



Faculty of Sciences
Department of Chemistry and Forensic Sciences

***GREWIA FLAVA TWIGS HERBAL EXTRACTS: PHYTOCHEMICAL,
ANTIOXIDANT, AND ANTIMICROBIAL EVALUATIONS***

by

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DECLARATION REGARDING THE WORK AND COPYRIGHT

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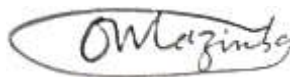


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DEDICATION

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LIST OF ABBREVIATIONS

μ: Microgram

Ace: Acetone

ANOVA: Analysis of variance

COSY: Correlated spectroscopy

DCM: Dichloromethane

DEPT: Distortions Enhancement by Polarization Transfer

Dis water: Distilled water

DPPH: 2, 2- diphenyl-1-picrylhydrazyl

Hex: *n*-Hexane

HMBC: Heteronuclear Multiple Bond Correlation

HMQC: Heteronuclear Multiple-Quantum Correlation

IC₅₀: Fifty percent inhibitory concentration

EtOAc: Ethyl acetate

FT-IR: Fourier-transform infrared spectroscopy

GC- MS: Gas chromatography coupled with mass spectrometer

MIC: Minimum inhibitory concentration

MeOH: Methanol

MS: Mass spectroscopy

NMR: Nuclear magnetic resonance

SDS: Sodium dodecyl sulphate

SEM: Standard error of the mean

UV- Vis : Ultraviolet- visible

ABSTRACT

The ethnopharmacological properties of the medicinal plants are ascribed to their bioactive phytochemicals. Thus, the purpose of this study was to assess the phytochemical profile, antioxidant, and antimicrobial activities of *Grewia flava* twig extract. Infusions of this plant part are consumed to assist with kidney problems, however, there is no scientific data on the phytochemical profile, biological properties of the extract to validate its folklore use. The *in vitro* antioxidant activity of *G. flava* twig extracts were evaluated using DPPH radical scavenging, reducing power and metal chelation assays and the total phenolic and flavonoid content in the extracts were determined and related to the antioxidant activity. Bioactive compounds were isolated and characterized from the twig extract on the basis of NMR and FT-IR spectroscopy. Isolates were subjected to antimicrobial screening against *P. aeruginosa*, *S. aureus*, *E. coli*, *B. subtilis*, *A. niger*, and *R. oryzae* using the agar well diffusion and microdilution methods. All crude extracts exhibited dose dependent DPPH radical quenching and chelating abilities with IC₅₀ values ranging from 15 to 496 µg/mL and from 97 to 428 µg/mL respectively. Methanol twig extract exhibited highest concentration of phenolic (250 mg GAE/ g) and flavonoids (64.54 mg QUE/ g), which explains the high antioxidant ability observed. The bioactivities of the extracts can be ascribed to the presence of different bioactive phytochemical compounds including alkaloids, flavonoids, saponins, steroids, glycosides, anthraquinones and tannins which were detected in the extracts. Phytochemical study of the twig extract afforded isolation of five compounds: Lupeol, β-sitosterol, stigmasterol, eicosyl protocatechuate and kaempferol-3-*O*-rutinoside from aqueous methanol twig extract. All compounds except lupeol are being reported for the first time from *G. flava*. Eicosyl protocatechuate exhibited highest antiradical activity among the isolated compounds with an IC₅₀ value of 120 µg/mL. Stigmasterol displayed inhibitory effect against *S. aureus*, *E. coli*, *A. niger* and *R. oryzae* with MIC values ranging from 0.40 to 1.70 mg/mL making it the most active isolated compound. Root and twig *n*-hexane extracts were subjected to GC-MS analysis, which identified a number of bioactive compounds like betulin, β-amyrin, palmitic acid, Lupenone, and phytol, highlighting the potential of the plant species as a botanical drug. In conclusion this study provides scientific evidence to justify the use of *G. flava* twigs in traditional medicine, suggestive that the plant part may serve as a medicinal agent.

CHAPTER 1

1. INTRODUCTION

1.1. Background

The use of herbal remedies for the cure and prevention of various ailments worldwide dates way back to ancient times. In developing countries, there is continued use of plants as medical remedies for different ailments, especially in rural areas. This might be attributed to their availability, efficacy, reliability, low expenses to acquire and minimal side effects in comparison to synthetic drugs (Jyoti *et al.*, 2015; Motamedi *et al.*, 2010; Motlhanka & Nthoiwa, 2013). The ethnopharmacological properties of the herbal/ green teas or medicinal plants in general are ascribed to their bioactive phytochemicals. These are constituted by tannins, terpenoids, alkaloids, anthraquinones, flavonoids, saponins (Dhawan & Gupta, 2017; Hutke & Naswale, 2017). Herbal tea possess a noteworthy phenolic compounds exhibiting strong antioxidant activity against free radicals and/or reactive oxygen species (ROS) found within the human body (Koch *et al.*, 2018; Li *et al.*, 2016). Failure to remove these ROS's leads to increase in the oxidative stress which is responsible for the emergence of chronic diseases like Parkinson's disease, cancer, diabetes, Alzheimer's disease and aging (Ahmed *et al.*, 2019; Chiavaroli *et al.*, 2011; Stobiecka *et al.*, 2022).

Literature shows that herbal phytochemicals can directly be used as drugs or lead compounds for the discovery, development, and synthesis of new synthetic medicinal drugs. In the context of the former, numerous phytochemicals have been isolated and are used in their natural form, for example aspirin, morphine, eugenol have been isolated from Willow tree bark, *Papaver somniferum* (*Opium poppy*), and Cinnamon leaves respectively, which are used for the treatment of numerous diseases, including inflammation. For the latter metformin, the famous antidiabetic drug which was developed from phytochemicals (guanidine and galegine) isolated from *Gallega officianalis* (Bailey, 2017; Błaszczuk *et al.*, 2021; Katiyar *et al.*, 2012; Kinaan *et al.*, 2015; Krishnamurti & Rao, 2016). *Grewia Flava* twig extract is amongst the commonly consumed infusions in different parts of Southern Africa for its medicinal benefits. However, despite its continued use, there is lack of knowledge on its phytochemical composition and biological properties. The current research will focus on the assessment of the

phytochemical composition (isolation and identification of the bioactive compounds), antioxidant and antimicrobial activities of *Grewia flava* twigs herbal tea.

1.2. Problem statement

There is continued use of indigenous medicines in developing countries (Botswana included) due to their medicinal benefits. Folklore documentation shows that *Grewia flava* twigs possess antioxidant and antimicrobial activities as they are used as remedies for certain illnesses (Lamola *et al.*, 2017). However, there is lack of information on the antioxidant, antimicrobial properties, and phytochemical composition to justify the use of the twig infusions. Furthermore, *Grewia flava* roots happen to be the most adopted plant part for diverse medicinal benefits, and the use of this plant part is not sustainable for the conservation of the plant species. Lastly there are concerns regarding the use of synthetic drugs as they present negative side effects to the human body compared to traditional medicine considered to be a much safer option.

1.3. Significance of the study

The generated information on the biological activities of specific phytochemicals from the twig extract of *Grewia flava* might pave way for their use either in their natural form for treating certain ailments, as lead compounds for the discovery and/or preparation of synthetic drugs and in plant extract supplementary infusions. Furthermore, this study might also provide nutritionist with needed information to further the research in the production of commercially packaged herbal tea. The research findings might also boost the confidence of the users (traditional healers, herbalist and even patients) who uses *Grewia flava* twigs for treating different ailments. Lastly, the knowledge can be used to promote the use of twigs over the roots which would be sustainable for the conservation of biodiversity.

1.3.1. Aim

The study aims to profile *G. flava* twig extract and fraction's phytochemicals and thereafter evaluate their antioxidant and antimicrobial activities.

1.3.2. Objectives

- i. To evaluate the phytochemical composition of *G. flava* extracts
- ii. To determine the *G. flava* crude extracts and fraction's antioxidant and antimicrobial activities
- iii. To identify the *G. flava* twig extract bioactive compounds.
- iv. To determine the *G. flava* twig non-polar extract composition using GCMS.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Introduction

This section aims to give insight on the research which has been conducted regarding *Grewia flava*. Research on the phytochemical composition, antioxidant and antimicrobial activities and the isolation of the bioactive compounds in the genus *Grewia* has been conducted over the years; however, *Grewia flava* seems to be inconspicuous since there is scarcity of information on its case.

2.2. Genus *Grewia*

Genus *Grewia* belongs to the angiosperm group comprising of about 400 species of shrubs and trees. The genus is found in warmer regions across the world which includes tropical Africa, Arabia, India, China, Malaysia, and Northern Australia (Dharmasoth & Battu, 2019; Haile *et al.*, 2020). Folkloric data shows that several plant species in the genus *Grewia* (*Malvaceae* family) are used as therapeutic agents to treat different ailments including malaria, diabetes, eye complaints, diarrhoea, inflammation, cough, piles, syphilitic ulceration, liver disorder, gonorrhoea, digestive problems, ulcerative colitis, heat stroke, typhoid fever and sore throats (Bari *et al.*, 2019; ELhassan *et al.*, 2021; Githinji *et al.*, 2020; Joshi *et al.*, 2013; Sinha *et al.*, 2015). The medicinal use of these plant species can be attributed to their appreciable antioxidants content, which are responsible for the anti-cancerous, antifungal, antioxidant, hepatoprotective and antibiotic activities previously reported from different studies (Kumar & Venkatachalam, 2016). Furthermore, studies have shown that various *Grewia* species have fairly high levels of phytochemicals like tannins, diterpenes, glycosides, triterpenoids, sterols, saponins, alkaloids, flavonoids and total phenolic content which supports their use in folk medicine (Akwu *et al.*, 2019; Ali *et al.*, 2017; Hutke & Naswale, 2017; Waliullah, Ghias Uddin, Abdur Rauf & Taj Ur Rehman, Sadiq azam, 2011).

Bioactive phytochemicals are the principal active compounds which give these plants their medicinal purpose and they are found in all parts of the plant although varying. The genus *Grewia* is mainly associated with the phytochemicals; triterpenoids, flavonoids and alkaloids (Arora, 2011). Research on the phytochemical profile of the *Grewia* species has enabled the separation and identification of different phytochemicals like steroids, lignans, triterpenes, flavones, and glycosides. Isolated bioactive compounds from the genus include quercetin (**1**), harman (**2**), microcosamine B (**3**), Sitosterol β -D-glucoside (**4**), stigmasterol (**5**), β -amyrin (**6**), friedelin (**7**), oleanolic acid (**8**), betulin (**9**), and glutaric acid (**10**) (Goyal, 2012; Khanal *et al.*, 2016; S. Kumar *et al.*, 2022; Ullah *et al.*, 2012). Table 1 shows some of the bioactive compounds which have been isolated from the *Grewia* genus, while the structures of the most active isolated compounds are shown in Figure 1.

Table 1: Isolated bioactive compounds from the genus *Grewia*

SN	Compound name	Class	Reported activity	Plant species	Plant part	Reference
1	Quercetin	Flavonol	Anti-HIV, anti-inflammatory, antimicrobial and antitumor	<i>G. asiatica</i>	Fruit	(Parvez <i>et al.</i> , 2020; Zia-Ul-Haq <i>et al.</i> , 2013)
2	Harman	β -Carboline	Antispasmodic and sedative	<i>G. bicolour</i>	Root	(Jaspers <i>et al.</i> , 1986)
3	Microcosamine B	Piperidine	Antidiabetic	<i>G. nervosa</i>	Root	(Meena <i>et al.</i> , 2017)
4	Sitosterol β -D-glucoside	sterol	Anti-inflammatory	<i>G. damine</i>	Leaves	(Jayasinghe <i>et al.</i> , 2004)
5	Stigmasterol	Sterol	Cytotoxic, antibacterial	<i>G. tiliaefolia</i> , <i>G. plagioplylla</i>	Stem bark	(Ahamed <i>et al.</i> , 2007; Githinji <i>et al.</i> , 2020)
6	β -Amyrin	Triterpene	anti-hyperlipidemic	<i>G. asiatica</i>	Leaves	(Abou Zeid & Sleem, 2005; Akram <i>et al.</i> , 2019)
7	Friedelin	Triterpene	Analgesic, anti-inflammatory, antipyretic, anticancer, anti-diabetic, antidiarrheal, antihypertensive, and antiulcer	<i>G. biloba</i> , <i>G. lasiocarpa</i> and <i>G. tiliaefolia</i> ,	Stem bark and leaves	(N. A. Akwu <i>et al.</i> , 2019; Dharmasoth <i>et al.</i> , 2022; Liu <i>et al.</i> , 2008;

						Malhotra <i>et al.</i> , 2020)
8	Oleanolic acid	Triterpene	phytotoxicity, anti-inflammatory, antibacterial, anticancer, and anti-inflammation	<i>G. optiva</i>	Stem bark	(Uddin <i>et al.</i> , 2011)
9	Betulin	Triterpene	Anti-malarial, antiviral (including anti-HIV), anti-inflammatory, antioxidant, cytotoxic, and phytotoxic	<i>G. optiva</i> , and <i>G. plagiophylla</i>	Stem bark	(Bari <i>et al.</i> , 2019; ELhassan <i>et al.</i> , 2021; Githinji <i>et al.</i> , 2020; Uddin <i>et al.</i> , 2011)
10	Glutaric acid	Fatty acid	Antioxidant	<i>G. optiva</i>	Root	(Bari <i>et al.</i> , 2019; Teoh, 2016)
11	kaempferol 3-O- β -neohesperidoside or Kaempferol-3-O- β -D-glucorhamnoside	Flavonol	Anticancer and antioxidant	<i>G. asiatica</i> and <i>G. damine</i>	Fruit and leaves	(Akdemir <i>et al.</i> , 2001; Koley <i>et al.</i> , 2020; S. Kumar <i>et al.</i> , 2022)
12	3',6',8' -trihydroxyl-4H chromen-4' -one	Flavonol	Anticancer and antioxidant	<i>G. optiva</i>	Root	(Bari <i>et al.</i> , 2019)
13	Cyanidin 3-galactoside	Anthocyanin	Antidiabetic, hepatoprotective and radioprotective	<i>G. asiatica</i>	Fruit	(S. Kumar <i>et al.</i> , 2022; Ullah <i>et al.</i> , 2012)
14	Isovitexin	Flavone	Anticancer, anti-inflammatory and antioxidant	<i>G. damine</i>	Leaves	(Ullah <i>et al.</i> , 2012)

15	Vitexin	Flavone	anticancer, anti-inflammatory, antioxidant and neuroprotective	<i>G. damine</i>	Leaves	(Ullah <i>et al.</i> , 2012)
16	6-Methoxyharman	β -Carboline	Antispasmodic and sedative	<i>G. bicolour</i>	Root	(Jaspers <i>et al.</i> , 1986)
17	6-Hydroxyharman	β -Carboline	Antispasmodic and sedative	<i>G. bicolour</i>	Root	(Jaspers <i>et al.</i> , 1986)
18	n-Methyl microcosamine B	Piperidine	Antidiabetic	<i>G. nervosa</i>	Root	(Meena <i>et al.</i> , 2017)
19	MicroGrewiapipe A	Piperidine	Antidiabetic	<i>G. nervosa</i>	Root	(Meena <i>et al.</i> , 2017)
20	HomomicroGrewiapipe	Piperidine	Antidiabetic	<i>G. nervosa</i>	Root	(Meena <i>et al.</i> , 2017)
21	<i>N</i> -methyl-6- β -(1,3,5-trienyl)-3- β -methoxyl-3- β -methyl piperidine	Piperidine	Insecticidal activity	<i>G. microcos</i>	Leaves	(Joshi <i>et al.</i> , 2013)
22	Grewin	Coumarinolignan	Antimalarial	<i>G. bilamellata</i>	Leaves, twigs, and Stem	(Ma <i>et al.</i> , 2006; Zhang <i>et al.</i> , 2016)
23	Cleomiscosin D	Coumarinolignan	Antimalarial	<i>G. bilamellata</i>	Leaves, twigs, and stem	(Ma <i>et al.</i> , 2006; Ullah <i>et al.</i> , 2012)

24	Bilagrewin	Neolignan	Antimalarial	<i>G. bilamellata</i>	Leaves, twigs, and stem	(Ma <i>et al.</i> , 2006; Zhang <i>et al.</i> , 2016)
25	Nitidanin	Neolignan	antimalarial	<i>G. bilamellata</i>	Leaves, twigs, and stem	(Ma <i>et al.</i> , 2006; Zhang <i>et al.</i> , 2016)
26	8-O-4'-Neolignanguaia-cylglycerol- β -coniferyl ether (erythro)	Neolignan	Antimalarial and cytotoxicity	<i>G. bilamellata</i>	Leaves, twigs, and stem	(Ma <i>et al.</i> , 2006; Ullah <i>et al.</i> , 2012)
27	8-O-4'-Neolignanguaia-cylglycerol- β -coniferyl ether (threo)	Neolignan	Antimalarial and cytotoxicity	<i>G. bilamellata</i>	Leaves, twigs, and stem	(Ma <i>et al.</i> , 2006; Ullah <i>et al.</i> , 2012)
28	Syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde)	Phenol	Uterotonic	<i>G. occidentalis</i>	Bark and wood	(Mulholland <i>et al.</i> , 2002)
29	2,6-dimethoxy-1-acetylquinol	Quinone	Antimalarial	<i>G. bilamellata</i>	Twigs, leaves, and stem	(Ma <i>et al.</i> , 2006)
30	3,5-Dihydroxy phenyl acrylic acid	Hydroxycinnamic acids	Antioxidant	<i>G. optiva</i>	Root	(Bari <i>et al.</i> , 2019)

31	Coniferaldehyde	Phenol	Memory boosters and uterotonic	<i>G. occidentalis</i>	Wood and bark	(Mulholland <i>et al.</i> , 2002)
32	Sinapaldehyde (E)-4-hydroxy-3,5-dimethoxycinnam aldehyde)	Phenol	Uterotonic	<i>G. occidentalis</i>	Wood and bark	(Mulholland <i>et al.</i> , 2002)
33	β -sitosterol glucopyranoside	sterol	Anti-cell proliferation	<i>G. flavescens</i>	Stem bark	(ELhassan <i>et al.</i> , 2021)
34	Stigmast-7-en-3-ol	sterol	Anti-hyperlipidemic	<i>G. asiatica</i>	Leaves	(Abou Zeid & Sleem, 2005; Zia-Ul-Haq <i>et al.</i> , 2013)
35	Daucosterol	sterol	Antiproliferative and phytotoxicity	<i>G. bilamellata</i> and <i>G. optiva</i>	Stem bark	(Uddin <i>et al.</i> , 2011)
36	β -Sitosterol	sterol	Antidiabetic, antifibrotic, anti-inflammatory, and antiproliferative	<i>G. bicolour</i> , <i>G. biloba</i> , <i>G. lasiocarp</i> , <i>G. optiva</i> , <i>G. tiliaefolia</i>	Leaves and roots	(Ahamed <i>et al.</i> , 2007; N. A. Akwu <i>et al.</i> , 2019; Bari <i>et al.</i> , 2019)
37	2 α ,3 β -dihydroxyolean-12-en-28- oic acid	Triterpene	Antimalarial	<i>G. bilamellata</i>	Twigs, leaves,	(Ma <i>et al.</i> , 2006)

					and stem	
38	Lanost-9(11)-en-12-one (17)	Triterpene	Anti-hyperlipidemic	<i>G. asiatica</i>	Leaves	(Abou Zeid & Sleem, 2005; Zia-Ul-Haq <i>et al.</i> , 2013)
39	Lupeol	Triterpene	Cardioprotective, nephroprotective, neuroprotective, anti-arthritis, anti-asthmatic, anti-cancer, antidiabetic, anti-inflammatory, antinociceptive, and antipyretic	<i>G. bicolour</i> , <i>G. damine</i> , <i>G. lasiocarpa</i> , and <i>G. tiliaefolia</i>	Leaves and stem bark	(Ahamed <i>et al.</i> , 2007; Geetha & Varalakshmi, 2001; Tsai <i>et al.</i> , 2016)
40	3 α ,20-lupandiol	Triterpene	Antimalarial	<i>G. bilamellata</i>	Twigs, leaves, and stem	(Ma <i>et al.</i> , 2006)
41	α -Amyrin	Triterpene	Antioxidant, antiplatelet, hepatoprotective, anti-inflammatory, antinociceptive, and anti-hyperlipidemic	<i>G. asiatica</i> , <i>G. flavescens</i> , and <i>G. villosa</i>	Fruit and leaves	(Abou Zeid & Sleem, 2005; Zia-Ul-Haq <i>et al.</i> , 2013)
42	α -Amyrone	Triterpene	anti-hyperlipidemic	<i>G. asiatica</i>	Leaves	(Abou Zeid & Sleem, 2005; Zia-Ul-Haq <i>et al.</i> , 2013)

43	Ursene-3,19,28-triol	Triterpene	Uterotonic	<i>G. villosa</i>	Bark and wood	(Ullah <i>et al.</i> , 2012)
44	Oleanonic acid	Triterpene	Hepatoprotective, uterotonic, and antitumor	<i>G. occidentalis</i>	Wood and bark	(Mulholland <i>et al.</i> , 2002)
45	29 (30) -lupene-3, 20-diol; 3 β -form, 29-Aldehyde	Triterpene	Anticancer, anti-cell proliferation activity	<i>G. flavescens</i>	Stem bark	(ELhassan <i>et al.</i> , 2021)
46	Ursolic acid	Triterpene	Antioxidant, phytotoxic, anti-microbial, anti-inflammatory, and anticancer	<i>G. optiva</i>	Stem bark	(Joshi <i>et al.</i> , 2013; Uddin <i>et al.</i> , 2011)
47	Lup-20(29)-en-3-ol	Triterpene	Cytotoxic,	<i>G. plagiophylla</i>	Stem bark	(Githinji <i>et al.</i> , 2020)
48	Betulinic acid	Triterpene	Anti-inflammatory, antioxidant, anticancer, and analgesic	<i>G. optiva</i>	Stem bark, and roots	(Uddin <i>et al.</i> , 2011)
49	(4Z, 12Z)-cyclopentadeca-4, 12-dienone	ketone	Anti-diabetic potential	<i>G. hirsuta</i>	Leaves	(Abirami & Natarajan, 2014)
50	Hexacosanol	Fatty acid	Anti-hyperlipidemic	<i>G. asiatica</i>	Leaves	(Abou Zeid & Sleem, 2005)

51	Heptacosanol	Fatty acid	Anti-hyperlipidemic	<i>G. asiatica</i>	Leaves	(Abou Zeid & Sleem, 2005)
52	Nonacosanol	Fatty acid	Anti-hyperlipidemic	<i>G. asiatica</i>	Leaves	(Abou Zeid & Sleem, 2005)
53	Docosanol	Fatty acid	Anti-hyperlipidemic	<i>G. asiatica</i>	Leaves	(Abou Zeid & Sleem, 2005)
54	Hexanedioic acid	Fatty acid	Antioxidant and antibacterial	<i>G. optiva</i>	Root	(Bari <i>et al.</i> , 2019; Teoh, 2016)
55	2 α ,3 β -dihydroxy-olean-12-en-28-oic acid	Fatty acid	Antimalarial	<i>G. bilamellata</i>	Twigs, and leaves	(Ma <i>et al.</i> , 2006)
56	α -Linolenic acid	Ester	Anti-inflammatory	<i>G. nervosa</i>	Leaves	(Uddin <i>et al.</i> , 2011)
57	2,6-dimethoxy-1-acetylquinol	quinol	antimalarial	<i>G. bilamellata</i>	Twigs, and leaves	(Ma <i>et al.</i> , 2006)
58	Luteloin	Flavonol	Anti-inflammatory and antioxidant	<i>G. mollis</i>		(Cox, 2003; Goyal, 2012)
59	Kaempferol-3-O-rutinoside	Flavonol	Anticancer and antioxidant	<i>G. tenax</i>	Fruits	(S. Kumar <i>et al.</i> , 2022; Saleh <i>et al.</i> , 2010)

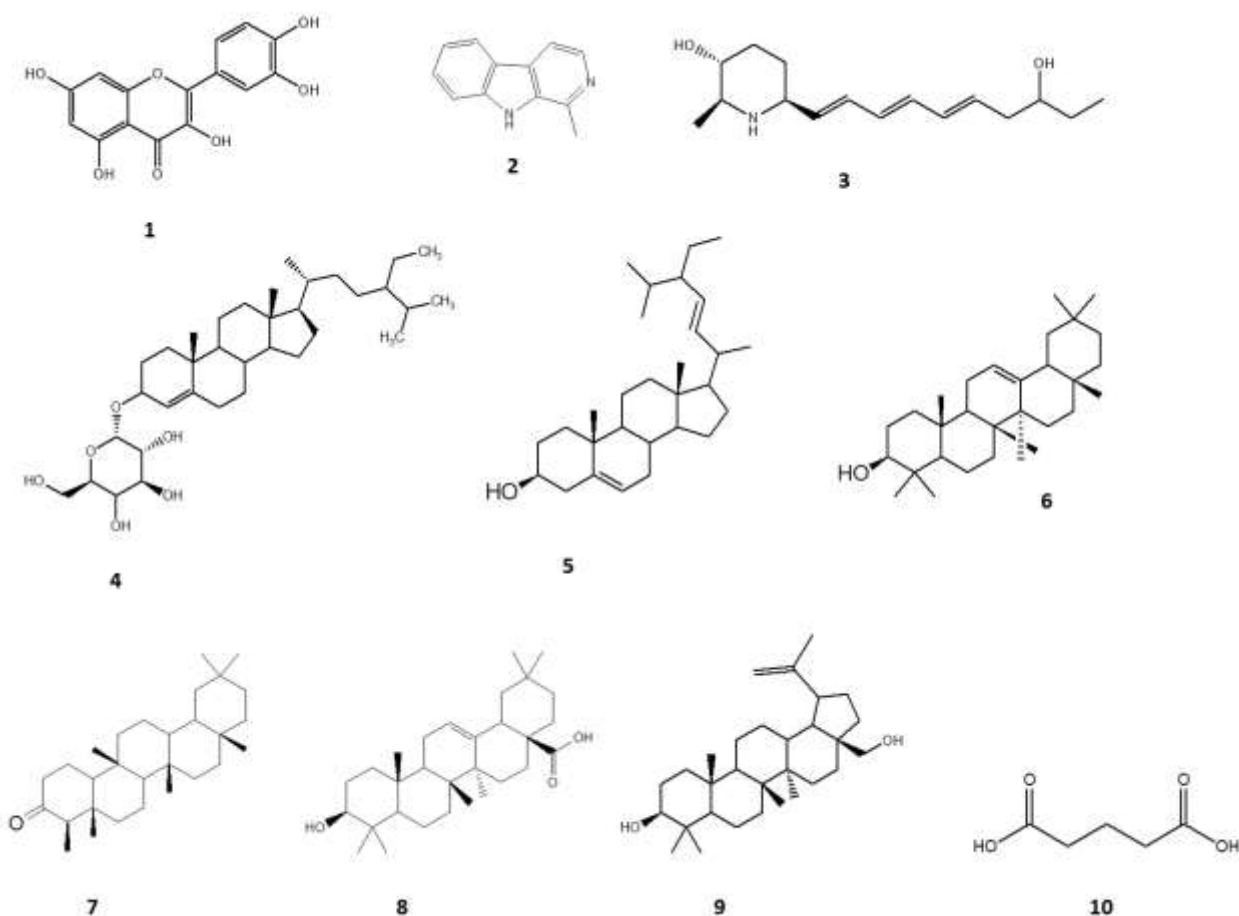


Figure 1: Structures of some of the isolated bioactive compounds from the genus *Grewia*.

2.3. *Grewia flava*

2.3.1. Botanical information

Grewia flava, commonly known as raisin tree, brandy bush in English. In Tswana it is referred to as *morethwa*, *moseme*, *ntewa* and is widely distributed in the drier bushland and deciduous woodland in the northern, central, and eastern parts of Botswana. It is also found in some parts of Southern Africa region, in South Africa, Zimbabwe, Namibia and Eswatini (Ulian *et al.*, 2019). *G. flava* is a compact shrub with greyish brown new twigs which becomes dark purple or black as they grow old, it often

reaches a height of 2-3 m tall. The plant has hairy leaves which appear greyish green on the upper side and light green underside. The plant has yellow star shaped flowers and bears edible globular fruits that becomes reddish brown when ripe (Bartsch, 2000; Lamola *et al.*, 2017). Figure 2 below shows the leaves, flower, fruit, twigs, and a whole plant.



