GSC Biological and Pharmaceutical Sciences, 2019, 06(03), 051–060

Available online at GSC Online Press Directory

GSC Biological and Pharmaceutical Sciences

e-ISSN: 2581-3250, CODEN (USA): GBPSC2

Journal homepage: https://www.gsconlinepress.com/journals/gscbps

(RESEARCH ARTICLE)

Spectroscopic analysis and anti-inflammatory effects of *Milicia excelsa* (Moraceae) leaf and fractions

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Publication history**:** Received on 15 February 2019; revised on 07 March 2019; accepted on 11 March 2019

Article DOI: https://doi.org/10.30574/gscbps.2019.6.3.0035

Abstract

This study evaluated the spectroscopic and anti-inflammatory properties of the ethanol leaf extract of *Milicia excelsa* and fractions. The anti-inflammatory activities of the extract and various organic fractions were investigated using bovine erythrocyte membrane stabilizing assay. Ultraviolet-visible (UV-VIS) and Fourier transform-infrared (FT-IR) methods were used to detect the characteristic peak values and their functional groups. The results showed that the crude extract and ethyl acetate fraction showed minimum percentage inhibitions of 0.88 ± 0.30 and 72.05 ± 0.45% and maximum percentage inhibitions of 81.66 ± 0.23% and 99.07 ± 0.30% respectively compared to the standard anti-inflammatory drug (Indomethacin) which exhibited minimum and maximum percentage inhibitions of 52.64 ± 0.83 and 75.51 ± 1.52% respectively. The UV-VIS profile showed the peaks ranging from 270 to 670 nm with the absorption values from 0.040 – 0.720 which could confirm the presence of aromatic compounds, alkaloids, flavonoids, unsaturated conjugated compounds in the plant. In addition, the FT-IR revealed diagnostic peaks around 3570-3200, 3000-2800, 2865-2845, 2850-2815, 2820-2780, 2070-2000, 1630-1750, 1640-1450, 1340-1250, 1200-1000, 800-700 nm^{-1} which could confirm the presence of phenols/alcohol/carboxylic acids, alkanes, diazocompounds, aldehyde /ketone/ amide/ester, alkenes/aromatic compounds, ethers/phosphate compounds, aliphatic/chloro compounds respectively in *Milicia excelsa.* This study therefore demonstrated that ethyl acetate fraction with the highest percentage inhibition contained bioactive principles that protected the erythrocyte membranes effectively from lyses and also exhibited both monophasic and biphasic modes. The study also produced the UV-VIS and FT-IR spectrum profile for *Milicia excelsa* leaf which could be used to identify the plant biomarkers and chemical markers.

Keywords: *Milicia excelsa*; Anti-inflammatory; Membrane stabilizing assay; Plant biomarkers

1. Introduction

Inflammation is a pathophysiological response of the body to injury, infection or destruction characterized by heat, redness, pain, swelling and disturbed functions [1]. It is a primary physiological defensive mechanism that assists the body in protecting itself against noxious stimuli, toxic chemicals, infection, burn or allergens [2]. Although inflammation is a bodily defensive mechanism, attendant complex events and mediators that are involved in the inflammatory reaction can induce, maintain or worsen many diseases [3]. The nonsteroidal anti-inflammatory drugs are the most

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commonly used anti-inflammatory drugs but with attendant side effects especially gastric irritation leading to gastric ulcers [4] [5]. Therefore, the development of potent anti-inflammatory drugs with fewer side effects is a step in the right direction.

The detection and identification of functional groups in phytochemicals present in medicinal plants by the use of UV-Visible (UV-Vis) and Fourier transform-infrared (FT-IR) spectroscopic techniques guarantee immense value, because of their simplicity and cost-effectiveness [6]. FT-IR is an established time-saving method known for its unique "fingerprint" of any compound and for the detection and identification of various functional groups in plant extracts [6] [7] [8]. However, the study of the phytochemicals of *Milicia excelsa* (*M. excelsa*) leaf extract and fractions and their anti-inflammatory effects are rare in the literature. Therefore, the main objective was to carry out the profiling of the functional groups of the phytochemicals present in *M. excelsa* leaf extract and fractions using UV-Vis and FT-IR spectroscopic techniques and to evaluate their anti-inflammatory properties.

