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Original Research Article (Experimental)

In vitro antiplasmodial activity, cytotoxicity, and gas chromatography — flame ionization detector metabolites fingerprint of extracts and fractions from *Tetrorchidium didymostemon*

Osamudiamen Ebohon [a, *], Francis Irabor [a], Nekpen Erhunse [b, c], Abigail Omagene [b], Ehimwenma Sheena Omoregie [b]

[a] Michael and Cecilia Ibru University, Faculty of Natural and Applied Sciences, Department of Biological and Chemical Sciences, Biochemistry Program, P.M.B. 100, Agbarha-Otor, Delta State, Nigeria
[b] University of Benin, Faculty of Life Sciences, Department of Biochemistry, Malaria Research, Molecular Biology and Toxicology Unit, P.M.B 1154, Benin City, Nigeria
[c] International Centre for Genetic Engineering and Biotechnology, Malaria Drug Discovery Research Group, New Delhi, 110067, India

A R T I C L E   I N F O

A B S T R A C T

Background: Tetrorchidium didymostemon is used as an antimalarial remedy in southern Nigeria.
Objective(s): This study was aimed at providing scientific validation for the use of *T. didymostemon* in the treatment of malaria in Nigeria.
Materials and methods: Plasmodium falciparum 3D7 (*Pf*3D7) strain was cultured and maintained in fresh O^+ human erythrocytes. Standard methods were used to evaluate *in vitro* antiplasmodial activity, cytotoxic effect on Vero cell line, phytochemical screening, and antioxidant capacity. Gas Chromatography — Flame Ionization Detector (GC-FID) metabolite fingerprinting of the most potent fraction was carried out.
Results: The methanol leaf extract had higher antiplasmodial activity ($IC_{50}Pf3D7 = 25 \pm 0.21$ μg/mL) in comparison with the stem bark extract (SBE) ($IC_{50}Pf3D7 = 50 \pm 0.94$ μg/mL). The n-hexane fraction of the leaf extract had the best antiplasmodial activity ($IC_{50}Pf3D7 = 3.92 \pm 0.46$ μg/mL) and selectivity index. This was followed by the dichloromethane ($IC_{50}Pf3D7 = 12.5 \pm 1.32$ μg/mL), ethyl acetate ($IC_{50}Pf3D7 = 35.0 \pm 4.80$ μg/mL), and hydromethanol fraction which was inactive ($IC_{50}Pf3D7 > 100$ μg/mL). All extracts and fractions were not toxic on Vero cell line ($CC_{50} > 1000$ μg/mL). The n-hexane and dichloromethane fractions had the highest amount of phytochemicals. GC-FID analysis revealed high amounts of kaempferol, α-pinene, camphor, humulene, azulene, and β-caryophyllene in the n-hexane fraction.
Conclusion: The results of our study validate the traditional use of *T. didymostemon* in the treatment of malaria in southern Nigeria. They also suggest that the phytoconstituent(s) responsible for the antiplasmodial activity of this plant may be more extractable in non-polar solvents.

1. Introduction

Malaria is one of the world's deadliest diseases caused by protozoan parasites of the genus *Plasmodium*. Sub-Saharan Africa disproportionately shares the brunt of the disease with children under 5 years and pregnant women being the sub-populations worst hit by the disease. In 2018, an estimated 228 million cases of malaria were reported worldwide and Africa accounted for 93% [1]. The same year, 405 000 deaths from malaria were recorded globally and this was lower than the 416 000 estimated deaths in 2017, and 585 000 in 2010 [1]. This decrease in deaths due to malaria may be a result of increased availability of antimalarial drugs and distribution of long-lasting insecticide treated nets. Despite the decreased malarial mortality in 2018, Africa still accounted for 94% of all malaria death of which Nigeria contributed 24% [1]. The

* Corresponding author.
  E-mail: ebohonosamudiamen@yahoo.com
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prevalence of *Plasmodium falciparum* (the most virulent parasite), in Africa plays a significant role in these deaths [2]. Several factors contribute to the increased malaria-related deaths in endemic regions, such as, temperature and climate that favour the growth of the parasite and its vectors, lack of good health facilities, poverty, poor hygiene, and sometimes religious beliefs.

P. falciparum is highly skilled at evading the toxic effects of antimalarials by generating resistance mutations. As a result, all antimalarials used till date in managing malaria and the resistant mutations are rendered useless [3–8]. Presently, there is no artemisinin resistance in Africa. However, a recent report from a study in Rwanda identified *Pfkelch13* R561H mutation in 7.4% of the studied population and this mutation was confirmed to drive artemisinin resistance *in vitro* [9]. Similarly, there are increasing numbers of therapeutic failures after regimen with artemisinin-based combination therapy (ACT) of artemether-lumefantrine in regions of Africa that have used this ACT for over 10 years [10–12]. These treatment failures have been linked to decreased parasite susceptibility to partner drugs [13,14].

Therefore, there is a need to identify novel, cheap, and effective alternate therapy to manage and treat this disease in order to avoid a global health crisis that may arise if the resistant parasite spreads to endemic region such as Africa. Such therapies should be alien to the parasite and fast-acting like the multi-stage artemisinins. Till date, traditional medicines have given rise to some of the most effective antimalarial drugs such as quinine and artemisinin. The use of herbs for the management of diseases in malaria-endemic regions is widespread [15,16]. Most people in rural areas use herbal medicine because it is cheap and readily available. They also believe that it's more effective than the orthodox drugs which in most cases are unaffordable. However, there is scarce scientific evidence to support the use of many of these plants. Thus, in Nigeria, there are several medicinal plants with traditionally acclaimed antimalarial benefits in need of scientific validation.