Figure 2: *Grewia flava*; whole plant (A), flowered plant (B), leaves, twigs, and flower and fruit (C and E), and berries (D).

2.3.2. Traditional uses

The plant has different uses with socio-economic and medicinal benefits. The plant can be relied upon as a source of income as its berries are sold in informal markets, Figure 3. Plant stems can be used for building and to make tools like knobkerries, axe and hoe handles (Badimo *et al.*, 2015; Mainah, 2001). Dried plant berries can be consumed in their natural form or can be used to prepare porridge for the Bushmen by soaking them in water, they can also be fermented to make a local Setswana intoxicating

wine called *khadi* (Figure 3) which is believed to have antioxidant activities (Motlhanka & Ngwako, 2009; Motlhanka *et al.*, 2018, 2020)



Figure 3: *Khadi* (A) and berries being sold in informal markets (B) (Motlhanka *et al.*, 2018)

Moreover, the plant is employed in the folklore medicine for alleviation of various health problems, herbal tea of the twigs is consumed frequently for its health benefits on the kidneys, roots are used to treat sexually transmitted infections and a mixture of stem bark, roots and milk is believed to help with stomach problems resulting from bacterial infections (Gololo, 2015; Mongalo *et al.*, 2022; Ulian *et al.*, 2019). Note that the term herb is not limited to herbaceous plants only, it can also be used to refer to the roots, bark, twigs, leaves, flowers, fruits and seeds of the medicinal shrubs and trees (Speisky *et al.*, 2006).

2.3.3. Phytochemical composition of *Grewia flava*

Phytochemicals are secondary metabolites with extensive spectrum of biological activities, enabling them to aid protection against different diseases and stress conditions as they possess antimicrobial,

antioxidant, anticoagulant and lipid lowering capabilities amongst others (Murugan *et al.*, 2022; Teoh, 2016). Therefore, the type of biological properties that the medicinal plant exhibits is dependent on the phytochemicals that constitute the extract. There isn't much attention that has been given to the study of phytochemical composition of *Grewia flava* extracts. The phytochemical detection of aqueous methanol leaf extract of *Grewia flava* indicated the presence of tannins, reducing sugars, saponins, alkaloids, anthraquinones, and flavonoids (Gololo *et al.*, 2016). Only three bioactive compounds which were separated and identified from the leaf and root extracts of *Grewia flava*, are described in Table 2 and their chemical structures displayed in Figure 4 (Gololo, 2015; Lamola, 2015).

Table 2: Compounds isolated from different *Grewia flava* extracts and their reported activities.

Isolated compound	Plant part	Extract	Class of compound.	Biological activity	Reference
Lupeol (39)	Leaves	Acetone leaf extract	Triterpenoid	Antimicrobial activity (<i>E. faecalis</i> , <i>B. cereus</i> MIC; 1.5 and 2.5 mg/mL respectively), cytostatic.	(Lamola, 2015)
	Roots	Mixture of Carbon tetrachloride, chloroform, and aqueous methanol fractions	Triterpenoid	Anti-inflammatory activity (compared better than quercetin) and antimicrobial activity (<i>Cryptococcus neoformans</i> , <i>Escherichia coli</i> , <i>Moraxella catarrhalis</i> , <i>Mycobacterium smegmatis</i> , <i>Mycoplasma hominis</i> , <i>Klebsiella pneumoniae</i> , <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i> , <i>Candida albicans</i> , <i>Bacillus cereus</i> , MIC; 10-250 µg/mL).	(Mongalo <i>et al.</i> , 2022)
Kaempferol-3-O-	Leaves	Methanol leaf extract	Flavonol	Good Antioxidant (qualitative) and antimicrobial activity	(Gololo, 2015)

rutinoside- 7-O- β - glucoside (60)				(<i>Escherichia coli</i> , <i>Staphylococcus aureus</i> , <i>Enterococcus faecalis</i> , <i>Pseudomonas aeruginosa</i> , MIC; 0.75-1.5 mg/mL)	
Taraxerol (61)	roots	Mixture of Carbon tetrachloride, chloroform, and aqueous methanol fractions	Triterpenoid	Anti-inflammatory activity (compared better than quercetin) and antimicrobial activity (<i>Cryptococcus neoformans</i> , <i>Escherichia coli</i> , <i>Moraxella</i> <i>catarrhalis</i> , <i>Mycobacterium</i> <i>smegmatis</i> , <i>Mycoplasma</i> <i>hominis</i> , <i>Klebsiella</i> <i>pneumoniae</i> , <i>Staphylococcus</i> <i>aureus</i> , <i>Pseudomonas</i> <i>aeruginosa</i> , <i>Candida albicans</i> , <i>Bacillus cereus</i> , MIC; 30-250 μ g/mL).	(Mongalo <i>et al.</i> , 2022)

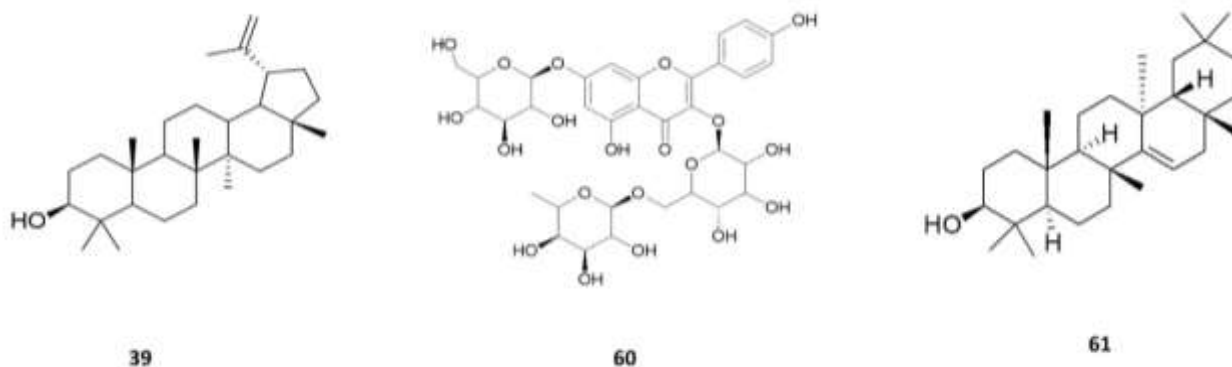


Figure 4: The structures of compounds (**39**, **60** and **61**) isolated from the leaf extracts of *Grewia flava*.

2.3.4. Antioxidant and antimicrobial activities

The continued use of synthetic antioxidants has raised safety concerns (e.g., immunosuppressant hypersensitivity, and allergic reactions) which has led to the need for the study and discovery of natural antioxidants which are believed to be much safer as they have minimum side effects. The biological effects of medicinal plants are linked to the ability of their bioactive phytoconstituents to adsorb, neutralize and inhibit disease causing free radicals and microbes (Karimi Alavijeh *et al.*, 2012; N. Stanković *et al.*, 2016). Polyphenols are the major compounds responsible for the antioxidant activities, thus the amount of the phenolic content in the extract can be directly correlated with the antioxidant/antimicrobial activity.

Research have been conducted on the antioxidant activities of *Grewia flava* leaf, root, fruit, and stem bark extracts. In all the reports 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was used. Antioxidant activities of the acetone, hexane, dichloromethane (DCM), and methanol leaf extracts were reported using the qualitative TLC-DPPH assay, which is based on the discolouration of the purple background of DPPH to yellow thus indicating the activity against the radical. All the extracts exhibited activity against the radical. The methanol and acetone extracts showed significant activity while the DCM and hexane extracts exhibited moderate activity. On the qualitative analysis of the antimicrobial

activity (using bioautographic method), acetone and hexane extracts showed good growth inhibition activity against all tested organisms, while activity exhibited by DCM extract was moderate (Gololo *et al.*, 2016).

The *Grewia flava* berries ethanol extract was reported to have exhibited a higher total phenolic content when compared to the water extract. This extract also indicated higher antioxidant ability against the DPPH radical. Ethanol extract showed a total phenolic content of 375.00 (μM of GAE/g), while the water extract activity was 110.00 (μM of GAE/g) (Masisi *et al.*, 2021). Table 3 and 4 shows the reported quantitative antioxidant and antimicrobial activities of *Grewia flava* extracts (leaf, root, bark, and berries), where DPPH radical scavenging assay and micro dilution technique were used to determine the IC_{50} and minimum inhibitory concentrations (MIC) values, respectively (Gololo *et al.*, 2017; Lamola *et al.*, 2017).

Table 3: IC_{50} values of *Grewia flava* extracts against DPPH radical.

Plant part	Solvent used	Antioxidant activity (IC_{50} mg/mL)
Leaves	Acetone	0.124 ^a
		0.227 ^b
Roots		0.070 ^a
Bark		0.251 ^a
Berries		0.222 ^a
Leaves	Methanol	0.105 ^b

Reference; (Lamola *et al.*, 2017)^a (Gololo *et al.*, 2017)^b

Table 4: Antimicrobial activities of *Grewia flava* extracts.

Extracting solvent	Plant part	Antimicrobial activity (MIC in mg/mL)					
		<i>B. Cereus</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. typhimurium</i>	<i>E. faecalis</i>	<i>P. aeruginosa</i>
Methanol ^a	Leaves	0.62	0.07	0.07	0.15	-	-
	Bark	0.15	0.31	0.31	1.25	-	-
	Root	0.31	0.07	0.07	0.15	-	-
Acetone ^a	Leaves	0.07	0.03	0.07	0.03	-	-
	Bark	0.31	0.07	0.15	0.62	-	-
	Root	0.07	0.03	0.07	0.62	-	-
	Berries	1.25	0.62	0.31	1.25	-	-
DCM ^b	Leaves	-	0.750	0.375	-	0.375	0.750
Acetone ^b	Leaves	-	0.750	3.00	-	0.375	0.750
n-Hexane ^b	Leaves	-	0.375	3.00	-	0.750	0.375

References; (Lamola *et al.*, 2017)^a (Gololo *et al.*, 2017)^b, -: not reported

2.4. Analysis of the Literature review on *Grewia flava*

The goal of the literature review was to draw attention to the work that has been done on the phytochemical composition, biological activities, isolation, and identification of bioactive compounds of *Grewia flava*. There is however scarcity of information regarding the biological properties and phytochemical composition and/or identification of compounds responsible for the properties which gives the plant twigs their medicinal value despite being used in traditional medicine. Hence this observation coupled with the non-sustainable usage of the roots in its infusions was used to rationalize the enactment of this study on *G. flava* twigs.

CHAPTER 3

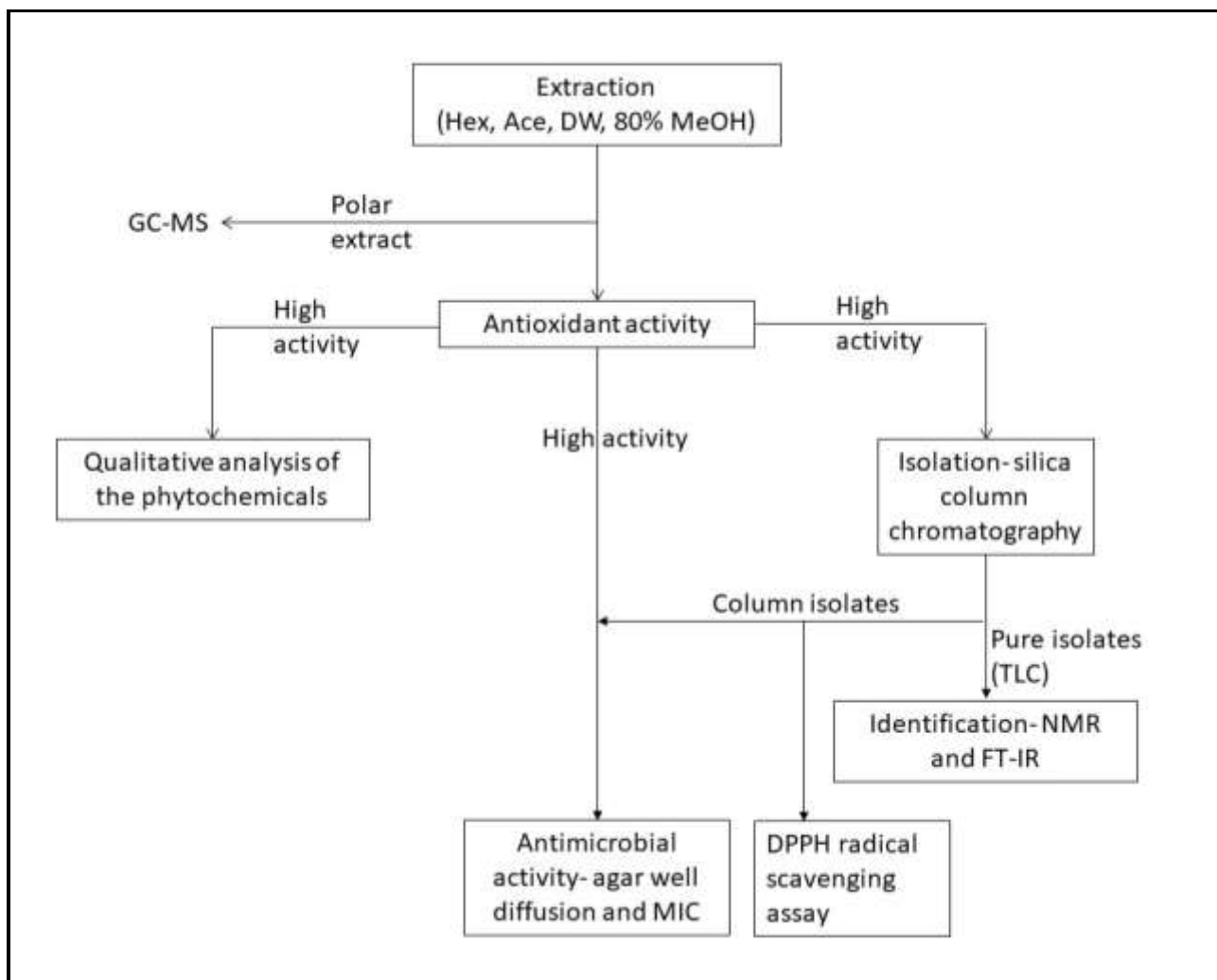
3. METHODS

3.1. Chemicals and general procedures

The following chemicals were used during this research; AR solvents (ethanol 99.9% Absolute fully denatured Batch no.20/322 Ethanol SA and methanol AR batch no.220917MT Rochelles Chemicals), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) 97% lot # S27679 ALDRICH, gallic acid 97.5-102.5% (titration) lot# SLBV4504, sodium carbonate anhydrous AR S2910, EDTA disodium salt AR E7350 MINEMA, ferric chloride AR batch no.FC155 Alpha chemicals, Folin-Ciocalteu reagent chemically pure Batch no. 270917FL Rochelle chemicals, HCl 35-36% batch no.HA829 Alpha chemicals, NaOH Chemalin, H₂SO₄ 98% Minera, Merck silica gel, 60-80 mesh, particle size (0.0400-0.0630 mm).

Thin-layer chromatography (TLC) on silica gel precoated aluminium foils (Sigma-Aldrich, Germany), and using *n*-hexane and ethyl acetate as solvent system and the spots were visualized in an iodine chamber or by exposure to UV lamp at λ_{254} nm. The IR spectra (ν cm⁻¹) (neat) were recorded in the spectral regions from 400 cm⁻¹ to 4000 cm⁻¹ on Thermo scientific Smart ITR Nicolet iS10. ¹H NMR spectra were run on a Bruker AC 400 MHz Spectrophotometer at the University of Botswana NMR center, while ¹³C NMR experiments were acquired at 100 MHz on the same spectrometer. CDCl₃, acetone-d₆ or DMSO-d₆ were used as solvents and chemical shifts (δ) were reported in parts per million (ppm) referenced to TMS as the internal standard. Coupling constants (*J*) were reported in Hz and referred to apparent peak multiplications.

3.2. Research design and summary



3.3. Sample collection and preparation

The twigs and roots of *Grewia flava* were sampled on 31-10-2020 from Mmashoro village in the Central district of Botswana. Coordinates: 21° 48' 15.9" S 26° 27' 22.4" E. The plant species was verified by Dr Andrew Muzila of the Herbarium, Department of Biological Sciences, University of Botswana, voucher number MZ002_2022. The medicinal plant was chosen based on its use and asserted effectiveness in folklore medicine. To remove any dirt residue on the samples, they were washed with water and left to dry in open air for two weeks. After drying the samples were ground into smaller pieces to increase surface area for efficient extraction.

3.4. Extraction

Maceration procedure was used to extract dried ground root and twig extracts of *Grewia flava* using *n*-hexane, acetone, distilled water (DW), and 80% methanol. To extract individual plant parts, 100 g of *G. flava* ground roots and twigs were extracted repeatedly with 500 mL of *n*-hexane (Hex), acetone (Ace), distilled water, and 80% methanol in water (80% MeOH). The extracts were filtered through Whatman No. 1 filter paper, concentrated with a rota-vapor (Heidolph, Germany), and dried under running fume hood. The extracts obtained were kept at 4°C until use.

3.5. Preliminary phytochemical screening

Chemical tests were performed to determine the occurrence of tannins, saponins, flavonoids, steroids, glycosides, anthraquinones and alkaloids in the 80% aqueous methanol root and twig extracts of *Grewia flava* using conventional techniques for the qualitative detection of these phytochemical groups (Maigoda *et al.*, 2022; Murugan *et al.*, 2022; Shaikh & Patil, 2020).

3.5.1. Test for Flavonoids (Shinoda Test)

Briefly about 5 mL of 10% ammonia solution was added to 2 mL of aqueous plant extract, followed by 1 mL of concentrated sulphuric acid. Orange-yellowish colouration indicated the occurrence of flavonoids (Murugan *et al.*, 2022).

3.5.2. Test for Steroids

To about 5 mL of the aqueous plant extract, 2 mL of both chloroform and sulphuric acid were added. The presence of steroids was noted due to the appearance of red colour in the lower chloroform layer. (Shaikh & Patil, 2020)

3.5.3. Test for glycosides

To about 1 mL of the aqueous plant extract, six drops of aqueous NaOH solution were added. The occurrence of glycosides in the extract was implied by the appearance of a yellow colour. (Shaikh & Patil, 2020).

3.5.4. Test for anthraquinones

A mixture of 4 mL of the plant extract solution and 10 drops of 10% ammonia solution was shaken vigorously, and the presence of anthraquinones was suggested by the emergence of the red colour (Shaikh & Patil, 2020).

3.5.5. Test for Alkaloids

Two milliliters of Wagner's reagent were added to one milliliter of the plant extract solution. The emergence of the reddish-brown color suggested the occurrence of alkaloids (Maigoda *et al.*, 2022).

3.5.6. Test for Tannins

About 3 drops of ferric chloride (10%) and 3 mL of distilled water were introduced in a test tube containing 1 mL of the plant extract solution. The formation of a green colour indicated the presence of tannins (Shaikh & Patil, 2020).

3.5.7. Test for saponins

Plant extract (1 g) was suspended in 10-20 mL of distilled water, thereafter, boiled for 2-3 mins until all the extract dissolved. The solution was left to cool to room temperature before shaking it for 5 mins, the existence of saponins was suggested by the appearance of foam lasting 10-15 minutes (Maigoda *et al.*, 2022).

3.6. Antioxidant activity assays

3.6.1. Total phenolic content

Folin-Ciocalteu assay was used to evaluate the total phenolic content of the crude extracts as described by (Cao *et al.*, 2020; Lfitat *et al.*, 2021) with some modifications. A calibration curve was created from the evaluations of varying concentrations of gallic acid (0.01- 0.05) mg/mL in 80% methanol. Briefly 1 mg/mL solution of the extracts was prepared in 80% methanol. In a 10 mL test-tube, 215 µL of the test sample was mixed with 2150 µL of 80% aqueous methanol followed by 215 µL of Folin-Ciocalteu reagent. After 3 minutes, 1 mL of 20% sodium carbonate solution was added thereafter shaking the mixture vigorously for 5 minutes and incubated for 2 hours before determining the absorbance of the supernatant solution at 725 nm using UV-Vis spectrophotometer. 80% aqueous methanol was used as the solvent blank. All the experiments were done in triplicates.

3.6.2. Total Flavonoid content

Total flavonoid content (TFC) of the crude extracts was examined using the aluminium chloride colorimetric method as described in Literature (Atere *et al.*, 2018; Hmamou *et al.*, 2022). Quercetin was employed as the standard. Exactly 1.0 mL of 1 mg/mL of the extract's solutions (prepared in 80% methanol) and 5% sodium nitrite solution were added in a 10 mL test-tube. The mixture was incubated for 5 minutes, thereafter 1.0 mL of 10% aluminium chloride and 2.0 mL of 0.1 M sodium hydroxide were added. The absorbance was determined at 510 nm after 30 mins of incubation at room temperature against the blank (80% methanol). All experiments were done in triplicates. Total flavonoid content of the extract was determined by formula shown below and represented as milligrams of quercetin equivalents per grams of the extract (mg QUE/ g of the extract).

$$X = q * \frac{V}{W}$$

X = Total flavonoid content in quercetin equivalents

Q = Quercetin concentration from the standard curve

V = Volume of the extract in millilitres

W = Mass of the crude extract

3.6.3. DPPH radical scavenging assay

The antioxidant activity was determined by the DPPH radical scavenging assay where the activity was compared to the activity of a known standard, ascorbic acid, following the literature protocol (Hmamou *et al.*, 2022) with modifications. Briefly, 75 μ L of the test sample (extracts and standard, dissolved in methanol at concentration range of (0.05 – 1 mg/mL) was mixed with 75 μ L 0.1 mM DPPH in methanol. The reaction mixture was incubated in the dark for 30 min at room temperature before measuring the absorbance at 550 nm using microplate reader. Methanol was used as a blank and each test was performed in triplicates. Percentage DPPH inhibition was determined using the following relationship:

$$\% \text{ DPPH Inhibition} = \left(\frac{A(\text{control}) - A(\text{sample})}{A(\text{control})} \right) * 100\%$$

Where A(control) is the absorbance of DPPH solution with no sample and A(sample) is the absorbance of DPPH with known concentration of the sample under study. The relationship between concentration and % inhibition, established using linear regression analysis was used to determine the IC₅₀ values.

3.6.4. Reducing power assay

Briefly about 1 mL of the test sample solution (extract/standard) was mixed with 0.5 mL of 96% ethanol, 5 mL H₂O, 1.5 mL of 1 M HCl, 1.5 mL of 1% ferricyanide solution, 0.5 mL of SDS (1%), and 0.5 mL of 0.2% ferric chloride hexahydrate. The reaction mixture was then incubated for 30 min in a water bath at 50°C. The mixture was cooled to room temperature before measuring the absorbance at 750 nm. Distilled water was used as the blank and ascorbic acid as the positive control and test were performed in triplicates. Reducing capacity was expressed as milligrams of ascorbic acid equivalents per grams of the extract dry matter (mg of AAE/g) (Berker *et al.*, 2010; Kanmaz *et al.*, 2020).

3.6.5. Metal chelation ability

The chelation ability of the extracts was estimated using the method by (Saliu & Olabiyi, 2017) with some modifications. Antioxidants from the plant extract compete with *O*-phenanthroline to form complexes with Fe²⁺ ions. *O*-phenanthroline forms red-coloured complexes with Fe²⁺ which can be quantified spectrophotometrically. Briefly 2 mL of the plant extracts and standard (0.1 – 1.0 mg/mL) were mixed with 1 mL of *O*-phenanthroline (0.05% in methanol) and 2 mL of FeSO₄ (1% in methanol). The mixture was thereafter incubated at room temperature for 40 mins before measuring absorbance at 510 nm against a control comprising of the same reaction mixture with the exception of the plant extracts. EDTA was used as a standard chelator and the experiment was done in triplicates. The percent chelation was calculated using the following equation.

$$\% \text{ Chelation} = \left(\frac{A(\text{control}) - A(\text{sample})}{A(\text{control})} \right) * 100\%$$

Where A(control) is the absorbance of the control solution and A(sample) is the absorbance of the solutions of samples under study. Thereafter the % chelation was plotted against concentration to determine the IC₅₀ values.

3.7. Antimicrobial activity

Two Gram-positive (*Bacillus subtilis*, and *Staphylococcus aureus*), two Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*), bacterial species and two fungal strains (*Aspergillus niger* and *Rhizopus oryzae*) were selected for antibacterial and antifungal assays respectively, the strains were obtained from the Microbiology Laboratory (Department of Biological Sciences and Biotechnology, BIUST). The bacterial and fungal strains were maintained on Muller Hinton agar plates and Potato Dextrose Agar at 4°C and sub-cultured on Muller Hinton broth and Potato Dextrose broth before use, respectively.

3.7.1. Agar well diffusion method

A preliminary evaluation of all the samples was performed using the modified agar well diffusion method by (Barberis *et al.*, 2020). Briefly 50 mL of Muller-Hinton agar was added into a petri dish and allowed to solidify. About 100 μ L of standardised inoculum (1.0×10^8 CFU/mL) of each strain was inoculated on the surface of the plate using sterilised glass cell spreader, thereafter, using 1000 μ L pipette tip, wells of 8 mm diameter were made in an agar plate. The test sample (100 μ L of 10 mg/L of the extract) was introduced into the well. The solution was allowed to diffuse before incubating the plates at 37°C and 30°C for 24 and 48 h, for bacteria and fungi, respectively. Ten percent DMSO was used as negative control and chloramphenicol was used as a positive control for the antibacterial test. After incubation, the microbial growth restriction was assessed by measuring the diameter of the zone of inhibition (in millimetres). The experiment was performed in triplicate and the results were presented as mean values $\pm$ SEM (Rakholiya & Chanda, 2014).

3.7.2. Determination of minimum inhibitory concentration (MIC)

The MIC was evaluated using the micro broth dilution method with two-fold serial dilution of test samples in liquid medium. The test was performed for extracts which showed activity against the test strains in the Kirby-Bauer diffusion assay. A twofold serial dilution of the test samples (2 mg/mL to 0.0313 mg/mL) were used for the assay. The colony suspension method was used to inoculate the microbial strains, resulting in a suspension with an optical density of about 0.4 at 600 nm (the log phase). The suspension was diluted to give a final concentration corresponding to 5×10^5 CFU/mL. (Wiegand *et al.*, 2008; Zamakshshari *et al.*, 2021). Briefly 50 μ L of Muller-Hinton broth, 25 μ L of the test samples (crude extracts and column fractions) and 25 μ L of the test organism suspension were added to each well to attain a total volume of 100 μ L in each well, 10% DMSO was used as a negative control and chloramphenicol was used as positive controls for antibacterial assay. The plates were incubated at 37°C and 30°C for 24 and 48 h, for bacteria and fungi respectively. Using a microplate reader, the optical density of the wells was established at 600 nm. The MIC was determined as the lowest concentration of the test sample that inhibits at least 80% of growth compared to the growth control (Alfarayeh *et al.*, 2022; Kowalska-krochmal & Dudek-wicher, 2021). The experiment was performed in triplicate and the results were presented as mean values $\pm$ SEM (standard error of the mean values).

3.8. Qualitative analysis using GC-MS

GC- MS analysis was performed using an HP-5 MS capillary column (Hewlett-Packard, CA, USA) (30m x 320 μ m x 0.25mm) coated with 5% phenylmethyl silicone, 95% dimethyl silicone in an Agilent 7890B GC system coupled to an Agilent 5977A mass spectrometer detector. The carrier gas was helium with a constant flow rate of 1 mL/min. The oven temperature was initially kept at 100 °C for 2 min then ramped at 10 °C/min to 30 °C then held isothermally for 30 min. Sample solutions of 0.3 mg/mL concentration in dichloromethane were injected manually at 250 °C, with an injection volume of 1.0 μ l in the splitless mode. The pressure and average velocity of the carrier gas were 3.1561 psi and 10.769 cm/s, respectively. Mass spectra were obtained by EI at electron energy of 70 eV (Ahuchaogu *et al.*, 2018; Phiri *et al.*, 2021).