Milicia excelsa (welw.) C.C. Berg popularly called African teak or Iroko belongs to the family Moraceae and it is a large deciduous tree growing up to 30 to 50 m high and naturally occurring in humid forests of West Africa [9]. Its various parts such as the latex, leaf, stem bark, root, fruit and ashes are used in African traditional medicine to prepare traditional medicines for the treatment of: malaria [10], mental illnesses [11] [12] [13], sexual dysfunction [14], rheumatism [15], stomach problems, hypertension, tumours and obstructions of the throat [12]. Biologically, the antibacterial [16], anti-amoebic [17], wound healing [18] and *in-vivo* anti-inflammatory properties of the stem bark extract have been demonstrated [19]. The present study reported the anti-inflammatory activity of *M. excelsa* leaf extract and fractions, using bovine red blood stabilization bio-assay; as well as the profile of the functional moieties of phytochemicals present that could be responsible for the anti-inflammatory effects using UV-Vis and FT-IR spectroscopic analysis.

2. Material and methods

2.1. Plant identification and authentication

Milicia excelsa leaves were collected within the campus of the Obafemi Awolowo University (OAU) Ile Ife Nigeria. It was identified and authenticated by Mr. G. A. Ademoriyo of the Herbarium Unit, Department of Botany, Faculty of Sciences, OAU, Ile-Ife and herbarium number Ife-17482 was obtained.

2.2. Preparation of plant materials

The leaves of *M. excelsa* were air dried at room temperature. The dried leaves were pulverized and 1.0 kg of the powder was extracted with 3 liters of 70% ethanol for 72 h. The marc was re-extracted once and the combined extract was concentrated *in vacuo* at a temperature of 40^{o}C to yield 70 g (7.0%) crude extract and coded (EME). Sixty grams of the crude extract was successively partitioned into n-hexane, ethyl acetate, n-butanol and aqueous fractions. The fractions were again concentrated *in vacuo* to give n-hexane, ethyl-acetate, butanol, and aqueous fractions [20].

2.3. Spectroscopic analysis

The UV-VIS spectrum of the ethanol leaf extract of *M. excelsa* and the various fractions were obtained with a UV spectrophotometer in the wavelength range of 190-900 nm. The FT-IR spectrum of each of the sample was mixed with spectra-grade potassium bromide (KBr) in the ratio of 1:100 and pressed to a pellet. The pressed pellet was immediately inserted into the sample holder of Perkin Elmer Spectrophotometer and was operated in the range 4000 – 400 cm^{-1}. Hence, the different functional groups present in each sample were detected from the spectral data obtained.

2.4. Preparation of drugs

The reference drug (Indomethacin, 1.0 mg/ml) and various extracts/fractions were prepared in isosaline (0.85% w/v NaCl) to give final concentrations of 0.1 – 0.5 mg/ml.

2.5. The bovine red blood cell preparation

The bovine red blood cell was prepared as previously described [21].

2.6. The membrane stabilizing activity assay

The assay for the membrane stabilizing activity of the ethanol leaf extract and fractions was carried out as previously described [21-23].

3. Results

3.1. Membrane stabilizing assay

Figures 1 A - E showed the percentage inhibitions of hemolysis of the red blood cell membrane by the ethanol leaf extract and various fractions of *M. excelsa* on bovine red blood cell exposed to both heat and hypotonic induced lyses. The ethanol extract showed minimum and maximum percentage inhibitions of 0.88 ± 0.30 and 81.66 ± 0.23%, the mode of responses of the erythrocyte were both monophasic and biphasic (Fig 1 A). The hexane fraction exhibited minimum and maximum percentage inhibitions of 20.10 ± 1.02 and 52.31 ± 0.27 %, the mode of response of the erythrocyte was both monophasic and biphasic (Fig 1 B). EAF exhibited minimum and maximum percentage inhibitions of 72.04 ± 0.45% and 99.07 ± 0.30%, the mode of response of the erythrocyte was both monophasic and biphasic (Fig 1 C). BF showed minimum and maximum percentage inhibition of 6.15 ± 0.74 % and 25.16 ± 1.63 %, the mode of response of the erythrocyte was both monophasic and biphasic (Fig 1 D) while the AF exhibited minimum and maximum percentage inhibition of 1.64 ± 0.31% and 50.15 ± 1.20%, the mode of response of the erythrocyte was both monophasic and biphasic (Fig 1 E). The standard anti-inflammatory drug (Indomethacin) exhibited minimum and maximum percentage inhibition of 52.65 ± 0.83 and 75.55 ± 1.52%, the mode of response of the erythrocyte was both monophasic and biphasic.

