the antimalarial property of Paludose™. This study was designed to evaluate the efficacy and safety of Paludose™ as an antimalarial herbal mixture in treating *Plasmodium berghei* infected mice.

MATERIALS AND METHODS

Ethical consideration: Ethical approval was obtained from Animal Care and Use Research Ethic Committee (ACUREC) of University of Ibadan (UI-ACUREC/18/0068). Standard guidelines and procedures for the use of animals in research, teaching and care was followed (NIH, 85-93 revised in 1985).

Experimental animals: Swiss mice, 8 weeks of age (18–22 g) were obtained from the animal house of the Institute for Advanced Medical Research and Training (IAMRAT). The mice were maintained in the experimental animal handling facilities of IAMRAT, under standard condition at room temperature with mouse cubes (ACE®) and water *ad libitum*.

Determination of acute toxicity of Paludose™: The oral acute toxicity of Paludose™ was estimated in two groups of three Swiss mice each weighing between 18-22g using a modified Organisation for Economic Cooperation and Development (OECD) 423 guidelines. The mice received orally, 20x and 50x (2.86 and 7.15 mL/kg) Paludose™ respectively. The animals were monitored for the first critical four hours, 24 hours and at day 14 while number of deaths per group were recorded. Also, gross behavioural changes such as feeding, paw-licking, stretching, hair erection, shivering, heat-seeking behaviour, mortality and other signs of toxicity were observed.

Parasite inoculation and treatment of infected mice
Chloroquine sensitive *P. berghei* NK65 obtained from MR4 maintained by serial passage in IAMRAT was used. Donor mice infected with a parasitaemia of 30% was used to infect experimental mice. Each mouse used in the experiment was inoculated with 1.0 x 10^6 parasitized red blood cells suspension in 0.9% NaCl (Kendall McGaw Laboratories Inc. IRVINE. CA. USA). The Rane's test described by Ryley and Peters (1995) was used. Briefly, seventy (70) Swiss mice were infected intravenously with 1 x 10^6 chloroquine sensitive *P. berghei* NK65 strain. The animals were assigned to 5 treatment groups to receive; 1x, 2x and 4x Paludose™ (0.143, 0.29 and 0.57 mL/kg; given twice daily for three days), artemether/lumefantrine (Standard drug; 0.5 mg/3.0 mg/mL twice daily for 3 days) or saline 2 mL/kg (negative control group; once daily for 3 days). The infected mice were treated orally 72 hr after parasite inoculation.

Assessment of antimalarial activity of Paludose™: Thin blood films were prepared from the tail vein of each mouse 24 hour after the last day of treatment (Day 4). Thereafter, on day 7, 9, 12, 14 and 21. Thin blood films were air-dried, fixed with methanol and stained with 10% freshly prepared Giemsa stain for 30 min. Slides were examined microscopically under oil immersion objective to identify and quantify parasitaemia. Inhibition of parasite growth in treatment groups was calculated in relation to parasite growth in the normal saline treated control group using the formula:

$$\% \text{ suppression of parasite growth} = 100 - \left[\frac{\text{mean parasitaemia of treated mice}}{\text{mean parasitaemia of control mice}} \times 100\right]$$

(Abiodun *et al*., 2016). Mortality was monitored and recorded daily.

Effects of Paludose™ on liver and kidney function on mice infected with *P. berghei*: Three mice from each of the treatment groups were euthanized on D 7, D 14 and D 21 post parasite inoculation to assess the safety of the treatment regimen. Whole blood was collected through cardiac puncture from each of the animals into plain bottles. Serum from the clotted blood was separated and stored at -20°C till needed for biochemical analysis. The liver and kidney function tests of the experimental mice were determined by estimating the level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea and creatinine using Randox® kits following the manufacturer instruction.

Determination of the weight of organs: The liver and kidney were removed on D 7, D 14 and D 21 post parasite inoculation from mice after induction of terminal general anaesthesia by inhalation of diethyl ether (Sigma Co., Germany). Organ wet weights were measured and compared with controls as indices for degree of hepatomegaly and renal enlargement.

Determination of survival rate: Mortality was monitored daily and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the 21 day of the experiment.