Tetrorchidium didymostemon (Baill.) Pax & K. Hoffm is an evergreen shrub belonging to the Euphorbiaceae family. It has drooping branches and can grow up to 25 m tall [17]. It is called *Iheni* (Edo-language, Nigeria) and *ofun oke* (Yoruba-language, Nigeria). *T. didymostemon* leaf and stem bark have several medicinal uses including its usage as an antimalarial and febrifuge. Extensive reports are available on the medicinal uses of *T. didymostemon* [17,18].

Phytoconstituents and antioxidant potential of methanol extracts of *T. didymostemon* leaf and stem bark have been elucidated in our laboratory (Ebohon et al.: Unpublished results). The sub-acute toxic effects of *T. didymostemon* using biochemical analysis and gene expression have been investigated [19]. In this study, scientific validation on the antiplasmodial activity of *T. didymostemon* leaf and stem bark was provided. To achieve this, *in vitro* studies were conducted to evaluate the antiplasmodial activity, cytotoxicity, and antioxidant activity of extracts and fractions from methanol leaf extract of *T. didymostemon*. Furthermore, GC-FID metabolite fingerprinting of the most potent fraction was also carried out.

2. Materials and methods

2.1. Collection of plant materials and authentication

Fresh samples of *T. didymostemon* (Baill.) Pax & K. Hoffm leaf and stem bark were collected from the wild in Urhokuosa village (6.452980, 5.802755) in Uhunmwonde Local Government Area of Edo State, Nigeria in March 2019. The plant was authenticated at the Department of Plant Biology and Biotechnology, University of Benin, Nigeria by Dr. H.A Akinnibosun and voucher specimen of the plant UBH$_T$439 was deposited at the herbarium of the same department.

2.2. Plant extraction

The leaf and stem bark of *T. didymostemon* were air-dried in the absence of sunlight and then pulverized. Al-Afifi et al. [20] method was followed for the extraction process. Three hundred grams of each macerated leaf with stem bark were soaked in air-tight containers with 2.5 L of methanol (99.8% purity) at room temperature for 72 h and stirred occasionally. The soaked macerated plant parts were filtered using Whatman No 1 filter paper into a clean flask; the resulting filtrates were concentrated using a rotary evaporator (RE 300, Bibby Scientific, UK) with reduced pressure at 45 °C and a freeze dryer was used to completely dry the extracts. The dried extracts were thereafter stored in vials at 4 °C till when needed. Methanol was chosen as an extraction solvent based on a previous study which reported that it can extract more phytochemicals in comparison to water, ethanol, chloroform, ether, and acetone [21].

2.3. Solvent fractionation of methanol extract of T. didymostemon

The methanol extract (15 g) of *T. didymostemon* leaf was fractionated with solvents of increasing polarity (n-hexane, dichloromethane, ethyl acetate, and hydromethanol) using a separatory funnel. The fractions obtained were concentrated using a rotary evaporator (RE 300, Bibby Scientific, UK) with reduced pressure at 45 °C. A freeze dryer was then employed for the complete concentration of the fractions.

2.4. Plasmodium falciparum culture and maintenance

The antiplasmodial activities of the crude methanol extracts and fractions from *T. didymostemon* were screened against the chloroquine sensitive *P. falciparum* 3D7 (*Pf*3D7) strain. All the chemicals used in this study, except Albumax II (Gibco, Invitrogen, USA), were obtained from Sigma−Aldrich Germany. Human O Rh+ red blood cells (RBCs) were used to culture the parasite using the method of Trager and Jensen [22]. The parasites were maintained at 3% hematocrit in human RBCs at 37 °C in a candle jar on complete culture medium (RPMI 1640) supplemented with 25 mM HEPES buffer (pH 7.4), 20 μg/mL of gentamycin, 0.5% albumax, 100 μM hypoxanthine, 0.2% D-glucose and buffered with 0.2% NaHCO₃. Fresh culture was maintained for two complete life cycles (96 h) before its usage in this study. D-sorbitol (5%) was used to synchronize the culture at ring stage. The synchronized culture was then used for the assay. Thereafter, percentage parasitaemia was measured using Giemsa-stained microscopy.

2.5. Antiplasmodial activity and cytotoxicity of the plant extracts and fractions

The growth inhibition of chloroquine-sensitive *Pf*3D7 by the plant extracts and fractions was evaluated using the Mark III test, as developed by the WHO [23] (detailed description of the method is available in the supplementary file, Text 1). Parasitaemia was estimated after 48 h incubation of *Pf*3D7 with plant extracts/fractions using Giemsa-stained microscopy and the average suppression of parasite growth was calculated. The IC$_{50}$ was determined by interpolation from growth inhibition curves after *Pf*3D7 exposure to fractions and extracts [24]. Rasoanaivo et al. [25] was used to grade the antiplasmodial activity of the fractions. According to this system, "extract is very active if IC$_{50}$ < 5 μg/mL, active if 5 < IC$_{50}$ < 50 μg/mL, weakly active 50 μg/mL < IC$_{50}$ < 100 μg/mL and inactive IC$_{50}$ > 100 μg/mL". Cell viability assays were performed using the Microculture tetrazolium (MTT) assay Mosmann [26]. The cytotoxicity of the plant extracts and fractions were assessed against Vero cell line (kidney cells from the African green monkey)

cultured in minimal essential medium (MEM) supplemented with 10% FBS, glutamine (2 mM), penicillin (100 units/mL) and streptomycin (100 μg/mL), HEPES (0.59%), NaHCO$_3$ (0.22%). All experiments were performed in triplicate (detailed description of method is available in supplementary file, Text 2).