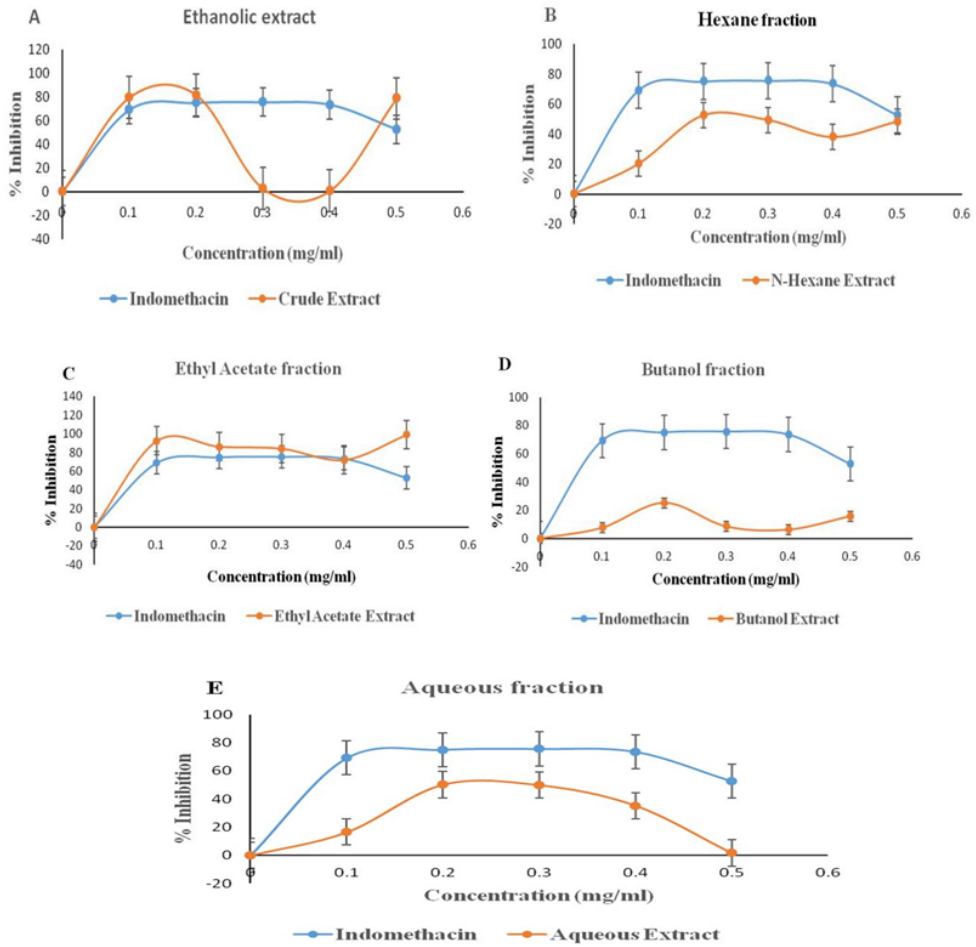

Figure 1 A - E Membrane stabilizing properties of ethanol extract (A); hexane fraction (B); ethyl acetate fraction (C); butanol fraction (D) and aqueous fraction (E) of *M. excelsa*; on bovine red blood cells subjected to both heat and hypotonic induced lyses. Each value represents the mean of four readings ± SEM.

3.2. UV-VIS spectral data interpretation

The extract and all the fractions of *M. excelsa* (Table 1) peak at the range of 234- 676 nm which revealed the presence of phenolics and alkaloids. The absorption peaks in the region of 400 – 420 nm found in the ethanol extract, HF and EAF revealed the presence of quinones and absorption in the UV region of 600-660 nm found in ethanol extract and revealed the presence of chlorophyll. The absorption peaks in the UV region of 220-280 nm found in ethanol extract and all the

fractions and in the range of 330- 420 nm found in ethanol extract; HF and EAF correspond to phenolic acids and derivatives.