Phytochemical components of Paludose™ by Gas chromatography-mass spectrometry (GC-MS) analysis: The phytochemical components of Paludose™ were identified using Agilent technologies 5975 MSD (Mass Spectrophotometer Detector) model. Software data analysis of GC-MS was used in data processing. The mobile phase and stationary phase are helium (99.99% purity) and the column model HP5 MS respectively. Separation of compounds and quantitative analysis performed on GC-MS components were conducted by a capillary column with a diameter of 0.320 mm, thickness 0.25 µm and a length of 30 m with a starting temperature of 60°C, rising by 10°C per minute until temperature reached is 240°C and ending time was 6 minutes. The injection volume is 1 microliter and the heater or detector temperature is 250°C. The sample was put in a vial bottle and placed in an auto injector sample compartment. The automatic injector injects the sample into the liner. The mobile phase pushes the sample from the liner into the column where separation takes place into different components at different retention time. The MS interpret the spectrum MZ (mass to charge ratio) with molar mass and structures. The identification of the compounds was achieved by comparing the data in the mass spectrum with existing data in the NIST 14. Library data Base (Adebayo *et al.*, 2017).

Data analysis: Data were presented as mean ± SEM (Standard error of mean). Percentage survival was calculated. Data obtained was statistically analyzed using one-way ANOVA followed by a post hoc test (Tukey's multiple comparison test). p-value < 0.05 was considered statistically significant

RESULTS

Acute Toxicity Tests: The median lethal dose LD_{50} of Paludose™ herbal mixture was estimated to be approximately > 7.15 mL/kg having shown no mortality at 2.86 and 7.15 ml/kg doses tested.

Antimalarial Activity: Parasitemia in the untreated control mice (negative control) ranged from 6.3% on day 4 to 50.9 % on day 21 post treatment. Parasitemia in animals infected with *P. berghei* and treated with 1x, 2x and 4x Paludose™ ranged from 4.11 to 49.28, 0.53 to 43.34 and 0.80 to 36.37 % day 4 to day 21 post treatment respectively (Table 1). In addition, parasitaemia in mice infected and treated with the standard drug AL ranged from 0.04 to 0.85 % day 4-21 post treatment. 2x and 4x Paludose™ produced significant suppression of parasite growth of 92 and 87 % respectively on day 4 post treatment, which was comparable to 99.3 % observed in infected mice treated with AL (Table 1). However, the initial significant suppression of parasite growth was not sustained for many days in infected mice treated with Paludose™ (Table 1).

Mean Survival Time of the Mice: The survival curve of mice infected with *P. berghei* and treated with Paludose™ is presented in fig.1. Mice in the negative control group had mean survival time of 15.6 ± 1.54 days. On the other hand, all the mice in AL control group survived till day 21. Mice treated Paludose™ had mean survival time ranging from 15.8 ± 1.46 to 19.0 ± 0.71 days. Treatment with 4x Paludose™ and AL significantly prolonged survival of *P. berghei* infected mice (fig. 1b and c).

Table 1:
Mean Parasitaemia and Percent Suppression of Parasite Growth in Mice Infected with *P. berghei* and Treated with Paludose ®

	Mean ± SEM and %SPG								
*Days	PAL 1X	% SPG	PAL 2X	% SPG	PAL 4X	% SPG	A/L	% SPG	Neg.Con
4	4.11±0.32	**34.54**	0.53±0.05	**91.53**	0.80±0.07	**87.2**	0.04±0.03	**99.3**	6.28±0.12
7	7.02±0.23	**34.07**	4.55±0.25	**57.26**	4.47±0.29	**58.03**	0.60±0.01	**94.35**	10.65±0.29
9	13.51±0.30	**12.94**	9.57±0.29	**38.32**	7.24±0.30	**53.33**	0.60±0.24	**94.30**	15.52±0.19
12	17.73±0.29	**8.76**	14.62±0.44	**24.76**	11.94±0.24	**38.55**	1.35±0.19	**93.04**	19.43±0.37
14	35.67±7.02	**10.20**	32.72±9.03	**17.62**	23.20±1.73	**41.59**	0.76±0.32	**97.53**	39.72±4.06
21	49.28±9.98	**3.08**	43.34±6.19	**14.77**	36.37±6.12	**28.48**	0.85±0.26	**98.32**	50.85±3.31