Lastly the identification of the phytochemical constituents was based on the interpretation of the mass spectrum using the National Institute Standard and Technology (NIST 2012 version) database. The structure, name and molecular weight of the compounds were determined by comparing the spectrum of the unknown constituent of the test sample with the spectrum of the known phytochemical compound in the NIST database. The identification was carried out similarly to reported studies. The identification of constituents was considered for spectra above 95% match. The relative percentage composition of each compound was calculated based on peak areas.(Adams, 2017; N. A. Akwu *et al.*, 2019; Hamid *et al.*, 2016)

3.9. Extraction and isolation of compounds from *Grewia flava* twigs

The dried and ground *G. flava* twigs (1535.21 g) were macerated in 5 L of 80% aqueous methanol for 3 days with occasional shaking. The extract was filtered and concentrated using a rotary evaporator (Heidolph, Germany) at 45°C and left to dry under running fume hood. This was repeated three times to end up with 147.31 g of dark brown gummy crude extract.

Briefly about 120 g of the extract was adsorbed on silica gel powder (20.00 g) and loaded onto a column packed with Merck silica gel, 60-80 mesh prepared in *n*-hexane. The column was subjected to different

solvent systems of increasing polarity, starting with graded mixtures of *n*-hexane: ethyl acetate (4:0, 4:1, 2:1, 4:3, 1:1) followed by graded mixtures of EtOAc:MeOH. (4:0, 4:1, 2:1, 4:3, 1:1). The column eluates were monitored by TLC (Silica gel 60, UV₂₅₄). Each time elutes of different colours (otherwise 150 mL) were collected and identical fractions were combined, dried, and kept aside. Fractions of similar R_f on TLC plate were pooled together.

White crystals were obtained upon concentration of fraction 2 under fume hood to obtain compounds **36** and **39** (35.71 mg). Fraction 3 (54.18 mg) was eluted on silica gel 60-80 mesh column using EtOAc: MeOH (4:1) and 10 fractions (a to j) were collected in 15 ml portions. Fractions (c-e) with TLC similarities were pooled together to obtain compound **5** (17.93 mg). Fraction 10 was concentrated resulting in a brown gum which afforded compound **62** while compound **59** was obtained as a brown paste from fraction 16. An overview of the compounds isolation from 80% aqueous methanol extract of *Grewia flava* twigs is shown in Figure 5. Other fractions on TLC analysis showed many spots under UV lamp suggesting the fractions may have many compounds, so they were not explored further. Table 5 shows all the column fractions obtained.

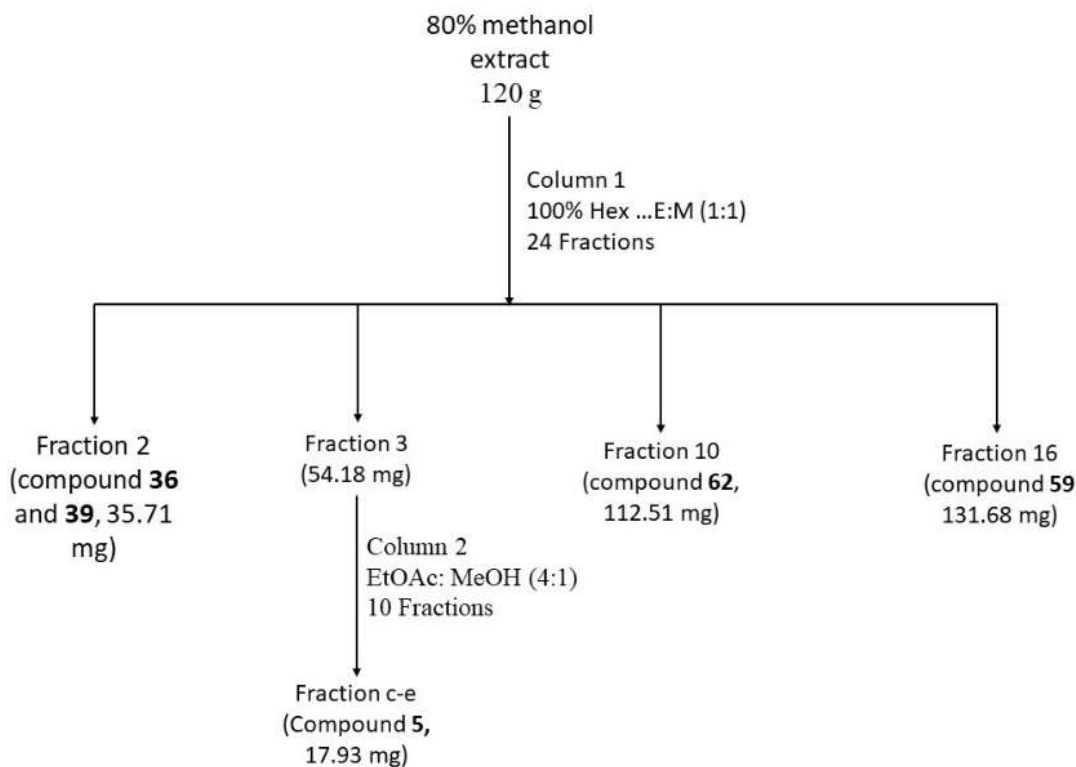


Figure 5: An overview of the compounds isolation from 80% methanol extract of *Grewia flava* twigs.

Table 5: Column fractions of the crude 80% methanol twig extract of *G. flava*.

Column fraction ID	Eluent composition
Fraction 1	Hex (100%)
Fraction 2	Hex: EtOAc (4:1, v/v)
Fraction 3	Hex: EtOAc (4:1, v/v)
Fraction 4	Hex: EtOAc (4:1, v/v)
Fraction 5	Hex: EtOAc (4:2, v/v)
Fraction 6	Hex: EtOAc (4:2, v/v)
Fraction 7	Hex: EtOAc (4:2, v/v)
Fraction 8	Hex: EtOAc (4:3, v/v)
Fraction 9	Hex: EtOAc (4:3, v/v)
Fraction 10	Hex: EtOAc (4:3, v/v)
Fraction 11	Hex: EtOAc (1:1, v/v)
Fraction 12	Hex: EtOAc (1:1, v/v)
Fraction 13	Hex: EtOAc (1:1, v/v)
Fraction 14	EtOAc (100%)
Fraction 15	EtOAc: MeOH (4:1, v/v)
Fraction 16	EtOAc: MeOH (4:1, v/v)
Fraction 17	EtOAc: MeOH (4:1, v/v)
Fraction 18	EtOAc: MeOH (4:2, v/v)
Fraction 19	EtOAc: MeOH (4:2, v/v)
Fraction 20	EtOAc: MeOH (4:2, v/v)
Fraction 21	EtOAc: MeOH (4:3, v/v)
Fraction 22	EtOAc: MeOH (4:3, v/v)
Fraction 23	EtOAc: MeOH (4:3, v/v)
Fraction 24	EtOAc: MeOH (1:1, v/v)

3.9.1. Physical and spectroscopic data for compounds

Mixture of lupeol **39** and β -sitosterol **36**

White crystals. Melting point 142-147 °C, IR $\nu_{\max}$ cm⁻¹: 3305.11, 2942.95, 2846.52, , 1452.19, 1035.62, 81. HRMS [M]⁺ *m/z* for **39**: 426.3852 calculated for C₃₀H₅₀O and **36**: 414.3862 calculated for C₂₉H₅₀O. ¹H and ¹³C NMR data (acetone-d₆) refer to Table 16 and 17, Appendix 1 and 2.

Stigmasterol **5**

White powder. Melting point 158-161 °C, IR $\nu_{\max}$ cm⁻¹: 3409.65, 2921.73, 2854.16, 1643.11, 1436.76, 1018.27, HRMS [M]⁺ *m/z* for **5**: 412.3725 calculated for C₂₉H₄₈O. ¹H and ¹³C NMR data (chloroform-d) refer to Table 18, Appendix 3 and 4.

Eicosyl protocatechuate **62**

Brown gum. IR $\nu_{\max}$ cm⁻¹: 3409.65, 2921.73, 2854.16, 1643.11, 1436.76, 1018.27, HRMS [M]⁺ *m/z* for **62**: 434.34 calculated for C₂₇H₄₆O₄; ¹H and ¹³C NMR data (Acetone-d₆) refer to Table 19, Appendix 5 and 6.

Kaempferol-3-O-rutinoside **59**

Brown paste. IR $\nu_{\max}$ cm⁻¹: 3292.01, 1637.33, 1604. 54, 1247.76, 1103.12, HRMS [M]⁺ *m/z* for **59**: 594.1585 calculated for C₂₇H₃₀O₁₅; ¹H and ¹³C NMR data (methanol-d₄) refer to Table 20, Appendix 7 and 8.

3.10. Statistical analysis

All experiments were performed in triplicates and the results were expressed as means $\pm$ SEM (n = 3). Statistical analysis was carried out using Microsoft Excel and Origin 9.0 professional software. Analysis for the difference was based on one way analysis of variance (ANOVA) followed by Tukey test. Values were regarded as statistically significant at P < 0.05. Pearson's correlation coefficients were determined to establish the relationship between antioxidants capacity and antioxidant activity.

CHAPTER 4

4. RESULTS AND DISCUSSIONS

The study investigated the phytochemical composition, antioxidant and antimicrobial activities of the twig and root extracts from *Grewia flava* medicinal plant.

4.1. Mass yield for different extracting solvents

One hundred grams of the ground roots and twigs of *G. flava* were extracted using acetone, *n*-hexane, distilled water and 80% methanol separately using maceration procedure. Distilled water extracted the highest mass of the extractible material from both the roots and twigs (29.88% and 15.72% respectively). The lowest mass of the extractible material was obtained with acetone in both scenarios, Table 6. When selecting extraction method, extraction yield should be considered since low yield is one of the limitations in natural products research, although high yield does not imply high concentrations of bioactive components, high extraction yield is required for an efficient process. In folklore tradition water is widely used as an extracting solvent although other organic solvents have shown better activity (Rakholiya & Chanda, 2014; Zamakshshari *et al.*, 2021). The difference in the extractive yields observed in the current study (Figure 6) can be explained by the extracting ability of each solvent, since they can extract distinct groups of phytochemicals found in the plant part under study. It can be noted that increase in the solvent polarity results in high extractive yield. To this end, conclusion can be drawn that both plant parts (twigs and roots) under study contain a considerable amount of polar compounds and the twigs also contain high content of non-polar compounds evidenced by high extractive yield for *n*-hexane (6.97%) extract.

The trend of the %yield for the extracting solvent was similar to values reported in literature (Gololo, 2015) where methanol was identified as the best extracting solvent (22.8%) followed by *n*-hexane (9.2%) and acetone (4.7%) for extracting the extractable mass from *Grewia flava* leaves. Furthermore, similar observation was also made (Lamola, 2015) where the extractive yield from the roots and bark were considerably more in methanol (8.7% and 5.4% respectively) than in acetone (1.4% and 1.3% respectively). The notable difference with the current study was that lower extractable yield from roots

was observed in distilled water (3.53%) compared to aqueous methanol (8.7%). It was noted that factors including the geographic location of the plant, the plant part under research, the season of sampling, and ultimately the extraction techniques employed can be used to explain variability in extract yields across different studies (Senhaji *et al.*, 2020).

Table 6: *Grewia flava* roots and twigs extracts yields.

Solvent	Mass extracted (g), (% yield)	
	Roots	Twigs
<i>n</i> -Hexane	0.383 (0.38%)	6.971 (6.97%)
Acetone	0.166 (0.17%)	0.534 (0.53%)
80% methanol	19.391 (19.39%)	10.319 (10.32%)
Distilled water	29.883 (29.88%)	15.718 (15.72%)

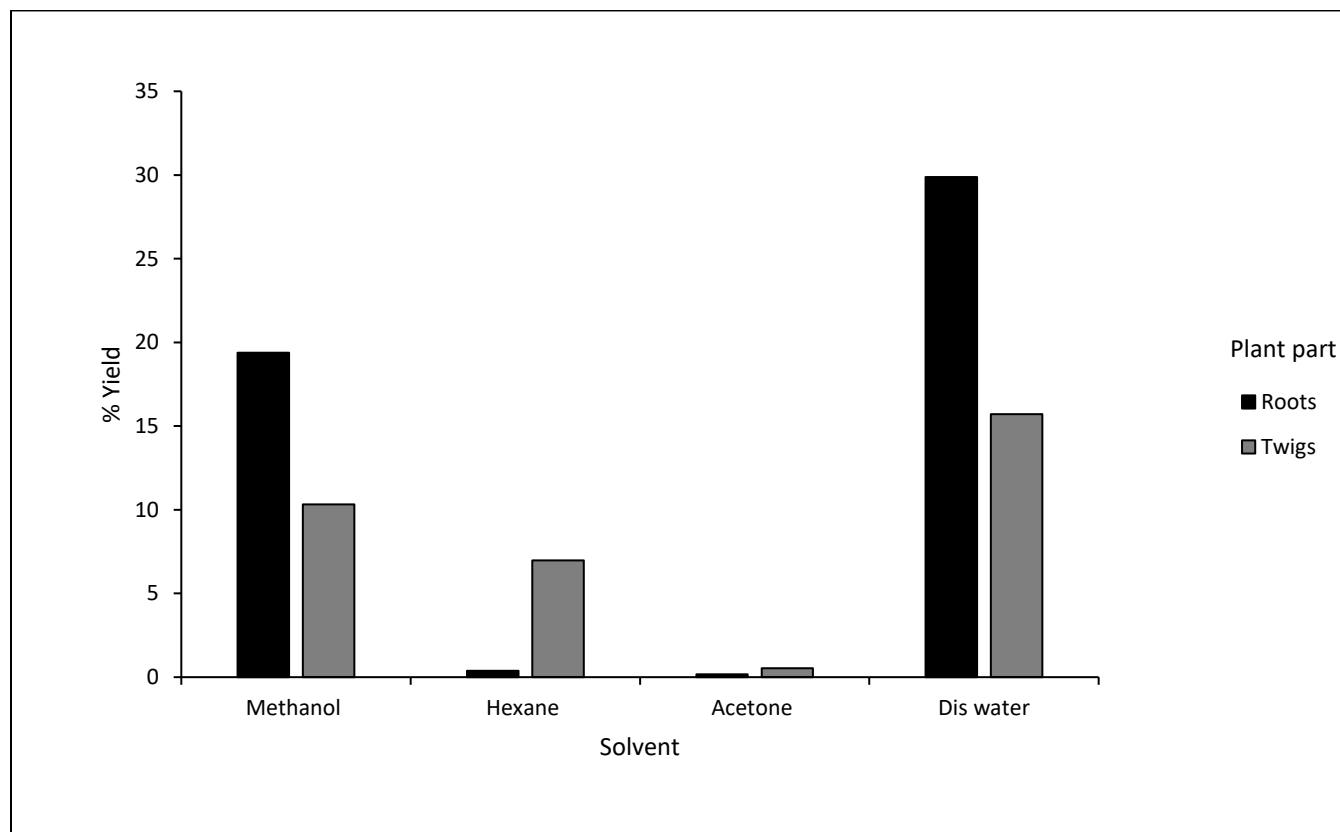


Figure 6: Extracts yield for different solvents

4.2. Antioxidant activities of the Crude extracts

The antioxidant abilities of *Grewia flava* *n*-hexane, distilled water, 80% methanol and acetone twig and root crude extracts were determined by estimating the total phenolic content (TPC) and total flavonoid content (TFC) thereafter determining the DPPH radical scavenging, metal chelation and reducing power abilities. All the experiments were done in triplicates. The extracts which exhibited high antioxidant activity was selected for isolation using column chromatography.

4.2.1. Total phenolic content

The biological activities of plant extracts have been linked to phenolic compounds that constitutes the extract, hence why it is imperative to estimate the total phenolic content (TPC). Gallic acid (GA) was used to create a standard curve that was based on the relationship between absorbance and concentration to determine the phenolic content of the *Grewia flava* extracts. From the analysis of the standard (GA), a linear calibration curve was generated, shown in Figure 7 with a linear equation, $y = 11.49x + 0.2422$, $R^2 = 0.9971$. The total phenolic content was expressed as milligrams of gallic acid equivalence per gram of the extract (mg GAE/g), and the results are displayed in Table 7. For the twig extracts, methanol extract (250 ± 2 mg GAE/g) exhibited high concentration of phenolics followed by distilled water extract (192 ± 2 mg GAE/g), with the *n*-hexane extract (43.1 ± 0.2 mg GAE/g) exhibiting the minimum TPC value, and for the root extracts (Table 7) similar trend was observed except for the aqueous extract showing the highest TPC compared to methanol extract.

Phenolic compounds have the potential to treat different ailments since they are believed to be potent antioxidant candidates, metal chelators, and free radical scavengers. These compounds range from simple phenolics, phenolic acids, hydroxycinnamic acid derivatives, anthocyanins, and flavonoids, all of which possess antioxidant, anti-inflammatory, antibacterial and anti-carcinogenic effects (Adebiyi *et al.*, 2017). High amounts of phenolics in all *Grewia flava* extracts indicates the potential of the plant to be used for a variety of medicinal purpose and explain its folklore use associated with phenolic compounds in literature (Bouarab-Chibane *et al.*, 2019; Nisar, 2021).

In contrast, ethanol fruit peel extracts have been reported to exhibit high TPC values than the water extract (Masisi *et al.*, 2021). High levels of phenolics were reported in polar solvents leaf extracts, methanol (4.800 ± 0.118) and acetone (3.200 ± 0.038) while the relatively non-polar extracts exhibited lower TPC value, hexane (0.130 ± 0.026) and dichloromethane (0.190 ± 0.103), expressed in percentage of tannic acid equivalence (TAE) per gram of the extract (Gololo *et al.*, 2017). Observations of the current study agree with the aforementioned literature data, where polar extracts exhibited high levels of phenolics, however significantly high values were reported in the current study which can be attributed to different factors like geographical location, season of harvest, genetic diversity and method of extraction used.

The same observations were also extended to different species in the genus, methanol extracts were reported to show high levels of TPC, 144.11 and 132.82 mg GAE/g from the leaf extracts of *G. asiatica* and *G. lasiocarpa* respectively (Akwu *et al.*, 2019; Sinha *et al.*, 2015). The ethanol stem and leaf extracts of *G. carpinifolia* exhibited relatively low amounts of TPC values, 14.85 and 19.08 mg GAE/g respectively (Adebiyi *et al.*, 2017). With its ability to extract high levels of lipophilic antioxidant compounds, methanol has the potential to better illustrate the antioxidant ability of the plant under study. The methanol extract of the commonly consumed green tea, rooibos has been reported to exhibit high TPC compared to water extract with values of 303 and 282 mg GAE/g respectively (Hussein *et al.*, 2022) which were slightly better than TPC values observed in the current report.

4.2.2. Total flavonoid content

Aluminium chloride colorimetric method was used to determine the total flavonoid content (TFC) of *Grewia flava* extracts. Linear calibration curve ($y = 18.983x + 0.0465$, $R^2 = 0.9963$, shown in Figure 8) was used to determine TFC values, which were represented as grams of quercetin equivalents per gram of extract (g QUE/g), the results are displayed in Table 7. For both the twig and root extracts, methanolic extract showed high levels of total flavonoid content (64.540 and 56.164 mg QUE/g respectively) while the lowest values of flavonoid content was found in the *n*-hexane extracts (9.106 and 5.639 mg QUE/g for the twig and root extract respectively). In contrast to the phenolic content lower values were recorded although following a similar trend in extraction capacity of the solvent, which was not far-fetched since flavonoids are a sub-class of phenolic compounds.

The potency of flavonoids as antioxidant, cardioprotective, immunoregulatory agents is dependent on the functional group arrangement about the flavonoid C₆-C₃-C₆ structure, mainly the position and number of highly reactive hydroxyl groups from ring B of the flavonoid aglycone structure (Aryal *et al.*, 2019; Kaur *et al.*, 2021; S. Kumar & Pandey, 2013). As far as the researcher is aware, there hasn't been any published research on the flavonoid content of *Grewia flava*. However, there are a few reports on the genus, reporting the determined TFC of 4.608 mg QUE/g from the methanol fruit extract of *G. asiatica* (Sinha *et al.*, 2015), methanol root extract of *G. optiva* were reported to have flavonoid content of 82.19 mg QUE/g (Zahoor *et al.*, 2020). Flavonoid content in the leaf and stem ethanol extracts of *G. carpinifolia* was estimated to be 9.00 and 13.22 mg QUE/g respectively. The significant difference can be attributed to genetic diversity between species. The flavonoid content in different rooibos water extracts to exhibit flavonoid concentration ranging from 36.22 to 65.25 mg QUE/g (Neamtiu & Oancea, 2018), in comparison to *Grewia flava* extracts of the current study exhibit similar flavonoid content values which indicates that the extract has a potential to be biological active.

Table 7: Total phenolic content and flavonoid content of *Grewia flava* twig and root extracts.

Solvent	Total phenolic Content (mg GAE/ g)		Total Flavonoid Content (mg QUE/ g)	
	Twig	Root	Twig	Root
80% Methanol	250 ± 2 ^a	217 ± 2 ^c	64.54 ± 0.04 ^a	56.16 ± 0.02 ^c
<i>n</i> -Hexane	43.1 ± 0.2 ^b	28 ± 1 ^f	9.106 ± 0.005 ^b	5.64 ± 0.01 ^f
Acetone	86.9 ± 0.8 ^c	80 ± 0.8 ^c	20.01 ± 0.05 ^c	16.22 ± 0.09 ^g
Distilled water	192 ± 2 ^d	230 ± 3 ^g	48. 86 ± 0.09 ^d	51.15± 0.03 ^h

All the results are represented as means of three individual experiments ± SEM (n=3). TPC was represented in milligrams of Gallic acid equivalence per gram of the dry matter (mg GAE/g) and TFC was represented in milligrams of quercetin equivalents per gram of dry matter (mg QUE/g). Results under each test that have a distinct superscript letter are statistically different ($p \leq 0.05$).

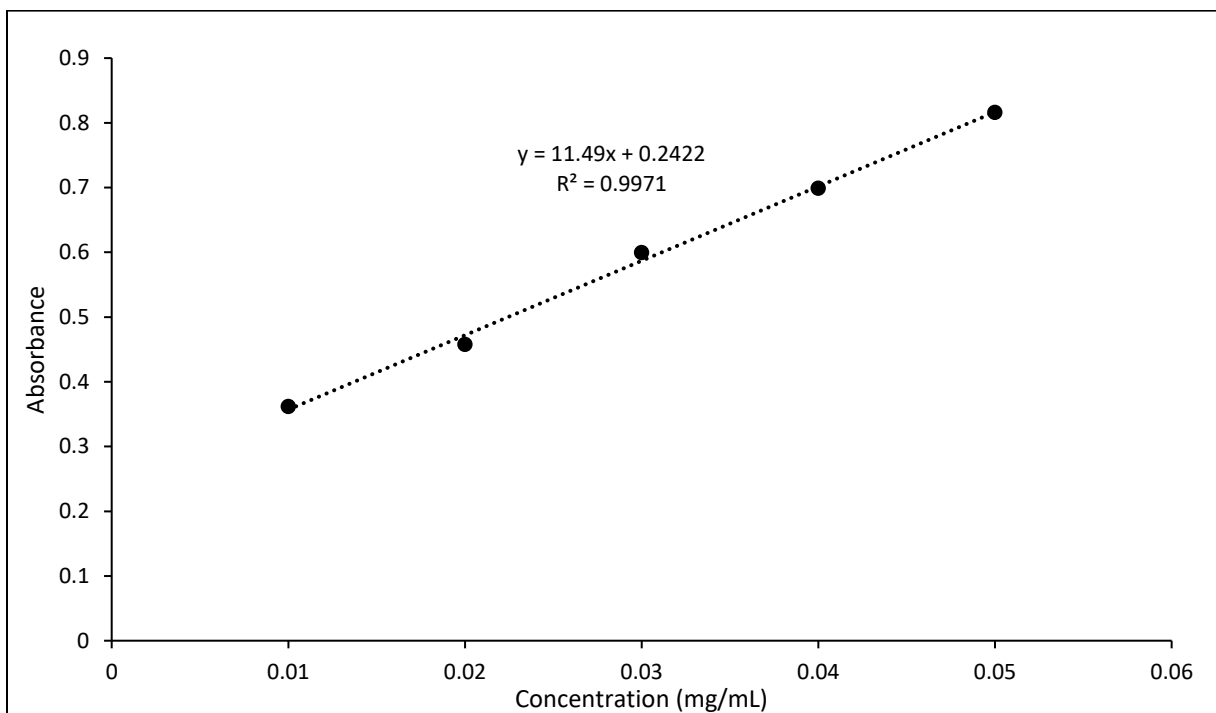


Figure 7: Total phenolic content regression curve for gallic acid

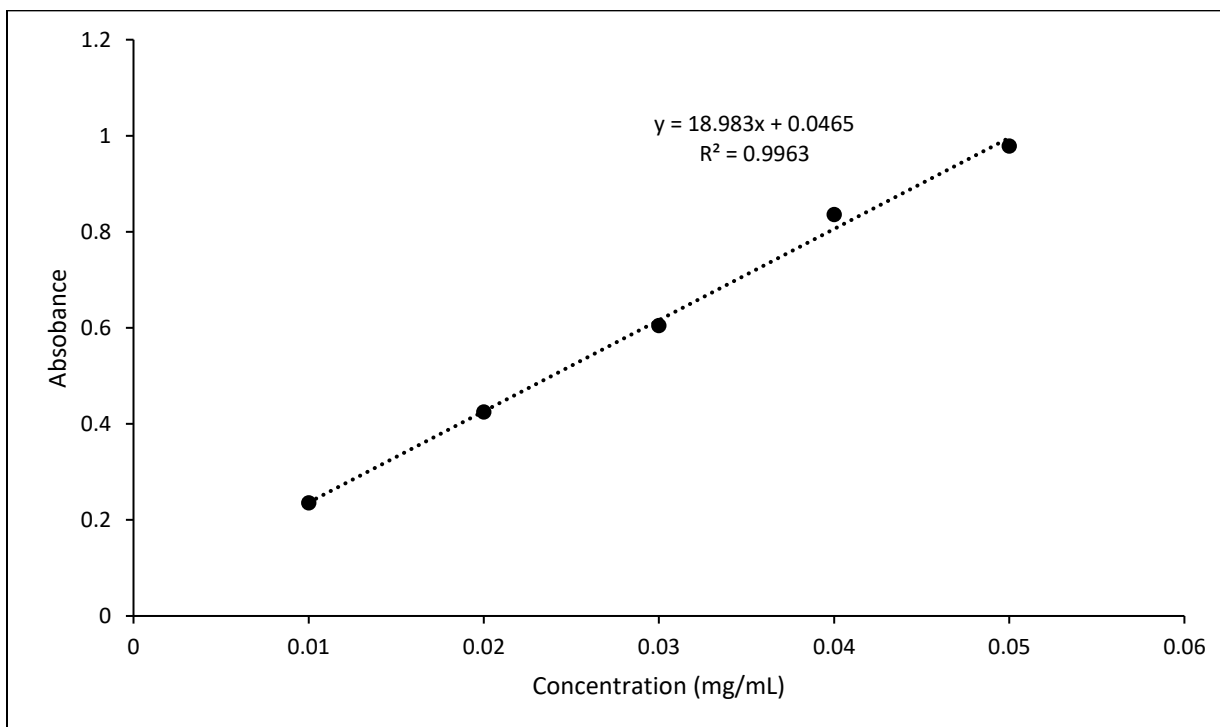


Figure 8: Total flavonoid content regression curve for quercetin

4.2.3. DPPH radical scavenging assay

The capacity of the extracts to scavenge free radicals was investigated using a widely adopted stable DPPH radical. The assay is dependent on the DPPH radical being reduced in the availability of antioxidants with hydrogen or electron donating potential, producing a non-radical product. Figure 9 presents the DPPH radical scavenging ability for both the crude extracts and the reference compound, the corresponding IC₅₀ values are tabulated in Table 8. All the extracts under study exhibited a dose dependant radical scavenging ability. Aqueous methanol extracts showed stronger DPPH scavenging ability with IC₅₀ value of 14.5 ± 0.7 µg/mL and 179 ± 2 µg/mL for the twig and root extracts respectively. It has been determined that there is a linear relationship between phenolic content and antioxidant activity, indicating that phenolics contribute immensely to the medicinal plant's antioxidant activity (Stanković, 2011). However, a peculiar observation was made, aqueous extracts (both root and twig) revealed low radical scavenging abilities, IC₅₀ values recorded were 265 ± 3 µg/mL and 495.0 ± 0.7 µg/mL respectively although high phenolic concentrations (Table 7) have been recorded for the extracts. On the other hand, *n*-hexane and acetone twig extracts also exhibited high activity with IC₅₀ values of 70 ± 1 µg/mL and 88.4 ± 0.9 µg/mL even though, relatively low concentrations of phenolics were recorded. TPC assay is susceptible to interferences like side reactions and the constituents in the matrix which could explain why no correlation between the two was observed (Rabeta & An Nabil, 2013). A known standard, Ascorbic acid showed activity with an IC₅₀ of 23 ± 1 µg/mL, however, 80% methanol twig extract presented stronger activity which shows the potential of *G. flava* twigs to act as antioxidant agent.

