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RESEARCH PAPER

Phytochemical Characterization and Invitro Evaluation of Antibacterial and Antioxidant Activities of *Dodonaea angustifolia* Leaves in Ambo District, Ethiopia

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Abstract

Medicinal plants contain a wide range of secondary metabolites that can be used to treat infectious diseases. Such natural sources can be a well-intentioned starting point in the search for new drugs since they are rich in phytochemicals that may possess antimicrobial and antioxidant activity. Thus, the objectives of this study were to characterize phytochemicals and to evaluate the antimicrobial and antioxidant activities of leaf extracts of Dodonaea angustifolia in a laboratory. The plant material was collected, dried, and powdered, then subjected to successively extraction with n-hexane, chloroform, and methanol using the maceration technique meanwhile it was concentrated by rotary vapor. The crude extracts were evaluated for their antibacterial activities against standard strains of Staphylococcus aureus (S. aureus), Streptococcus pyogenes (S. pyogenes), Escherichia coli (E. coli), and Klebsiella pneumonia (K. pneumonia). All extracts were assessed with different concentrations (200mg/ml,100mg/ml,50mg/ml,25mg/ml). The chloroform extract has shown superior activities against all the strains at all concentrations. The extracts were also screened for potential antioxidant activity based on the method of competitive reaction of radicals with the antioxidants. It was evaluated using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and the methanol extract of the leaves exhibited the highest antioxidant activity in DPPH. Based on their TLC profile, phytochemical screening, and antibacterial test of the chloroform extract was subjected to fractionation using column chromatography over silica gel. It was eluted with n-hexane containing increasing amounts of ethyl acetate and has resulted in compounds denoted as DA1, DA2, DA3, and DA4. Among these, the pure compound DA3 was characterized by using the spectroscopic technique (1H-NMR, 13C-NMR, and DEPT-135 and FTIR), and the structure was proposed as β–Stigmasteryl (9Z, 12Z) - pentadeca-9,12-dienoate. The most dominant compounds obtained from chloroform crude extracts of gas chromatography-mass spectroscopy analysis were Caryophyllene, β-Phellandrene, and α-Pinenenoic acid. The findings from the phytochemical screening tests of the plant leaves indicate that these leaves are abundant in secondary metabolites. This abundance correlates with enhanced antioxidant activity, which in turn contributes to improved antibacterial activity.

Keywords: Antibacterial, Antioxidant, Phytochemical, Medicinal plants, Dodonaea angustifolia

Introduction

Medicinal plants are regarded as a source of diverse bioactive compounds with possible biological functions. According to the World Health Organization, traditional medicine is the totality of knowledge, skills, and practices based on theories, beliefs, and experiences unique to various cultures. Whether or not these practices can be rationalized, they are utilized in the maintenance of health as well as in the prevention, diagnosis, improvement, or treatment of physical and social imbalances. Traditional medicine relies heavily on real-

world experience and observation, which have been passed down from generation to generation. This body of knowledge is characterized by its cultural specificity and diverse applications, reflecting the unique historical and social contexts from which it arises. While the efficacy of traditional medicine may vary, its significance in the health practices of many communities cannot be understated. In summary, traditional medicine encompasses a wide range of practices that are deeply rooted in cultural beliefs and experiences, playing a crucial role in healthcare for numerous populations around the world (WHO, 2013). Traditional medicine, which is mostly based on plants, has frequently been supported by phytochemical analyses, pharmacological research, and clinical trials, sparking additional research on medicinal plants around the globe. More research is needed to ensure the effectiveness and safety of traditional medicine and methods employed by traditional medicine practitioners and customers. However, occasionally, utilizing conventional medications can be harmful (Beshah *et al*., 2020).

Understanding the specific chemical components of a medicinal plant is essential for refining extraction processes, grasping pharmacological activity, and identifying potential toxicity and drug interactions. For example, Aloe vera is one of the most widely used medicinal plants worldwide. It is highly valued for its healing, anti-inflammatory, and soothing properties, particularly in treating skin conditions such as burns, wounds, and irritation. Beyond skincare, Aloe vera is also used in traditional medicine for its digestive and immune-boosting benefits. Its widespread use spans many cultures, making it one of the most recognized medicinal plants globally (Surjushe, *et al*.,2008). In addition, Dodonaea plants could deliver several medical advantages; native people crosswise the plant's area have used them. It is a popular traditional drug that can be ingested or administered topically to treat a variety of ailments. Recent investigations on phytochemicals have confirmed a positive link between several active phytoconstituent groups and ethnopharmacological use (Anode *et al*., 2018). The leaves of Dodonaea angustifolia are recognized for their rich content of flavonoids and their derivatives, which serve as the primary bioactive compounds. Therefore, the study aimed to extract phytochemicals with antifungal and antibacterial properties from the stem bark of Monsonia angustifolia and the leaves of Dodonaea angustifolia (Beshah *et al*., 2020).

Infectious diseases caused by bacteria, fungi, viruses, and parasites continue to pose a threatening challenge to public health. The indiscriminate and incorrect use of current antimicrobial medications is blamed for the rising problem of bacteria developing resistance to antimicrobial treatments. Drug resistance presents an ever-increasing global health threat that involves all major microbial pathogens and antimicrobial drugs. These are difficult to treat and are responsible for a variety of infectious diseases (Salam *et.al*.,2023). The formation of reactive oxidative species (ROS) can lead to oxidative stress and the destruction of unsaturated lipids, DNA, proteins, and other essential molecules. ROS plays an important role in aging and the development of degenerative and chronic diseases such as atherosclerosis and cancer. The harmful and pathological effects of free radicals can be blocked by antioxidants. Antioxidants inhibit oxidation, reduce the concentration of free radicals in the body, and chelate metal ions to prevent lipid peroxidation (Putri *et al*., 2018). Therefore antioxidants are important in maintaining good health and there is a growing interest in the investigation of the antioxidant activity of secondary metabolites from medicinal plants for compounds with higher potency and lower toxicities than the synthetic ones currently available (Bhatti *et al*., 2015).