2.6. Qualitative phytochemical screening

Trease and Evans [27], Sofowora [28] and Harborne [29] methods were used for the phytochemical screening.

2.7. In vitro antioxidant activity of the fractions

The method of Brand-Williams et al. [30] with slight modifications from Omoregie and Okugbo's method [31] was used to evaluate the free-radical scavenging capacity of the fractions against 1,1–diphenyl–2–picrylhydrazyl (DPPH) radical. The method of Prieto et al. [32] was used to evaluate phosphomolybdate reduction capacity (PRC) while ferric-reducing antioxidant power (FRAP) assay was conducted on the fractions using the protocol of Benzie and Strain [33].

2.8. GC-FID metabolite fingerprinting of the most potent fraction (n-hexane)

The phytochemical fingerprints (flavonoids, terpenes, alkaloids, terpenoids, and volatile organic constituents) of the n-hexane fraction were determined using gas chromatography with flame ionization detector (detailed description of methods and conditions are available in supplementary file, Text 3). Gas chromatography analysis was carried out on HP 6890 Powered with HP ChemStation Rev. A 09 01[1206] Software. Bioactive metabolites were identified based on comparison of the retention times of the peaks with those of the corresponding reference standards (Supelco Inc.) mixtures used. Flavonoids extraction was carried out by following the method described by Millogo-Kone et al. [34], terpenes/terpenoids by Ortan et al. method [35], and alkaloids extraction by Ngounou et al. method [36].

2.9. Statistical analysis

Results from this study were expressed as Mean ± SEM. One-way analysis of variance (ANOVA) test was used to determine significant differences between the means. Post hoc multiple comparison test was done using Tukey's HSD (honest significant difference). Statistical significance was declared when the P value was less than 0.05. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) for Windows, version 16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. In vitro antiplasmodial activity and cytotoxicity of methanol extracts of T. didymostemon and the resulting fractions from the leaf extract

In vitro antiplasmodial capacity of methanolic extracts of *T. didymostemon* leaf and stem bark as well as the resulting fractions from the leaf extract on *Pf*3D7 strain are shown in Fig. 1. The leaf extract of *T. didymostemon* had a higher antiplasmodial activity when compared with the stem bark extract (SBE). Parasite growth inhibition of fractions was dose-dependent and n-hexane showed higher ability to inhibit parasite growth in comparison with the other fractions (Figs. 1 and 2). The leaf extract had a lower IC$_{50}$ value in comparison with the SBE (Table 1). Amongst the fractions from

the leaf extract, n-hexane fraction had the least IC$_{50}$ *Pf*3D7 which corresponds to the highest antiplasmodial activity while the hydromethanolic fraction was inactive having the highest IC$_{50}$ value (Table 1).

The cell viability of the Vero cell line upon exposure to methanol extracts of *T. didymostenmon* leaf and stem bark as well as partitioned fractions of methanolic extracts of *T. didymostemon* leaf is shown in Fig. 1. The cell viability was more than 50% in the extracts and fractions at all the concentrations used (1.95–1000 μg/mL). The selectivity index of the leaf extract was higher than the SBE (Table 1). Amongst the fractions, the n-hexane fraction had the highest selectivity index, while the ethyl acetate fraction had the lowest. The selectivity index indicates the cytotoxic selectivity of the extracts and fractions against *Plasmodium* parasite cells versus normal cells.

3.2. Phytochemical composition and in vitro antioxidant activity of the partitioned fractions of methanol extracts of T. didymostemon leaf

Table 2 shows the phytoconstituents present in the partitioned fractions of methanol extract of *T. didymostemon* leaf. Quinones, terpenoids, alkaloids, and sterols were detected in all the fractions (n-hexane, dichloromethane, ethyl acetate, and hydromethanol). However, flavonoids and cardiac glycosides were detected only in the n-hexane, dichloromethane, and ethyl acetate fractions. Phenolics and tannins were detected only in the dichloromethane fraction. The results of the DPPH radical scavenging activities of the partitioned fractions of *T. didymostemon* methanol leaf extract are shown in Fig. 3 with IC$_{50}$ values shown in Table 1. The dichloromethane fraction showed the best DPPH radical scavenging ability with corresponding lower IC$_{50}$ value, which was closely followed by the ethyl acetate fraction and n-hexane fraction. The PRC value was higher in the dichloromethane fraction when compared with the other fractions, while ethyl acetate fraction had the highest ferric reducing antioxidant power in contrast to the other fractions (Table 1).