The results obtained conforms to literature observations (Masisi *et al.*, 2021) that indicated that *Grewia flava* has scavenging ability against DPPH radical. The scavenging ability of plant extract revealed with IC₅₀ values for the acetone root, bark, leaf, and fruit extracts that ranged from 0.070 to 0.251 mg/mL (Lamola *et al.*, 2017). In comparison 80% methanol extract exhibited better activity against DPPH radical compared to acetone extracts. Methanol leaf extract was previously reported to show scavenging ability with IC₅₀ value of 0.105 mg/mL compared to 0.227 mg/mL for the acetone leaf extracts (Gololo *et al.*, 2017). The effectiveness of the all the crude extracts to scavenge the DPPH radical suggested that all extracts contain compounds with the ability to donate protons to free radicals. Also, it substantiates the extracts' effectiveness in a dose-response manner.

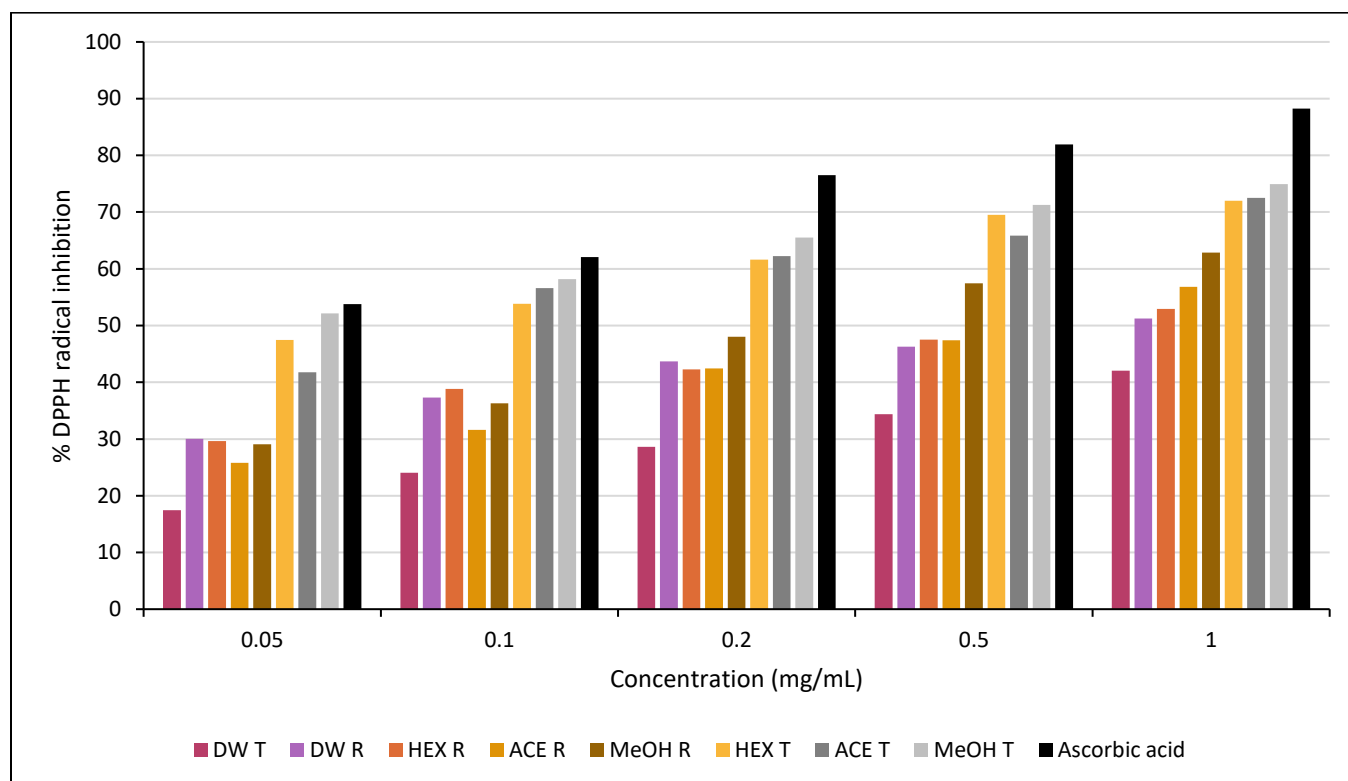


Figure 9: Percentage DPPH radical scavenging ability of *G. flava* crude extracts. Ascorbic acid was employed as a reference standard. DW= distilled water, T= twigs, R= roots.

Table 8: Inhibitory concentrations of the crude extracts against DPPH radical

Sample	IC ₅₀ (µg/mL)	
	Root	Twig
<i>n</i> -Hexane	286 ± 1 ^a	70 ± 1 ^e
Acetone	268 ± 2 ^b	88.4 ± 0.9 ^f
80% methanol	179 ± 2 ^c	14.5 ± 0.7^g
Distilled water	265 ± 3 ^b	495.0 ± 0.7 ^h
Ascorbic acid	23 ± 1 ^d	

All the results are represented as means of three individual experiments ± SEM (n=3). Highlighted in bold compare better to reference standard. Significant differences exist between values with distinct superscript letters ($p \leq 0.05$).

4.2.4. Metal chelation ability

Oxidative stress may be prevented by chelation of transition metal ions since excessive amounts of these ions in the body results in the formation of radicals, supported by the ability of metal ion Fe^{2+} to gain or lose electrons (Gulcin & Alwasel, 2022; Sudan *et al.*, 2014). Iron (II) chelation assay was used to establish the chelation ability of the crude extracts. *O*-phenanthroline has the ability to quantitatively form complexes with Fe^{2+} giving a red-coloured solution, which will be restricted by the availability of other chelating agents causing the red colour to fade. Therefore, measurement of the red colour difference allows for the estimation of chelation ability (Saliu & Olabiyi, 2017).

The dose-response graphs for the chelating ability of the extracts and standard are presented in Figure 10. Results show that all plant extracts and reference standard (EDTA) have a dose dependent response since increase in concentration resulted in increased chelation ability. The assay illustrated that aqueous methanol extracts possess strong chelation ability with IC_{50} values of $98 \pm 7 \mu\text{g/mL}$ and $110 \pm 24 \mu\text{g/mL}$ for the twig and root extracts respectively although the chelation ability was significantly lower than that of EDTA ($70 \pm 4 \mu\text{g/mL}$) as positive standard (Table 9). Nonetheless this high activity can be linked to the extract's higher than average concentration of phenolics and flavonoids compared to other extracts. Phenolic compounds have been reported to form complexes with Fe^{2+} consequently halting the radical generation that leads to the maturation of various neurological disorders, including Alzheimer's and Parkinson's disease. (Obboh, 2009; Sudan *et al.*, 2014). In addition, it was also observed that in all scenarios twig extracts exhibited high chelation ability when compared to root extracts except for the aqueous extract where root extract ($141 \pm 11 \mu\text{g/mL}$) showed significantly high chelation ability ($P < 0.05$) compared to twig extract ($196 \pm 16 \mu\text{g/mL}$). In this regard it can be concluded that organic solvents have better extracting power from the twigs compared to the roots and vice versa for the aqueous solvent.

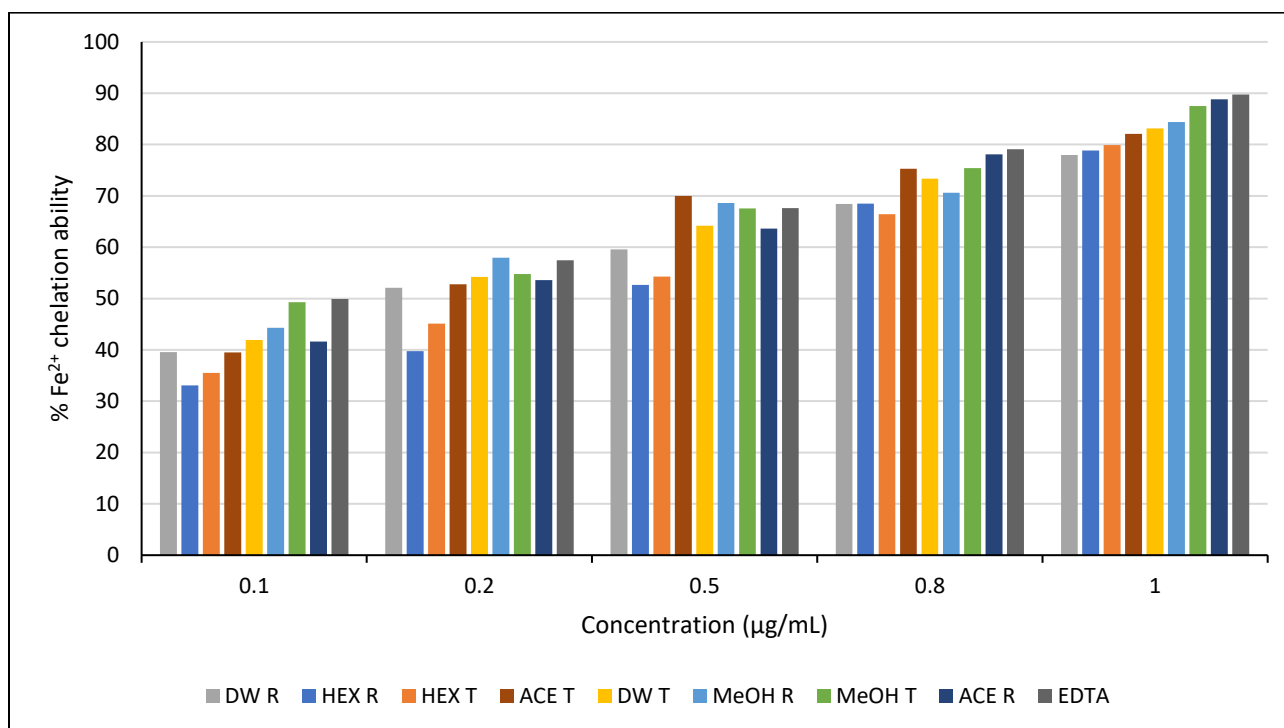


Figure 10: Percentage metal chelation ability of *G. flava* crude extracts (T = twig, R= root). EDTA was used as a standard chelator. DW= distilled water.

Table 9: Metal chelation ability of *Grewia flava* crude extracts.

Sample	IC ₅₀ (µg/mL)	
	Root	Twig
<i>n</i> -Hexane	428 ± 15 ^a	382 ± 42 ^f
Acetone	208 ± 21 ^b	199 ± 42 ^b
80% methanol	110 ± 24 ^c	98 ± 7 ^c
Distilled water	141 ± 11 ^d	196 ± 16 ^b
EDTA	70 ± 4 ^e	

All the results are represented as means of three individual experiments ± SEM (n=3). Significant differences exist between values with distinct superscript letters

Hexane extracts exhibited weak chelation abilities in both scenarios with IC₅₀ values of 382 ± 43 µg/mL and 428 ± 15 µg/mL for the twig and root extract respectively. From these observations it can be

concluded that the plant extracts contain rather less non-polar bioactive phytochemical compounds. Although all the extracts exhibited lower activity compared to the standard chelator, they demonstrated moderate chelation abilities therefore the use of phytochemicals from *G. flava* extracts to chelate metal ions can be of therapeutic significance.

4.2.5. Reducing Power Capacity.

The ability of the crude extracts to reduce Fe^{3+} to Fe^{2+} ions was assessed using the reducing power assay this indicates the ability of the phytochemicals to donate electrons, an approach used to estimate radical scavenging activity (Vasyliov *et al.*, 2020). The intensity of the Prussian blue colour of the solution formed (measured at 750 nm) can be used to monitor the amount of phytochemical- Fe^{2+} complexes that have formed. Reducing power capacity values given in Table 10 as grams of ascorbic acid equivalents per gram of extract (g AAE/g) were estimated based on the linear calibration curve ($y = 1.466x + 0.019$, $R^2 = 0.993$) shown in Figure 11. Results shows that aqueous methanol extracts have a stronger electron donating ability, with twig extract presenting reducing power of 745 ± 1 mg AAE/g while root extract exhibited reducing ability of 637 ± 3 mg AAE/g. This trend was expected since the extracts ability to reduce ions depends on the availability of the phytochemicals which exert their antioxidant activity by donating electrons thereby quenching free radicals. The reducing capacity of the plant extract may be ascribed to the existence of polyphenolic compounds, flavonoids and tannins (Atere *et al.*, 2018). From prior observations (in Section 4.2.1 and 4.2.2) it was noted 80% methanol extracts have high levels of flavonoids and phenolic compounds compared to other extracts. These results corroborate previous findings that indeed the reducing capacity of the plant extracts is correlated with phenolic content (Sagbo, 2015).

The result of this study reveals that twig extracts exhibited high reducing power compared to root extracts in all scenarios except for the distilled water extracts, where twig extract (183 ± 2 mg AAE/g) had lower reduced capacity compared to root extract (247 ± 3 mg AAE/g). In this study it was also observed that twig extracts exhibited high reducing power compared to root extracts in all scenarios except for the aqueous extracts, The reducing power of the reference standard was revealed to be dependent on the dose as shown in Figure 1. All of the twig extracts except for the aqueous extract exhibited significantly high reducing capacity with IC_{50} values of 745 ± 1 , 433 ± 3 and 450 ± 6 mg

AAE/g for acetone, *n*-hexane and 80% methanol, respectively which implies good reducing potential. Therefore, from this observation it can be deduced that *Grewia flava* twigs have the potential to produce antioxidants that can prevent pathogenesis of degenerative diseases from free radicals and also partially validate its use in folklore traditions.

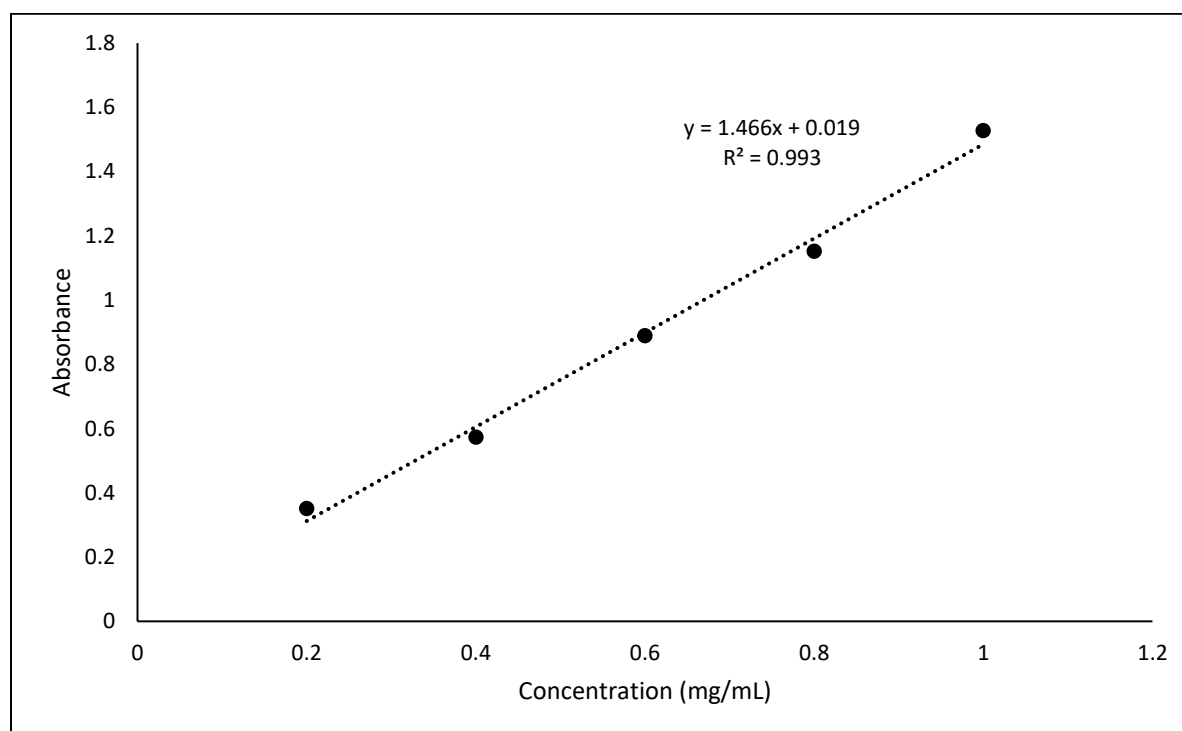


Figure 11: Reducing power regression curve for Ascorbic acid.

Table 10: Reducing power ability for *Grewia flava* twig and root extracts.

Extract	Reducing power capacity (mg AAE/g)	
	Root	Twig
Distilled water	247 ± 3 ^a	183 ± 2 ^d
80% methanol	637 ± 3 ^b	745 ± 1 ^e
Acetone	281 ± 1 ^c	433 ± 3 ^f
<i>n</i> -Hexane	252 ± 1 ^a	450 ± 6 ^g

All the results are represented as means of three individual experiments ± SEM (n=3). Significant differences exist between values with distinct superscript letters

4.2.6. Correlation between antioxidant capacity and antioxidant activities

Pearson's correlation coefficient analysis was performed (Table 11 and Figure 12 to 15) to establish the link between TPC and TFC with antioxidant activities. Literature data shows that phenolic and flavonoid compounds exhibit important biological properties like anti-inflammatory, antioxidant, and antibacterial activities (Nisar, 2021). Due to the ideal structure of these compounds, they are able to donate electrons or hydrogen atoms to free radicals, thereby stabilizing them (Aryal *et al.*, 2019). A linear strong positive relationship between TPC and metal chelation was established with correlation coefficients in the range of 0.951 to 0.994, and for TPC with DPPH radical scavenging activity ($r = 0.973$ to 0.996), all determined at 95% confidence level. These observations concur with previous reports highlighting phenolic compounds as the primary components involved in the antioxidant activities of medicinal plants due to significant correlation between the two (Rabeta & An Nabil, 2013). Similarly, a strong positive correlation was also observed between TFC and metal chelation, DPPH radical scavenging ability with correlation's coefficients in the range of 0.938 to 0.998. Comparing the coefficients (r values) it can be deduced that both the phenolic and flavonoid compounds foster the antioxidant activities of the extracts equally.

Table 11: Pearson's correlation coefficients between total phenolic content, total flavonoid content and antiradical and chelation ability.

Sample	Metal chelation ability		DPPH radical scavenging ability	
	TPC	TFC	TPC	TFC
80% methanol root	0.959	0.938	0.987	0.987
80% methanol twig	0.994	0.977	0.992	0.977
Distilled water root	0.967	0.959	0.996	0.988
Distilled water twig	0.993	0.993	0.992	0.986
Acetone root	0.991	0.998	0.993	0.982
Acetone twig	0.951	0.951	0.973	0.970
<i>n</i> -Hexane root	0.986	0.988	0.994	0.988
<i>n</i> -Hexane twig	0.987	0.986	0.984	0.992

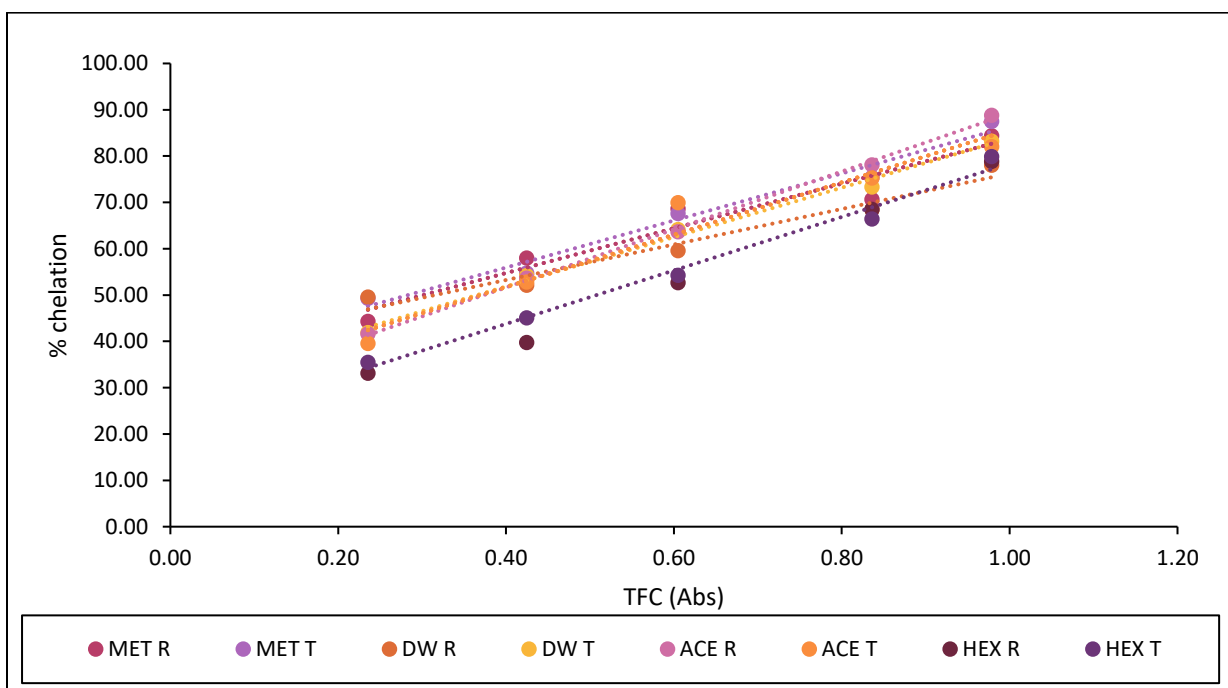


Figure 12: Correlation between metal chelation ability and flavonoid content. DW= distilled water, T= twigs, R= roots, MET= 80% methanol.

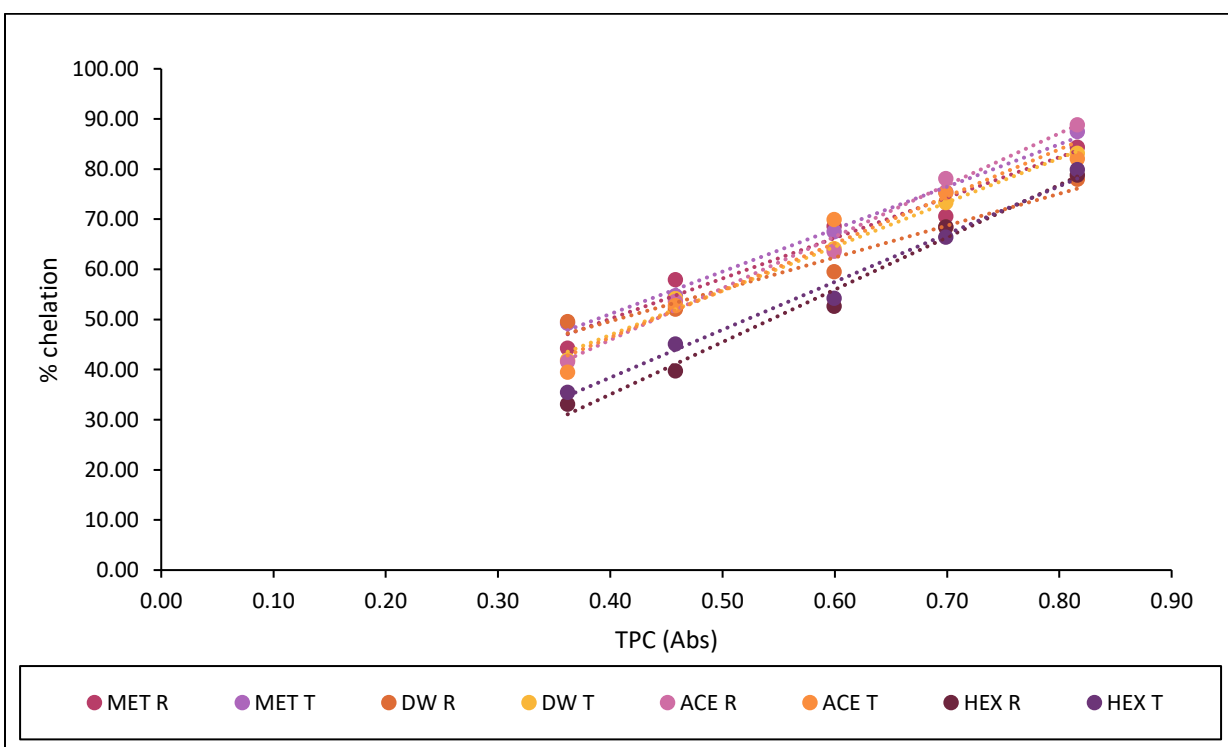


Figure 13: Correlation between metal chelation ability and phenolic content. DW= distilled water, T= twigs, R= roots, MET= 80% methanol.

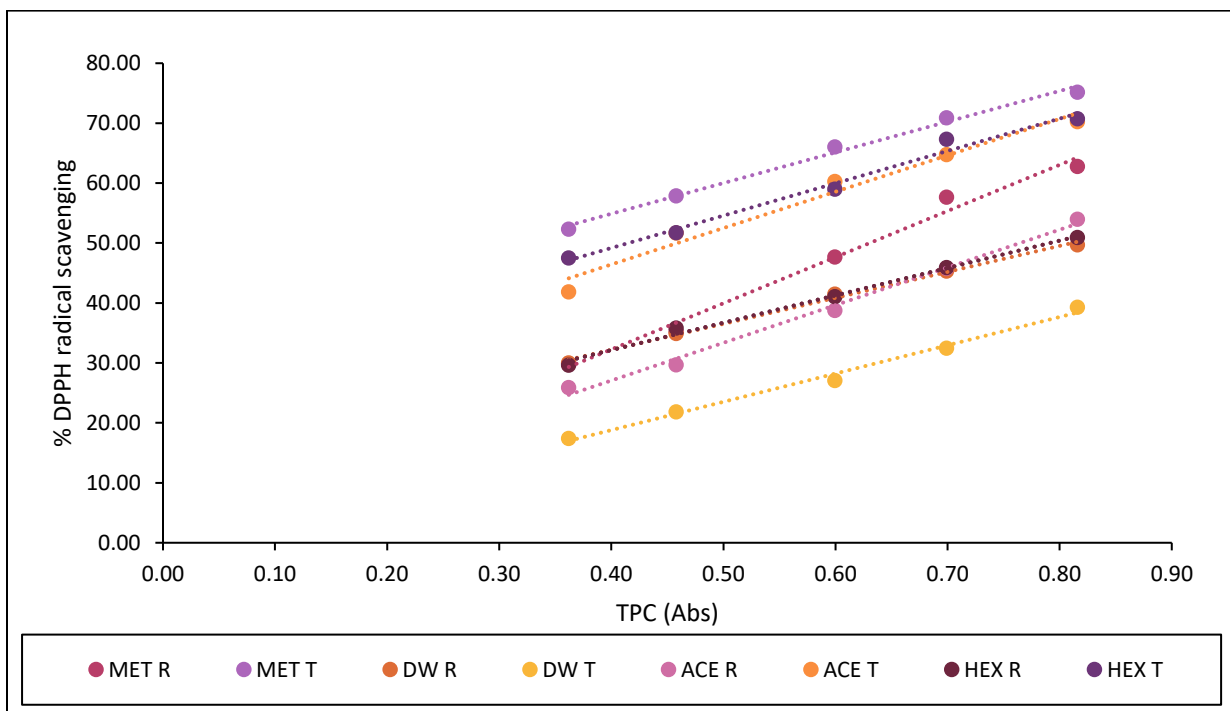


Figure 14: Correlation between DPPH radical scavenging ability and phenolic content. DW= distilled water, T= twigs, R= roots, MET= 80% methanol.

