Ilorin Journal of Science
FAPSCON 2024 PROCEEDINGS, 2024, pp. 229 – 236 (Printed in Nigeria)
ISSN: 2408 – 4840 © 2024 Faculty of Physical Sciences, University of Ilorin
https://doi.org/10.54908/iljs.fapscon.2024.11.03.1030

ILORIN JOURNAL OF SCEINCE

ILJS-24-080 (SPECIAL EDITION)

Phytochemical Analysis and Anti-malarial Activities of the Ethanol Extract of the Leaves of *Cassia Fistula*

Ameen O. M. and Muhammad F. A.

Department of Chemistry, Faculty of Physical Sciences, University of Ilorin, Nigeria.

Abstract

The Synthetic drugs are expensive, scarce, and time-consuming, while plant-derived substances are bio-friendly, cheap, culturally accepted, and compatible with the human body system with minimal side effects. This study aims to investigate the phytochemical and anti-malaria activities of the Ethanol Extract of *Cassia Fistula* Leaves.
The pulverized leaves of Cassia Fistula were extracted with ethanol and investigated for phytochemicals and characterized with Gas Chromatography-Mass Spectroscopy (GCMS). The extracts were further fractionated into n-hexane and dichloromethane fractions which were tested against malaria parasites using an animal model.
The results of the phytochemical analysis indicate the presence of alkaloids, tannins, saponins, phenolic compounds, flavonoids, carbohydrates, proteins, triterpenoids and glycosides in the ethanol extract. The antimalarial analysis results revealed some activities in the dichloromethane (1.2 % inhibition) fraction, while the n-hexane fraction was 0.2 % inhibition. The results of the GCMS analysis indicate the presence of cis-Vaccenic acid, Hexadecanoic acid, Octadecanoic acid, Hexadecanoic acid, ethyl ester, Octacosanol, 1-Heneicosanol. These studies have revealed the potential of the extracts of the leaves of *C. fistula* as a candidate for the development of new useful drugs.

Keyword: Anti-malarial, Phytochemical, Cassia Fistula, and GCMS

1. Introduction

Exodus of most of the drug manufacturing companies in Nigeria has made treatment of disease, ailment or wound to be expensive using synthetic drugs. The high cost of treatment has left the poor masses of Nigeria heavily reliant on the use of medicinal plants for the treatment of the disease (Oladeji *et al.*, 2020). Over the years, plant extracts have been used as traditional or alternative drug solutions for various diseases, with 25% of plant species used globally (Maximus *et al.*, 2021). Plant isolates are used due to their low cost, availability, and nontoxic properties, including malaria.

Malaria is a prevalent disease in humid, middle-income countries, sub-Saharan Africa, Southeast Asia, and South America, affects sociocultural, economic, and health, with 19 million cases and 17 million deaths in Africa (Oladeji *et al.*, 2020). Malaria infections in Africa are being controlled through various factors, including the use of medicinal plants with potential antimalarial phytomedicine (Ekasari *et al.*, 2021). Although several compounds have achieved success at treating malaria diseases with plant extracts, the emerging threats of drug resistance by some plasmodium species call for the development of new molecules with novel bioactive features (Oladeji *et al.*, 2020).
Previous studies on plants like Alstonia boonei, C. spectabilis DC, and C. gigantean have shown significant antimalarial activity in mice, with plant extracts containing essential compounds for

Corresponding Author: Ameen, O. M.
Email: moameen@unilorin.edu.ng; fatima4joy@gmail.com