3.3. GC-FID metabolite fingerprinting of n-hexane fraction (most active fraction)

GC-FID metabolite fingerprinting revealed that terpenes and flavonoids were the most abundant phytochemicals in the n-hexane fraction (Table 3 and 4). Flavonoid content of the n-hexane fraction showed high amount of kaempferol (Table 3). Luteolin, naringenin, quercetin, and myricetin were also present in relatively high quantity. Table 4 shows the presence of terpenes in the n-hexane fraction. The terpenes azulene, α-pinene, β-pinene, camphor, borneol, and humulene were present abundantly in the n-hexane fraction. Pinene-2-ol, γ-terpinene, neryl acetate, and β-caryophyllene were also present in relatively high amount. Terpenoid profile of the fraction revealed higher amount of α and β-amyrin and triterpene lupeol when compared with taraxerol and bauerenol acetate (Supplementary file, Table S1). Alkaloids and volatile organic compounds in the n-hexane fractions were low and the results are shown in Supplementary file, Tables S2 and S3 respectively. The alkaloids coniceine, cassine, spectaline, and coniine occurred in relatively high amounts as compared to other alkaloids in this study. 2-methyl butenoic acid, chloroethyl vinyl ether, 2-methyl butanoic acid, and 2-methyl butenoic acid ethyl ester were present in abundance as compared to other volatile organic compounds. GC-FID spectra of the flavonoids, terpenes, terpenoids, alkaloids, and volatile organic compounds are shown in Fig. 4.
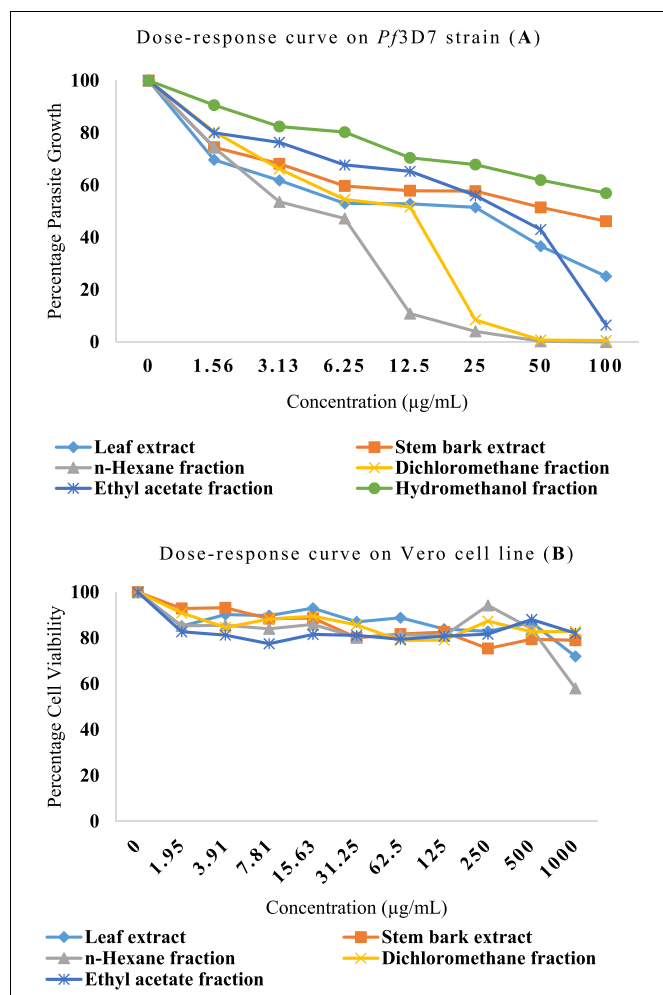
Fig. 1. Dose−response curves of *T. didymostemon* leaf and SBEs as well as the resulting fractions from the leaf extract on growth of *Pf*3D7 strain (A) and viability of Vero cell line (B). *n* = 3 wells/concentration.

4. Discussion

In vitro methods allow researcher to screen large amount of samples as well as those with low yield for activities such as antiplasmodial activity. This enables the researcher to shortlist plants and compounds with activities for *in vivo* studies. Apart from eliminating the interference of the host system *in vivo, in vitro* screening also cuts short the time spent on drug discovery.

T. didymostemon leaf and SBEs inhibition of parasite growth was dose-dependent. However, the leaf extract had more potency and it gave a better parasite inhibition (IC$_{50}$ *Pf*3D7 = 25 ± 0.21 µg/mL). This greater potency of the leaf extract may be as a result of the presence of higher amounts of *Plasmodium* parasite sensitive bioactive compounds. The leaf extract has been noted to have higher amount of phytochemicals relative to the SBE (Ebohon et al.: Unpublished results). Based on Rasoanaivo et al. [25] classification, the leaf extract can be said to be active while the SBE is weakly active. *T. didymostemon* methanol extracts had higher antiplasmodial activity against *Pf*3D7 strain when compared with methanolic extract of *Bidens pilosa* leaves (IC$_{50}$ = 266.77 ± 0.49 µg/ mL) [37]. However, the aqueous (IC$_{50}$ = 14.31 ± 1.37 µg/mL) and ethyl acetate (IC$_{50}$ = 8.18 ± 2.15 µg/mL) extract of *B. pilosa* leaves had a better inhibition of *Pf*3D7 strain growth when compared with *T. didymostemon* extracts [37]. The distribution of bioactive

compounds in medicinal plants is unique and depends on the geographic distribution of the plant, the plant part used, species, age and sometimes time of collection. This may explain the differences in activity in the leaf and stem bark. The antiplasmodial property of medicinal plants have been linked mainly to secondary metabolites such as alkaloids, sesquiterpene lactones and quassinoids [38,39]. To provide more details on the observed antiplasmodial capacity of the leaf extract, the leaf extract was fractionated with solvents of different polarity and the resulting fractions were tested for antiplasmodial activity and cytotoxicity.