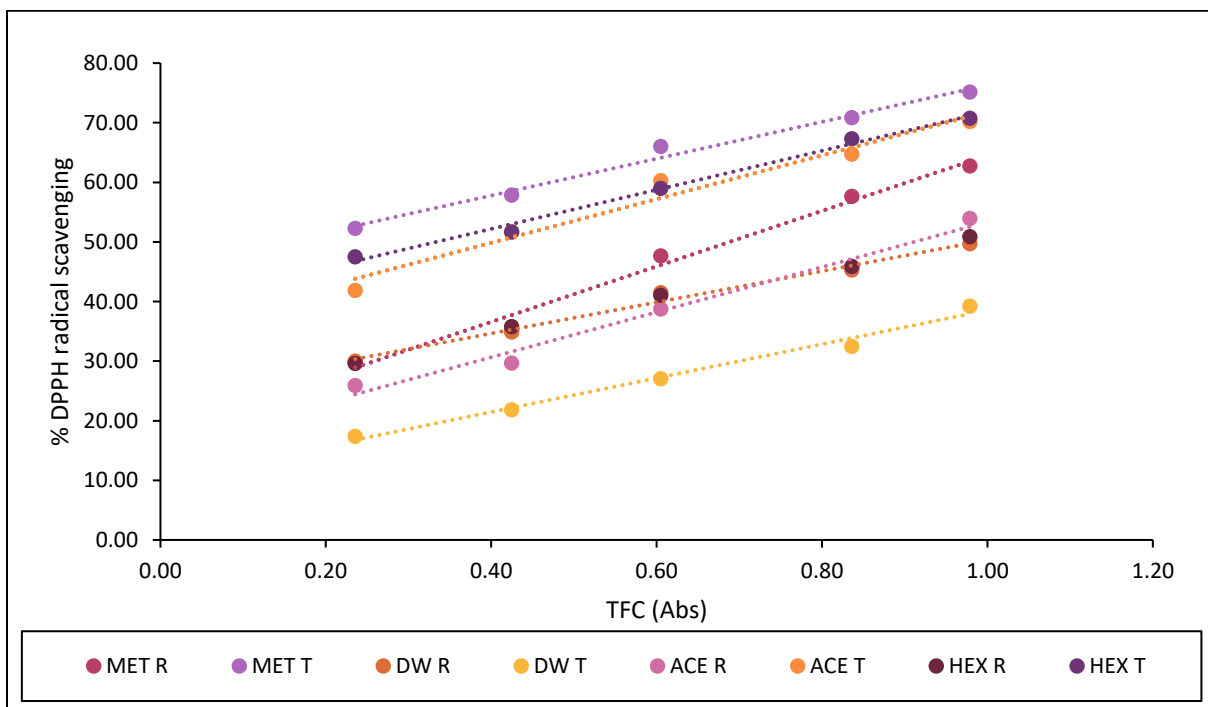


Figure 15: Correlation between DPPH radical scavenging ability and flavonoid content. DW= distilled water, T= twigs, R= roots, MET= 80% methanol.

4.2.7. Analysis of antioxidant activity

This section sought to evaluate the antioxidant properties of crude extracts of different solvents. The crude extracts exhibited a broad spectrum of antioxidant activities with 80% methanol extracts exhibiting high antioxidant activity compared to other extracts in most antioxidant assays performed therefore it was selected for further analysis.

4.3. Preliminary phytochemical composition

Grewia flava 80% methanol twig and root extracts were subjected to qualitative phytochemical analysis to detect the presence of tannins, flavonoids, glycosides, steroids, anthraquinones, and saponins. The preliminary screening was done by carrying out a set of qualitative characterization reactions which mainly depend on precipitation and coloration phenomena. The results presented in Table 12 shows the presence of alkaloids, flavonoids, saponins, steroids, glycosides, anthraquinones and tannins in both the twig and root extracts. However, the extent of the observations noted varied between the extracts which implies the difference in phytochemical concentration between the two samples. The varying phytochemical makeup of the plants parts under investigation could suggest difference in response to phytochemical biosynthetic triggers and/or pathogenic susceptibility (H. Li *et al.*, 2012). This could also explain why different plants parts of the same plant can be used for assisting with different ailments in folklore medicine, as the biological activities of medicinal plants have been linked to their phytochemical composition.

Table 12: Qualitative phytochemical composition of the root and twig extracts of *Grewia flava*

Phytochemical group	Root extract	Twig extract
Steroids	+	+
Tannins	+	+
Flavonoids	+	+
Glycosides	+	+
Alkaloids	+	+
Saponins	+	+
Anthraquinones	+	+

+: indicates presence, -: indicates absence.

Plants with diverse classes of phytochemicals are generally found to have superior biological activities. Thus, the strength and range of the biological properties that a plant may display is mainly influenced by the quantity and diversity of phytochemicals a plant possess (Akinseye, 2017). Phytochemical analysis of the root and twig extracts established the occurrence of important phytochemical groups with biological properties of medicinal value (Table 12). The presence of detected phytochemicals explains the use of the plant as a remedy for different ailments. Most phytochemicals assist the body in combating illnesses and microbial invasion through their antioxidant abilities. For instance, alkaloids are chemical substances with a range of pharmacological properties, like antimalarial, anticancer, psychotropic, antibacterial, antihyperglycemic, anaesthetics and anti-inflammatory activities, the well-known used drugs include morphine, strychnine, quinine, ephedrine, and nicotine. (Ezeonu & Ejikeme, 2016; Heinrich *et al.*, 2021; Yeung *et al.*, 2018). Flavonoids play a crucial role in the prevention of the development of diseases like atherosclerosis, carcinogenesis, unstable angina, obesity, thrombosis, and diabetes, activity enabled by the phenolic hydroxyl group in their structure (Ballard & Maróstica, 2018; Choudhary *et al.*, 2021; Chowdhury *et al.*, 2017; Rehman *et al.*, 2013; Saliu & Olabiyi, 2017; Sharma *et al.*, 2020). Terpenoids have shown significant bioactive properties such as antiviral, analgesic, antibacterial, antimalarial, anti-cancer, anti-helminthic, anti-inflammatory and cholesterol synthesis inhibition properties (Agidew, 2022; Nath & Yadav, 2016). Tannins are known to show activities which include; antimicrobial, antitumor, antiviral and has shown to selectively suppress HIV replication (Akinseye, 2017; Ezeonu & Ejikeme, 2016). Literature shows that, hypocholesterolemic, anti-diabetic, cholesterol-lowering, cytotoxic, anti-bacterial, and anti-viral properties in plants extracts has been attributed to the presence of saponins (Akhtar *et al.*, 2018; Bailly & Vergoten, 2020; Biswas *et al.*, 2021).

Plant species of the genus *Grewia* have exhibited appreciable amounts of alkaloids, anthocyanins, quinones, lignans, flavonoids, saponins and tannins, phenolic acids, terpenoids, and isoflavones, with flavonoids being the major group in the genus. (N. A. Akwu *et al.*, 2019; Goyal, 2012; S. Kumar *et al.*, 2022). For example, phytochemical studies of the leaf extract of *G. tenax* revealed that it includes lipids, alkaloids, triterpenoids, sterols, flavonoids, saponins, and tannins in addition to diterpenes and glycosides. (Adam, 2019). Furthermore the main phytochemicals reported in the *G. tiliifolia* include proteins, saponins, lipids, steroids, phenolic compounds, flavonoids, alkaloids, triterpenoids, and tannins (Malhotra *et al.*, 2020). Similar phytochemicals including anthocyanins have been reported in *G. asiatica* (Akram *et al.*, 2019; Parul *et al.*, 2013; Sinha *et al.*, 2015). The identified phytochemicals

in the current study (Table 12) suggest similar phytochemical composition to other plants species in the genus evidenced by reported data.

The results of the current study confirm earlier findings on the occurrence of tannins, saponins, flavonoids, anthraquinones and alkaloids in the *G. flava* extracts, (Gololo *et al.*, 2016) while this is the first study reporting glycosides and steroids from the plant although having being reported in the genus, extending conclusion on the plant specificity on phytoconstituents composition to the genus (Koche *et al.*, 2010).

4.4. Biological activities of the column fractions/ isolated compounds

Fractionation of 80% methanol twig extract yielded a total of 24 fractions which were all tested for antioxidant and antimicrobial activities. The antimicrobial activity was determined using the Agar well diffusion and microdilution method, while the antioxidant activity was determined using the DPPH radical scavenging assay. The antibacterial and antifungal activities of the crude extracts (methanol twig and root extracts) and column fractions/separated compounds were assessed. Antimicrobial activity was initially investigated using the agar well diffusion method which measured the activity of the test samples as zone of inhibition, followed by the determination of the minimum inhibitory concentration (MIC), using microdilution method. MIC was determined for samples with zones of inhibition ≥ 15 mm only since they show susceptibility otherwise, < 15 mm was considered an intermediate activity and no zone represent resistance or no activity (Rakholiya & Chanda, 2014; Senhaji *et al.*, 2020).

4.4.1. Antioxidant activity

Medicinal plants are known to exhibit various biological properties amongst which include antioxidant activity, which is mainly attributed to the phytochemical compounds like phenolic compounds, flavonoids and tannins (Alam *et al.*, 2013). Antiradical abilities of the column fractions reported as the IC₅₀ (lowest dose needed to quench at least 50% of the radical) are presented in Table 13. Column fraction 14, EtOAc (100%) revealed to be the most effective in quenching the DPPH radical with an IC₅₀ of 10.6 ± 0.3 $\mu\text{g/mL}$, indicating the fraction to be more potent than the crude extract (80% MeOH

twig extract) which exhibited an IC_{50} value of $14.5 \pm 0.7 \mu\text{g/mL}$. In this regard it can be concluded that fraction 14 contains the main antioxidant components from the extract. In addition to Fraction 14, Fraction 1, Hex (100%), Fraction 23, EtOAc: MeOH (4:3, v/v) and Fraction 24, EtOAc: MeOH, (1:1, v/v) ($10.6 \pm 0.3 \mu\text{g/mL}$ to $18 \pm 2 \mu\text{g/mL}$) exhibited high radical quenching ability which compares better to the reference standard used, Ascorbic acid ($23 \pm 1 \mu\text{g/mL}$). However, the identified fractions (2, 3 and 16) displayed a significantly ($P < 0.05$) poor radical quenching ability compared to the reference standard and the crude extract, recording IC_{50} values ranging from $221 \pm 4 \mu\text{g/mL}$ to $339 \pm 2 \mu\text{g/mL}$.

Observations made suggest that the antioxidant activity of the crude extract is likely due to the presence of both polar and less polar phytochemicals, such as phenolic compounds, flavonoids, tannins, and sterols. These phytochemicals are known to possess strong antioxidant activity, which can help to protect against cardiovascular disease, Parkinson's disease, Alzheimer's disease, and other degenerative illnesses. (Alam *et al.*, 2013; N. Stanković *et al.*, 2016). All the fractions displayed a dose dependent DPPH scavenging ability except for fraction 9, Hex: EtOAc (4:3, v/v) which did not exhibit any activity at the tested concentration.

Table 13: Antioxidant activity of the column fractions and the isolated compounds.

Column fraction	IC ₅₀ (µg/mL)
Fraction 1	18 ± 2^a
Fraction 2 (36 and 39)	226 ± 4 ^b
Fraction 3 (5)	221 ± 4 ^b
Fraction 4	119 ± 3 ^c
Fraction 5	824 ± 1 ^d
Fraction 6	348 ± 2 ^{e, m}
Fraction 7	131 ± 1 ^{e, f}
Fraction 8	135 ± 2 ^{f, g}
Fraction 9	NA
Fraction 10 (59)	120 ± 3 ^{e, h}
Fraction 11	131 ± 1 ^{f, h}
Fraction 12	26 ± 1 ^{a, i}
Fraction 13	182 ± 1 ^j
Fraction 14	10.6 ± 0.3^k
Fraction 15	44 ± 2 ^{l, p}
Fraction 16 (62)	339 ± 2 ^m
Fraction 17	147.3 ± 0.8 ^g
Fraction 18	491 ± 4 ⁿ
Fraction 19	77 ± 2 ^o
Fraction 20	27 ± 2 ⁱ
Fraction 21	37 ± 2 ^p
Fraction 22	49 ± 1 ^l
Fraction 23	15.1 ± 0.6^{a, k}
Fraction 24	18 ± 2^{a, k}
Ascorbic acid	23 ± 1 ^q

All the results are represented as means of three individual experiments ± SEM (n=3). Highlighted in bold compare better to reference standard. ND: No activity at the tested concentrations. Significant differences exist between values with distinct superscript letters (p < 0.05).

4.4.2. Antimicrobial activity

The results for the antimicrobial effect of the plant extracts and column fractions against four pathogenic bacterial strains and two fungal strains were summarized in Table 14 and Figure 16 (zones of inhibition) and Table 15 and Figure 17 (MIC values). Both MeOH twig and root extracts were found to be active against all test organisms under study with zones of inhibition ranging from 20.33 ± 0.66 to 31.00 ± 2.00 mm and 16.00 ± 0.58 to 26.67 ± 1.20 mm for the twig and root extracts respectively. Twig extract exhibited relatively high activity compared to root extract against all test organisms except with *P. aeruginosa* and *R. oryzae*. This observation was attributed to elevated levels of flavonoids in the twig extract. Flavonoids have been reported to possess the ability to inhibit nucleic acid synthesis, energy metabolism, attachment and biofilm formation, cytoplasmic membrane function, alteration of the cell membrane and attenuation of microbe's pathogenicity all leading to high antimicrobial activity (Bouarab-Chibane *et al.*, 2019; Xie *et al.*, 2015). The observation was substantiated by the GC-MS detection of different bioactive compounds like Phytol, stigmasterol, β -amyirin, lupen-3-one and campesterol from the twig extract (Table 21). From literature, susceptibility test results for stigmasterol (100 μ g/mL) showed microbial growth inhibition with 23 mm to 30 mm zone of inhibition against *MRSA*, *S. aureus*, *S. faecalis*, *E. coli*, *P. fluorescens*, *C. albicans*, *C. krusei* (Yusuf *et al.*, 2018).

The reference standard, chloramphenicol showed activity against all tested bacterial species with regions of inhibition from 28.67 ± 0.88 to 35.33 ± 0.88 mm while the negative control (10% DMSO) did not show any inhibition in all tested strains. Column fractions 14 and 24 were the most effective against all tested microorganisms with inhibition regions ranging from 15.67 ± 0.44 to 30.67 ± 0.33 mm. Fraction 2 (compound **36** and **39**) showed growth inhibition against *E. coli*, *A. niger* and *R. oryzae* with 18.67 ± 1.45 mm, 14.00 ± 0.67 mm, and 14.33 ± 0.67 mm inhibition zones respectively. Fractions 3 (compound **5**) and 4 exhibited activity against *S. aureus*, *E. coli*, *A. niger*, and *R. oryzae*. *P. aeruginosa* and *R. oryzae* were susceptible to Fraction 16 (compound **59**) and 20 with 18.67 ± 1.67 to 20.17 ± 0.58 mm zones of inhibition. Fractions 6, 11, 13, 17, 18, 19 and 21 were inactive against all test microbes while the other remaining fractions exhibited intermediate activity against at least one microbe.

The MIC values of the samples were determined using the broth microdilution method and the results are presented in Table 15. From the results obtained it can be seen that the samples possess different degrees of antibacterial activities with MIC values between 0.02 mg/mL and 1.70 mg/mL. In phytomedicine plant extracts are considered to have a substantial activity when they show an MIC < 1 mg/mL (Gibbons, 2005; Gololo *et al.*, 2017), while MIC < 0.1 may be taken as “excellent” antibacterial activity (Mulaudzi *et al.*, 2011). In this study extracts with MIC < 0.1 mg/mL were deemed to have good antibacterial activity. The crude extracts exhibited a broad spectrum of antimicrobial activity (MIC range; 0.11 ± 0.00 to 0.96 ± 0.02 mg/mL) against all tested strains, although none had an MIC value lower than 0.1 mg/mL which was a breakpoint for good activity. Fraction 24 showed strong antimicrobial activity against *B. subtilis* and *P. aeruginosa* with MIC values of 0.06 and 0.09 mg/mL respectively. The other notable strong activity was that of fraction 14 against all test strains with MIC values ranging from 0.29 to 0.02 mg/mL. The two fractions exhibited activities that were significantly better than the parent extract, suggesting that they contain a higher concentration of bioactive compounds.

It was worth noting that *P. aeruginosa*, a gram-negative strain and opportunistic pathogen exhibited susceptibility to more column fractions compared to other bacterial strains. General observation has been that Gram-negative bacteria were more susceptible to the extracts and column fractions which contradicts the general consensus where Gram-negative was reported to be resistant against plants extracts, which has been attributed to the intricate and multilayered structure of the Gram-negative cell wall, making it inaccessible by the phytochemical compounds (Rakholiya & Chanda, 2014; Sagbo , 2015). It must be noted that this was a significant observation since most of the studies tend to seek antioxidants which are active against resistant microbes. The observation made corroborate finding made by (Rakholiya & Chanda, 2014) where Gram-negative bacterial strains displayed high susceptibility towards the *M. charantia* extracts (aerial part, peel, pulp and seed) compared to Gram-positive bacteria. *S. aureus* was the most susceptible Gram-positive bacteria. The fungi *R. oryzae* growth was inhibited by more column fractions compared to *A. niger*. Different strains of bacteria responded differently to isolated compounds and column fractions, which may be attributed to the differences in the cell wall composition and genetic makeup of plasmids transferred among microbial strains (Tamokou *et al.*, 2009). The observed antimicrobial activities of the extracts and column fractions were attributed to the presence of bioactive compounds like flavonoids, phenolic compounds, and tannins. It is in this regard that *Grewia flava* extracts are considered to be effective herbs and these results form part of the explanation for the use of the plant parts in folklore traditions.

Table 14: Antibacterial activity of *Grewia flava* crude extracts and column fractions

Extracts	Zone of Inhibition (mm)					
	Bacterial strains				Fungal strains	
	<i>S. aureus</i>	<i>E. coli</i>	<i>B. subtilis</i>	<i>P. aeruginosa</i>	<i>A. niger</i>	<i>R. oryzae</i>
MeOH twig extract	30.0 ±0.6 ^a	31 ±2 ^a	28.3 ±0.3 ^a	20.3 ±0.7 ^a	24.3±0.3 ^a	22.7 ±0.7 ^a
MeOH root extract	27 ±1 ^{a, b}	24.3 ±0.9 ^b	18.3 ±0.7 ^b	24 ±2 ^{a, b}	16.0± 0.6 ^b	21.3 ±0.3 ^{a, b}
10% DMSO	ND	ND	ND	ND	ND	ND
Chloramphenicol	35.3 ±0.9 ^c	34.3 ±0.9 ^a	28.7±0.9 ^a	31.3 ±0.3 ^c	NT	NT
Fraction 1	ND	9.0± 0.6 ^c	ND	ND	ND	9.0± 0.6 ^c
Fraction 2 (36 and 39)	ND	19 ±2 ^b	ND	ND	14.0±0.7 ^{b, c}	14.3±0.7 ^d
Fraction 3 (5)	22.7 ±0.9 ^{b, d}	21.3 ±0.3 ^b	ND	ND	13.5±0.5 ^{c, d}	15.2±0.4 ^{d, e}
Fraction 4	20.3 ±0.9 ^d	19.3 ±0.9 ^b	ND	ND	15.2±0.4 ^{b, f}	15.5±0.3 ^{d, e}
Fraction 5	ND	9.3± 0.3 ^c	ND	ND	ND	10.7±0.3 ^{c, f}
Fraction 6	ND	ND	ND	ND	ND	ND
Fraction 7	ND	ND	ND	ND	11.3±0.3 ^{d, e}	12.3± 0.3 ^{d, f}
Fraction 8	ND	ND	ND	ND	8.3± 0.3 ^d	9.3± 0.7 ^{c, f}
Fraction 9	ND	ND	ND	ND	ND	8.3± 0.3 ^c
Fraction 10 (62)	ND	ND	ND	ND	11.7± 0.3 ^{c, d, e}	10.7± 0.3 ^{c, f}
Fraction 11	ND	ND	ND	ND	ND	ND
Fraction 12	ND	ND	ND	ND	8.3± 0.3 ^d	ND
Fraction 13	ND	ND	ND	ND	ND	ND
Fraction 14	29 ±2 ^a	24 ±2 ^b	25 ±0 ^c	28±1 ^{b, c, d}	23±1 ^a	15.7±0.4 ^{d, h}

Fraction 15	ND	ND	ND	ND	14.7±0.7^b	11.5±0.3 ^f
Fraction 16 (59)	ND	ND	ND	19 ±2^a	ND	19±2^{b, c, h, i}
Fraction 17	ND	ND	ND	ND	ND	ND
Fraction 18	ND	ND	ND	ND	ND	ND
Fraction 19	ND	ND	ND	ND	ND	ND
Fraction 20	ND	ND	ND	20.2 ±0.6^a	ND	20.0±0.6^{a, i}
Fraction 21	ND	ND	ND	ND	ND	ND
Fraction 22	8.7±0.3 ^e	10.7 ± 0.3 ^c	8± 0 ^e	ND	8.3± 0.3 ^d	ND
Fraction 23	10.7± 0.3 ^e	8±0 ^c	12.0± 0.6 ^f	8.7± 0.7 ^e	13.0± 0.6 ^{c, e}	ND
Fraction 24	30.7 ±0.3^{a, c}	21 ±2^b	23.3 ±0.3^c	25 ±2^{a, d}	21.3±0.7^{a, f}	20.2±0.4^{a, i}

The values in bold shows susceptibility (zone of inhibition ≥ 15 mm), ND: Not detectable at the tested concentrations. Negative control = 10% DMSO, Positive control = chloramphenicol, NT, represents “not tested”. Significant differences exist between values with distinct superscript letters ($p \leq 0.05$). Fractions 1-24 were from the Twig 80% MeOH crude extract column chromatography.

Table 15: MIC of aqueous methanol extracts and column fractions from *Grewia flava*

Extracts	MIC (mg/mL)					
	Bacterial strains				Fungal strains	
	<i>S. aureus</i>	<i>E. coli</i>	<i>B. subtilis</i>	<i>P. aeruginosa</i>	<i>A. Niger</i>	<i>R. Oryzae</i>
80% MeOH twig extract	0.52± 0.06 ^a	0.26 ± 0.04 ^a	0.13±0.01 ^a	0.96± 0.02 ^a	0.17± 0.00 ^a	0.20± 0.02 ^a
80% MeOH root extract	0.52 ± 0.00 ^a	0.30± 0.03 ^a	0.11±0.00 ^a	0.86 ±0.13 ^a	0.18± 0.01 ^a	0.27± 0.01 ^{a, b}
Fraction 2 (36 and 39)	NT	0.7± 0.2 ^b	NT	NT	0.15± 0.00 ^a	0.56± 0.02 ^c
Fraction 3 (5)	0.47 ± 0.01 ^b	1.70 ± 0.06 ^c	NT	NT	0.42 ± 0.06 ^b	0.40± 0.01 ^{b, d}
Fraction 4	1.03 ± 0.06 ^c	1.4 ±0.2 ^c	NT	NT	0.49± 0.06 ^b	0.50±0.05 ^{c, d}
Fraction 14	0.30 ± 0.04 ^d	0.28 ± 0.02 ^a	0.02 ± 0.00^b	0.31± 0.04 ^b	0.29± 0.03 ^c	0.21± 0.03 ^a
Fraction 15	NT	NT	NT	NT	0.42± 0.05 ^b	NT
Fraction 16 (59)	NT	NT	NT	0.52± 0.01 ^c	NT	0.18± 0.08 ^a
Fraction 20	NT	NT	NT	0.921± 0.15 ^a	NT	NT
Fraction 24	0.25± 0.03 ^c	0.34± 0.02 ^a	0.06± 0.01^b	0.09 ±0.01^d	0.26± 0.02 ^{a, c}	0.03± 0.00^e

MIC values are presented as mean of three individual replicates ±SEM. NT, represents “not tested. Significant differences exist between values with distinct superscript letters ($p \leq 0.05$).

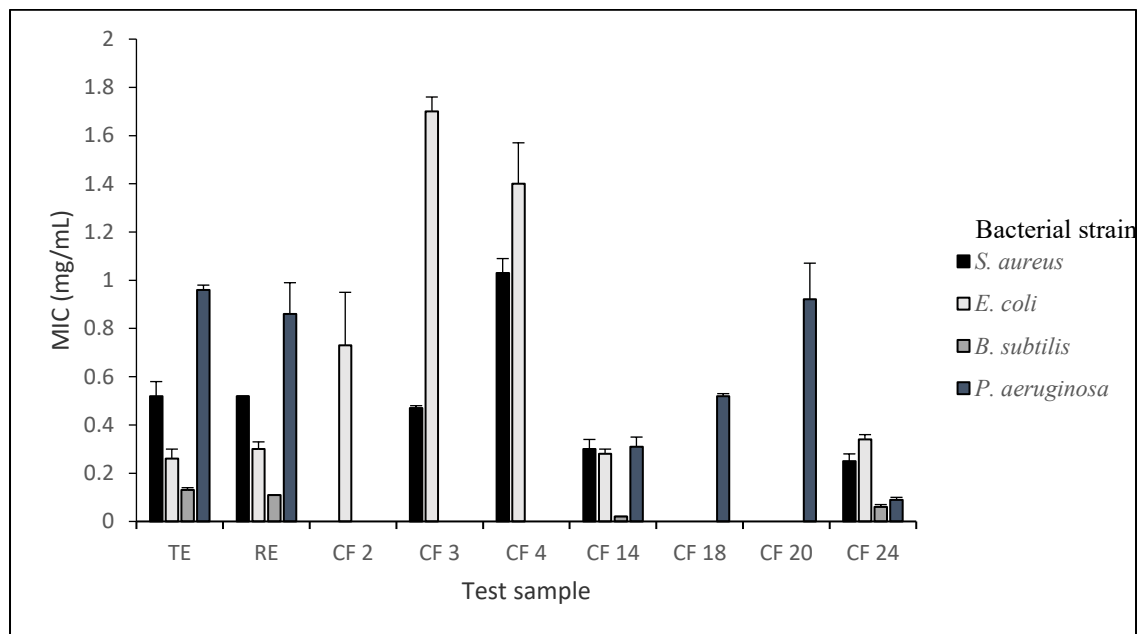


Figure 16: Comparative analysis of MIC values of Aq MeOH crude extracts and column fractions of *Grewia flava* against four bacterial strains. TE= Twig extract, RE= Root extract, CF= Column fraction.

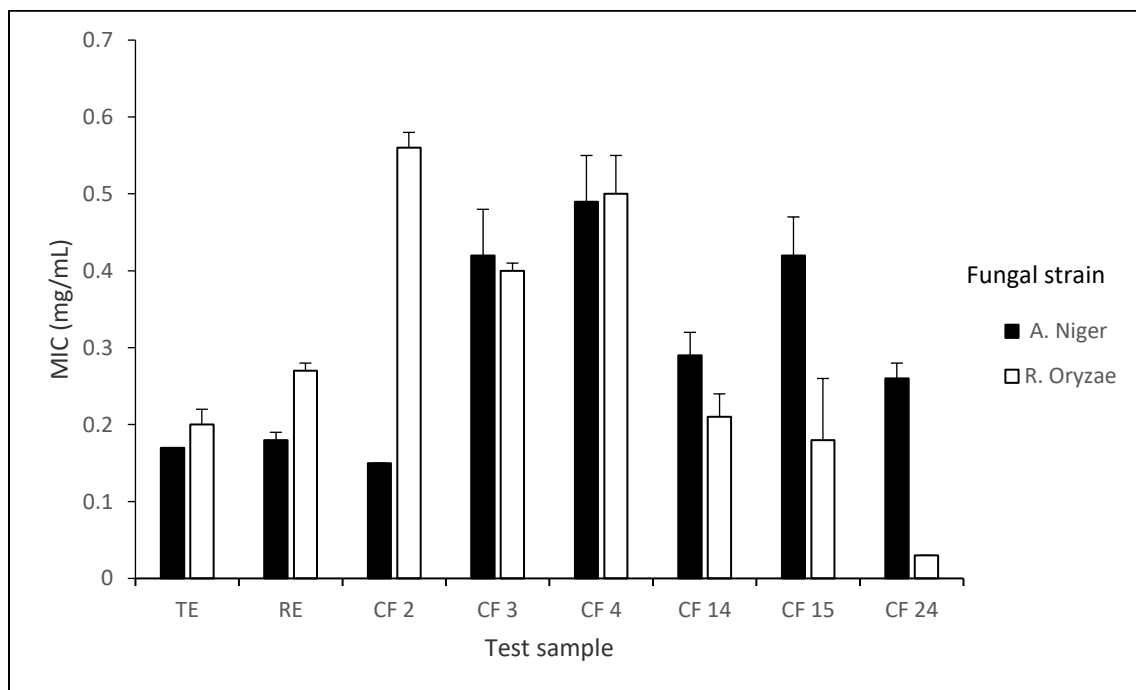


Figure 17: Comparative investigation of the MIC values of Aq MeOH crude extracts and column fractions against two fungal strains. TE= Twig extract, RE= Root extract, CF= Column fraction.