**Days - post treatment day, PAL 1X, 2X & 4X - Paludose (0.143, 0.286 & 0.572 mg/kg), % SPG - percent suppression of parasite growth, AL- artemether/lumenfantrine, Neg.Con - negative control*

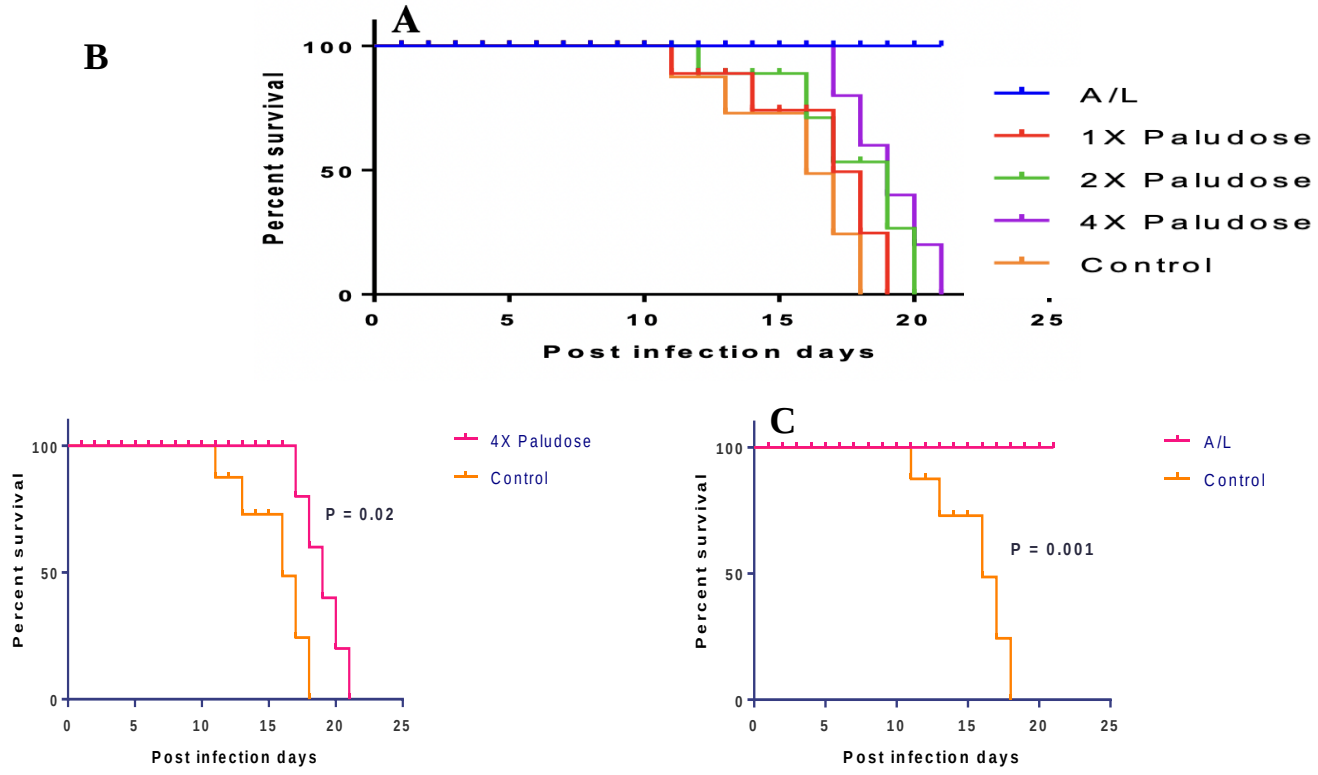


Figure 1:
Survival Curve of Mice Infected with *P. berghei* and Treated with Paludose ®
AL- artemether/lumenfantrine, Paludose 1X, 2X & 4X - (0.143, 0.286 & 0.572 mg/kg respectively). ***1A.*** *Survival curve of P. berghei infected mice in the control and treatment groups.* ***1B..****Comparison of survival curve of P. berghei infected mice teated with 4X Paludose and control (untreated mice).* ***1C.*** *Comparison of survival curve of P. berghei infected mice teated with AL and control (untreated mice)*