Our results revealed that the n-hexane fraction had the highest potency and closely following n-hexane was dichloromethane, ethylacetate and hydromethanol fractions. It was observed that as the polarity of the solvent used for fractionation increased, the antiplasmodial activity of the resulting fractions decreased. Hence, the n-hexane fraction (with less polarity) was observed to be the most active while the hydromethanol fraction (with high polarity) was inactive. Therefore, the bioactive constituents that mainly account for this observed antiplasmodial activity of the methanol extract of *T. didymostemon* leaf may be more soluble in the nonpolar solvent such as n-hexane. The n-hexane fraction may be considered very active based on the Rasoanaivo et al. [25] classification. The n-hexane fraction in our study had a better antiplasmodial growth inhibition (IC$_{50}$ *Pf*3D7 = 3.92 µg/mL) when compared with all the fractions of crude husk extract of *Zea mays* (IC$_{50}$ *Pf*3D7 = 8.46 to >100 µg/mL) [40].

In a review conducted by Bero et al. [41] and Lawal et al. [42] alkaloids, flavonoids, terpenes, and terpenoids were identified as being responsible for the antiplasmodial activity of several medicinal plants. Indeed, phytochemical screening revealed very high amount of terpenes in the n-hexane fraction compared with the other fractions. Hence, the antiplasmodial activity of the methanol leaf extract may be associated with non-polar terpenoids and terpenes. This may explain why the n-hexane fraction had the highest antiplasmodial activity even when other polar fractions had terpenoids and terpenes. In other words, to harness the antiplasmodial properties of *T. didymostemon* leaf extract, the usage of non-polar extracting solvents like n-hexane is suggested.

The extracts and fractions from *T. didymostemon* were not toxic to Vero cell line with CC$_{50}$ values of the extracts and fractions greater than 30 µg/mL; which according to the classification of Malebo et al. [43] on cytotoxicity, may be regarded as non-cytotoxic. This observation has thrown more light on the possible high level of safety of *T. didymostemon*. Selectivity indices of the fractions demonstrated high selectivity indices value suggesting their potential as a safer therapy [44]. Amongst the fractions, n-hexane had the highest selectivity index and thus, was not only more toxic to the parasite, but also seems to be the safest therapy. These observations validate the safety of this plant and support its traditional use in the management of the malaria. Phytochemical screening of the fractions revealed high level of phytochemicals in the dichloromethane fraction followed by the n-hexane fraction. The presence of phenolics in the dichloromethane fraction may have been responsible for its high antioxidant activity. Phenolics are known to be excellent scavenger of free radicals.

This study have shown n-hexane fraction (IC$_{50}$ = 3.92 ± 0.46 µg/ mL) to be the most active against *Plasmodium* parasite when compared with other fractions; DCM (IC$_{50}$ = 12.5 ± 1.32 µg/mL), EA (IC$_{50}$ = 35.0 ± 4.80 µg/mL) and HM (IC$_{50}$ > 100 µg/mL). Hence, we decided to fingerprint the metabolites present in this fraction using GC-FID. Various flavonoids, terpenes, alkaloids, quinones, terpenoids, and volatile organic compounds were detected in the n-hexane fraction of which some are known to possess antiplasmodial activity. Terpenes and flavonoids were the most
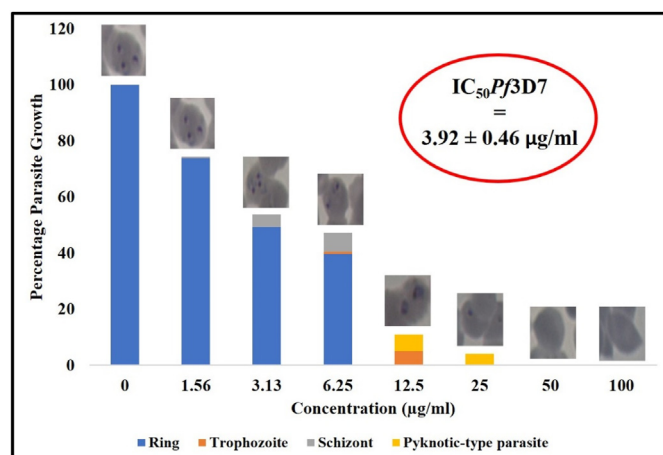
Fig. 2. Parasite growth inhibition of n-hexane fraction of methanol extract of *T. didymoostemon* leaf showing the different stages. Representative images of Giemsa-stained smears are given on each bar.

kinase-3β (GSK3β) [48]. The enzyme GSK3β is believed to play a role in the host response to malaria infection [49]. Hence, the antiplasmodial activity of the n-hexane fraction may probably be linked to the inhibition of GSK3β in the parasite by kaempferol. Furthermore, the presence of myricetin and quercetin in the n-hexane fraction may have also contributed to its antiplasmodial properties. Myricetin isolated from *Limonium caspium* [50] and quercetin isolated from *Mezoneuron benthamianum* leaf [51] have been reported to have antiplasmodial activity.

Terpenes were the most abundant bioactive constituents found in the n-hexane fraction (Table 4). Amongst these terpenes, α-pinene, camphor, borneol, azulene, and humulene were in high amount. Terpenes have shown to inhibit the synthesis of dolichol in the trophozoites and schizont stages of the *Plasmodium* parasite through the inhibition of isoprenyl diphosphate synthase [52]. Furthermore, the parasiticidal activity of β-pinene and β-caryophyllene which were present in the n-hexane fraction has been tied to endoperoxidation [53]. Synergy between these terpenes or their individual actions may be responsible for the antiplasmodial activity of the n-hexane fraction.

Table 1
CC$_{50}$ Vero cell line, IC$_{50}$ parasite, selectivity indices and antioxidant capacity of methanol extracts of *T. didymostemon* leaf and stem bark as well as the resulting fractions from the leaf extract.