4.5. Identification of the isolated bioactive compounds

4.5.1. Structure elucidation of fraction 2

Fraction 2 was dried and yielded white crystals with a melting point of 142-147 °C. The FT-IR spectrum showed absorption band at 3305.11 cm^{-1} indicating the hydroxyl group (-OH) band, which was confirmed by the appearance of the secondary alcohol C-O stretch at 1035.62 cm^{-1} . The availability of the aliphatic C-H was confirmed by the absorptions appearing at 2942.95 and 2846.52 cm^{-1} and the absorption at 1452.19 cm^{-1} were attributed to the cyclic CH_2 . Interpretation of Fraction 2 ^1H -MNR spectra suggested that the fraction was a mixture of two compounds, lupeol **39** (major compound) and β -sitosterol **36** (minor compound). With almost the same polarities these compounds were isolated as a mixture. The identity of individual compounds was elucidated by analysing the 1D and 2D NMR spectral data and comparison to literature data.

4.5.1.1. Fraction 2 major compound, Lupeol 39

The ^1H -NMR spectrum (Appendix 1) of fraction 2 showed a major signals at δ_{H} 3.14, representing the oxymethine proton (H-3), it also shows peaks at δ_{H} 4.70 and 4.56 indicating the olefinic protons (H_a -29 and H_b -29 respectively). The spectrum shows the presence of singlets at δ_{H} (0.75, 0.79, 0.82, 0.86, 0.96, 1.04, 1.69) attributed to the seven methyl groups (H-24, H-28, H-25, H-23, H-27, H-26, and H-30 respectively). ^{13}C NMR spectrum (Appendix 2) showed signals at δ_{C} 150.63 and 109.14, assigned to the olefinic carbons C-20 and terminal $=\text{CH}_2$, C-29 respectively. The oxymethine carbon (C-3) signal appeared at δ_{C} 77.69, and signals at δ_{C} (38.72, 40.75, 37.04, 42.67 and 42.82) were assigned to other quaternary carbons (4, 8, 10, 14 and 17 respectively). The contrast of the obtained NMR spectral data to literature data, (Table 16) suggests some similarities consistent to that of Lupeol **39**, Figure 18 (Jain & Bari, 2010; Oliveira *et al.*, 2012; Shwe *et al.*, 2019).

Reports show that lupeol **39** exhibits a spectrum of antioxidant activities like antiasthma, antidiabetic, anti-inflammatory, cardioprotective, hepatoprotective properties, anticancer, protective effect during low density lipoprotein oxidation (Ahamed *et al.*, 2007; Dharmasoth *et al.*, 2022; ELhassan *et al.*,
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2021; Tsai *et al.*, 2016). Isolation of lupeol **39** has been reported before, from other plant species in the genus and *Grewia flava* leaves (S. Kumar *et al.*, 2022; Lamola, 2015).

Table 16: ^1H and ^{13}C NMR chemical shifts for Fraction 2 (Lupeol) major peaks in acetone- d_6 .

Position	C/H	^1H NMR (δ_{H})	^{13}C NMR (δ_{C})	^1H NMR (δ_{H}) Lit**	^{13}C NMR (δ_{C}) Lit**
1	CH_2	0.86 (bs), 1.41-1.63*10H	38.63	0.90, 1.65*	38.85
2	CH_2	1.41-1.63*10H	27.69	1.52, 1.67*	27.44
3	CH	3.13 (m)	77.68		79.00
4	C		38.72		38.70
5	CH	0.83*	55.24	0.67*	55.29
6	CH_2	1.41-1.63*10H	18.21	1.37, 1.52*	18.31
7	CH_2	1.28-1.36* 10H	34.23	1.39*	34.27
8	C		40.75		40.83
9	CH	1.28-1.36* 10H	50.42	1.25*	50.43
10	C		37.04		37.16
11	CH_2	1.28-1.36* 10H	20.76	1.20, 1.40*	20.92
12	CH_2	0.86 (br s), 1.41-1.63*10H	25.11	1.06, 1.62*	25.13
13	CH	1.41-1.63*10H	38.09	1.66*	38.05
14	C		42.67		42.82
15	CH_2	1.41-1.63*10H	27.82	1.05, 1.60*	27.41
16	CH_2	1.28-1.36* 10H 1.41-1.63*10H	35.38	1.35, 1.45*	35.58
17	C		42.83		42.99
18	CH	1.28-1.36* 10H	48.13	1.36, 1.37*	48.30
19	CH	2.44 (ddd, $J=5.6$ Hz, 5.6 Hz, 11.0 Hz)	47.93	2.40 (m), 1.45*	47.98
20	C		150.62		150.97
21	CH_2	1.28-1.36* 10H 1.96 (3H, m)	29.60	1.3*, 1.91(m)	29.84
22	CH_2	1.11 (br s) 1.28-1.36* 10H	39.56	1.18, 1.37*	40.00
23	CH_3	1.07 (s)	27.90	0.90 (s)	27.98
24	CH_3	0.75 (s)	15.25	0.76 (s)	15.36
25	CH_3	0.82 (s)	16.50	0.83 (s)	16.11
26	CH_3	1.04 (s)	15.75	1.03 (s)	15.97
27	CH_3	0.96 (s)	14.06	0.94 (s)	14.60
28	CH_3	0.78 (s)	17.05	0.79 (s)	17.99
29	CH_2	a) 4.70 (d, $J=1.8$ Hz) b) 4.56 (d, $J=1.8$ Hz)	109.13	4.57, 4.69 (d, $J=1.9$ Hz)	109.31
30	CH_3	1.69 (s)	18.62	1.67 (s)	19.30

Assignments were confirmed by COSY, HMQC, HMBC and DEPT experiments, *:Overlapping signals, literature reference **: (Shwe *et al.*, 2019).

4.5.1.2. Fraction 2 minor compound β -Sitosterol **36**

Analysis of the 1D and 2D NMR spectral data lead to the identification of the minor Fraction 2 peaks. The ^1H NMR spectrum of Compound **2** (Appendix 1) shows a key peak at δ_{H} 5.18 indicating the availability of the olefinic methine proton (H-6) and the multiplet peak signal at δ_{H} 3.37 representing H-3 proton of the sterol moiety. The ^{13}C NMR (Appendix 2) shows signals for the olefinic carbons, C-6, and C-5 at δ_{C} 124.62, and 139.51 respectively, which confirms the presence of steroid skeleton. Protons were assigned based on HMQC and HMBC correlations, which assisted in delineating the structure. The oxymethine proton H-3 was assigned to the carbinolic carbon (C-3) appearing at δ_{C} 77.66. Although many peak were overlapped by the major compound, the structure of Fraction 2 minor compound was subsequently elucidated to be consistent with that of sitosterol **36** (Figure 18) based on the comparison of the spectral data to the previous studies, Table 17 (Chaturvedula & Prakash, 2012; Erwin *et al.*, 2020; Jamila *et al.*, 2014; Kamal *et al.*, 2016).

This is the first study to identify β -sitosterol **36** from *G. flava* twigs, however, its isolation has been reported from the *G. tiliaefolia* and *G. plagioplylla* stem barks (Ahamed *et al.*, 2007; Githinji *et al.*, 2020). The compound has reportedly been linked to different biological actions such as anti-inflammatory, antioxidant, antidiabetic, antitumor therapeutic effect activity (Babu & Jayaraman, 2020; Bao *et al.*, 2022)

Table 17: ^1H and ^{13}C NMR chemical shifts for Fraction 2 (β -sitosterol) minor peaks in acetone- d_6 .

Position	C/H	^1H NMR (δ_{H})	^{13}C NMR (δ_{C})	^1H NMR (δ_{H}) Lit***	^{13}C NMR (δ_{C}) Lit**
1	CH_2	*	38.66	1.85 (m)	37.2
2	CH_2	*	29.16	1.95 (m)	29.6
3	CH	3.37 (m)	77.65	3.52 (m)	71.8
4	CH_2	2.43 (td)	41.97	2.24 (dd, $J = 1.44, 1.06$ Hz) and 2.38, t)	42.3
5	C		139.51		140.7
6	CH	5.18 (t)	124.62	5.36 (t)	121.7
7	CH_2	*	31.02	1.99 (m)	29.6
8	CH	*	33.57	2.00(m)	31.9
9	CH	*	47.69	0.94 (m)	50.1
10	C		36.76		36.5
11	CH_2	*	20.80	1.02 (m,)	21.1
12	CH_2	*	39.54	1.16 (m)	39.8
13	C		41.94		42.3
14	CH	*	59.06	1.00 (m)	56.6
15	CH_2	*	23.19	1.06 (m) and 1.58 (m)	24.3
16	CH_2	*	28.77	1.66 (m) and 1.25 (m)	29.2
17	CH	*	55.38	1.09 (m)	55.1
18	CH_3	*	15.23	0.85 (s)	12.2
19	CH_3	*	20.80	0.82 (s)	19.0
20	CH	*	39.96	1.35 (m)	40.4
21	CH_3	*	22.84	0.92 (d, $J=5.12$ Hz)	21.2
22	CH_2	*	36.7	1,33 (m)	36.1
23	CH_2	*	28.96	1.16 (m)	28.9
24	CH	*	50.42	0.94 (m)	51.2
25	CH	*	32.85	1.66 (m)	31.6
26	CH_3	*	26.46	0.83(d, $J=11$ Hz)	21.2
27	CH_3	*	17.44	0.84 (br s)	19.8
28	CH_2	*	27.27	1.25 (m)	25.4
29	CH_3	*	14.06	0.85 (t)	12.0

Assignments were confirmed by COSY, HMQC, HMBC and DEPT experiments, *: Peaks overlapped by fraction 2 major peaks in Table 16, Literature reference **: (Jamila *et al.*, 2014), *** (Erwin *et al.*, 2020).

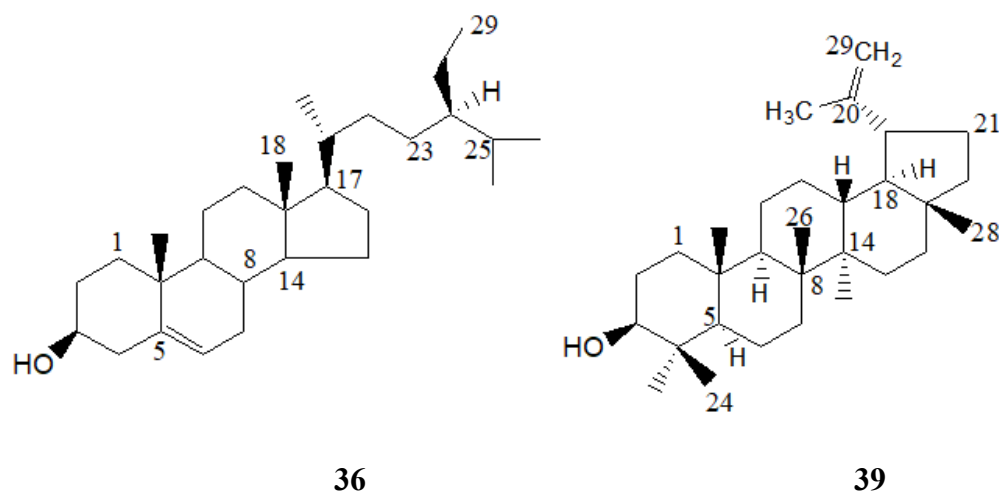


Figure 18: Structures of β -Sitosterol **36** and Lupeol **39** from fraction

The antioxidant activity test revealed low DPPH radical scavenging ability with an IC_{50} value of $226 \pm 4 \mu\text{g/mL}$, however, both the compounds have been previously reported to exhibit antioxidant activities, with β -sitosterol **36** exerting its activity through modulation of antioxidant enzymes (ELhassan *et al.*, 2021; Erwin *et al.*, 2020) while lupeol **39** has been linked to hepatoprotective and anticancer properties resulting from its ability to protect the membrane permeability and direct scavenging of the free radicals (Santiago & Mayor, 2014). Lupeol **39** (88.40%) has previously exhibited better DPPH radical scavenging ability at $800 \mu\text{g/mL}$ compared to the known reference standard, ascorbic acid (82.37%) (Tchimene *et al.*, 2016). Additionally, the compounds reportedly possess antimicrobial properties. In this study the fraction 2 was found to be effective in inhibiting the growth of *E. coli*, *A. niger* and *R. oryzae* with zones of inhibition; $18.67 \pm 1.45 \text{ mm}$, $14.00 \pm 0.67 \text{ mm}$, and $14.33 \pm 0.67 \text{ mm}$ respectively while *S. aureus*, *B. subtilis*, and *P. aeruginosa* exhibited resistance towards the compound since no zone clear zone of inhibition were observed. However moderate activity of lupeol **39** at $30 \mu\text{g/disc}$ in inhibiting the growth of *B. subtilis*, *E. coli* and *S.aureus* has been reported before (Rosandy *et al.*, 2021). This observation supports the activity of fraction 2 against *E. coli* on the other hand it contradicts observation made regarding *B. subtilis*, and *S. aureus* resistance. The resistance of *P. aeruginosa* against lupeol **39** has also been reported by (N. Akwu *et al.*, 2020). Similarly, sitosterol reportedly exhibit growth inhibition activity against *S. aureus*, *B. subtilis*, and *K. pneumoniae* with MIC values of 25, 12, and 25 mg/mL respectively. However, no signs of activity were observed with *C. albicans*, *E. coli*, and *P. aeruginosa* (Alawode *et al.*, 2021; Pierre Luhata & Usuki, 2021). The MIC values of the fraction 2 against the susceptible microbes were established to be 0.73 ± 0.22 , 0.15 ± 0.00 , $0.56 \pm 0.02 \text{ mg/mL}$ for *E. coli*, *A. niger* and *R. oryzae* respectively.

4.5.2. Structure elucidation of stigmasterol 5

Fraction 3 was dried and yielded white crystals with a melting point of 158-161°C. FT-IR spectrum showed absorption band at 3409.65 cm⁻¹ suggestive of the hydroxyl group (-OH), which was supported by the availability of the secondary alcohol C-O stretch at 1018.27 cm⁻¹. The availability of the aliphatic C-H was deduced from the observed absorption at 2921.73 and 2854.16 cm⁻¹, absorptions at 1436.76 cm⁻¹ were attributed to the cyclic CH₂. The other absorption at 1643.11 was assigned to the C=C stretch. The ¹H NMR spectrum of Compound **5** (Appendix 3) shows peaks at δ_H 4.96, 5.11 and 5.63 indicating the presence of olefinic methine protons (H-22, H-23, and H-6 respectively) and the multiplet peak at δ_H 3.61 for H-3 proton of the sterol moiety. The ¹³C NMR (Appendix 4) shows signals at δ_C 126.10, 129.49, 135.59 and 138.09 for the olefinic carbons (C-6, C-23, C-22, and C-5 respectively), of which confirmed the presence of steroid skeleton. The oxygenated methine carbon (C-3) was assigned to a carbon signal appearing at δ_C 70.52. The analysis and comparison of NMR spectral data (Table 18) to literature values (Erwin *et al.*, 2020; Kamal *et al.*, 2016) resulted in the identification of fraction 3 compound as stigmasterol **5**, Figure 19. There are studies reporting the isolation of stigmasterol from other plants species in the genus *Grewia* (Goyal, 2012; S. Kumar *et al.*, 2022), however it's isolation and identification from *G. flava* is being reported for the first time.

Table 18: ^1H (400 MHz) and ^{13}C NMR (100.6 MHz) chemical shifts for fraction 3 (stigmasterol) in chloroform-d.

Position	C/H	^1H NMR (δ_{H})	^{13}C NMR (δ_{C})	^1H NMR (δ_{H}) Lit**	^{13}C NMR (δ_{C}) Lit**
1	CH ₂	1.82-1.98* 4H	38.29	1.85 (m)	37.39
2	CH ₂	1.82-1.98* 4H	31.93	1.95 (m)	32.02
3	CH	3.61 (m)	70.52	3.52 (m)	71.93
4	CH ₂	2.43 (m)	40.26	2.24 (dd, $J = 1.44$; 1.06) and 2.38, t)	42.42
5	C		138.09		140.87
6	CH	5.62 (br s)	126.10	5.36 (t)	121.84
7	CH ₂	2.25- 2.36* 3H	31.44	1.99 (m)	31.78
8	CH	2.25- 2.36* 3H	32.41	2.00(m)	32.05
9	CH	0.73 – 0.77* 10H	50.03	0.94 (m)	50.26
10	C		36.34		36.64
11	CH ₂	0.73 – 0.77* 10H	21.22	1.02 (m,)	21.22
12	CH ₂	1.18-1.26* 9H	40.26	1.16 (m)	39.82
13	C		42.98		42.35
14	CH	0.73 – 0.77* 10H	51.22	1.00 (m)	56.99
15	CH ₂	1.18-1.26* 9H	24.73	1.06 (m) and 1.58 (m)	24.45
16	CH ₂	1.47-1.57* 5H	29.11	1.66 (m) and 1.25 (m)	29.07
17	CH	0.73 – 0.77* 10H	54.69	1.12 (m)	56.08
18	CH ₃	0.73 – 0.77* 10H	11.98	0.85 (s)	12.13
19	CH ₃	1.13 (br s)	19.81	1,01 (s)	19.54
20	CH	1.18-1.26* 9H	41.79	1.16 (m)	40.65
21	CH ₃	0.96 (d, $J = 6.7$ Hz)	21.42	1.03 (d, $J = 7.2$ Hz, 3H)	21.23
22	CH	4.95 (dd $J = 15.2$, 8.6 Hz)	135.59	5.00 (dd, $J = 1.73$ Hz and 1.72 Hz)	138.46
23	CH	5.10 (dd, $J = 15.2$, 8.5 Hz)	129.48	5.15 (dd, $J = 1.75$ Hz and 1.73 Hz)	129.39
24	CH	1.47-1.57* 5H	51.22	1.55 (m)	51.38
25	CH	1.47-1.57* 5H	31.88	1.45 (m)	32.03
26	CH ₃	0.84 (d, $J = 6.4$)	26.41	1.02 (d, $J = 13$ Hz)	21.21
27	CH ₃	0.73 – 0.77* 10H	19.04	0.84 (br s)	19.97
28	CH ₂	1.18-1.26* 9H	25.38	1.16 (m)	25.56
29	CH ₃	0.61 (m)	12.20	0.81 (t)	12.41

Assignments were confirmed by COSY, HSQC, HMBC and DEPT experiments, overlapping signals: *; Literature reference **: (Erwin *et al.*, 2020)

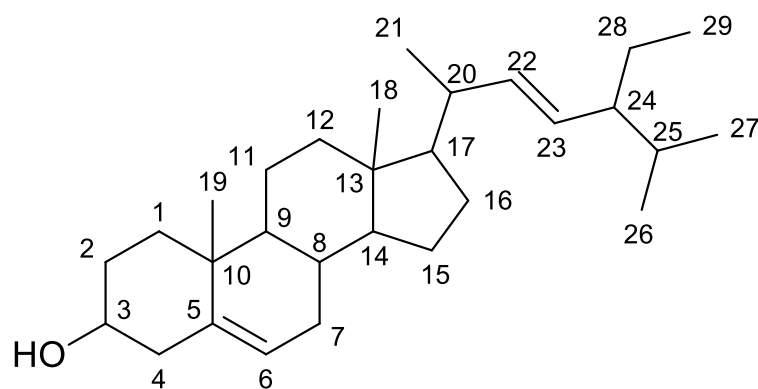


Figure 19: Structure of stigmasterol **5**

The radical scavenging ability of the compound against DPPH radical was poor with an IC_{50} value of $221 \pm 3.83 \mu\text{g/mL}$, however, stigmasterol **5** have been previously reported to display moderate radical quenching ability against DPPH radical which can be linked to the presence of electron rich oxygen found in the hydroxyl group of the compound (Abdollahnezhad *et al.*, 2021). Stigmasterol **5** has been reported to exhibit anti-inflammatory, antimicrobial activity and cytotoxic effect against cancer cells. The antimicrobial activity of the compound has been linked to disruption of the cell membrane (Suttiarporn *et al.*, 2015). Compound **5** exhibited different levels of antimicrobial activities against all test microbes except for the *B. subtilis* and *P. aeruginosa* which proved to be resistant. Zones of inhibition for the susceptible microbes ranged from $13.50 \pm 0.50 \text{ mm}$ to $22.67 \pm 0.88 \text{ mm}$ (Table 14). The highest activity was recorded against *S. aureus* (22.67 mm), a causative agent for different health issues like pneumonia, skin infections, and other fatal infections, while the least sensitive was *A. niger* (13.50 mm). Previously stigmasterol **5** has exhibited a very good antimicrobial activity against *S. aureus* (12.5 $\mu\text{g/mL}$), *MRSA* (6.25 $\mu\text{g/mL}$), *E. coli* (25 $\mu\text{g/mL}$), *Pseudomonas fluorescens* (25 $\mu\text{g/mL}$), *Streptococcus faecalis* (12.5 $\mu\text{g/mL}$), *Candida albicans* (25 $\mu\text{g/mL}$), *Candida krusei* (12.5 $\mu\text{g/mL}$) (Yusuf *et al.*, 2018). The susceptibility testing of the current study revealed low MIC values ($0.40 \pm 0.01 \text{ mg/mL}$ to 1.70 mg/mL , Table 15) which are considered to be good since most are below 1 mg/mL. The differences in the degree of susceptibility are attributable to concentrations difference across studies. The current study exhibits the presence of bioactive compound, stigmasterol **5** which is reported for the first time from *G. flava*.

4.5.3. Structure elucidation of eicosyl protocatechuate **62**

Fraction 10 (compound **62**) was obtained as brown gum. FT-IR spectrum showed absorption band at 3338.30 cm^{-1} indicating the presence of phenolic hydroxyl group (-OH), which was supported by the presence of the aromatic C=C at 1448.33 cm^{-1} . A carbonyl functionality appeared at 1714.46 cm^{-1} and a sharp peak was observed at 1025.98 cm^{-1} representing the ester C-O bond. Analysis of the ^1H -MNR spectrum exhibited an aromatic ABX proton spin system with resonances at δ_{H} 7.47 (1H, t, $J = 2.1\text{ Hz}$, H-2), 7.58 (1H, d, $J = 8.6\text{ Hz}$, H-5) and 7.27 (dd, $J = 8.6, 2.1\text{ Hz}$, H-6) proposing a 1,3,4- trisubstituted benzene ring. The spectrum shows the presence of the oxymethylene protons resonating at δ_{H} 3.6 (H-1'). A broad singlet signal at δ_{H} 1.27–1.37 integrating for 32 protons was observed, suggestive for the presence of hydrocarbon long straight chain and another broad singlet signal appearing at δ_{H} 0.87 was attributed to the terminal methyl protons (H-20'). Interpretation of the ^{13}C -NMR revealed the presence of an ester functional group which was deduced from the presence of the carbonyl carbon signal appearing at δ_{C} 173.75 and the oxymethylene carbon at δ_{C} 55.43. The carbon signal at δ_{C} 13.47 which was ascribed to the terminal methyl group and the carbon signals observed between 22.44 and 33.29 ppm attributable to methylene carbons substantiated the presence of long hydrocarbon straight chain. Analysis of the spectrum further reveals the presence of oxygenated aromatic carbons, with signals appearing at δ_{C} 147.99 (C-4) and 138.22 (C-3), three aromatic methine carbon signals at δ_{C} 118.97 (C-5), 124.03 (C-2) and 124.56 (C-6) and an aromatic quaternary carbon at δ_{C} 120.88 (C-1).

The assignments above were assisted with the HMBC spectrum where the proton signal at δ_{H} 7.47 showed cross peaks with the carbonyl carbon at δ_{C} 173.75 and the quaternary carbon at δ_{C} 120.88. Moreover, methylene protons at δ_{H} 2.26 (C-2') showed a correlation with the carbon at δ_{C} 173.75 and δ_{C} 33.29. Comparison of NMR spectral data (Table 19) to literature data revealed that compound **62** was a protocatechuic acid ester derivative (Grajeda-Iglesias *et al.*, 2016; Huang *et al.*, 2003; Zeraik *et al.*, 2014). The ^1H -NMR and ^{13}C -NMR spectra are shown in Appendix 5 and 6. The proposed structure of compound **62** is shown in Figure 20, and the compound has been identified as a protocatechuic acid alkyl ester, Eicosyl protocatechuate (3,4-dihydroxybenzoic acid Eicosyl ester). There is no literature data on the occurrence of this compound in *Grewia flava*, or any other plant species, however ethyl protocatechuate which is also a protocatechuic acid derivative has been isolated from the ethanolic extract of peanut seed testa (Huang *et al.*, 2003) and eicosyl has been identified as a plant oil component

(Grafakou *et al.*, 2022). Ferulic acid eicosyl (hemicosanyl ferulate) which is the closest isolated compound to **62** has been reported from *Synadenium glaucescens* Pax, and *Aristolochia kankauensis* (Rwegoshora *et al.*, 2022; Wu *et al.*, 1994)

Table 19: ^1H (400 MHz) and ^{13}C NMR (100.6 MHz) chemical shifts for compound **62** (3,4-dihydroxybenzoic acid eicosyl ester) in acetone- d_6

Position	C/H	^1H NMR (δ_{H})	^{13}C NMR (δ_{C})	^1H NMR (δ_{H}) Lit ^{**}	^{13}C NMR (δ_{C}) Lit ^{**}
1	C		120.88		115.3
2	CH	7.47 (t, $J = 2.1$ Hz)	124.02	7.58 (d, $J = 2.0$ Hz)	120.8
3	C		138.32		145.1
4	C		147.99		150.4
5	CH	7.57 (d, $J = 8.6$ Hz)	118.96	6.91 (d, $J = 8.6$ Hz)	121.7
6	CH	7.27 (dd, $J = 8.6, 2.1$ Hz)	124.56	7.57 (dd, $J = 2.0, 8.6$ Hz)	116.2
7	C		173.74		165.7
1'	CH ₂	3.63 (m)	55.42	4.22 (t, $J = 6.6$ Hz)	64.0
2'	CH ₂	2.29 (m)	34.66	1.82–1.58 (m, 2H)	22.1–31.3
3'	CH ₂	1.58 (br s)	34.25	1.55–1.12 (m)	22.1–31.3
4'–19'	CH ₂	1.27–1.37 (br s, 32H)	22.44 – 33.29	1.55–1.12 (m)	22.1–31.3
20'	CH ₂	0.87 (br s)	13.47	0.87 (t, $J = 6.5$ Hz)	13.7

Assignments were confirmed by COSY, HSQC, HMBC and DEPT experiments. ^{**}Literature data: (Grajeda-Iglesias *et al.*, 2016; Huang *et al.*, 2003; Zeraik *et al.*, 2014).