treatment, including tannins, flavonoids, steroids, phenols, and esters (Ekasari *et al.,* 2021; Maximus *et al.,* 2021; Chidiebere *et al.,* 2020). Recently, *Cassia fistula* has been used as a drug for hepatoprotective, anti-inflammatory, analgesic, hepatoprotective, antibacterial, antitussive, antifungal, wound healing properties, ant-cancer and in relieving the symptoms of asthma, leprosy, ringworm, heart-related disorders and fever (Mwangi *et al.,* 2021; Kulkarni *et al.,* 2015; Raji *et al.,* 2015; Bargah and Kushwaha, 2017). *Cassia fistula* (Amaltas), a golden shower tree belongs to the Leguminosae family with 8-15 m in height. *Cassia fistula* stem is greenish grey, reddish brown root, compound leaf, 3-8 pairs of leaflets *with* rod-shaped fruits having pulp and bright yellow flowers. The healing properties of different plant species have contributed exceptionally to the derivation as well as the development of several traditional herbal medicines (Oladeji *et al.,* 2020). This is because the plant contains bioactive compounds that are rich in tannins, flavonoids, glycosides, carbohydrates, stearic acids, oleic, oxalic, linoleic, oxyanthraquinones and anthraquinones derivatives. Therefore, this study aims to investigate the phytochemical analysis and antimalarial activities of ethanol leave extract of cassia fistula.

2. Materials and Methods

2.1 Preparation of Plant Material

Fresh *Cassia fistula* leaves were collected from the Vice Chancellor Lodge and identified at the herbarium of the Department of Plant Biology, University of Ilorin, Ilorin with a voucher number UILH/001/1020 assigned to the plant. The leaves sample was shade-dried at room temperature, grounded to a fine powder with a mechanical crusher and stored in a polythene bag before use. The sample (1kg) was defatted with N-hexane. The n-hexane extracts were concentrated, weighed and stored in a sample bottle until required for use. The marc was obtained from the n-hexane extract with ethanol. The extract was concentrated on a rotary evaporator (United Nations Database Inventory 064681), weighed, and kept in a sample bottle until further analysis.

2.2 Phytochemical Screening

The different qualitative chemical tests were carried out on the aqueous extract using standard procedures to identify the constituents (Usha and Bopaiah, 2011). The presence of alkaloids, tannins, saponins, anthraquinones, anthocyanides, brown precipitates, phenolic flavonoids, and golden yellow flavonoids in a mixture was confirmed using various tests. The presence of turbid orange, whitish-yellow, cream-colored precipitates, dark green tannins, saponins, pinkish-colored anthraquinones, pale pink anthocyanides, brown precipitates with lead acetate, and golden yellow flavonoids was confirmed. Other compounds present include Carbohydrates, Proteins, Steroids, Terpenoids, Cardiac glycosides and Phlobatannins.

2.3 Antimalarial Analysis

The antiplasmodial activity of ethanol extract, dichloromethane, and n-hexane fraction was tested on Wister rats infected with plasmodium berghei, and artesunate was administered to mice. The mice were administered 4 mg/kg of artesunate on the first day and subsequently 2 mg/kg (Arise *et al.,* 2012). The study involved 25 male Swiss albino mice, weighing 20 g, from Ogbomosho and transferred to the Zoology Department at the University of Ilorin. They were acclimatized, kept in well-ventilated cages, and fed Vital Feed, which contains cereals, vegetables, proteins, vitamins, minerals, and enzymes. The percentage composition of each nutrient present in the feed is as follows; crude protein 14%, fat 7%, crude fibre 10%, calcium 1%, phosphorus 0.35% and metabolized energy 250 kcal/kg.

2.4 Parasite Collection and Inoculation

Mice infected with *P. berghei* were obtained from the Institute of Advanced Medical Research and Training, College of Medicine, University of Ibadan, Ibadan. The volume of infected blood to anticoagulant used was 0.2 ml at 1:5 (Kabiru *et al,* 2013). Microscopic examination of Giemsa-stained blood smears from mice's caudal tip revealed 20-25% parasitemia in donor mice. Mice were inactivated by cervical dislocation and

infected with 0.9% normal saline blood. Infected blood was injected into experimental mice, confirming the presence of parasites in the blood.

2.5 Blood Smear Preparation

Parasitemia was checked by thin blood smears collected from the candal tip of infected mice every two days and prepared on microscope slides before, during and after infection The slides were air-dried in the absence of dust and fixed in absolute methanol, stained for 15 minutes in 5 % solution of Giemsa stain, rinsed with water and air dried. This was later viewed under the light microscope (magnification x 100).