Samples	CC$_{50}$ Vero cell line (µg/mL)	IC$_{50}$ *Pf*3D7 (µg/mL)	Selectivity Index	IC$_{50}$ DPPH (µg/mL)	PRC	FRAP
SBE	1365.30 ± 18.87[a]	50 ± 0.94[a]	27.31	N/A	N/A	N/A
LE	1747.26 ± 12.45[b]	25 ± 0.21[b]	69.89	N/A	N/A	N/A
HEX	1078.61 ± 34.3[c]	3.92 ± 0.46[c]	275.16	87.09 ± 1.42[a]	105.39 ± 2.19[a]	355.33 ± 10.00[a]
DCM	2502.15 ± 19.9[d]	12.5 ± 1.32[d]	200.17	64.19 ± 2.31[b]	182.09 ± 0.25[b]	326.89 ± 15.51[a]
EA	1815.33 ± 25.7[b]	35.0 ± 4.80[e]	51.87	67.66 ± 2.22[b]	90.64 ± 1.43[a]	404.22 ± 12.25[b]
HM	N/A	>100	N/A	105.80 ± 3.14[c]	41.02 ± 1.09[c]	379.83 ± 15.50[a]
DOX	0.62 ± 0.05[e]	N/A	N/A	N/A	N/A	N/A
ASC	N/A	N/A	N/A	1.07 ± 0.04[d]	N/A	564.33 ± 7.21[c]

Values are mean ± SEM, n = 3/group. Values in the same column with different superscripts are significantly different (P < 0.05). Phosphomolybdate reduction capacity (PRC) is expressed as ascorbic acid equivalent/g fraction. FRAP is expressed as µM Fe (II)/g of the extract. DPPH = 1,1−Diphenyl−2−picrylhydrazyl, SBE = Stem bark extract, LE = Leaf extract, HEX= Hexane fraction, DCM = Dichloromethane fraction, EA = Ethyl acetate fraction, HM= Hydromethanol fraction, DOX = Doxorubicin, ASC = Ascorbate, N/A = Not available. Selectivity Index = CC$_{50}$ Vero cell line/IC$_{50}$ *Pf*3D7.

Table 2
Phytochemical screening of the partitioned fractions from methanol extracts of *T. didymostemon* leaf.

Bioactive compounds	HEX	DCM	EA	HM
Phenolics	−	+	−	−
Flavonoids	+	++	+	−
Quinones	+	++	+	+
Terpenes	+++	+++	++	+
Alkaloids	+	+	−	−
Cardiac glycosides	+	++	+	−
Sterols	+	++	+	+
Tannins	−	+	−	−

KEY: +++ = Very high; ++ = High; + = Less; − = Not detected. Where: HEX = hexane, DCM = dichloromethane, EA = Ethyl acetate and HM = hydromethanol.

abundant phytochemicals in the n-hexane fraction and they may be responsible for its antiplasmodial activity. Some flavonoids with antiplasmodial activity have the ability to inhibit fatty acid synthesis in parasite [45] and also the influx of L-glutamine and myoinositol into infected erythrocytes [46]. GC-FID analysis revealed that the flavonoid, kaempferol was the most abundant in the n-hexane fraction (Table 3). Studies have shown that kaempferol has antiplasmodial activity. Somsak et al. [47] reported the antimalarial activity of kaempferol and its combination with chloroquine in *Plasmodium berghei*-infected mice. The molecular basis for the antiplasmodial activity of kaempferol has been linked to its ability to inhibit malaria parasite enzyme; glycogen synthase

The n-hexane fraction had a low amount of terpenoids (Supplementary file, Table S1). However, lupeol (terpenoids) present in the fraction may have also played a role in its antimalarial activity by working in synergy with other bioactive metabolites. Lupeol isolated from ethyl acetate fraction of *Cassia siamea* is responsible for the antimalarial activity of this plant [54]. The antiplasmodial activity of α-amyrin, a terpenoids identified in the n-hexane fraction has also been reported [55]. Similarly, alkaloids were not in abundance in the n-hexane fraction (Supplementary
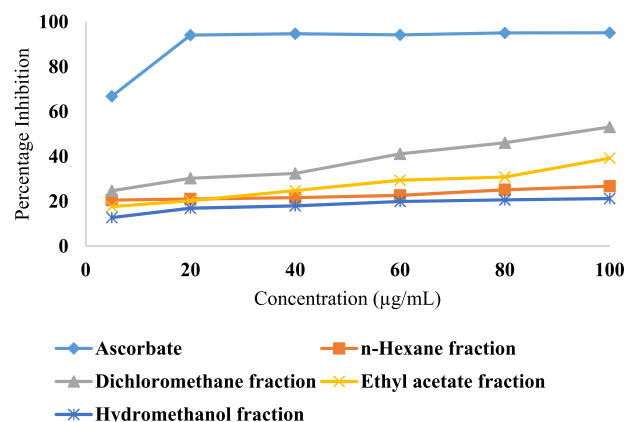

Fig. 3. DPPH radical scavenging activity of fractions from methanol extract of *T. didymostemon* leaf. n = 3/group.

Table 3
Amounts of the various flavonoids detected in the n-hexane fraction.