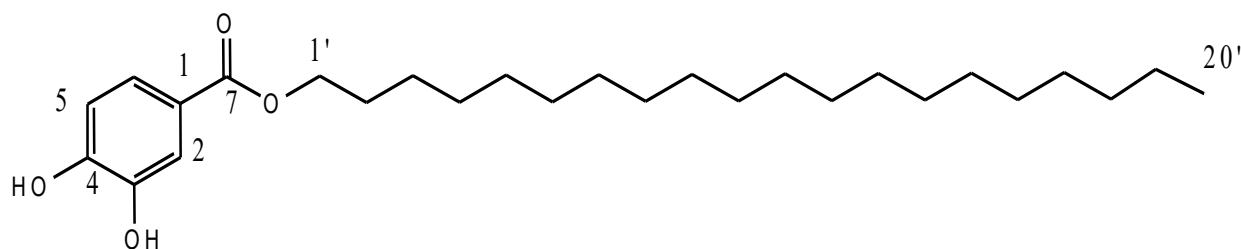


Figure 20: Structure of compound **62**

Protocatechuic acid and its derivatives isolated from different plant species has exhibited varying degrees of antioxidant activities, showed by the DPPH radical scavenging ability presented by the isolated protocatechuic acid derivative **62** with an IC_{50} value of $120 \pm 2.74 \mu\text{g/mL}$ (Table 13). This can be linked to the aromatic hydroxyl groups with the potential to either donate electrons or hydrogen. Protocatechuic acid derivatives have previously been reported to exhibit better antiradical properties compared to the parent compound, which was attributed to increased solubility in organic solvents as a result of lipophilization (Grajeda-Iglesias *et al.*, 2016). Esterification of protocatechuic acid has showed to be a great tool in improving its biological properties. A correlation between inhibitory potency and increase in the carbon chain length of the ester was reported (de Faria *et al.*, 2012). Compound **62** exhibited intermediate inhibitory effect against the fungal species under study with zones of inhibition of 11.7 ± 0.3 and 10.7 ± 0.3 mm against *A. niger* and *R. oryzae* respectively (Table 14), while all bacterial strains were resistant to the compound. Nonetheless, protocatechuic acid derivatives has been reported to exhibit antibacterial properties, ethyl protocatechuate exhibited inhibitory effect against *S.aureus* with MIC values ranging from 64 to 1024 $\mu\text{g/mL}$ (Miklasińska *et al.*, 2015).

4.5.4. Structure elucidation of Kaempferol 3-*O*-rutinoside **59**

Fraction 16 dried to give a brown gummy paste. The IR spectrum of the fraction showed a broad absorption band at 3292.01 cm^{-1} suggesting the availability of OH and another one at 1637.33 cm^{-1} representing the carbonyl group. The C=C stretch was observed at 1604.54 cm^{-1} . The identity of the compound was elucidated by the analysis of 1D and 2D NMR spectral data and reviewing of literature data. The $^1\text{H-NMR}$ spectrum of compound **59** (Appendix 7) revealed the presence of peak signals consistent with flavonoids and glucoside moieties. The $^1\text{H-NMR}$ spectrum exhibits two meta doublets

($J = 3.7$ Hz) at δ_H 5.13 (1H, H-6) and 5.41 (1H, H-8) which suggests a 5,7-dihydroxy substituted A-ring. The two *ortho*-doublets ($J = 8.2$ Hz) appearing at δ_H 6.76 (2H, H-3' and H-5') and 7.13 (2H, H-2' and H-6') gave evidence for the 4'-hydroxyl group on B-ring. Peak signals observed in the aliphatic region indicated the presence of 3-O-rutinoside moiety, exhibited by the appearance of two anomeric protons at δ_H 4.51 (1H, H-1'') and 4.13 (1H, H-1''') for the glucoside and rhamnosyl moieties respectively and the signals at δ_H 0.92 (3H, H-6''') for the rhamnosyl methyl protons (Aisyah *et al.*, 2017; Sakaguchi *et al.*, 2013; Tram *et al.*, 2016). There was an observed interference from the hydroxyl groups in the δ_H 2.86- 3.90 ppm range. The carbonyl carbon, C-4 was assigned to the peak appearing at δ_C 176.74 in the ^{13}C -NMR spectrum (Appendix 8). Other key peaks for the aglycone moiety carbons were observed at δ_C 167.49 and 159.52×2 for the oxygenated carbons C-7, C-4' and C-5 respectively. (Osw & Hussain, 2020). The comparison of literature data with NMR spectra data (Table 20) led to the identification of fraction 16 compound as kaempferol-3-O-rutinoside **59**, with a proposed structure shown in Figure 21 (Osw & Hussain, 2020). Occurrence of the compound has been previously reported in other plant species and *Grewia tenax* Forssk (Saleh *et al.*, 2010) while this is the first report of the compound from *Grewia flava* plant.

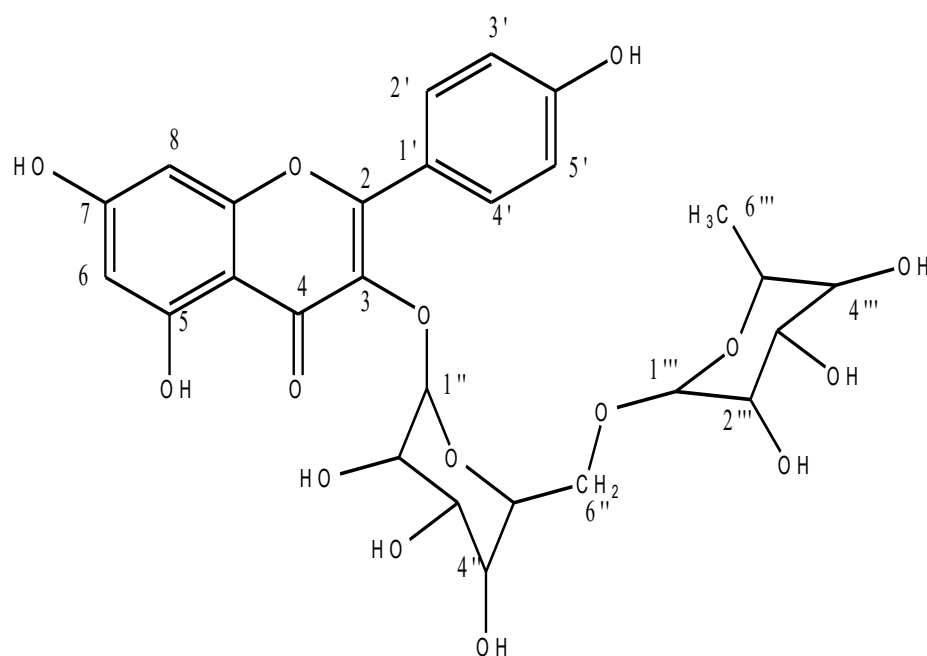


Figure 21: Structure of kaempferol-3-O-rutinoside **59**

Table 20: ^1H (400 MHz) and ^{13}C NMR (100.6 MHz) chemical shifts for compound **59** (kaempferol-3-*O*-rutinoside) in methanol- d_4 .

Position	C/H	^1H NMR (δ_{H})	^{13}C NMR (δ_{C})	^1H NMR (δ_{H}) Lit**	^{13}C NMR (δ_{C}) Lit**
Aglycone moiety					
2	C		158.94		156.49
3	C		131.91		133.20
4	C		176.73		177.34
5	C		159.51		161.17
6	CH	5.13 (d, $J = 3.7$)	92.54	6.20 (d, $J = 1.8$ Hz)	98.76
7	C		167.48		164.34
8	CH	5.41 (d, $J = 3.7$)	92.23	6.40 (d, $J = 1.8$ Hz)	93.76
9	C		156.30		156.80
10	C		103.89		103.90
1'	C				120.86
2' & 6'	CH	7.11 (d, $J = 8.2$)	129.42	8.00 (d, $J = 8.8$ Hz)	130.85
3' & 5'	CH	6.78 (d, $J = 8.1$)	115.32	6.88 (d, $J = 8.8$ Hz)	115.08
4'	C	-	159.51		159.91
Glucoside moiety					
1''	CH	4.50 (d, $J = 7.76$)	101.74	5.30 (d, $J = 7.3$ Hz)	101.35
2''	CH	3.65-3.73* 6H	74.26	3.16 (m)	74.16
3''	CH	3.65-3.73* 6H	77.86	3.21 (m)	76.36
4''	CH	2.93 (t)	69.89	3.05 (m)	69.89
5''	CH	3.65-3.73* 6H	75.34	3.24 (m)	75.73
6''	CH ₂	3.44 (dd, $J = 3.8$ Hz, 9.8 Hz)	67.94	3.70 (bd, $J = 10.0$ Hz), 3.30 (bd, $J = 10.0$ Hz)	66.87
Rhamnosyl moiety					
1'''	CH	4.13 (s)	97.81	4.37 (bs)	100.76
2'''	CH	3.65-3.73* 6H	70.31	3.24 (m)	70.33
3'''	CH	3.65-3.73* 6H	70.42	3.68 (m)	70.58
4'''	CH	3.15 (t)	71.78	3.10 (m)	71.81
5'''	CH	3.65-3.73* 6H	69.34	3.68 (m)	68.23
6'''	CH ₃	0.96 (m)	13.07	0.98 (d, $J = 6.1$ Hz)	17.71

Assignments were confirmed by COSY, HSQC, HMBC and DEPT experiments, overlapping signals:

*, Literature data** (Osw & Hussain, 2020)

Kaempferol-3-O- β -rutinoside **59** reportedly possess the ability to inhibit the inflammation-related gene expression in LPS-induced macrophages (Hwang *et al.*, 2019). The antiradical ability of the compound against DPPH radical was found to be poor with an IC₅₀ value of 339 ± 2.22 μ g/mL, nonetheless, previously the compound was reported to exhibit strong DPPH quenching ability with IC₅₀ value of 30.962 ± 1.5317 μ g/mL which has been linked to its anticancer properties as it targets cancer promoting free radicals (Nascimento *et al.*, 2018). This study investigated the antimicrobial activity of the compound, where only two microbes, *P. aeruginosa* and *R. oryzae* displayed susceptibility with zones of inhibition of 18.67 ± 1.76 (Table 14) for both. The good antibacterial activity of compound **59** against *P. aeruginosa* proved by low MIC value (0.52 ± 0.01 mg/mL) it is encouraging and worthy of mention since *P. aeruginosa* is linked to most infections in humans especially in those hospitalised. The bacterial strain has been implicated in pneumonia, surgical site, and blood infections therefore this observation highlights a potential of the compound in development of novel bioactive compounds. Kaempferol-3-O- β -rutinoside **59** has been reported to exhibit good antimicrobial activity against the *Klebsiella pneumoniae* bacterial strains at concentrations of 32-64 μ g/mL, which is comparable to the positive control ofloxacin (Özçelik *et al.*, 2008).

4.6. GC-MS analysis of *Grewia flava* root and twig extracts

The *Grewia flava* *n*-hexane extracts were analysed for their chemical constituents using GC-MS. Constituents were identified by comparing their MS spectra to those of known compounds, stored in the GC-MS library (NIST database, 2012 version). Twenty-three compounds were identified from the root extract while twenty-eight were identified from the twig extract. Fatty acids (48.62%) were the major constituents of twig extract, followed by the triterpenes (16.20%) and the fatty acids esters (12.83%), and similar trend is also observed for the root extract with fatty acids (34.26%) being the predominant constituents followed by triterpenes (26.81%) and fatty acid esters (19.17%). Various bioactive compounds like lupeol, hexadecenoic acid, β -sitosterol, α -amyrin, betulin and phytol were identified. The predominant phytochemical constituents in the root extract were lupeol (23.58%), *n*-hexadecanoic acid (14.58%), oleic acid (10.14%), hexadecanoic acid, methyl ester (9.75%), 9-Octadecenamide, (Z)- (7.87%). From the twig extract major compounds identified were 9,12-octadecadienoic acid (25.85%), Octacosane (17.98%), *n*-hexadecanoic acid (17.34%), hentriacontane (7.88%), hexadecanoic acid, and methyl ester (5.15%). All the compounds detected are presented in Table 20 and 21, for the root and twig extract respectively, along with their retention time (RT),

molecular formula, molecular weight (MW) and peak area percentage (Rel %). The chromatograms of both the root and twig extracts are shown in Figure 22 and 23 respectively.

Lupeol **39** and γ -sitosterol were present in both the twig and root extracts (Table 20 and 21), these bioactive compounds have also been reported from the chloroform extract of *G. lasiocarpa* stem bark (Akwu *et al.*, 2019). Phytol, was only identified from the twig extract, has been reported before from the leaf extracts of *G. nervosa*, *G. pubescens*, and *G. lasiocarpa* (Kumar *et al.*, 2022). Betulin and squalene were identified from the root extract, have been previously reported from the methanol extracts of *G. tiliaefolia* (Badami *et al.*, 2004; Malar *et al.*, 2017). Fatty acid, hexadecanoic acid, which has been identified as one of the predominant compounds in both the root and twig extract has also been reported to be present in the methanol extract of the leaves and stem bark of *G. lasiocarpa* (Akwu *et al.*, 2019) and from the *G. pubescens* leaf extracts (hexane, methanol, and ethyl acetate) (Hamid *et al.*, 2016). β -amyrin was identified from the twig extract, this bioactive compound has also been detected from the methanol extract of *G. tiliaefolia* bark (Malar *et al.*, 2017). A peculiar constituent, chlorpyrifos was identified from the twig extract (Table 21), since this is not a common plant species constituent its presence was attributed to contamination. Chlorpyrifos is a widely used pesticide, contamination is suspected since sampling was done in communal farming area. Therefore, sampling from different locations is recommended to confirm if this compound occurs in the plant extract or it is an environmental contamination.

Literature survey for the biological properties of the identified constituents revealed a spectrum of biological properties for some of the identified compounds. For instance lupeol **39** reportedly has the ability to stimulate programmed cell death in human leukaemia cells, protective effect of hypercholesterolemia associated with renal injury, and antitumor activity (Singh *et al.*, 2018). Palmitic acid (*n*-Hexadecanoic acid) , is known to exhibit nematicide, pesticide, hemolytic 5-alpha reductase inhibitor, antioxidant and hypo-cholesterolemic properties (Gavamukulya *et al.*, 2015). According to previous studies, friedelan-3-one, a terpene, possess anti-inflammatory, antifeedant, and hepatoprotective properties. and it also showed antibacterial activity against *MRSA*, *S. aureus*, *H. pylori*, *S. pneumonia*, and *E. coli*.(Odeh *et al.*, 2016). Betulin, a pentacyclic triterpene alcohol, has been reported to show anticancer, anti-inflammatory, antioxidant, antineoplastic, anti-HIV and phytotoxic properties (Elhassan *et al.*, 2021; S. Kumar *et al.*, 2022).

Table 21: Phytochemical constituents detected from the *n*-hexane extract of *Grewia flava* root

No	Compounds	Rt/min	M. mass	M. Formula	Rel%
1	2-(4-Pyridyl)-4-methylthiazole-5-carboxylic Acid	12.528	220	C ₁₀ H ₈ N ₂ O ₂ S	2.50
2	4,5,6,7-Tetramethyl-2H-isoindole	12.804	268	C ₁₂ H ₁₅ N	0.81
3	Diisobutyl phthalate	13.14	278	C ₁₆ H ₂₂ O ₄	2.15
4	Hexadecanoic acid, methyl ester	13.651	270	C ₁₇ H ₃₄ O ₂	9.75
5	1,4,4,5,7,8-Pentamethyl-6-chromanol	13.781	220	C ₁₄ H ₂₀ O ₂	0.58
6	n-Hexadecanoic acid	14.051	256	C ₁₆ H ₃₂ O ₂	14.58
7	1,1,7,7-Tetramethyl-s-hydrindacene	14.31	214	C ₁₆ H ₂₂	0.48
8	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	15.287	296	C ₁₉ H ₃₄ O ₂	2.85
9	11-Octadecenoic acid, methyl ester	15.34	296	C ₁₉ H ₃₆ O ₂	3.06
10	Methyl stearate	15.557	298	C ₁₉ H ₃₈ O ₂	0.87
11	9,12-Octadecadienoic acid (Z, Z)-	15.669	280	C ₁₈ H ₃₂ O ₂	5.84
12	Oleic Acid	15.722	282	C ₁₈ H ₃₄ O ₂	10.14
13	Octadecanoic acid	15.881	284	C ₁₈ H ₃₆ O ₂	1.19
14	Hexadecanamide	16.075	255	C ₁₆ H ₃₃ NO	4.76
15	Heptacosane	16.198	380	C ₂₇ H ₅₆	0.26
16	9-Octadecenamide, (Z)-	17.645	281	C ₁₈ H ₃₅ NO	7.87
17	Octadecanamide	17.828	283	C ₁₈ H ₃₇ NO	4.50
18	Diisooctyl phthalate	19.128	390	C ₂₄ H ₃₈ O ₄	1.01
19	Squalene	21.174	410	C ₃₀ H ₅₀	0.82
20	γ-Sitosterol	25.445	414	C ₂₉ H ₅₀ O	0.90
21	Friedelan-3-one	25.721	426	C ₃₀ H ₅₀ O	0.90
22	Lupeol	26.627	426	C ₃₀ H ₅₀ O	23.58
23	Betulin	32.268	442	C ₃₀ H ₅₀ O ₂	0.61
	Total				100

Rt-retention time on HP-5MS column, Rel%: relative percent composition based on peak area

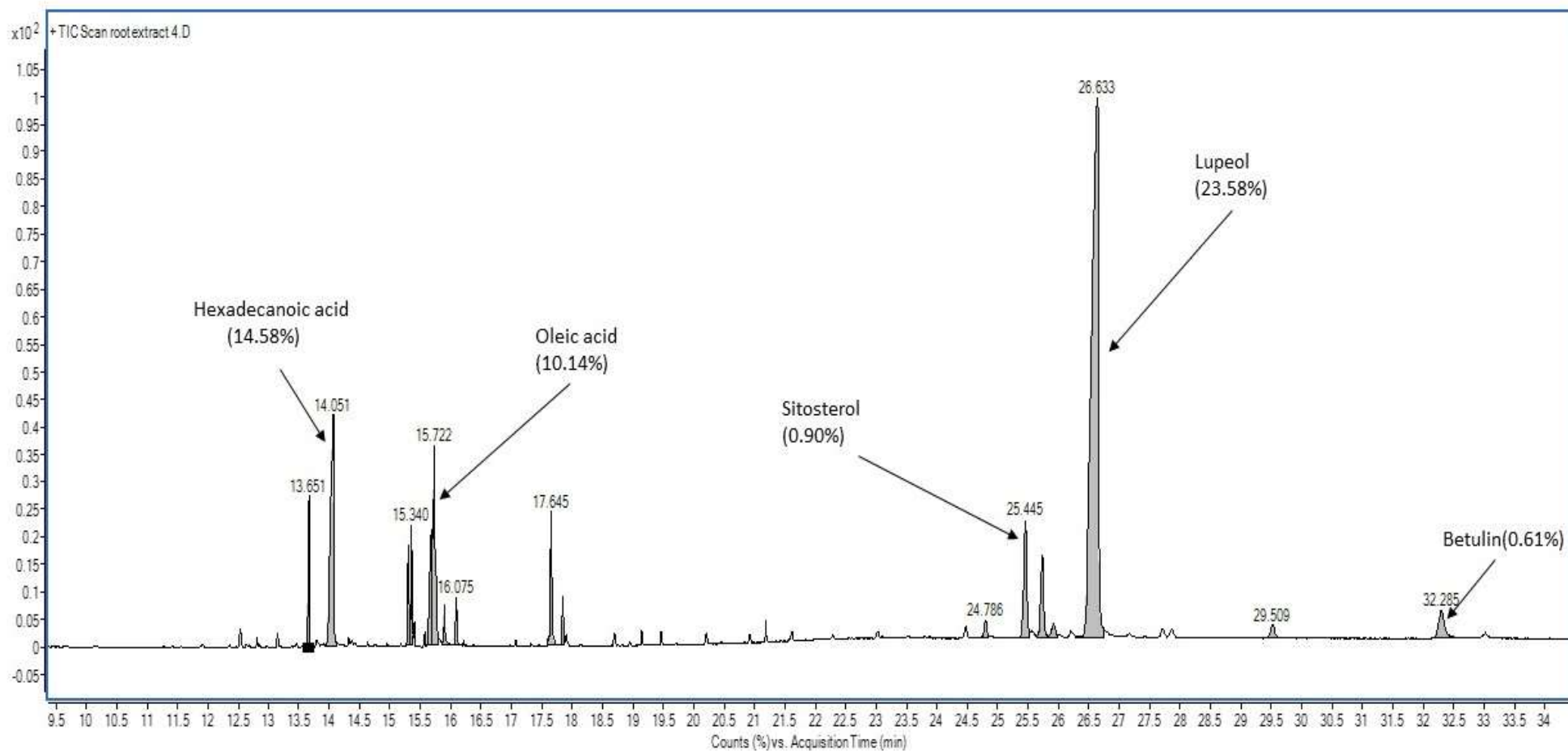


Figure 22: GC-MS chromatogram of the *n*-hexane extract of *Grewia flava* roots

Table 22: Phytochemical constituents detected from the *n*-hexane extract of *Grewia flava* twigs.

No	Compounds	Rt/min	M. mass	M. Formula	Rel %
1	Tetradecanoic acid	11.934	228	C ₁₄ H ₂₈ O ₂	0.63
2	2-Pentadecanone, 6,10,14-trimethyl-	12.839	268	C ₁₈ H ₃₆ O	0.27
3	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	13.139	278	C ₁₆ H ₂₂ O ₄	0.29
4	Hexadecanoic acid, methyl ester	13.651	270	C ₁₇ H ₃₄ O ₄	6.99
5	n-Hexadecanoic acid	14.139	256	C ₁₆ H ₃₂ O ₂	16.99
6	Chlorpyrifos	14.451	349	C ₉ H ₁₁ Cl ₃ NO ₃ PS	5.24
7	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	15.298	294	C ₁₉ H ₃₄ O ₂	2.57
8	9,12,15-Octadecatrienoic acid, methyl ester, (Z, Z,Z)-	15.363	292	C ₁₉ H ₃₂ O ₂	1.94
9	Phytol	15.457	296	C ₂₀ H ₄₀ O	0.51
10	Methyl stearate	15.563	298	C ₁₉ H ₃₈ O ₂	0.45
11	9,12-Octadecadienoic acid (Z, Z)-	15.780	280	C ₁₈ H ₃₂ O ₂	27.19
12	Octadecanoic acid	15.957	284	C ₁₈ H ₃₆ O ₂	3.51
13	7-Methyl-Z-tetradecen- 1-ol acetate	16.204	268	C ₁₇ H ₃₂ O ₂	0.53
14	Eicosanoic acid, methyl ester	17.316	326	C ₂₁ H ₄₂ O ₂	0.37
15	12-Methyl- <i>E,E</i> -2,13- octadecadien-1-ol	17.598	280	C ₁₉ H ₃₆ O	0.39
16	9-Octadecenamide, (Z)-	17.639	281	C ₁₈ H ₃₅ NO	0.39
17	Tetradecane, 2,6,10- trimethyl-	18.686	240	C ₁₇ H ₃₆	0.24

18	Diisooctyl phthalate	19.133	390	C ₂₄ H ₃₈ O ₄	0.29
19	Tetratetracontane	20.192	618	C ₄₄ H ₉₀	0.37
20	Tetracosanoic acid, methyl ester	20.433	382	C ₂₅ H ₅₀ O ₂	0.21
21	Hentriacontane	21.615	436	C ₃₁ H ₆₄	4.01
22	Octacosane	23.056		C ₂₈ H ₅₈	9.48
23	Campesterol	24.474	400	C ₂₈ H ₄₈ O	0.54
24	Stigmasterol	24.856	412	C ₂₉ H ₄₈ O	1.37
25	γ-Sitosterol	25.462	414	C ₂₉ H ₅₀ O	5.53
26	β-Amyrin	25.927	426	C ₃₀ H ₅₀ O	1.27
27	Lupenone	26.192	424	C ₃₀ H ₄₈ O	0.67
28	Lupeol	26.533	426	C ₃₀ H ₅₀ O	6.82
	Total				99.06

Rt-retention time on HP-5MS column, Rel%: relative percent composition based on peak area

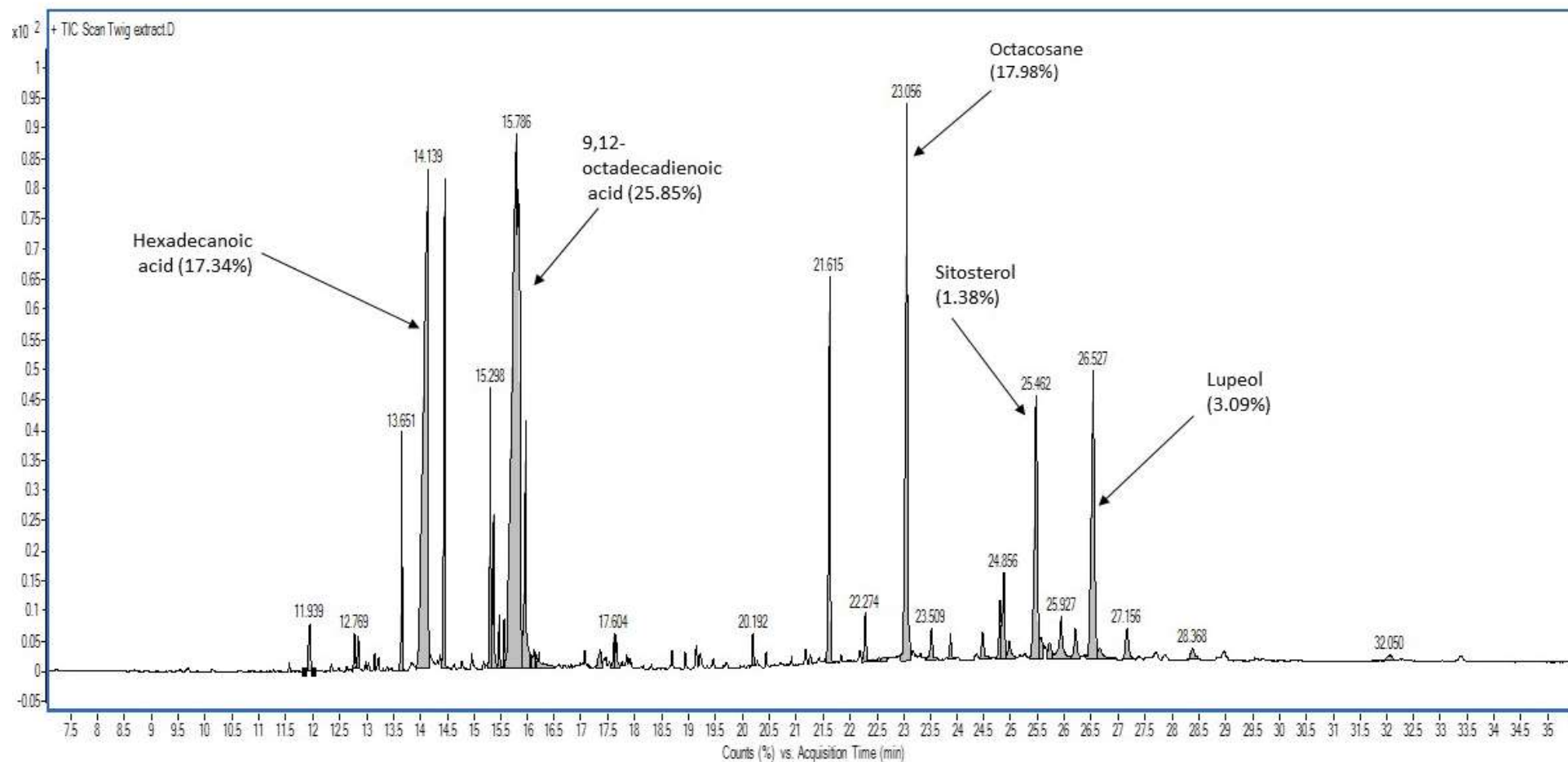


Figure 23: GC-MS chromatogram of the *n*-hexane extract of *Grewia flava* twigs.