2.6 Animal Treatment

Rane's 4-day curative test was employed to evaluate the anti-plasmodial activity of ethanol extract, dichloromethane and n-hexane fraction. After two weeks of acclimatizing, the mice were divided into five groups of five mice each; The first group was not infected with the parasite and this was used for normal (negative control), while each mouse in the remaining three groups was injected with 0.1ml of blood containing $1x10^7$ *P. berghei* strain NK 65- infected RBCs via the intra-peritoneal route and distributed.

2.7 Parasitemia Estimation

Parasitemia level was checked by microscopic examination at the Central Research Laboratory, University of Ilorin, Kwara State. Percentage parasitemia was determined by counting the number of parasitized erythrocytes out of the three random fields of the microscope from each slide. The average percentage of parasitemia was calculated using equation 1.

$$\%\text{Parasitemia} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC count}} \times 100 \quad (1)$$

2.8 Percentage Inhibition

Percentage inhibition was calculated by the aid of equation 2 (Adetutu *et.al*, 2016).

$$\%\ \text{Inhibition} = \frac{Mean\ \%\ Parasitemia\ of\ Day\ 0 - Mean\ Parasitemia\ of\ Day\ 4}{Mean\ \%\ Parasitemia\ of\ Day\ 0} \quad (2)$$

2.9 Determination of Mean Survival Time (MST)

Mean survival time (MST) is a key metric for assessing the efficacy of anti-malarial drugs, with active drugs showing a survival time greater than non-treated mice, monitored daily (Oliveira *et al*, 2009). This was determined using equation 3.

$$\text{MST} = \frac{Sum\ of\ Survival\ Time\ of\ all\ Mice\ in\ a\ Group\ (Days)}{Total\ Number\ of\ Mice\ in\ that\ Group} \quad (3)$$

2.10 Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analysis was carried out on the sample of three fractions, ethanol, n-hexane and dichloromethane extract at Shimzadu Training Centre for Analytical Instruments Lagos State, Nigeria, using GCMS-QP2010SE SHIMADZU, JAPAN model.

3. Result and Discussion

3.1 Fractions from the crude Ethanol extract

The crude ethanol extract was packed in the column using silica gel, and three fractions were obtained from column chromatography using n-hexane 100% was added to obtained elutes followed by n-hexane/DCM (3:1) with volume 200 ml respectively.

3.2 Phytochemical Analysis

The results obtained by qualitative phytochemical screening for primary and secondary metabolites in the ethanol leaves extract of *C. fistula* have been summarized below in Table 1.

Table 1: Results of Phytochemical Screening of The Ethanol Leave Extract of *Cassia Fistula*

S/No.	Name of the Phytochemical	Qualitative Test	Ethanol Extract
1.	Alkaloids	Mayer's test	+
		Dragendorff's Test	+
2.	Tannins	Alkaline Reagent	+
3.	Saponin	Foam test	+
4.	Anthraqunonies		–
5.	Anthocyanosides		–
6.	Phenolic Compound (flavonoids)	lead acetate	+
7.	Flavonoids	Alkaline Reagent Test	+
8.	Carbohydrates	Benedicts test	+
		Molisch's test	+
		Fehlings's test	+
9.	Proteins	Millon's test	+
10	Steroids	Salkowski's test	–
11	Triterpenoids	Thionyl chloride Test	+
12	Glycoside	Keller-Kiliani Test	+
13	Phlobatannins		–

KEY: (+): Presence, (**–**): Absence

Alkaloids, tannins, saponins, and phenolic compounds are secondary metabolites found to have antimicrobial, antioxidant, and free radical scavenger properties. Flavonoids, also known as vitamin P, are plant modifiers. Steroids, also known as analgesic agents, have been shown in clinical studies for their anti-inflammatory and analgesic properties (Usha and Bopaiah, 2011).

3.3 Minimum Inhibitory Concentration (MIC) Analysis

The result of Minimum Inhibitory Concentration (MIC) analysis on ethanol extracts, n-hexane fraction and dichloromethane fraction are presented in Table 2 to Table 2 and 3.