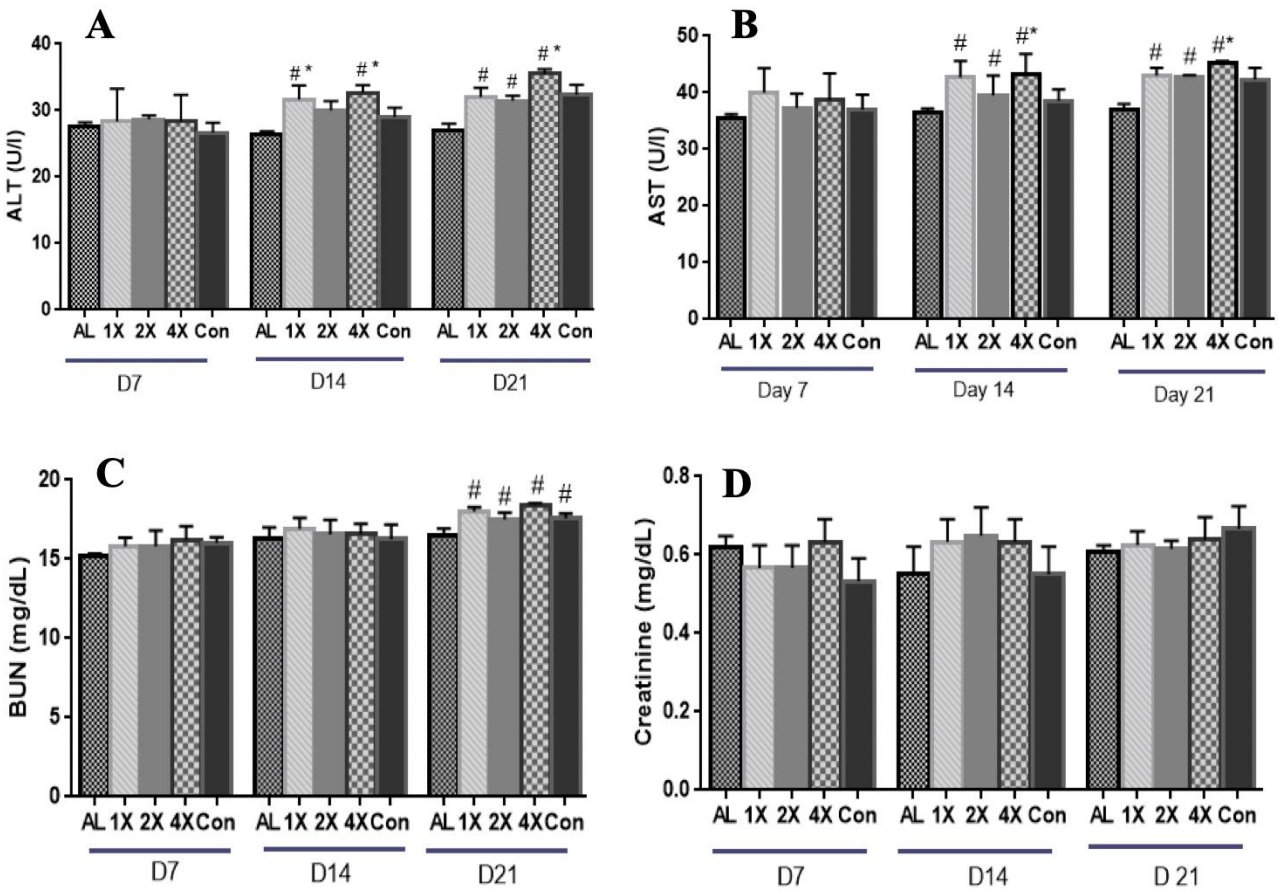


Figure 2:
Post treatment liver and kidney function test in mice infected with *P. berghei* NK65 and treated with PALUDOSE™
AL- artemether/lumenfantrine, 1X, 2X & 4X - Paludose (0.143, 0.286 & 0.572 mg/kg respectively), con - negative control. Data are expressed as mean ± SEM (n=5), # - AL vs other treatment groups $P \leq 0.05$.; * - negative control vs other treatment groups on D14 and D21

Liver function Test in Mice Infected and Treated with Paludose™: Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in mice infected with *P. berghei* NK65 treated with Paludose™ , AL and normal saline (-ve control) are shown in fig. 2a & b. The serum ALT and AST levels were significantly higher ($p < 0.05$) in the untreated group and groups treated with Paludose™ compared to AL group on D14 and D21 respectively.