CHAPTER 5

5. CONCLUSION

The present study provides scientific evidence to validate the usage of *G. flava* twigs infusion in folklore medicine. Phytochemical profiling of the extracts revealed the presence of different bioactive phytochemical compounds with a broad-spectrum of biological activities similar to other plants species of the same genus. The extracts also demonstrated potent antioxidant and antimicrobial activities against different microorganisms including *E. coli* and *P. aeruginosa* which have been linked to renal infections. Some of the column fractions exhibited strong antioxidant and antibacterial activities compared to the parent extract which shows concentration of the bioactive compounds. Notably the study led to the isolation of eicosyl protocatechuate **62** for the first time from any plant species. In addition to this, β -sitosterol **36**, stigmasterol **5**, and kaempferol-3-*O*-rutoside **59** are reported for the first time from *G. flava*, while lupeol **39** has been reported before. The isolated compounds exhibited moderate antioxidant and antimicrobial properties which shows the potential to be developed into a more potent therapeutic agents. Furthermore, the research findings suggest that *G. flava* twigs can be used as an alternative to the roots since the two extracts exhibited comparable phytochemical profile and bioactivities. The study support the sustainable use of *G. flava* twigs which contributes to the conservation of biodiversity. Overall, the study contributes to the understanding of the potential health benefits of *Grewia flava*.

5.1. Recommendations

The plant species is recommended as a potential plant of medicinal value; however, more studies should be conducted to isolate and identify more bioactive compounds since some of the uncharacterized column fractions exhibited better activity than the isolated compounds. Derivatization of the isolated compounds should be conducted in attempt to enhance the biological properties of these compounds. LC-MS profiling of the polar extract's metabolites will reveal an array of important phytochemicals. More antioxidant assays that could reveal the modes of action of the plant extracts and fractions such as beta-carotene-linoleic acid, and hydrogen peroxide should be done. Since the plant is used as folklore herbal tea, nutritional studies of the plant are recommended to unearth its nutritional benefits. Lastly cytotoxicity of the plant can be determined to establish the safety of the plant extracts and fractions.

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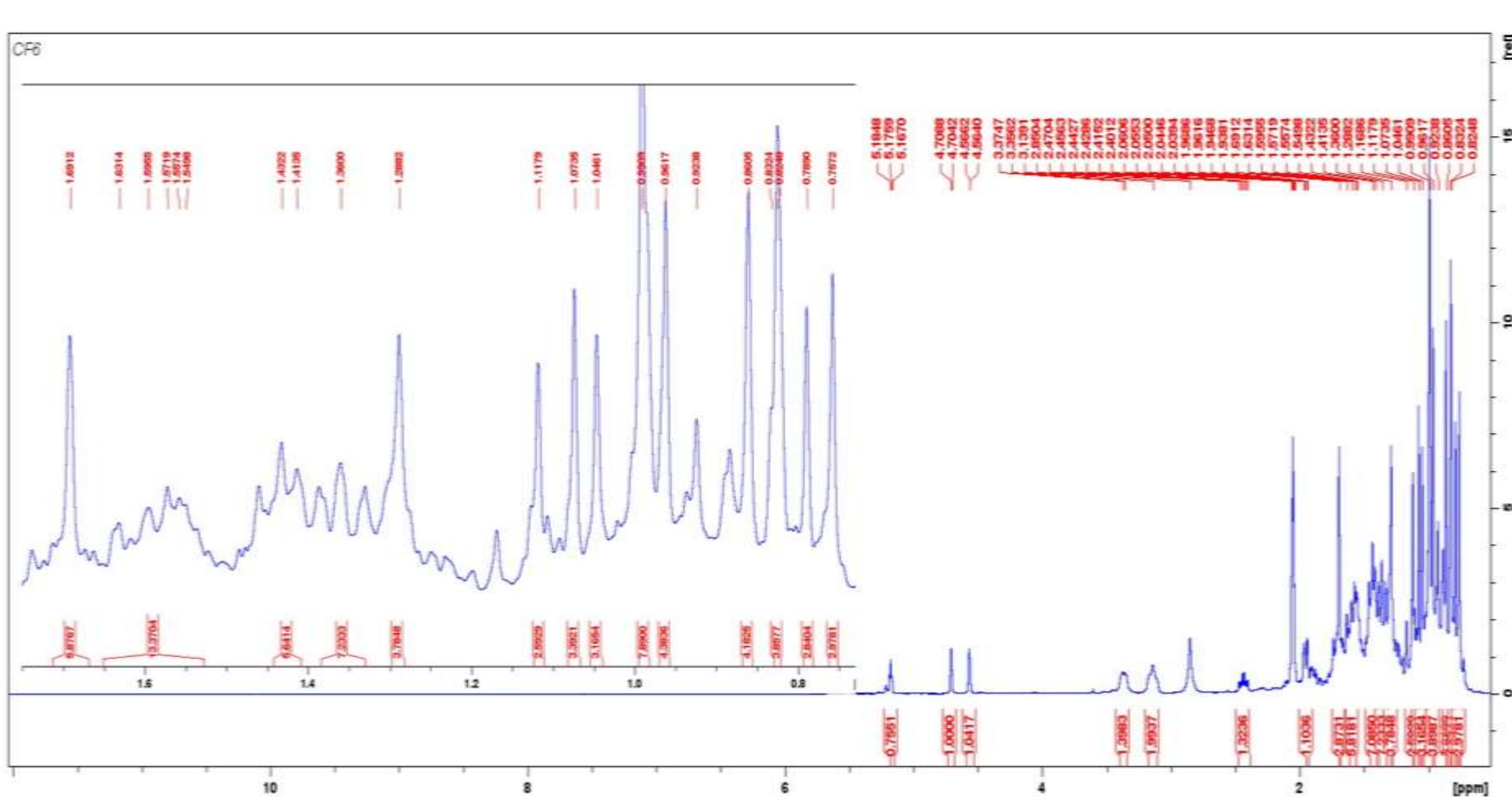
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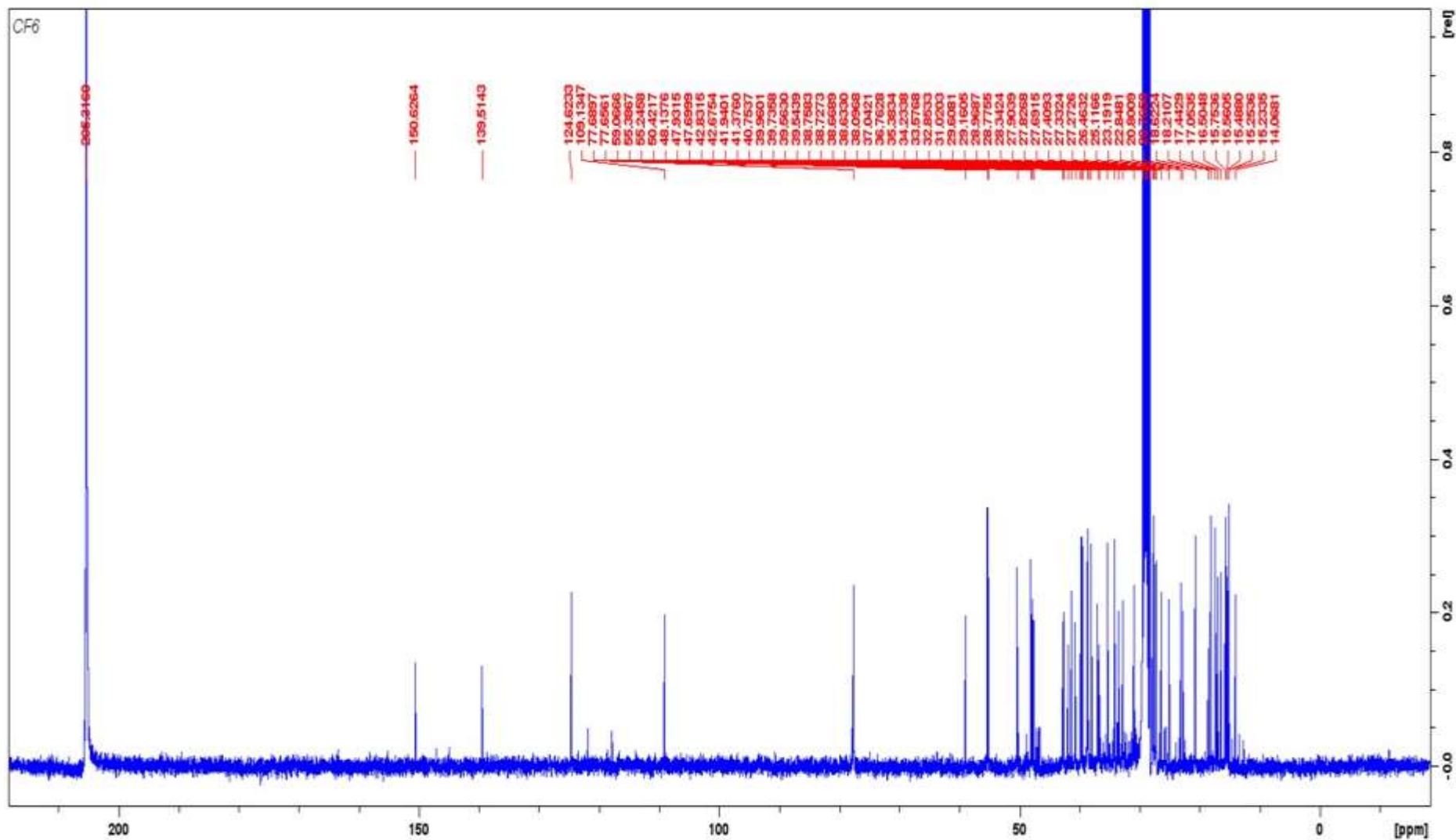
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7. APPENDICES

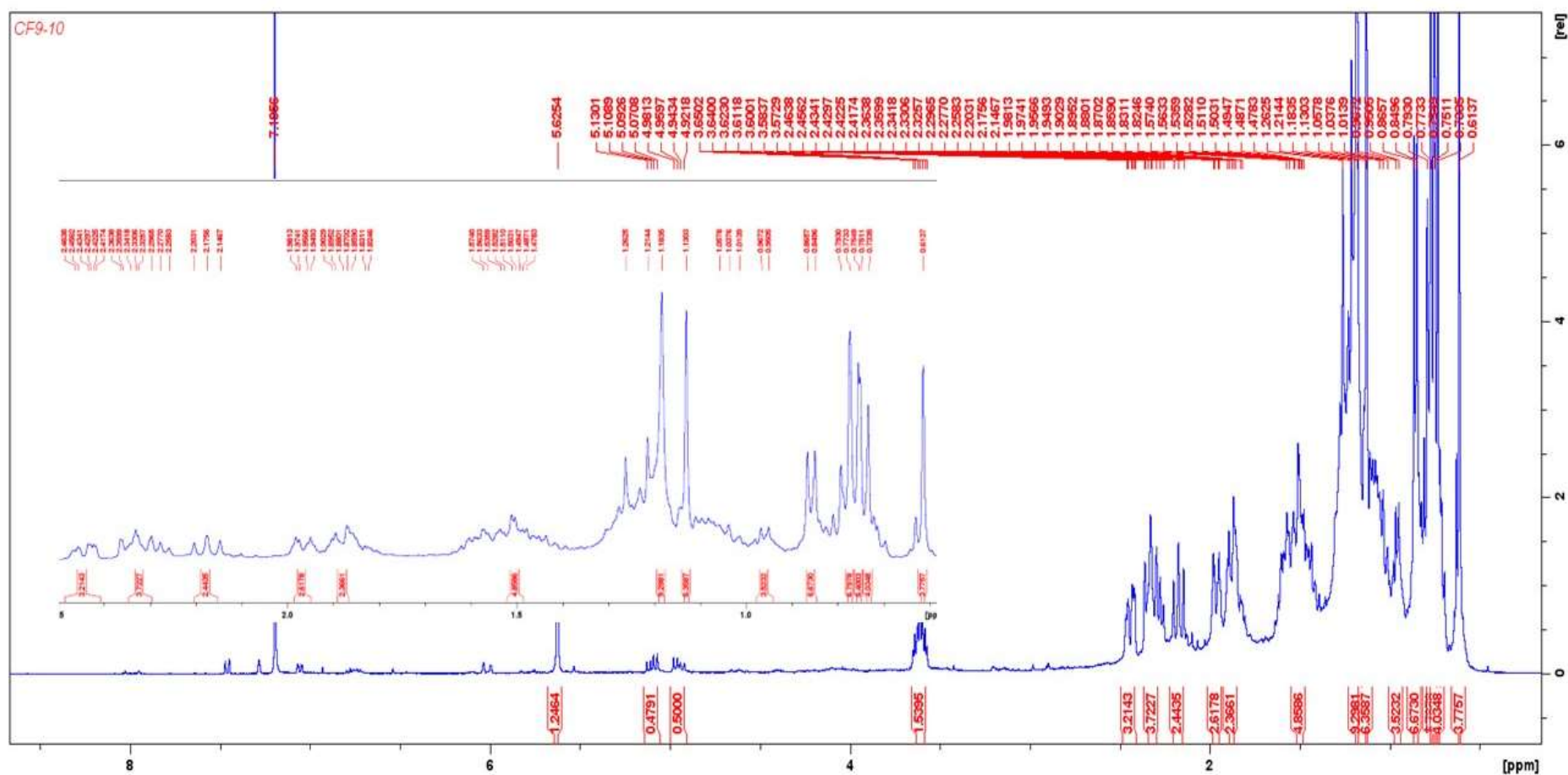
Appendix 1: ^1H -NMR (400 MHz) spectra for fraction 2 (**4** and **39**) in acetone- d_6



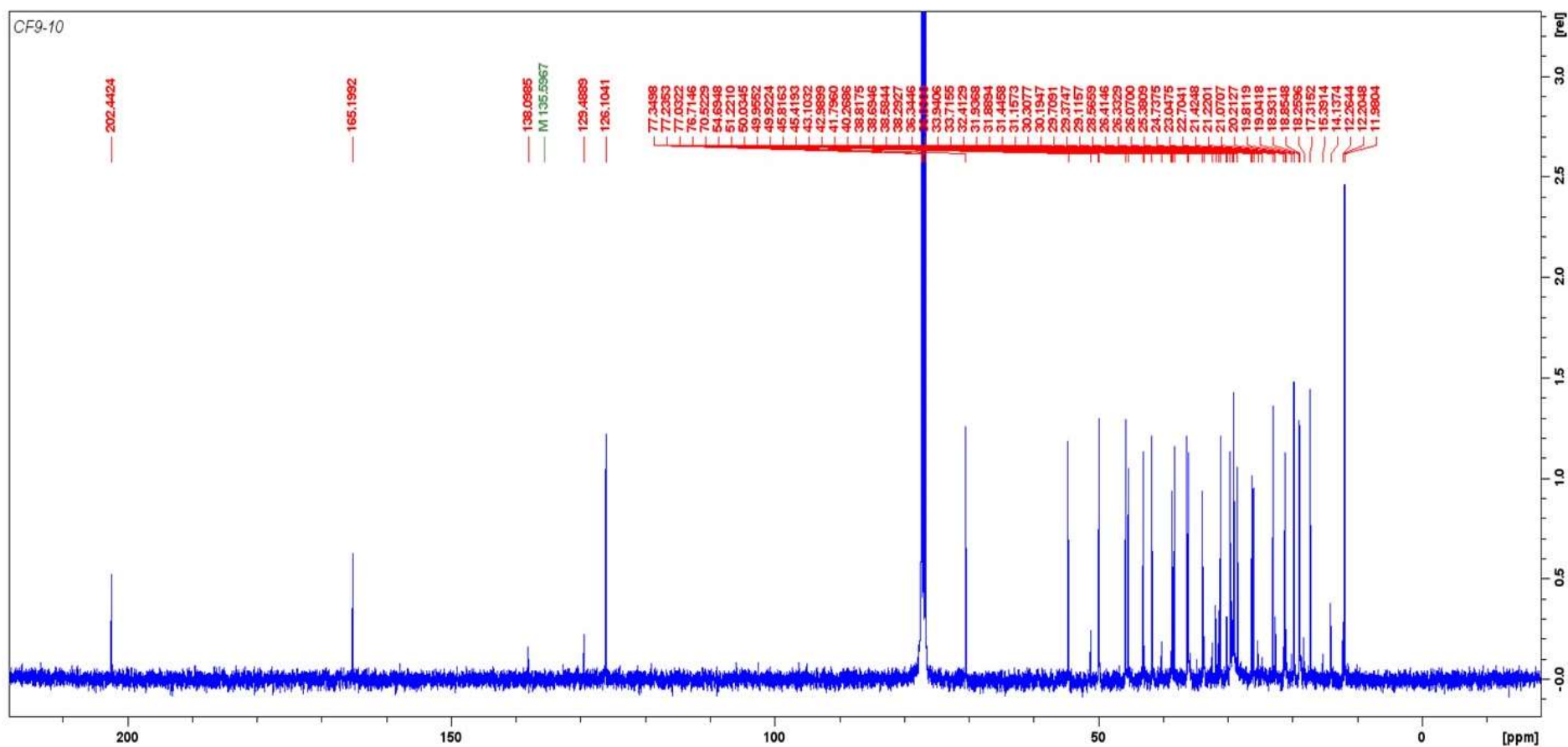
Appendix 2: ^{13}C -NMR (100.6 MHz) spectra for fraction 2 (**4** and **39**) in acetone- d_6



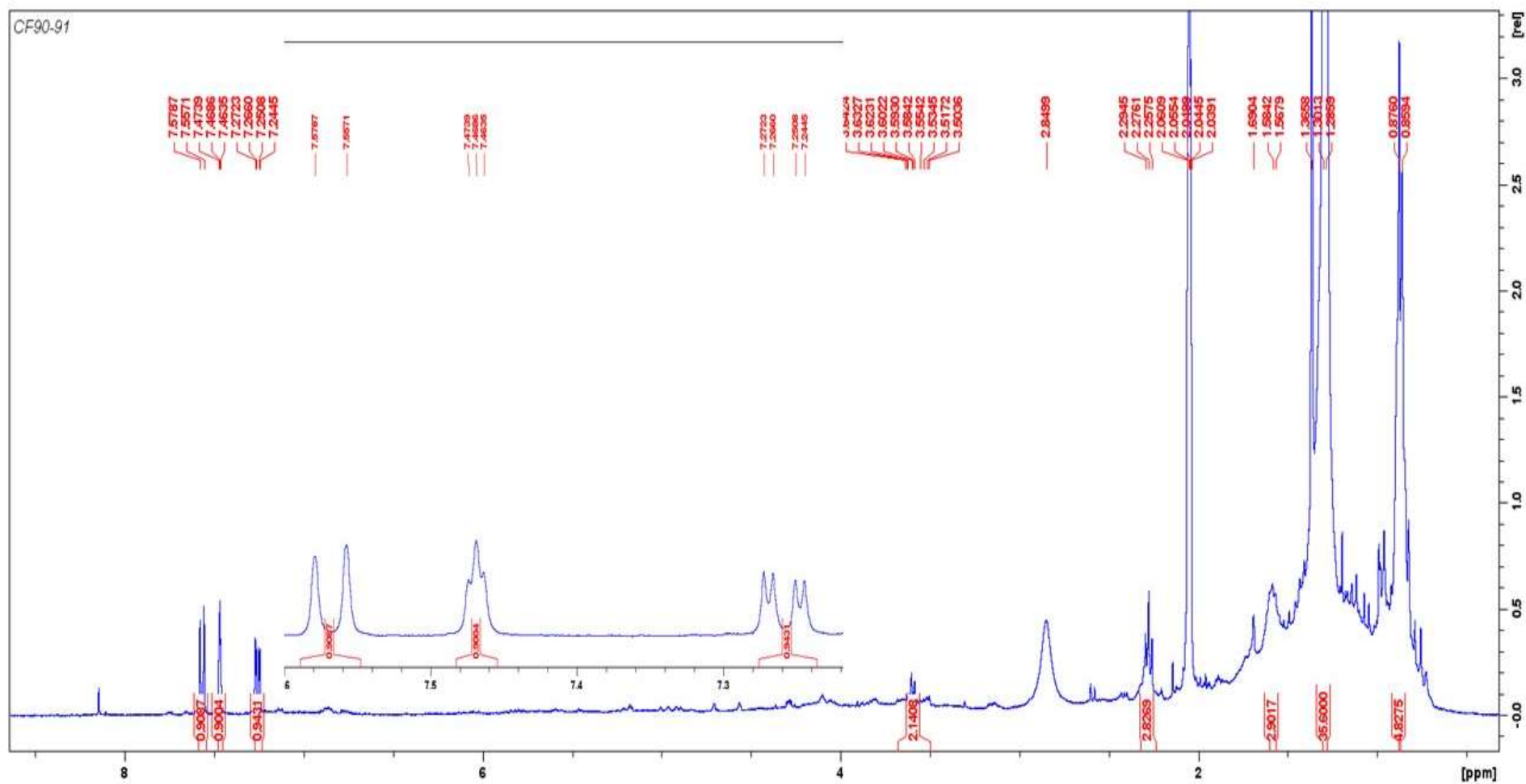
Appendix 3: ^1H -NMR (400 MHz) spectra for fraction 3 (**5**) in chloroform-d



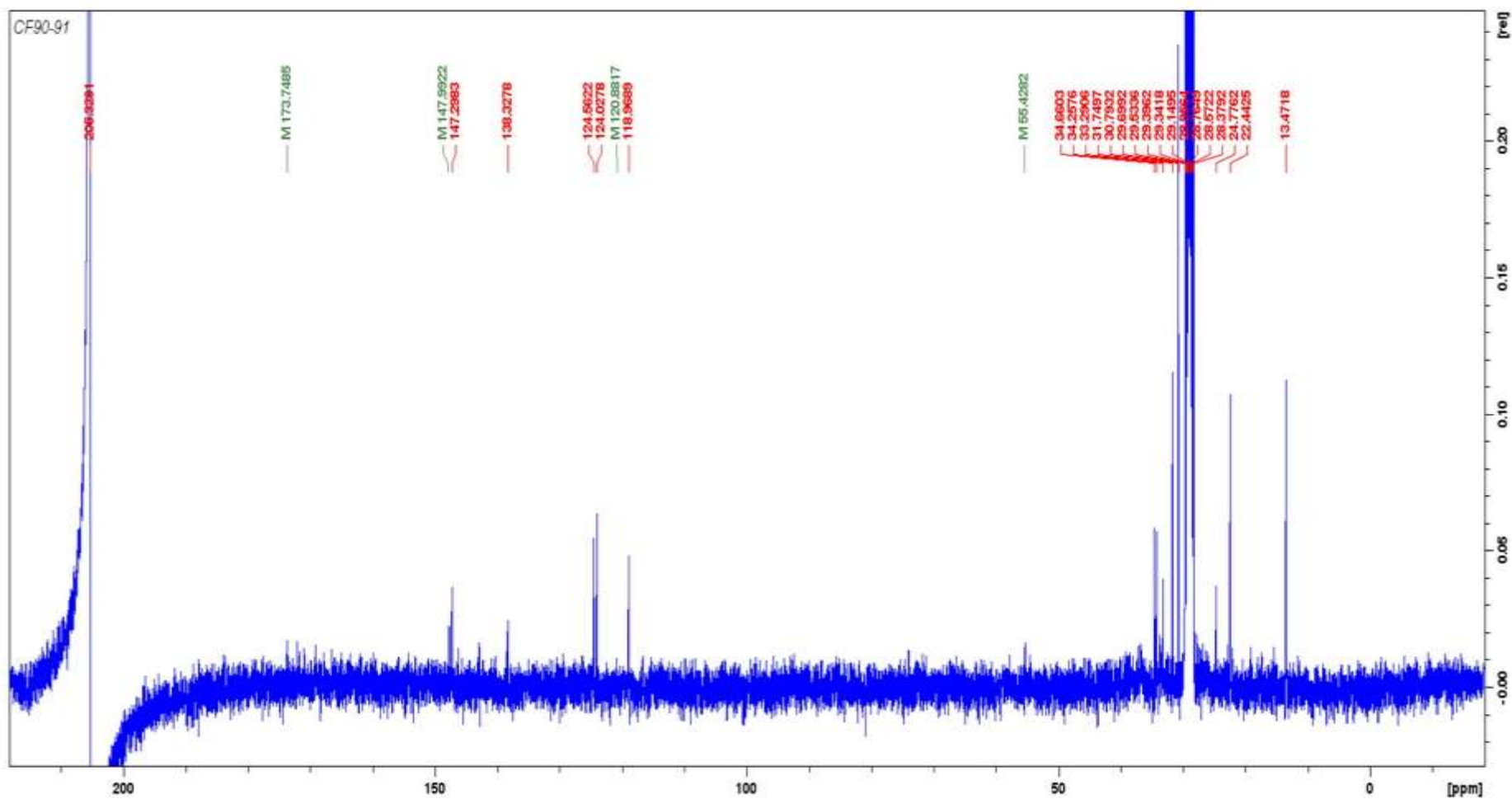
Appendix 4: ^{13}C -NMR (100.6 MHz) spectra for fraction 3 (**5**) in chloroform- d



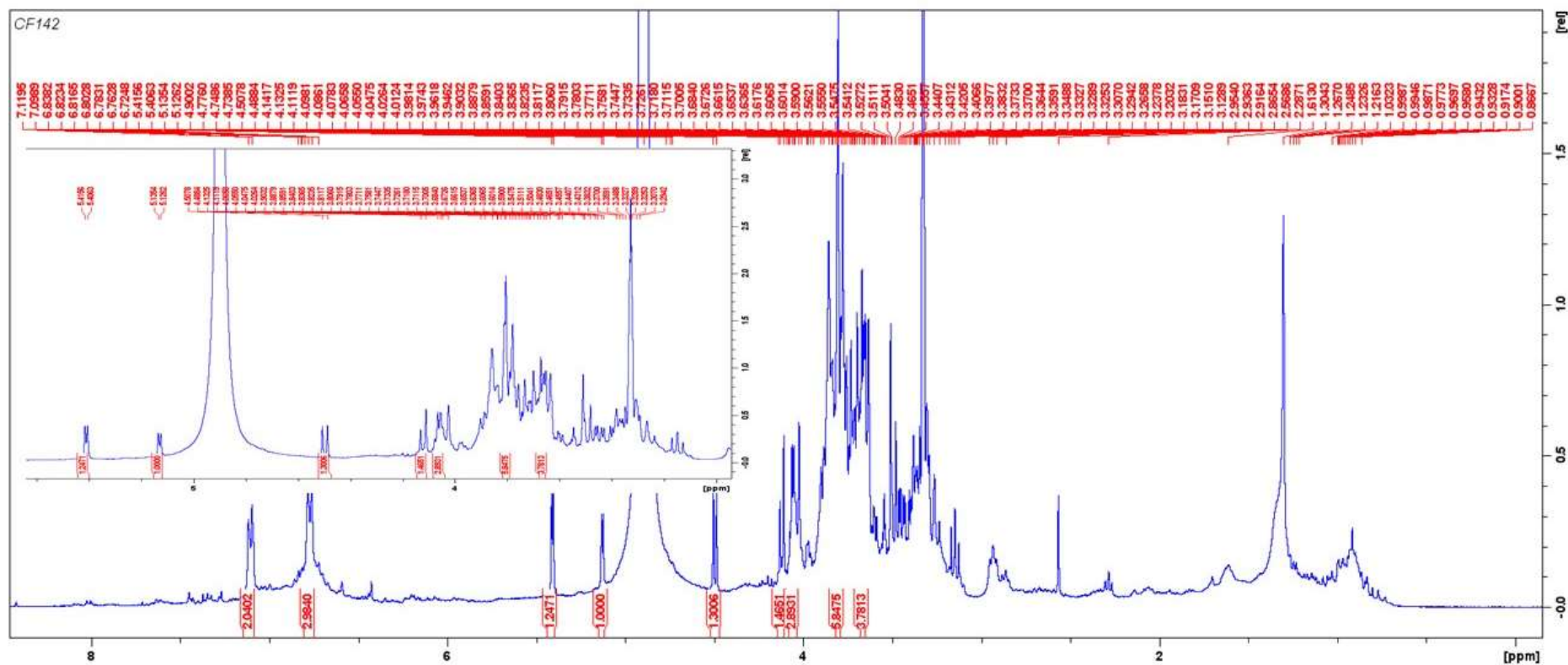
Appendix 5: ^1H -NMR (400 MHz) spectra for fraction 10 (**62**) in acetone- d_6



Appendix 6: ^{13}C -NMR (100.6 MHz) spectra for fraction 10 (**62**) in acetone- d_6



Appendix 7: ^1H -NMR (400 MHz) spectra for fraction 16 (**59**) in methanol- d_4



Appendix 8: ^{13}C -NMR (100.6 MHz) spectra for fraction 16 (**59**) in methanol- d_4