Table 2: Minimum Inhibitory Concentration Results of the Ethanol Leaf Extract of *C. fistula*

Concentration in (mg/ml)	S.a	E. c	B.sub	Ps.a	Kleb	Sal	C.a	A.n	Pen	Rhiz
25. 0	–	–	–	–	–	+	+	+	+	+
12.5	–	+	–	+	+	+	+	+	+	+
6.25	–	+	+	+	+	+	+	+	+	+
3.125	+	+	+	+	+	+	+	+	+	+

Table 3: Result of Minimum Inhibitory Concentration of N-hexane Fraction of the Ethanol Leaf Extract of *C. fistula*

Concentration in (mg/ml)	S.a	E. c	B.sub	Ps.a	Kleb	Sal	C.a	A.n	Pen	Rhiz
25. 0	+	+	+	+	–	–	–	+	+	+
12.5	+	+	+	+	+	+	+	+	+	+
6.25	+	+	+	+	+	+	+	+	+	+
3.125	+	+	+	+	+	+	+	+	+	+

KEY: – ⇒ No growth of the organism i.e. Inhibition occurred at that concentration.
+ ⇒ Growth of the organism occurred i.e. No Inhibition at that particular concentration.

The Result of Minimum Inhibitory Concentration of Dichloromethane Fraction of the Ethanol Leaf Extract of *C. fistula* was obtained to be all positives (+) for 25.0, 12.5, 6.25, and 3.125 mg/ml.

At the concentration of 25.0 mg/ml, the ethanol leave extract shows inhibition for *Staphylococcus aureus, Estierichia coli, Bacillus subtillis, Pseudomonas aeruginosa, Klebsiellae pneumonae* (bacteria) and no inhibition for *Salmonella typhi* (bacteria) and *Candida albicans*, *Aspergillus niger*, *Penicillium notatum*, *Rhizophus stoloniter* (fungi). At the concentration of 12.5 mg/ml, the extract shows inhibition for *Staphylococcus aureus* and *Bacillus subtillis* and no inhibition for *Estierichia coli, Pseudomonas aeruginosa, Pseudomonas aeruginosa, Klebsiellae pneumonae* (bacteria) and *Candida albicans*, *Aspergillus niger*, *Penicillium notatum*, *Rhizophus stoloniter* (fungi). At the concentration of 6.25 mg/ml the extract shows no inhibition for all tested organisms except *Staphylococcus aureus* (bacteri). At the concentration of 25.0 mg/ml the N-hexane fraction shows inhibition for *Klebsiellae pneumonae* and *Salmonella typhi* (bacteria) and no inhibition for *Staphylococcus aureus, Estierichia coli, Bacillus subtillis, Pseudomonas aeruginosa* (bacteria) and *Aspergillus niger*, *Penicillium notatum*, *Rhizophus stoloniter* (fungi). At concentrations of 12.5, 6.25 and 3.125 mg/ml the fraction shows inhibition for all tested organisms (bacteria and fungi). At concentrations of 25.0, 12.5, 6.25 and 3.125 mg/ml the dichloromethane fraction shows inhibition for all tested organisms (bacteria and fungi).

The MIC results showed all extracts effectively inhibited the growth of test organisms, with ethanol extract showing the most inhibition showed in Figure 1 and Figure 2. However, Gram-negative bacteria showed higher resistance, possibly due to the outer membrane composition (Santhi *et al.*, 2015).

3.4 Antimalarial Screening Result

The ethanol leaf extracts, n-hexane fraction and dichloromethane fraction of *C. fistula* were tested against the malaria parasite using animal model.

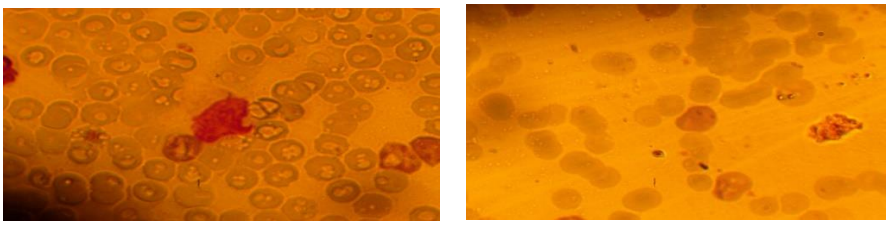


Figure 1: Microscopic Slide Showing Mice Red Blood Cells (A) infected with the Malaria Parasite, (B) after Treatment with the Ethanol leaf extracts