Understanding the fundamental chemical procedures involved in the screening of bioactive small-molecule compounds presents a substantial challenge for researchers working on medication development. Synthetic medication's declining efficacy and increased toxicity are exacerbating the problem.

Researchers are now looking to herbal medicines for therapy because it is now recognized that they are crucial in the development of effective treatments (Anand *et al*., 2019). The leaf content of Dodonaea angustifolia is known for the presence of flavonoids and their derivatives, which are the major bioactive components (Beshah *et al*., 2020).

Though traditional medicinal plants in particular Dodonaea angustifolia have long been used across cultures, there is a lack of comprehensive scientific validation regarding their bioactive compounds, efficacy, and safety. Furthermore, the rising global threat of antimicrobial resistance and oxidative stress-related diseases underscores the need for more targeted studies on the phytochemical properties of these plants. Limited research has thoroughly investigated the specific mechanisms by which these plants' bioactive compounds function as antimicrobial agents and antioxidants, especially in comparison to synthetic drugs. Therefore, this research targeted to fill the gap by identifying and characterizing the bioactive compounds of Dodonaea angustifolia for its antibacterial and antioxidant activities. Hence, the expected outcome is to provide scientific evidence supporting its traditional uses, highlighting its potential as an alternative treatment for infectious diseases and oxidative stress-related conditions. Additionally, the study aims to contribute to drug development efforts by exploring the therapeutic potential of this plant in addressing drug resistance and toxicity concerns associated with conventional medications.

Materials and methods

Chemicals and apparatus

Solvents and chemicals used for this study were methanol (98%, Merck), n-hexane (99%, Sigma), Chloroform (95%, Sigma), Silica gel 60-120 mesh, DPPH, DMSO, and ethyl acetate. The other chemicals used for phytochemical screening were 1% ammonia, 5% Ferric chloride, sodium hydroxide, potassium hydroxide, Hydrochloric acid, sulfuric acid, Wagner's reagent (KI and I2), Standard drugs discs such as ciprofloxacin(10µg) disk and L-ascorbic acid(10µg) for comparison of biological activities were purchased from the commercial market as required with standard purity.

Items used for extraction and isolation of compounds include Rota vapor (Labo Rota 4000, Heidolph Instrument), Orbital mini-shaker, Incubator TLC plates, Erlenmeyer flask, Vacuum pumps for filtration (Germany), Watchman filter papers-No1, Funnels, Beakers, Petri dish of medium size, column chromatography larger and smaller size, Test tube, Water bath, Refrigerators, Stand, Round bottom flask, Spatula, Stirrer, Sterile Cotton /Gauze, Analytical balance, Capillary tube, Oven, Ruler, Pencil, Pen, Reagent bottle, Pasture pipette, Dropper, and others.

Plant collection and identification

The plant material, D. angustofolia local name 'Itacha' (Afan Oromo), and 'Kitkita' (Amharic) was collected from their natural habitat, Ambo Town, Ambo referral hospital in January 2023. The plant was identified by the Botanist Mr. Wayessa Fikadu, and the plant was deposited at the Biology Department, College of Natural Computational Science, Ambo University, Ethiopia.

Extraction of compounds

The plant material samples of D. angustifolia were surface rinsed with tap water then with distilled water to remove surface dust and other solid contaminants and then dried under shade. The shade-dried leaves were ground to powder using an electric blender. The powdered plant material was stored in a plastic bag until used for extraction.

The powdered leaves (500 g) were macerated for 48hr through occasional shaking at 230 rpm using an orbital mini-shaker with n-hexane at room temperature. The extract was filtered by Whatman no.1 filter paper mediated by vacuum pumps and concentrated by a rotary evaporator at a reduced temperature (40 °C). The marc left was further extracted using chloroform and

then concentrated by the same procedure. Similarly, the marc from chloroform extraction was further extracted by the same procedure using methanol, filtered, and concentrated to get methanol extract. TLC analyzed the crude extracts (n-hexane, chloroform, and methanol) (Putri *et al*., 2018).

Phytochemical screening

Preliminary phytochemical screening tests on crude extracts and different fractions of the plant were carried out. Qualitative screening of various extracts of the plant was performed for the identification of various classes of chemical constituents in the plant based on the standard procedures with slight modifications as required (Kumar *et al.*, 2014).

Test for alkaloids

Wagner's reagent: A few drops of Wagner's reagent (purchased) were added to each extract of 1mL and observed for the formation of a reddish-brown precipitate which may indicate the presence of alkaloids (Guyasa *et al*., 2018)

Test for anthraquinones

One milliliter of plant extracts from each solvent was added in a dry test tube and 10 ml of chloroform was added and shaken for 5 min. The extract solution was filtered, and the filtrate was shaken with an equal volume of 10%v/v ammonia solution. A pink-violet or red color in the ammoniacal layer indicates the presence of anthraquinones (Anode *et al*., 2018).