Kidney Function Test in Mice Infected and Treated with Paludose™: Serum BUN levels in mice treated with *P. berghei* NK65 and treated with Paludose™, AL and -ve control are shown in fig. 2c. There was a slight significant rise in serum BUN levels in the paludose treated and –ve control groups, on D21 when compared with AL ($p \leq 0.05$). Furthermore, serum creatinine levels in mice infected with *P. berghei* NK65 and treated with Paludose™, AL and –VE control are shown in fig. 2d. There was no significant rise in serum creatinine levels except slight increase in the -ve group on D 21 that was not statistically significant (fig. 2d).

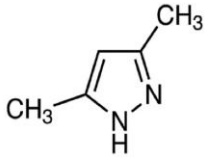
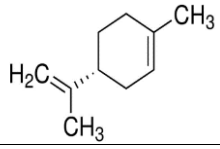
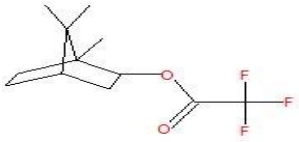
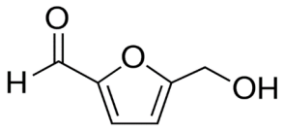
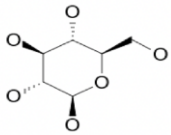
Gas Chromatography-Mass Spectrometry: Figure 3 shows the chromatogram of Paludose™ revealed by GC-MS. The five major compounds identified in the GC-MS are 3,5-Dimethylpyrazole, Limonene, Borneol,5-Hydroxymethylfurfural, β-D-Glucopyranose. The retention time, percentage abundance and structures of the compounds are presented on Table 2

DISCUSSION

The effectiveness of antimalarial treatment has been hampered by the emergence of parasite resistance to almost all available antimalarial drugs, including more recently the artemisinin class of drugs (Menard and Dondorp, 2017). There is thus an urgent need to develop newer antimalarial drugs and drug combinations. There is growing interest in the use of plants for medicinal purpose over the years due to their easy preparation procedures, cost effectiveness and fewer side effects (Hai *et al*., 2015). Phytochemicals have been shown to possess significant antiplasmodial activity (Abiodun *et al.,* 2011; Udobang *et al*., 2017; Vetvicka and Fernandez Botran, 2018). Many studies on the use of medicinal plants as antimalarial in Africa have reported varying results from high promising activities to very low effects against plasmodium species (Lawal *et al.,* 2015; Abiodun *et al*., 2016; Sulaiman *et al*., 2017). Nevertheless, natural products from plants remain a reliable source of phytomedicine for the treatment of certain ailments (Shuaib *et al*., 2019).

Paludose™, a herbal mixture used in the treatment of malaria and other febrile condition was investigated in this study using a mouse model of *P. berghei.* Paludose™ produced up to 11- fold suppression of parasitaemia in infected mice 24 hrs after 3 days of treatment. Despite the initial suppression of parasite growth observed with the treatment of Paludose™, recrudescence occurred after day 3 and parasitaemia increased steadily.

Table 2:
Phytochemical components of PALUDOSE™ as revealed by GC-MS spectra

S/No	Compound Name	Area percentage (%)	Retention Time (Min)	Molecular structure
1.	3,5-Dimethylpyrazole	6.51	4.489	
2.	D-Limonene Limonene	21.44	6.526	
3.	Borneol, L-α -Terpineol	2.83	9.450	
4.	5-Hydroxymethylfurfural	61.76	11.218	
5.	β-D-Glucopyranose,	7.47	16.099	