Phytocomponents	Amount (mg/100 g)	Area	Amount/Area	Retention time (min)
(+) − Catechin	9.43×10^{-1}	9.66	9.77×10^{-2}	13.74
Resveratrol	7.79×10^{-7}	5.52	1.41×10^{-7}	15.16
Genistein	3.97×10^{-7}	2.55	1.55×10^{-7}	15.49
Daidzein	5.12×10^{-7}	3.30	1.55×10^{-7}	15.62
Apigenin	2.24×10^{-1}	2.77	8.10×10^{-2}	15.74
Daidzein	6.40×10^{-8}	0.41	1.55×10^{-7}	16.30
Butein	9.74×10^{-9}	0.06	1.55×10^{-7}	16.60
Biochanin	1.23×10^{-6}	7.62	1.61×10^{-7}	17.16
Naringenin	2.51	0.02	137.36	17.28
Luteolin	3.75	40.03	9.36×10^{-2}	17.77
Kaempferol	17.70	76.72	2.31×10^{-1}	18.05
(−) − Epicatechin	1.22×10^{-3}	75.21	1.62×10^{-5}	19.52
(−) − Epigallocatechin	7.88×10^{-5}	6.75	1.17×10^{-5}	20.59
Gallocatechin	1.07×10^{-5}	22.26	4.80×10^{-7}	21.82
Quercetin	3.50	57.63	6.07×10^{-2}	22.60
(−) − Epicatechin-3-gallate	3.27×10^{-5}	7.96	4.11×10^{-6}	22.85
(−) − Epigallocatechin-3-gallate	$4 39 \times 10^{-7}$	0.15	2.84×10^{-6}	23.57
Isorhamnetin	7.98×10^{-7}	15.84	5.04×10^{-8}	23.96
Robinetin	6.25×10^{-7}	3.96	1.57×10^{-7}	24.18
Myricetin	2.17	2.74	7.91×10^{-1}	24.79
Baicalein	2.67×10^{-7}	1.73	1.55×10^{-7}	25.16
Nobiletin	3.44×10^{-7}	2.23	1.55×10^{-7}	25.48
Baicalin	2.22×10^{-7}	1.42	1.56×10^{-7}	25.86
Isoquercetin	1.42×10^{-3}	2.38	5.95×10^{-4}	26.01
Tageretin	1.71×10^{-7}	1.10	1.56×10^{-7}	26.50
Artemetin	1.32×10^{-7}	0.85	1.55×10^{-7}	26.84
Silymarin	2.21×10^{-7}	1.43	1.55×10^{-7}	26.95
Hesperidin	5.67×10^{-8}	0.08	6.93×10^{-7}	28.38

Table 4
Amounts of the various terpenes detected in the n-hexane fraction.

Phytocomponents	Amount (mg/100 g)	Area	Amount/Area	Retention time (min)
Azulene	15.38	158.27	9.71×10^{-2}	9.43
α-Pinene	44.03	22.35	1.97	9.87
β-Pinene	2.89×10^{-3}	24.78	1.16×10^{-4}	10.80
cis-Ocimene	2.95×10^{-5}	48.70	6.05×10^{-7}	12.17
Myrcene	8.79×10^{-5}	51.72	1.70×10^{-6}	13.04
Allo Ocimene	1.04×10^{-4}	35.07	2.95×10^{-6}	13.15
Pinene-2- ol	3.37	124.60	2.70×10^{-2}	13.79
α-Thujene	1.54×10^{-4}	27.80	5.55×10^{-6}	14.17
γ-Terpinene	3.82	8.17	4.68×10^{-1}	14.90
Citral	1.54×10^{-4}	70.58	2.18×10^{-6}	15.11
Camphor	33.27	15.96	2.08	15.29
Neral	2.19×10^{-4}	15.72	1.39×10^{-5}	15.34
1,8-Cineole	7.19	16.34	4.40×10^{-1}	15.57
Borneol	21.51	18.89	1.14	17.56
Linalool	8.25×10^{-5}	6.75	1.22×10^{-5}	17.67
Citronellal	8.25×10^{-5}	3.59	2.30×10^{-5}	18.26
Nerol	8.25×10^{-5}	0.77	1.08×10^{-4}	18.39
α-Terpineol	1.14×10^{-4}	3.74	3.05×10^{-5}	18.71
Terpinen-4-0l	4.99×10^{-1}	1.14	4.39×10^{-1}	18.99
Citronellol	1.51×10^{-4}	0.23	6.51×10^{-4}	19.49
α-Terpinenyl acetate	3.57×10^{-2}	0.83	4.33×10^{-2}	21.13
Geranyl acetate	8.99×10^{-2}	0.21	4.28×10^{-1}	21.33
Ethyl cinnamate	2.45×10^{-4}	0.27	9.13×10^{-4}	21.49
Borneol acetate	9.46×10^{-2}	0.42	2.24×10^{-1}	21.69
Neryl acetate	2.43	1.04	2.33	21.78
Germacrene B	3.61×10^{-2}	0.93	3.87×10^{-4}	22.23
β-Caryophyllene	5.80	0.58	9.93	22.40
Cyperene	6.04×10^{-5}	4.06	1.49×10^{-5}	23.38
[6] − Shogaol	2.34×10^{-3}	4.73	4.94×10^{-4}	24.12
α-Copane	8.86×10^{-5}	45.42	1.95×10^{-6}	25.06
Valencene	1.30×10^{-4}	13.66	9.54×10^{-6}	27.60
Humulene	35.08	14.93	2.35	27.79
β-Selinene	1.04×10^{-4}	79.48	1.31×10^{-6}	28.62
Aromadendrene	1.37×10^{-4}	43.91	3.12×10^{-6}	28.96
γ- Muurolene	1.32×10^{-4}	92.14	1.43×10^{-6}	29.54