Test for glycosides

One milliliter of plant extracts from each solvent was dissolved in 5 ml of glacial acetic acid containing 1 drop of ferric chloride solution. This was then under-layered with 1 ml of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of glycosides (Anode *et al*., 2018)

Test for flavonoids

Alkaline reagent Test: The extracted sample is treated with a few drops of sodium hydroxide solution. The formation of an intense yellow color, which becomes colorless with the addition of dilute acid, indicates the presence of flavonoids (Kumar *et al*., 2014).

Test for phenolic

Ferric chloride test: 1 ml of the extract was treated with a few ml of 5% neutral ferric chloride. A dark blue or bluish-color product showed the presence of tannins or phenolic compounds (Beshah *et al*., 2020).

Test for saponins

About 1 ml of the extract was diluted separately with 20 ml of distilled water and shaken in a graduated cylinder for 15 minutes. A 1 cm layer of foam indicated the presence of saponin (Geetha, 2014).

Test for steroids

Two milliliters of acetic anhydride were added to 2 ml of plant extract of each solvent sample along with 2 ml sulfuric acid. Color change from violet to blue or green indicates the presence of steroids (Kumar *et al*., 2014).

Test for tannins

One milliliter of plant extracts from each solvent was added to 20 ml of water in a flask and a few drops of 0.1% ferric chloride were added and observed brownish-green or blue-black coloration (Beshah *et al*., 2020).

Test for terpenoid

Five milliliters of chloroform and 3 ml of concentrated sulfuric acid were added to 1 ml of plant extract from each solvent in the test tube. The appearance of the monolayer of reddish brown color indicates the presence of triterpenoids (Beshah et al., 2020).

Isolation of compounds

As described earlier, the crude extracts were analyzed by TLC to choose the best mobile phase for column chromatography. Then, 10 g of Chloroform crude was adsorbed on an equal amount of silica gel (mesh size 60-120) and subjected to silica gel chromatography (150 g silica gel) using n-hexane for column packing and elution. The experiment was carried out with an increasing gradient of ethyl acetate in n-hexane as eluent (Table 1). Different fractions were collected and concentrated in a fume hood. The composition of the fractions collected was monitored by TLC and characteristics color. The spots were detected by their UV absorption and fluorescence under 254 and 365 nm respectively, as well as by spraying 1% vanillin in sulfuric acid and iodine chamber for UV inactive constituents. Fractions that showed the same Rf value and the same characteristic color on TLC were combined and further purified. The column was then eluted with the increasing polarity of the solvent system. Purification of compounds was done using column chromatography over silica gel (60-120 mesh). The NMR spectrum was measured using Bruker Avance 400 spectrometer operating at 400 MHz, using $CDCl_3$ as a solvent and TMS as an internal standard for recording chemical shifts in ppm respectively..

Table 1. Chromatographic fractionations of the chloroform leave crude extract.

Solvent system	Ratio of solvent	Fractions collected	Volume (mL) in each fraction
n-hexane	100%	F1-F14	25
n-hexane/EtOAc	90:10	F15-F34	25
n-hexane/EtOAc	80:20	F35-F44	”
n-hexane/EtOAc	70:30	F45-F51	”
n-hexane/EtOAc	60:40	F52–F64	,,
n-hexane/EtOAc	50:50	F65 –F73	“
n-hexane/EtOAc	40:60	F74 –F86	,,
n-hexane/EtOAc	30:70	F 87-F98	,,
n-hexane/EtOAc	20:80	F98 –F110	,,
n-hexane/EtOAc	10:90	F111 –F118	,,
n-hexane/EtOAc	0:100	F119 –F125	,,

Purification of compounds from fractions

TLC analysis of the fractions was done and some of the fractions that have the same TLC profiles were combined. Further purification was done using repeated column chromatography and Preparative Thin-Layer Chromatography (PTLC) to get a single pure compound from the collected fractions.

Characterization of the isolated compound

Column chromatographic separation of the chloroform extracts of the leaf of D. angustofolia has resulted in the isolation of four compounds which are coded as DA1, DA2, DA3, and DA4. The purity of the isolated compounds was monitored by TLC analysis. The structural elucidation of the compound DA1 and DA3 was performed by NMR (1HNMR, 13CNMR, and DEPT-135) at Addis Ababa University, IR spectroscopic at Addis Ababa Science and Technology University and comparison with reported literature data.

Antibacterial activity

Preparation of test solutions

Test solutions were prepared by dissolving 200 mg of each of the crude extracts in 1 mL of dimethyl sulfoxide. The standard microorganisms used for antibacterial activities of the crude extracts were two gram-positive bacteria (Staphylococcus aureus (ATCC25923), and Streptococcus pyogenes (ATCC2228) and two gram-negative bacteria (Escherichia coli (ATCC25922) and Klebsiella

pneumoniae (ATCC700603). These were gained from the Department of Biology, Microbiology Laboratory, Ambo University. Chloramphenicol disk was used as a positive control for the antibacterial susceptibility test whereas a solvent dimethyl sulfoxide served as a negative control test.

Preparation of Inoculum

The microbial stock cultures were maintained at 4 °C on the slopes of Muller Hinton Agar, MHA. Active cultures for experiments were prepared by transferring a loopful of cells from the stock culture to the test tubes containing Muller Hinton broth and incubated without agitation for 24 h at 37 °C. To 5 mL of Muller Hinton Broth, 100µL of culture was inoculated.