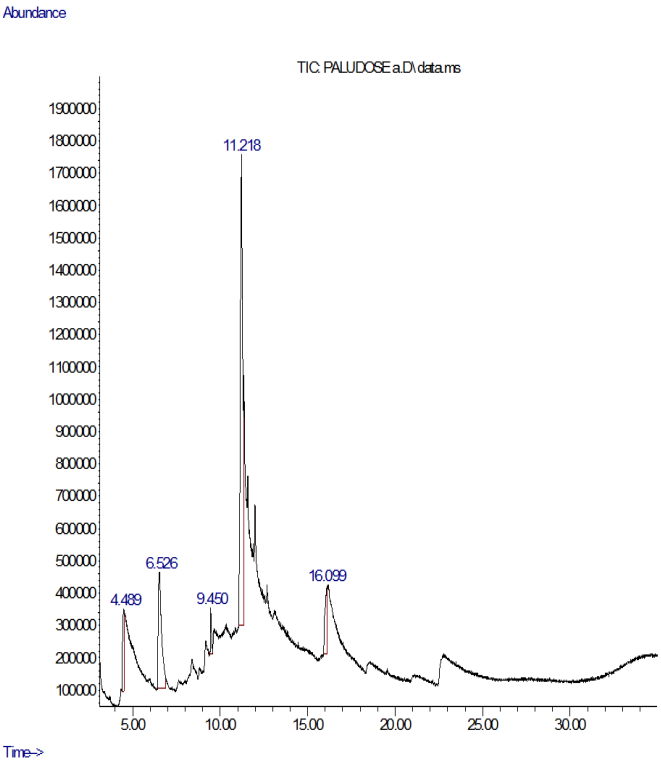


Figure 3:
The gas chromatography-mass spectrometry (GC-MS), chromatogram of PALUDOSE™

The short duration of antiplasmodial activity observed in this study is contrary to the claims of the indigenous people who uses Paludose™ for the treatment of malaria. It is a well-known fact that people living in malaria endemic region are semi-immune thus possessing some background immunity against malaria infection. The intersection of immunity to plasmodial and antimalarial drug activity is poorly understood (Falade *et al*., 2018; Baird, 2005). It is possible that a low antimalarial activity observed is sufficient to clear the infection in semi-immune individual. Furthermore, reports of some plant extracts locally used as antimalarial remedies alleviating the symptoms associated with malaria without having any significant effects on the parasite have been documented (Abiodun *et al*., 2016; Ajaiyeoba *et al*., 2006). More so, plant compounds that suppress or partly inhibit the growth of the parasite as well as those that stimulate the immune system or provide symptomatic cure and reverse some pathologic features of malaria infection are reported to enhance antiplasmodial activity in semi-immune individuals living in endemic areas (Falade *et al*., 2018). Therefore, this mixture may augment the immune system thus clearing the parasites and in a way showing antimalarial activity. However, using this herbal remedy to treat malaria in children and pregnant women may be dangerous because of their naïve (children) or compromised (pregnant women) immune status. Also, Paludose™ has a short duration of activity, which may not be a good monotherapy option especially in the era of antimalarial drug resistance.

It is well known that malaria disease causes weight loss (Belay *et al*., 2018). The herbal mixture was able to maintain the well-being of the mice at the highest dose of the mixture, as weight change on day 0 and day 14 was not significant in the presence of malaria disease in contrast to the other two lower test doses, which has significant weight loss. It was also observed that Paludose™ extended the survival of the mice treated with the highest dose. This extended survival may be multifactorial. This may be due to the significant reduction in parasitemia at the high doses of Paludose™ in part and also the high concentration of certain phytochemicals present in the mixture that improves the well being of mice in the presence of malaria infection. (Martey *et al.*, 2013).

In addition, Paludose™ appears to be safe in the acute toxicity test even up to 50 times the recommended dose. The kidneys and liver function test did not reveal any significant elevation compared to controls on day 7. The liver is the major organ of metabolism and excretion of bile, detoxification, enzyme and protein synthesis, making it a target site for toxic chemical by concentrating their metabolites in it at high doses resulting in organ damage and subsequent failure, (Enechi *et al*., 2019). It is not clear if the rise in the serum ALT and AST on day 14 and 21 in infected mice treated with Paludose™ has any clinical significance. This is because the rise in the serum ALT and AST on day 14 and 21 might be as a result of the recrudescence infection and not the effect of Paludose™. Since the antimalarial activity of the herbal remedy was short and the change in ALT and AST levels was similar to the untreated control. It has been reported that ALT and AST are raised in severe malaria infection (Jain *et al*., 2016).