Table 1 UV-VIS peak values of crude extract and various organic fractions of *Milicia excelsa* leaf

S/N.	Wavelength (nm)	Absorption
Ethanol extract		
1.	270 (Flavonoids) - (band I)	1.450
2.	305 (Flavonoids) - (band II)	0.880
3.	410 (terpenoids)	0.615
4.	670 (chlorophyll)	0.230
Hexane fraction		
1.	280 (Flavonoids) - (band I)	0.250
2.	350	0.155
3.	410 (terpenoids)	0.105
4.	670 (chlorophyll)	0.065
Ethyl acetate fraction		
1.	310 (Flavonoids) - (band II)	0.450
2.	350 (Flavonoids) - (band II)	0.105
3.	405 (terpenoids)	0.040
Butanol fraction		
1.	280 (Flavonoids) - (band I)	0.720
2.	330 (Flavonoids) - (band II)	0.410
3.	420 (terpenoids)	0.205
Aqueous fraction		
1.	280 (Flavonoids) - (band I)	0.750
2.	350 (Flavonoids) - (band II)	0.460
3.	405 (terpenoids)	0.220

3.3. FT-IR spectral data

3.3.1. The ethanol extract

Ethanol (EME) leaf extract exhibited a characteristic band at 3334.1, 2946.5, 2834.5, 1654.9, 1449.8, 1410.8, 1112.6 and 1017 cm^{-1} indicating the presence of OH, C-H stretch aliphatic, C-H, C=O, C=C-C aromatic ring stretch, OH alcoholic, P=O stretch and C-O-C stretch groups respectively (Table 2).

3.3.2. Hexane fraction (HF)

The hexane fraction exhibited a characteristic band at 3354.6, 2922.2, 2853.3, 2023.9, 1707.1, 1459.3, 1377.3,1313.9, 1243.1, 1161.1, 1034.3, 780.9 cm^{-1} indicating the presence of OH, C-H stretch aliphatic, $CH(CH_2)$, CNN stretch, C=O, C=C-C aromatic ring stretch, OH alcoholic, OH alcoholic , C-N, C-O, C-O-C and C-Cl stretch groups respectively (Table 2).

3.3.3. Ethyl acetate fraction (EAF)

Ethyl acetate fraction (EAF) leaf extract exhibited a characteristic band at 3324.8, 2942.7, 2830.0, 2038.0, 1656.8, 1448.1, 1420.1 and 1019.4 cm^{-1} indicating the presence of OH, C-H stretch aliphatic, C-H, CNN, C=O, C=C-C, OH alcoholic and phosphate ion groups respectively (Table 2).

3.3.4. Butanol fraction (BF)

Butanol fraction (BF) leaf extract exhibited a characteristic band at 3322.9, 2944.8, 2832.8, 1654.9, 1448.1, 1410.8, 1112.6 and 1019.4 cm^{-1} indicating the presence of OH, C-H stretch aliphatic, C-H, C=O, C=C-C aromatic ring stretch, OH alcoholic, P=O stretch and phosphate ion groups respectively (Table 2).

3.3.5. Aqueous fraction (AF)

Aqueous fraction (AF) leaf extract exhibited a characteristic band at 3321.1, 2944.6, 2832.8, 1662.4, 1448.2, 1412.7, 1112.6 and 1019.4 cm^{-1} indicating the presence of OH, C-H stretch aliphatic, C-H, C=O, C=C-C aromatic ring stretch, OH alcoholic, P=O stretch and phosphate ion groups respectively (Table 2).

Table 2 FTIR profile of *Milicia excelsa* leaf extract and various organic fractions