Agar well diffusion method

Agar Well diffusion assay with MHA medium was used to analyze the antimicrobial activities of n-hexane, Chloroform and Methanol crude extracts of D. angustifolia leaves. Muller Hinton Agar was melted and then cooled and finally poured into sterile Petri dishes to get a solid plate. Then, the fresh culture of bacteria was used for inoculum preparation. Using a sterile cotton swab, bacterial cultures were swabbed on the surface of sterile agar plates. The dried extracts of test solution (200mg/mL, 100mg/mL, 50mg/mL, and 25mg/mL) in dimethyl sulfoxide were prepared, and Sterilized 5mm wells were inoculated with 50µL of crude extracts of each concentration and placed on the surface of agar plates inoculated with a microbial culture. The diameter of the inhibition zones was measured in millimeters. Triplicate was kept in each case and average values were taken.

Antioxidant Investigation Test

Evaluation of the DPPH radical scavenging assay

Antioxidants play an important role as health protection factors. Scientific evidence suggests that antioxidants reduce the risk of chronic diseases including cancer and heart disease the DPPH assay was used to assess the free radical scavenging activity of crude and isolated pure compounds. With this method the radical scavenging power of an antioxidant compound was measured with the principle of a decrease in absorbance due to the donation of hydrogen from the sample to DPPH radical to produce stable DPPH-H and the color change from purple to yellow was observed as shown in (Figure 1) below (Kalbessa *et al*., 2019).

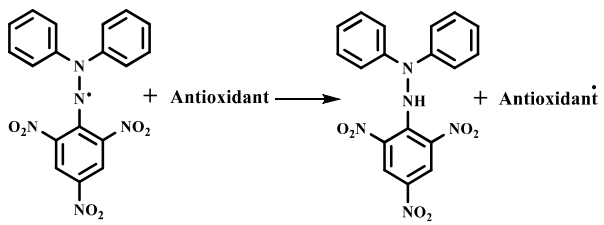


Figure 1.The structure of the DPPH radical and the scavenged radical.

The radical scavenging properties of the n-hexane, chloroform, and methanol extracts of D. angustifolia leaves were determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity as described by (Brand-Williams *et al*., 1995). Sample stock solutions (200 mg/mL) were diluted to final concentrations of 100, 50, 25, 12.5, and 6.25 mg/mL in ethanol. 3mL samples were added to 1 mL of 50mg freshly prepared in 250mL ethanol DPPH. The mixture was shaken and left to stand at room temperature in the dark. After 30 min, absorbance was measured at 517nm against a blank containing ethanol using the UV-Vis spectrophotometers. Ascorbic acid was used as a positive control (as a standard) with a similar concentration to the plant test sample.

$$\% \text{ free radical scavenging activity} = \frac{A-B}{A} \text{ X } 100$$

Where: - A = is the absorbance of pure DPPH in oxidized form

B = is the absorbance of a sample taken after 30 minutes of reaction with DPPH.

Results and Discussion

The yield of Dodonaea angustifolia leaves extract.

Successive extraction of 0.5 Kg leaves powder of Dodonaea angustifolia gave the highest yield with methanol (22 g) followed by chloroform (15 g) and n-hexane (6.5 g) as shown in Table 3

$$\% \text{ Yield} = \frac{\text{Mass of extract}}{\text{Mass of sample}} * 100 \ldots \ldots \ldots . eq1$$

Table 2. Percent yield of different solvent extracts of D. Angustifolia leave.

Solvent system/ Compounds	Mass of extract/Compounds (g)	% Yield
n-Hexane	6.5	1.3
Chloroform	15	3
Methanol	22	4.4

As Table 2 states, among the three solvents used for extraction, the methanol extract gives the highest yield in the crude extract of the leaf plant. This observation is common to the extraction of most bioactive compounds and relates to the ability of methanol to dissolve polar compounds and also some non-polar groups. Besides water, methanol with a polarity index of 5.1 was chosen as the extraction solvent for the extraction of bioactive compounds (Abdisa and Kenea, 2020)

Phytochemical analysis of crude extracts

Table 3. Phytochemical analyses of crude extracts

S. N	Phytochemicals	Type of tests	Color	Solvent system		
				hexane	chloroform	Methanol
1	Alkaloids	Wagner's	reddish brown	+	+	-
2	Anthraquinone	Chloroform	A pink violet	-	+	+
3	Glycosides	Ferric chloride test	brown ring	+	-	-
4	Flavonoids	Alkaline test	intense yellow	+	+	-
5	Phenolics	Ferric chloride test	bluish black	-	+	+
6	Tannins	Ferric chloride test	brownish green	-	+	+
7	Terpenoids	Sulfuric acid	reddish brown	+	+	+
8	Saponin	Water	White	-	+	+
9	Steroids	Sulfuric acid	blue or green	+	+	-

Key: (+) - Phytochemicals were detected, (-) - Phytochemicals were not detected.

Table 3 shows the phytochemical screening test performed on crude leaf extracts of n-hexane, chloroform, and methanol extracts of the D. Angustifolia plant. Most of the secondary metabolites were found in chloroform extract as compared to the other two extracts according to the phytochemical screening result. Glycosides were absent in both chloroform and methanol extracts, but flavonoids alkaloids, and steroids were not only found in methanol extract, again phenols and tannins are found in chloroform and methanol except in n-hexane extract whereas terpenoids were found in n-hexane, chloroform, and methanol extracts possess a variety of biological activities. Flavonoids have been reported to have both antibacterial and antifungal activities. The flavonoids and terpenoids properties in plants have been reported to exert multiple biological effects including antioxidant, free radical scavenging abilities, and reduction in cell damage. Alkaloids present in both stems and leaves play a metabolic role in controlling development in living systems (Geetha *et al.*, 2016).