The kidney is a major excretory and osmoregulatory organ of mammals, and this makes it a target for toxic chemical by concentrating xenobiotics and their metabolites in high levels resulting in the immediate organ failure or delayed malfunctioning (Enechi *et al*., 2019). Kidney function test, blood urea and nitrogen indicates excessive breakdown of protein in the body, while creatinine is formed from breakdown of products of creatinine phosphate in muscles and it's usually in prolonged breakdown. The maintenance of the creatinine level in mice indicate that Paludose™ might contain phytochemicals that are reno-protective (Enechi *et al*., 2019; Martey *et al.*, 2013). However, the rise in levels of BUN on day 21 similar to the negative group might be due to malaria disease progression since the herbal remedy is short acting

Five compounds were identified in Paludose™ using GCMS. The most abundant compound is 5-Hydroxymethylfurfural. a reducing sugar found in honey and various processed foods. It is reported to have anti-oxidative (Zhao *et al*., 2013), anti-allergic (Yamada *et al.*, 2011), anti-inflammatory (Kitts *et al.*, 2012), anti-hypoxic (Li *et al*., 2011), anti-sickling (Abdulmalik *et al.*, 2005) and anti-hyperuricemia effects (Lin *et al.*, 2012; Shapla *et al.*, 2018). D-Limonene was the second most abundant compound in the herbal mixture. D-Limonene and its derivatives are colourless liquid aliphatic hydrocarbon classified as cyclic monoterpene. They are major components in the oils of citrus fruit peels. The D isomer occurs in nature as the fragrance of oranges a flavouring agent in food manufacturing (Miller *et al*., 2011). It is used to prevent cancers (Soulimani *et al.*, 2019), treat bronchitis, used as anti-inflammatory compound (Rufino *et al*., 2015), immune regulation via gene signaling thereby protecting the body against invasive pathogens (Lappas and Lappas, 2012). The compound is used in foods, beverages and chewing gums as a detoxifying agent (Soulimani *et al.*, 2019, Miller *et al*., 2011). D-limonene has been shown to arrest malaria parasite development and inhibits iso-prenylation of proteins in *P. falciparum* (Erasto and Viljoen, 2008; Saito *et al*., 2016). The antiplasmodial activity observed here might be as a result of D-limonene.

Furthermore, β-D-Glucopyranose, the 3rd compound identified by GC-MS analysis of Paludose™ is a simple monosaccharide, an energy source for living organism. It also has anti-plasmodial activity and has 100% protection against formalin killed erythrocyte stages of *Plasmodium berghei* after simultaneous intravenous glucan injections (Vetvicka and Fernandez-Botran, 2018). It is naturally occurring and found in fruits and other parts of plants in its free state. It is used therapeutically in fluids as nutrients replacement (Vetvicka and Fernandez-Botran, 2018). In addition, 3,5-dimethylpyrazole was also identified, which appears to be a synthetic compound. It is used as precursors for the preparation of liquid crystals in the synthesis of polymer as ligands in transition metal complexes (Pundir *et al*., 2017). Lastly, Borneol, L-α –Terpineol a bicyclic monoterpenoid alcohol was also identified in the herbal mixture. It is an aromatic spice, used in food and folk medicine. Monoterpenes identified in some medicinal plants and fruits like borneol, α-terpineol, terpine-4ol, have been found to have antiplasmodial activity by inhibiting isoprenyl-diphosphate synthase (Udobang *et al*., 2017). Borneol, L-α –Terpineol interferes with the fatty acid biosynthesis (FAS II) of the plasmodium, and also inhibit entry of L-glutamine and Myo-inositol into infected red blood cells, thereby elevating erythrocyte oxidation and interfere with plasmodium protein synthesis (Udobang *et al*., 2017).

Other constituents in Paludose™ according to the manufacturer but not identified by GC-MS were aldehyde tans-cinnamic, cinnamyl acetate, eugenol, trans-methoxycinnamaldehyde, and levamenthol. The limitation of using GC-MS is that polar constituents might not be detected. Only non-polar and few moderately polar compounds can be detected using GC-MS. Thus, it will be also important to use HPLC-MS.

In conclusion, the result obtained in this study indicates that Paludose™ herbal mixture possess moderate antiplasmodial activity in a mouse model of *P. berghei* infection. However, the activity is short-lived. The herbal mixture appears to be safe. Some of the phytochemical constituents present in this herbal mixture were previously reported to possess antimalarial activity. Thus, the antimalarial activity observed in this study might be as a result of these constituents

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