Extract/fraction	Frequency (Wave number cm^{-1}) Test sample	Frequency (Wave number cm^{-1}) Reference article	Bond Assignments	Functional groups present
Ethanol extract				
1	3334.1	3570 - 3200	O-H stretch, H- bonded	phenols, alcohol, polyhydroxy compounds
2	2946.5	3000 – 2800	H-C-H stretch	alkanes
3.	2834.5	2850 – 2815	C-H stretch	methoxy methyl ether
4.	1654.9	1690 – 1640	C = O stretch	amide
5.	1449.8	1510 - 1450	C = C – C aromatic stretch	aromatic compounds
6	1410.8	1410 – 1310	O-H bend	phenols or tertiary alcohol
7.	1112.6	1200 – 1100	P=O stretch	phosphate compounds
8.	1017.6	1050 – 1000	C – O –C stretch	ether
Hexane fraction				
1.	3354.6	3570 – 3200	O-H stretch, H- bonded	phenols, alcohol, polyhydroxy compounds
2.	2922.2	3000 – 2800	H-C-H stretch	alkanes
3.	2853.3	2865 – 2845	$CH(CH_2)$	lipids, protein
4.	2023.9	2100 – 2000	-CNN stretch	diazocompounds
5.	1707.1	1750 – 1705	C = O stretch	ketone
6.	1459.3	1510 – 1450	C = C – C aromatic stretch	aromatic compounds
7	1377.3	1410 – 1310	O-H bend	phenols or tertiary alcohol
8.	1313.9	1410 – 1310	O-H bend	phenols or tertiary alcohol
9.	1243.1	1340 – 1250	C-N stretch	aromatic primary amine
10.	1161.1	1200 – 1125	C – O stretch	tertiary alcohol
11.	1034.3	1075 – 1020	C – O – C	ethers
12.	780.9	800 – 700	C – Cl stretch	aliphatic chloro compounds
Ethyl acetate fraction				
1.	3324.8	3570 – 3200	O-H stretch, H- bonded	phenols, alcohol, polyhydroxy compounds
2.	2942.7	3000 – 2800	H-C-H stretch	alkanes
3.	2830.9	2850 – 2815	C-H stretch	methoxy methyl ether
4.	2038.9	2070 – 2030	-CN2 stretch	diazomethylene compounds
5.	1656.8	1690 – 1640	C = O stretch	amide
6.	1448.1	1510 – 1450	C = C – C aromatic	aromatic compounds
7.	1420.1	1420 – 1330	O-H bending	alcohol
8.	1114.5			
9.	1021.3	1054 – 1019	phosphate ion	phosphate compounds
Butanol fraction				
1.	3322.9	3570 – 3200	O-H stretch group, H-bonded	phenols, alcohol polyhydroxy compounds
2.	2944.8	3000 – 2800	H-C-H stretch	alkanes
3.	2832.8	2850 – 2815	C-H stretch	methoxy methyl ether
4.	1654.9	1690 – 1640	C = O stretch	amide
5.	1448.1	1510 – 1450	C = C – C aromatic stretch	aromatic compounds
6.	1410.8	1410 – 1310	O-H bend	phenols or tertiary alcohol
7.	1112.6	1200 – 1100	P=O stretch	phosphate compounds
8.	1019.4	1054 – 1019	phosphate ion	phosphate compounds
Aqueous fraction				
1.	3321.1	3570 – 3200	O-H stretc, H- bonded	phenols, alcohol, polyhydroxy compounds
2.	2944.6	3000 – 2800	H-C-H stretch	alkanes
3.	2832.8	2850 – 2815	C-H stretch	methoxy methyl ether
4.	1662.4	1670 – 1600	C = O stretch	amides
5.	1448.2	1510 – 1450	C = C – C aromatic stretch	aromatic compounds
6.	1412.7	1410 – 1310	O-H bend	phenols or tertiary alcohol
7.	1112.6	1200 – 1100	P=O stretch	phosphate compounds
8.	1019.4	1054 – 1019	phosphate ion	phosphate compounds