GC-MS analysis of chloroform crude extract

The results of the GC-MS analysis of chloroform crude extract are tabulated in Table 4, the medicinal roles of each compound in GC-MS are indicated in Table 4 and Figure 2 represents the GC-MS profile of the chloroform crude extract of D. *angustifolia*.

Table 4. The gas chromatography-mass spectrometry profile of crude extract of *D. angustifolia* showing retention time, name of the compound, molecular formula, and peak area

S/No	RT	Name of compounds	Formula	Area
1	4.938	α-Pinene	$C_{10}H_{16}$	1.63E+09
2	5.141	Camphene	$C_{10}H_{16}$	34977900
3	5.487	β-Pinene	$C_{10}H_{16}$	1.78E+08
4	5.589	β-Myrcene	$C_{10}H_{16}$	58487358
5	5.8	α-Phellandrene	$C_{10}H_{16}$	26685672
6	5.876	3-Carene	$C_{10}H_{16}$	46397952
7	6.036	p-Cymene	$C_{10}H_{14}$	87956996
8	6.104	β-Phellandrene	$C_{10}H_{16}$	6.46E+08
9	6.433	γ-Terpinene	$C_{10}H_{16}$	59839074
10	6.594	o-Cymene	$C_{10}H_{14}$	21163491
11	6.864	Linalool	$C_{10}H_{18}O$	81287266
12	7.151	Benzene,1,2,3,5- tetramethyl	$C_{10}H_{14}$	47775103
13	7.675	Isoborneol	$C_{10}H_{18}O$	56734208
14	7.776	Terpinen-4-ol	$C_{10}H_{18}O$	55929327
15	7.827	2-Hexadecanol	$C_{16}H_{34}O$	67012396
16	7.903	α-Terpineol	$C_{10}H_{18}O$	93152935
17	8.435	2-Butanone, 4-phenyl-	$C_{10}H_{12}O$	46922242
18	9.744	α-Copaene	$C_{15}H_{24}$	2.60E+08
19	10.191	Caryophyllene	$C_{15}H_{24}$	4.22E+08
20	10.664	γ-Muurolene	$C_{15}H_{24}$	2.52E+08
21	10.867	di-t-butyl-phenol	$C_{14}H_{22}O$	2.48E+08
22	11.044	γ-Cadinene	$C_{15}H_{24}$	1E+08
23	11.095	δ-Cadinene	$C_{15}H_{24}$	1.67E+08
24	11.619	1-Hexadecanol	$C_{16}H_{34}O$	1.82E+08
25	11.821	Isoaromadendrene epoxide	$C_{15}H_{24}O$	2.28E+08
26	14.009	1-Octadecanol	$C_{18}H_{38}O$	1.81E+08
27	14.752	Neophytadiene	$C_{20}H_{38}$	2.54E+09
28	15.52	Methyl 10,11- tetradecadienoate	$C_{15}H_{26}O_2$	7.94E+08
29	18.037	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	2.04E+08

The details presented in Table 4 show that there are twenty-nine very important molecules such as α-Pinene, Camphene, β-Pinene, β-Myrcene, α-Phellandrene, 3-Carene, p-Cymene, β-Phellandrene, γ-Terpinene, P-Cymene, Linalool, Benzene, 1,2,3,5- tetramethyl, Isoborneol, Terpinen-4-ol, 2-Hexadecanol, α-Terpineol, 2-Butanone, 4-phenyl-, α.-Copaene, Caryophyllene, γ-Muurolene, di-t-butyl-phenol, γ-Cadinene, δ-Cadinene, 1-Hexadecanol, Isoaromadendrene epoxide, γ-Muurolene, 1-Octadecanol, Neophytadiene, Methyl 10,11-tetradecadienoate and Hexadecanoic acid, ethyl ester which have medicinal values such as antioxidant and antimicrobial. These molecules indicate the medicinal roles of Dodonaea recognized by ethno-pharmacological uses.

The medicinal roles of some of the molecules such as α-Pinene, Camphene, α-Phellandrene, 3-Carene, γ-Terpinene, P-Cymene, Linalool, Terpinen-4-ol, α-Terpineol, α-Copaene, Caryophyllene, γ-Muurolene, γ-Cadinene,

Isoaromadendrene epoxide, Hexadecanoic acid, Neophytadiene.

Table 5. The medicinal roles of each molecule shown in the gas chromatography-mass spectrometry profile leaves of Dodonaea angustifolia chloroform crude extract