file, Table S2). Coniceine, cassine, spectaline, and coniine made up large percentage of the alkaloid contents of this fraction. The antiplasmodial activity of alkaloids such as quinine and chloroquine are well-documented [56,57]. In our study, there was a wide range of volatile organic compounds in the n-hexane fractions (Supplementary file, Table S3). These compounds may or may not have contributed to the antiplasmodial potency of the n-hexane fraction.
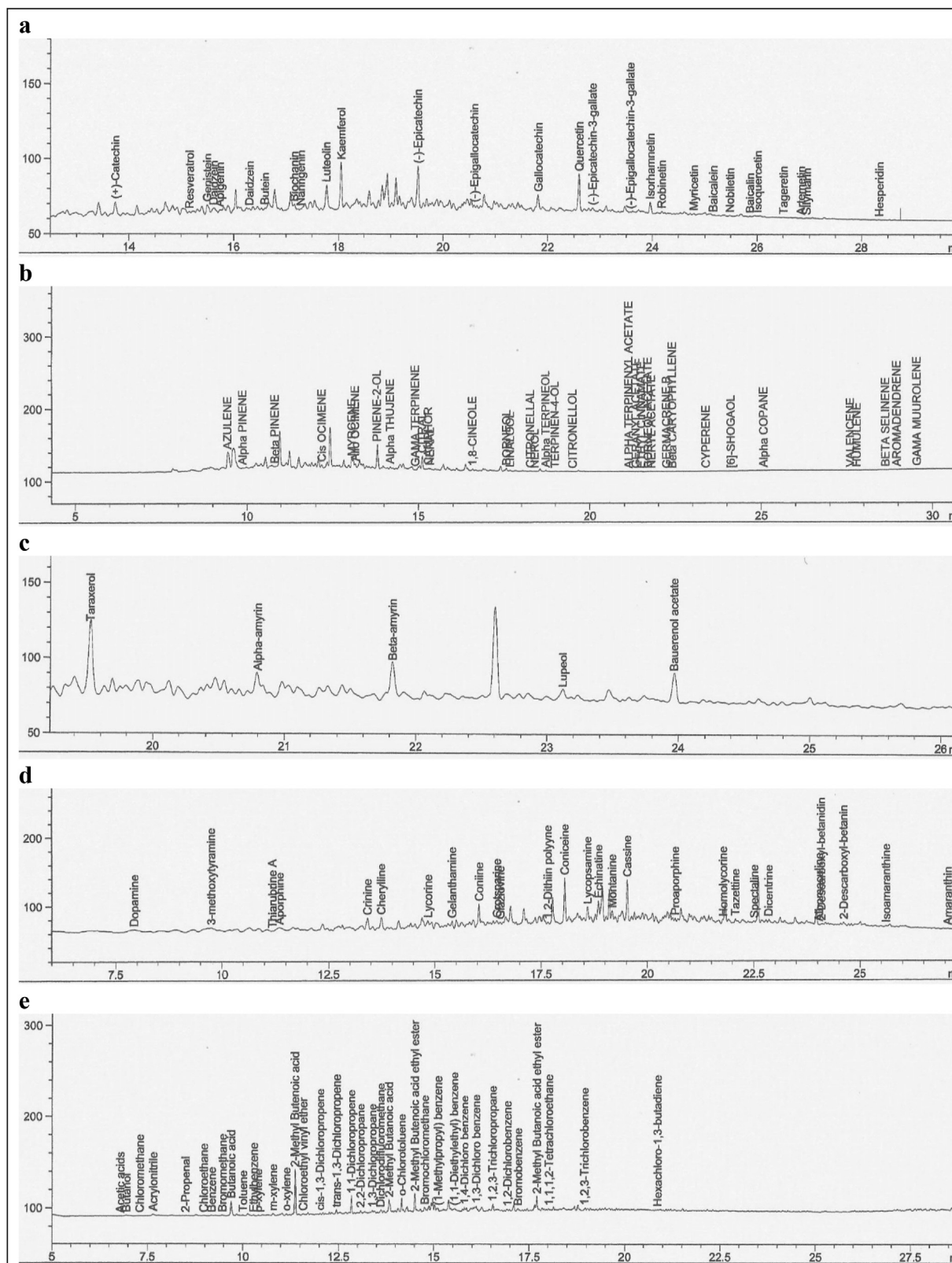
Fig. 4. GC-FID chromatograms of bioactive compounds in n-hexane fraction: **a** = Flavonoids, **b** = Terpenes, **c** = Terpenoids, **d** = Alkaloids, **e** = Volatile organic compounds.

5. Conclusion

The antiplasmodial activity of the n-hexane fraction and by extension methanol leaf extract may be due to the presence of high amounts of flavonoids (kaempferol) and terpenes (α-pinene, β-pinene and β-caryophyllene) which have been reported to have antiplasmodial properties. These compounds may therefore act singly or in synergy to bring about the antimalarial benefits

ascribed to *T. didymostemon*. The study suggests that the phyto-constituent(s) responsible for the antiplasmodial activity of this plant may be more extractable in non-polar solvents. Isolation, purification, and characterization of the bioactive compound(s) responsible for this antiplasmodial activity are recommended.

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Conflict of interest

None.

Author contributions

OE: Contributed in conceptualization of the study, data curation, formal analysis, investigation, resources, validation and writing the original draft of the manuscript. **FI:** Contributed in conceptualization of the study, validation, investigation and resources. **NE:** Contributed in conceptualization of the study, validation, investigation and resources. **AO:** Contributed in investigation and resources. **ESO:** Contributed in conceptualization of the study, review and editing of the manuscript, supervision and project administration. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaim.2021.05.004.

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