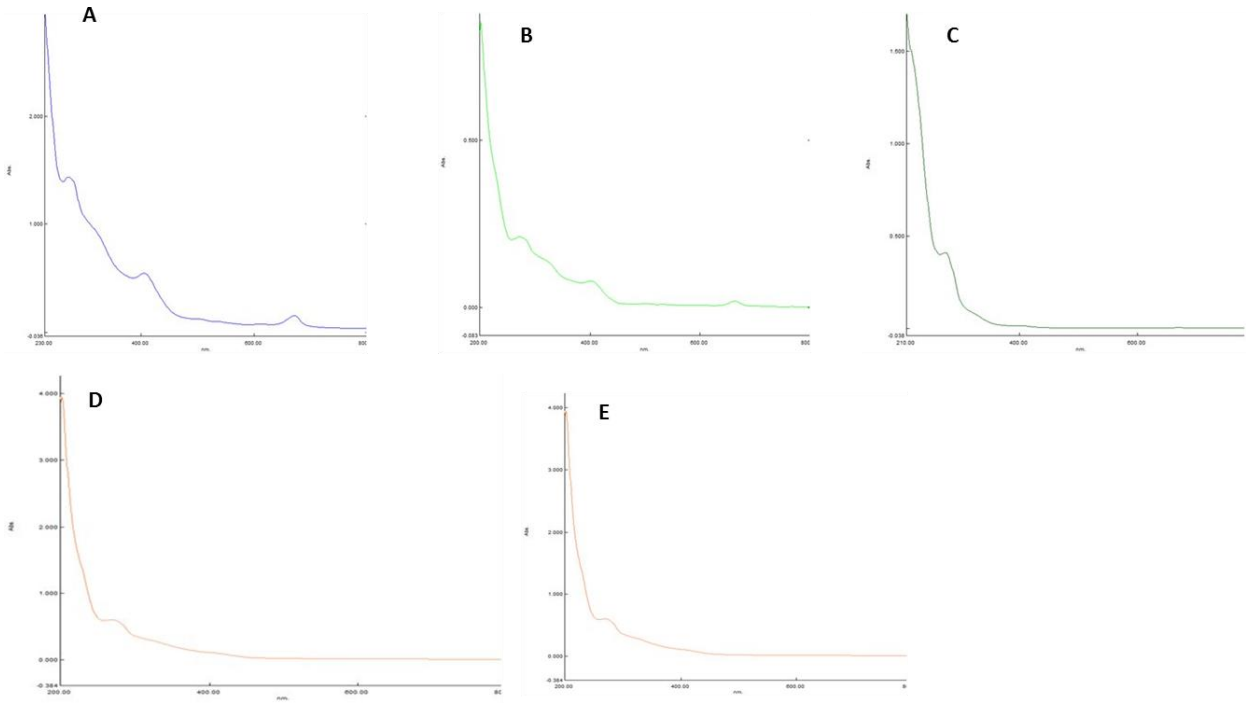

Figure 2 A – E UV-VIS spectrum profile of ethanol leaf extract and fractions of *Milicia excels*. A - Ethanol leaf extract, B - n- hexane fraction, C - Ethyl acetate fraction, D - n-butanol fraction, E - Aqueous fraction.