SN	Compound	Medicinal role	References
1	Camphene	Antibacterial, antifungal, anticancer, antioxidant, antiparasitic, antidiabetic, anti-inflammatory, and hypolipidemic activities.	(Hachlafi *et al.*, 2021)
2	3-Carene	anti-inflammatory, antimicrobial, and anxiolytic effect	(Woo *et al*., 2019)
3	α -Copaene	the cytotoxic, genotoxic/ antigenotoxic, and antioxidant/oxidant activity	(Türkez *et al*., 2014)
4	Caryophyllene,	anti-inflammatory, antiviral, and immunomodulatory properties	(Jha *et al.*, 2021)
5	Isoaromadendreneepoxide	general toxicity, anti-proliferative, antibacterial, and antioxidant properties	(Movahhedin *et al*., 2017)
6	Linalool	antimicrobial, anti-inflammatory, anticancer, antioxidant properties, and central nervous system	(Agrawal *et al*, 2018)
7	γ-Muurolene	antibacterial, antifungal, and antiviral activity	(Perigo *et al*., 2016)
8	Neophytadiene	Antibacterial, antifungal, antioxidant, anti-inflammatory, antidiuretic, antidiarrheal, lowering blood LDL-C level, insulin level booster, antiproliferative, and anti-cancer.	(Pratama *et al*., 2019)
9	P-Cymene	Antioxidant, anti-inflammatory, antiparasitic, antidiabetic, antiviral, antitumor, antibacterial, and antifungal activities, and act as an analgesic, ant nociceptive, immunomodulatory, vasorelaxant, and neuroprotective agent.	(Balahbib *et al*., 2021)
10	Terpinen-4-ol	anxiolytic analgesic, sedative, and anticonvulsant activity	(Nóbrega *et al*., 2014)
11	α-Phellandrene	analgesic, anti-inflammatory, bactericidal, veridical, fungicidal, antiparasitical, anti-malarial, anticancer, antioxidant, anticonvulsant, and antifungal activity	(Lima *et al*., 2012)
12	α-Terpineol	Possess anticonvulsant activity in animal experiments and is widely used in the perfumery, cosmetic, and soap industries. It is also used as a scenting agent in household products (e.g., disinfectant sprays)	(De Sousa *et al*., 2007)
13	γ-Cadinene	noticeable antimicrobial, and antioxidant activities and largely utilized in perfumes, make-up products, and sanitary products, in the food industry as food additives, in dentistry as natural remedies, and in agriculture as green pesticides	(Zeng *et al*., 2011)
14	γ-Terpinene	possess Diuretic, Antioxidant Activity, Ant-diabetic Anti-convulsant activity, Sedative Hypnotic Activity, Antimicrobial Activity, Anti-mutagenic, Anthelmintic activity	(Patel *et al*., 2011)

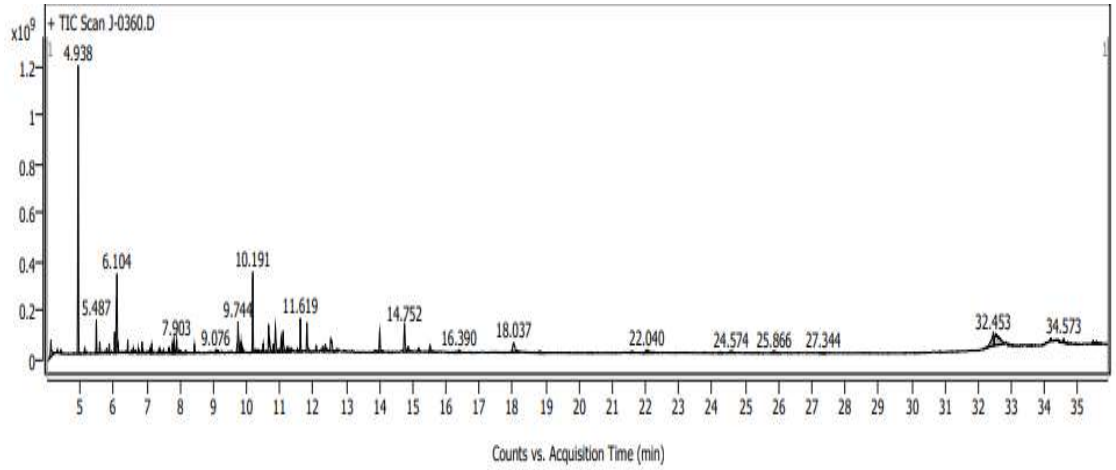


Figure 2: The gas chromatography-mass spectrometry graph of D. angustifolia.

Isolated Compounds

A total of 125 fractions each with 25 mL volume were collected. Among these fractions, based on the TLC profile, a fraction (F18 -34, 65mg) with an RF value of 0.72 labeled as DA -1 and (F 52 - 64,20 mg) with an RF value of 0.64 labeled as DA-2 solid yellow pure compounds were obtained as pure compound based on TLC profile. Again, some fractions (F 111-118) of the first elution were collected for further purification based on their TLC similarities (F 111-118) and combined, mixed with silica gel, and subjected into small columns. Then, elution was continued with n-hexane in ethyl acetate for further purification. Fraction (F 65 -69, 19mg) with RF value of 0.38, labeled as DA-3 Orange semi-solid pure compound obtained by elution with 9:1 ethyl acetate and n-hexane. Fraction (F70-73, 9mg) with an RF value of 0.8, labeled as a DA-4 greenish compound was obtained with 100% ethyl acetate. The level of separation was monitored by TLC analyses. At the same time, the detection of the spot was done using UV (254 and 366 nm).

Structural elucidation of isolated compounds

In the case of the good separation thin layer chromatography, the effect of phytochemical screening and antibacterial activities than the other two solvents, the chloroform fractions of the leaf part of Dodonea Angustifolia was subjected to repeated silica gel column chromatography which led to the isolation of four compounds coded as DA1 (65mg), DA2 (20 mg), DA3 (19mg), and DA4 (9mg), The purity of the isolated compounds was monitored by TLC analysis. Here is the description of the characterization of each compound. Fractions of (F52-64, 20 mg) with RF of hexane /EtOAc 7: 3 DA-2 (yellowish), and DA4 (greenish 100% ethyl acetate). Among these, currently, only two compounds (DA1 and DA3) were analyzed for structural determination. Structural elucidation of these compounds was performed by NMR (^{1}H NMR, ^{13}C NMR, and DEPT-135), FTIR, and GC-MS spectroscopic and comparison with reported literature data. However, the isolated compound DA1 has a good effect on FTIR and no good effects on NMR (1HNMR, ^{13}C NMR, and DEPT-135) spectroscopies.