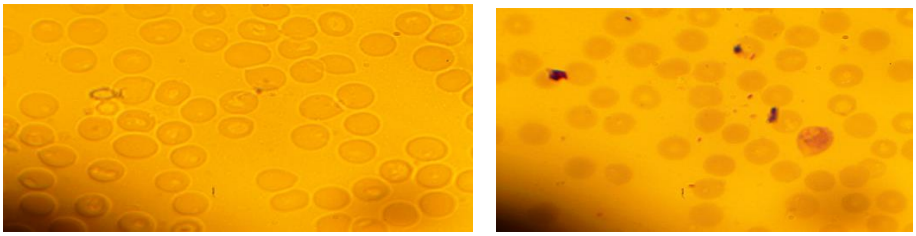


Figure 2: Microscopic Slide Showing Mice Red Blood Cells after being Treated with (C) the n-hexane, (D) Dichloromethane fraction of the Ethanol leaves Extract of *C. fistula*.

The experimental mice showed improvement in red blood cells infected with plasmodium parasite after two days of treatment with extracts, and on Day 4, they appeared healthy, as shown in Table 4 for antimalarial analysis.

Table 4: Comparison of Anti-Plasmodial Activities and Percentage inhibition of ethanol leaf extracts, n- hexane fraction and dichloromethane fraction

Group	Day 0	Day 2	Day 4	% Inhibition
Negative	0.00%	0.00%	0.00%	100%
Positive	420.70%	386.36%	352.02%	0.00%
Standard	250.00%	153.25%	139.67%	64.53%

0.6mg/kg of ethanol extract	22.2%	32.7%	33.3%	0.5%
0.6mg/kg of n-hexane fraction	25.0%	21.2%	20.0%	0.2%
0.6mg/kg of dichloromethane fraction	13.3%	30.4%	29.6%	1.2%

The results presented in the Table 4 indicate that there is an increase in parasite density of the untreated infected positive group whereas a significant decrease in parasite densities was observed in the artesunate-treated (positive control) group. However, secondary metabolites such as flavonoids, cardiac glycosides and steroids are responsible for antiplasmodial activity of the medicinal plant.

3.5 Gas Chromatography Mass Spectroscopy (GCMS)

The GC-MS analysis result in Figure 3 revealed the presence of 15 compounds in the ethanol leaf extracts shown in Table 5, 30 compounds in the n-hexane fraction, and 28 compounds in the dichloromethane fraction.

Table 5: Result of GCMS analysis of the Ethanol Leaf Extract of *C. fistula*

Peak Number	Compound Name	Retention Time (minute)	%Yield	Mass Spectral
1	Ethyl 2-hydroxybenzyl sulfone	12.348	0.29	136, 115, 107, 77
2	Methyl 4-O-methyl-d-arabinopyranoside	13.253	0.21	87, 73, 57, 45
3	Dodecanoic acid	14.061	0.21	171, 157, 129, 115
4	Tridecanoic acid	15.718	1.55	185, 171, 157, 143
5	4-O-Methylmannose	16.036	2.66	145, 116, 87, 73
6	Cis-Z-11,12-Epoxytetradecan-1-ol	16.227	0.59	123, 109, 95, 57
7	Tetradecanoic acid, 12-methyl-, methyl ester	16.860	0.47	101, 87, 74, 55
8	n-Hexadecanoic acid	17.199	24.43	157, 143, 129, 115
9	Eicosanoic acid	17.313	0.76	171, 157, 143, 129
10	Phytol	18.100	4.90	135, 109, 95, 55
11	Androstan-3-one, 17-hydroxy-1,17-dimethy	18.155	4.67	174, 161, 147, 121
12	cis-Vaccenic acid	18.360	32.91	193, 125, 98, 83
13	Octadecanoic acid	18.480	22.87	284, 241, 185, 171
14	γ.-Sitosterol	23.199	1.05	414, 396, 381, 329
15	1-Chloroeicosane	23.854	2.23	316, 178, 105, 73