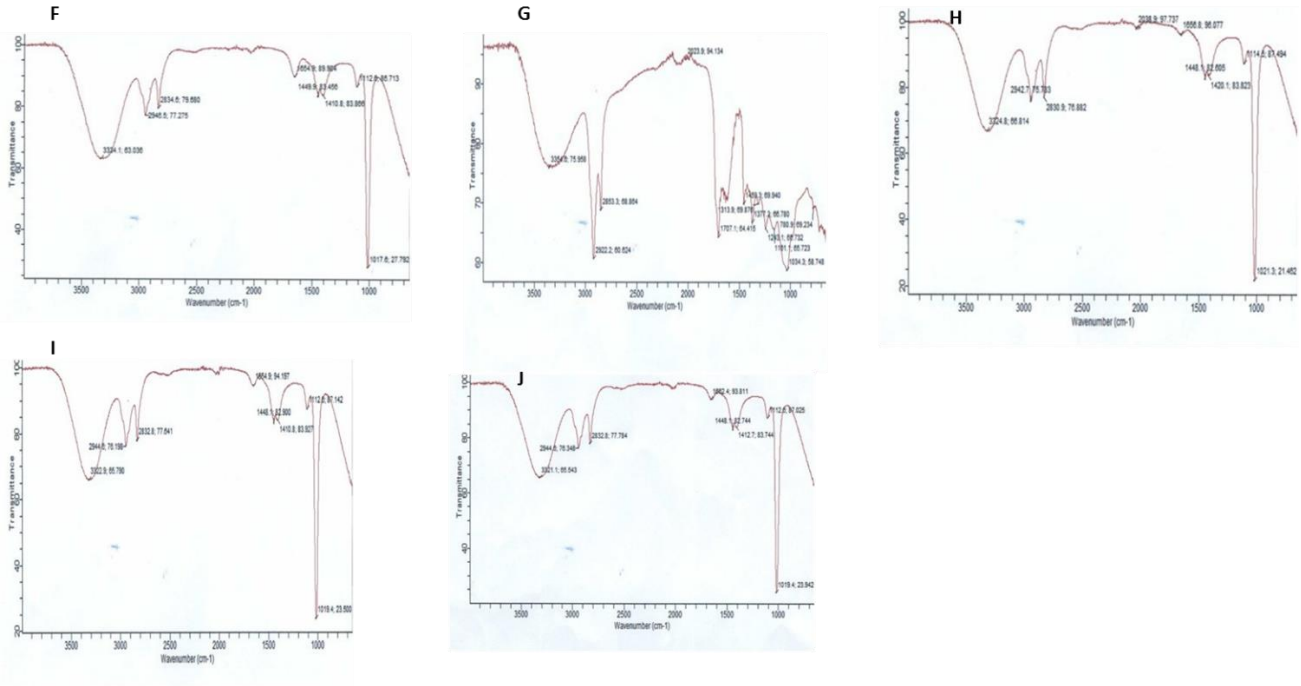

Figure 2 F – J FTIR spectrum profile of ethanol leaf extract and fractions of *Milicia excels*. F - Ethanol leaf extract, G - n-hexane fraction, H - Ethyl acetate fraction, I - n-butanol fraction, J - Aqueous fraction.

4. Discussion

Plants derived drugs have been demonstrated to contain principles that possess the ability to facilitate the stability of biological membranes when exposed to induced lyses [21] [22]. The result of this investigation presented the membrane stabilizing activities of *M. excelsa* extract and fractions (Figures 1A - E). The extract and fractions have varying degrees of inhibition of the hemolysis of bovine erythrocyte cells. Ethanol extract (Figure 1A) and EAF (Figure 1C) showed the

highest percentage inhibition of 81.66 ± 0.23% and 99.07 ± 0.30% respectively, hence, they are considered highly potent against heat and hypotonic induced lyses. Both ethanol extract and the ethyl acetate fraction therefore, contained principles that protected the erythrocytes membranes effectively. Moreover, ethyl acetate fraction offered the highest protection against induced lyses. The activities of the ethanol extract and ethyl acetate fraction were comparable to that of a standard anti-inflammatory drug (Indomethacin).

An earlier report has suggested that the mechanism of action of the membrane stabilizing effect of the extracts of *Lantana camara* and its fractions as well as the reference anti-inflammatory agent could be connected with binding to the erythrocyte membranes with subsequent alteration of the surface charges of the cells [23]. This binding could have prevented the physical interaction with aggregating agents or promote dispersal by mutual repulsion of like charges which are involved in the red blood cells hemolysis [23]. Therefore, the anti-inflammatory effect of *Milicia excelsa* ethanol extract and fractions could as well be due to this mechanism.

Recent research efforts toward drug discovery from medicinal plants are a multifaceted approach that combines botanical, phytochemical, biological and molecular techniques [24]. Plant based drugs have been reported to have an array of secondary metabolites like alkaloids, flavonoids, phenols, peptides, steroids, tannins and other phytocompounds [25]. Bioactive principles in medicinal plants are responsible for their therapeutic activities such as anti-carcinogenic, anticholinergic, anti-diabetic, anti-inflammatory, anti-leprosy activities, anti-malarial, anti-microbial, anti-oxidant among others [26].

The UV-VIS profile of the crude plant extract and fractions showed the peaks ranging from 270 to 670 nm with the absorption values from 0.040 – 0.720 (Table 1). The two absorption maxima in the ranges 230-285 nm (band I) and 300-350 nm (band II) are diagnostically characteristic of the presence of flavonoids while maximum absorptions in the range from 260 to 300 nm are characteristic of alkaloids occurrence [27]. Furthermore, the peaks ranging from 400-710 nm is characteristic of the presence of terpenoids and unsaturated conjugated compounds. Hence the UV-VIS profile of *M. excelsa* revealed the presence of flavonoids, alkaloids, terpenoids, and unsaturated conjugated compound.