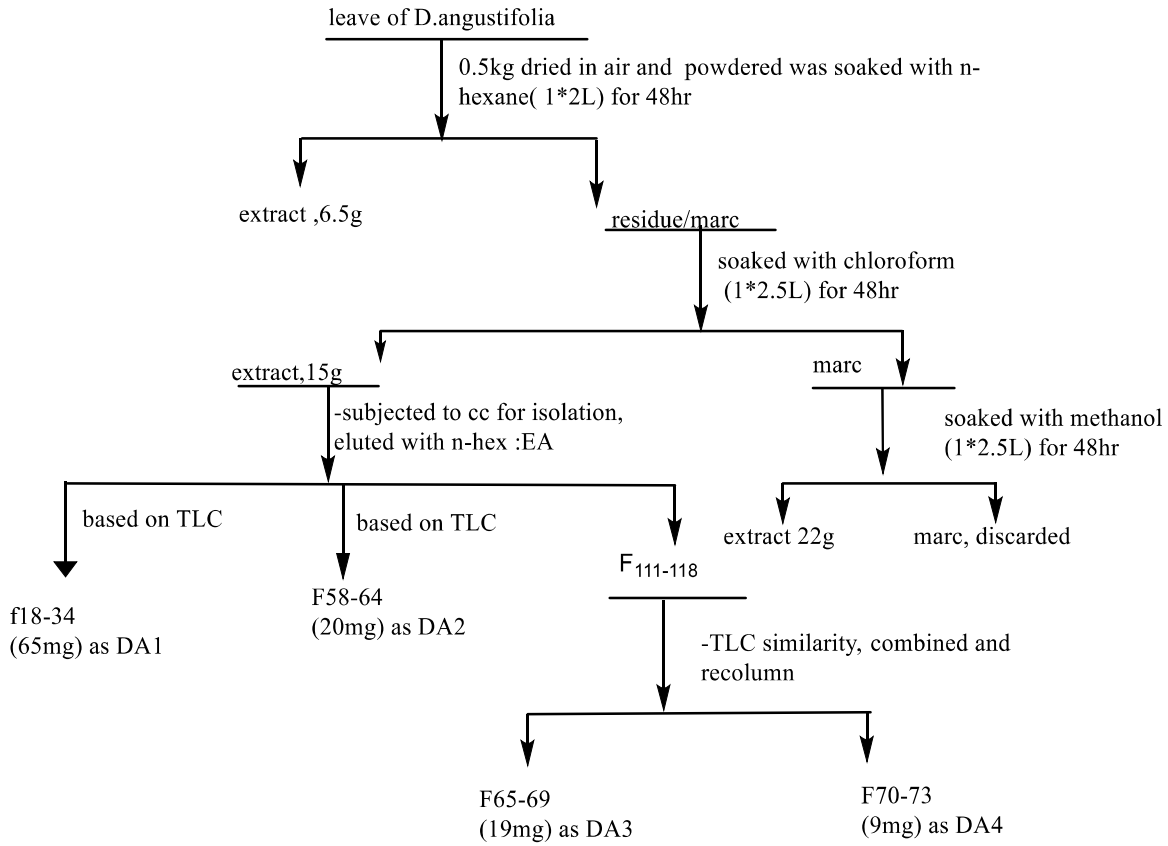


Figure 1: Flow chart for extraction leaves of *D. Angustifolia* and isolation pure compound

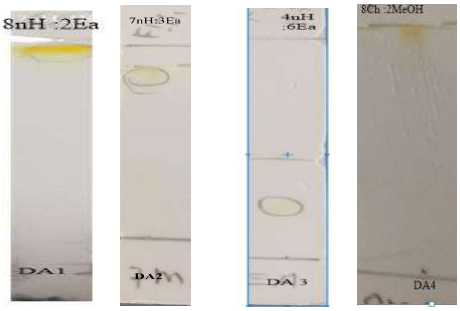


Figure 4: The TLC profiles of isolated compounds from a leave of D. angustifolia.

Characterization of compound DA3

DA3 was obtained as an Orange semi-solid from the CC of the chloroform fraction of the leaf extracts of D. Angustifolia.

FTIR characterization of DA3

The FTIR spectrum of compound DA3 in Table 6 displayed absorption bands indicating the presence of the carbonyl stretch C=O of aliphatic esters (1724 cm^{-1}), the methyl and methylene C-H stretching a sharp peak at 2919 cm^{-1} and 2830cm^{-1}, respectively and 11757 cm^{-1} shows the C–O single bond. The possible frequency ranges of these functional groups are listed in Table 6.

Table 6. Typical Infrared (IR) absorption frequencies of the compound DA3

Observed frequency (cm^{-1})	Possible Frequency Range (cm^{-1})	Function type
24	1730–1715	The carbonyl stretches C=O of aliphatic esters
2919, 2852	2850–2950	the methyl C–H stretching a sharp Peak
1157	1000 – 1300	for C–O single bond
721	675–1000	–CH vibration of an unsaturated part.

NMR characterization of DA3

The ^{1}H-NMR spectrum (Table 7) of compound DA3 showed the presence of resonances for three olefinic methine protons at δ_H 5.35 (^{1}H, dd, H-6), 5.15 (^{1}H, dd, H-22), and 5.02 (^{1}H,d, H-23); a carbonyl proton at δ_H 3.53 (m); and six methyl protons at δ_H 0.70 (s), 0.80 (t), 0.83 (d), 0.84 (d), 1.01 (s) and 1.02 (d).