The results of the GCMS of ethanol leaf extract revealed fifteen major peaks shown in Figure 3 indicating that fifteen compounds were present in the sample in which the prevailing compounds are cis-Vaccenic acid (32.91 % yield), n-Hexadecanoic acid (24.43 % yield) and Octadecanoic acid (22.87 % yield). The results of the GC-MS of n-hexane fraction revealed thirty major peaks indicating that thirty compounds were present in the sample in which the prevailing compounds are cis-Vaccenic acid (13.82 % yield) [1], Hexadecanoic acid, ethyl ester (13.14 % yield) and n-Hexadecanoic acid (12.65 % yield). The results of the GC-MS of Dichloromethane fraction revealed twenty-eight major peaks indicating that twenty-eight compounds were present in the sample in which the prevailing compounds are cis-Vaccenic acid (28.14 % yield), n-Hexadecanoic acid (22.4 % yield) and Octadecanoic acid (21.36 % yield).

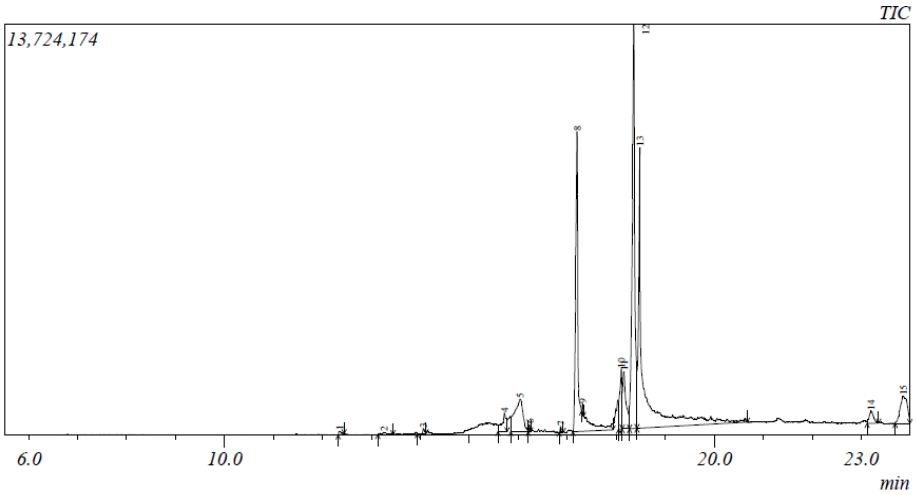
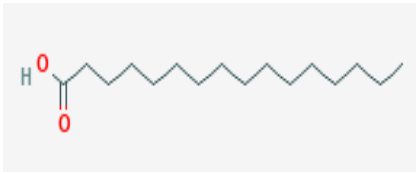


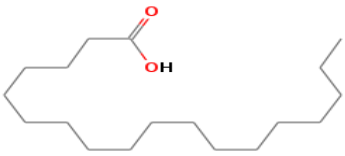
Figure 3: Result of GC-MS analysis of the Ethanol Leaf Extract of *C. fistula*

The structures of these prevailing compounds are presented in figure 4.

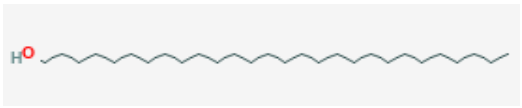
cis-Vaccenic acid [1]



n-Hexadecanoic acid [2]



Octadecanoic acid [3]



Octacosanol [5]

Figure 4: Structures of The Prevailing Compounds Present in The Ethanol Leaf Extract of *C. fistula*

4. Conclusion

The ethanol extract of *Cassia fistula* has a high potential for anti-malaria and antimicrobial activity. These findings provide insight into the usage of the leaves in traditional treatment and provide opportunities to explore their potential applications in the treatment of innumerable health disorders. Phytochemical studies have shown the presence of Alkaloids, Tannins, Saponins, Phenolic Compounds, Flavonoids, Carbohydrates, Proteins, Triterpenoids and Glycosides which are of great importance as a source of new useful drugs.

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