FT-IR spectroscopic analysis of the crude and various organic fractions (Table 2) revealed that, the peak at 3321-3354.6 cm^{-1} assigned as hydrogen bonded O-H or N-H stretching is attributable to the existence of phenols, alcohols, and polyhydroxyl compounds or amines. However, it is unambiguously clear that amine is not present as revealed by the absence of very sharp peak either doublet or singlet which represent primary and secondary amine respectively in this region. The peaks at 2922.2-2946.5 cm-1 assigned as sp3 H-C-H stretch are attributable to alkanes; 2853.3 cm^{-1} assigned as $CH(CH_2)$ stretch are due to alkane, lipids, and protein; 2830.9 -2834.6 cm^{-1}, designated as OCH_3, C-H stretch are due to the manifestation of methoxy/methyl ether in the plant chemical constituent; The vibrations between 2023.9-2038.9 cm^{-1} ascribed as -CN≡N stretch are attributable to diazomethylene compounds or diazo compounds; 1707.1 cm^{-1} allotted as C = O stretch are attributable to the presence of either carbonyl of an aldehyde or ketone while or amide 1654.9-1662.4 cm^{-1} assigned to C = O stretch are attributable to amide. Consequently, the conspicuously missing frequencies at 1735-1760 cm^{-1} from the IR spectrum unequivocally revealed the absence of an ester carbonyl in the chemical constituents of the plant. Furthermore, the peaks at 1449.8 cm^{-1} assigned to C = C – C aromatic stretch is attributed to the occurrence of aromatic compounds in the plant chemical constituents. In addition, the frequency peaks at 1410.8 cm^{-1} due to O-H bend is characteristics of either alcoholic or phenols or tertiary alcohols while the peaks at 1021.3 – 1161 cm^{-1} due to C – O – C or P=O stretch are characteristics of either ethers or phosphate compounds; 1017.6 cm^{-1} assigned to C – O –C stretch are attributable to occurrence of either ether or carboxylic acid while the peak at 780.9 cm^{-1} designated as C-Cl stretch are attributable to the incidence of aliphatic chloro compounds. It is therefore not surprising that *M. excelsa* is employed in the treatment of inflammation, rheumatism, hypertension, tumors, wound healing as previously highlighted; since phenolic and polyhydroxyl phytochemicals and alkaloids or nitrogen present in *M. excelsa* as revealed by the FT-IR have been reported in various studies to exhibit anti-inflammatory, antihypertensive, antimicrobial, antiviral, antimutagenic, and antitumor, effects among others [28-37].

The result of this finding further revealed that only the ethyl acetate fraction of *M. excelsa* leaf extract demonstrated excellent anti-inflammatory potential against bovine erythrocyte lyses. It is worth mentioning that it is the only fraction that gave absorption band of 2038 cm^{-1} (in the range of 2000 - 2100 cm^{-1}) which corresponded to the possible presence of diazo compounds (CNN), such as diazoalkanes, diazo ketones, and diazo esters and CH_3O substituted diphenyldiazomethanes to the stretching vibration of the group [38-39]. Therefore, CH_3O substituted diphenyldiazomethanes either in additivity, synergism or counter interaction with other phytochemicals in ethyl acetate fraction may be responsible for the conferment of excellent anti-inflammatory effect observed against bovine erythrocyte lyses.

5. Conclusion

In conclusion, the study demonstrated that the *M. excelsa* leaf extract possessed bioactive functional groups with excellent membrane stabilizing property and anti-inflammatory effects, thus justifying some of their ethnomedicinal uses. However, further chromatographic isolation and characterization of the chemical constituent(s) of the leaf extract, and especially the ethyl acetate fraction with the highest membrane stabilizing property and the *in vivo* studies will be carried out in future study.

Compliance with ethical standards

Acknowledgments

The authors are grateful to Professor E. M Obuotor of the Department of Biochemistry, Faculty of Sciences, Obafemi Awolowo University, Ile-Ife, Osun State for using his laboratory for the anti-inflammatory assay reported in this study.

Disclosure of conflict of interest

Authors declared no competing interests.

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How to cite this article

Akinpelu LA, Olawuni IJ, Ogundepo GE, Adegoke AM, Olayiwola G and Idowu TO. (2019). Spectroscopic analysis and anti-inflammatory effects of *Milicia excelsa* (Moraceae) leaf and fractions. GSC Biological and Pharmaceutical Sciences, 6(3), 51-60.