The ^{13}C NMR spectrum gave 44 signals; containing 7 methyl (CH_3) groups, 18 methylene (CH_2), 15 methine (CH), seven olefinic, and four quaternary carbon atoms which are corroborated by DEPT-135 spectrum further indicating the steroidal nature of the compound. C-NMR spectral data (Table 7) indicated resonances for twenty-nine carbon atoms with the following functionalities: four olefinic carbons (δδ 122.2(C-6), 129.6(C-23), 138.5(C-22), 141.2(C-5)); a carbonyl carbon (δ179.4); seven methine carbons (δδ 31.9 (2 x), 40.5, 50.1, 51.2, 55.9, 56.8); two quaternary carbons (δδ 36.5, 42.19); nine methylene carbons (δδ 21.06, 24.4, 25.4, 28.9, 31.6, 31.9, 37.2, 39.7, 42.27); and six methyl carbons (δδ 12.04, 12.3, 18.97, 19.4, 21.09, 21.2).and for ester bonded unsaturation substituent is 130.6(C-13'), 130.4(C-10'),128.5(C-9'), 128.3(C-12'). These are characteristic resonances of stigmasterol compounds and ester bonded substituent.

The signals at 19.8 and 12.5 correspond to the angular carbon atom at C-15' and C-14' respectively and the peaks at (44.6and 36.9 ppm) and (141.2 and 178.9 ppm) were assignable to saturated and unsaturated quaternary carbon atoms, which are confirmed by the absence of corresponding signals from the DEPT-135 spectrum. The resonance at δ72.4 (C-3) is due to the C-3 β-hydroxyl group further suggesting the compound is a stigmasterol derivative (Haque *et al*., 2019).

From the DEPT-135, a peak at 12.5, 19.8, 21.6, 18.9, and 12.7 ppm is consistent with six methyl carbon atoms. Peaks at 37.7, 32.3, 42.64, 32.4, 20.9, 40.2, 25.2, 29.9, and 25.8 showed methylene carbon atoms. Furthermore, peaks at 72. 4, 32.3, 50.6, 57.3, 56.4, 40.9, 51.7, 31.9, 138.7, and 129.7 indicated the presence of sp^3 and sp^2 carbon atoms.

Therefore, based on spectroscopic data (Table 7) and compared with the reported literature (Haque *et al*., 2019),(Horník *et al*., 2013),(Feyera Fufa *et al*., 2018) the compound was proposed to be a stigma sterol derivative, β–Stigmasterol (9Z, 12Z) - pentadeca-9,12-dienoate.

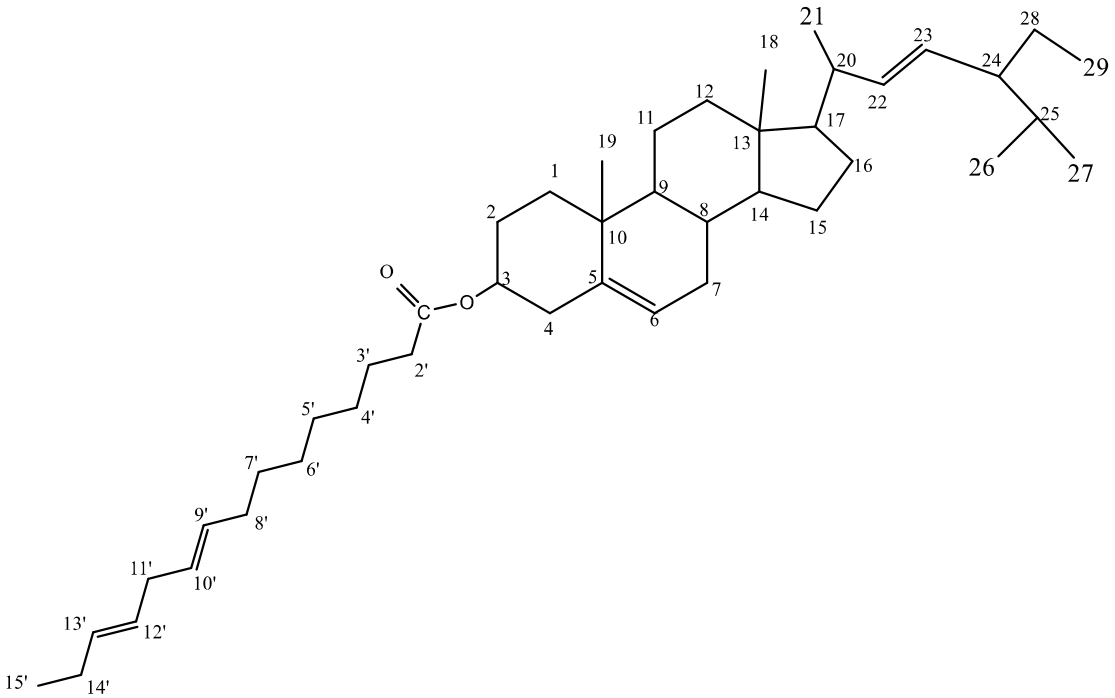
Evaluation of antibacterial activities of crude extract of D.Angustifolia.

Agar well diffusion methods

The antibacterial activities of the crude extract of D. Angustifolia against four bacterial species namely two- gram-positive bacteria Staphylococcus aurous (AT25923), and Streptococcus pyogenes (AT2228). And gram-negative bacteria Escherichia coli (AT25922) and Klebsiella pneumonia (AT700603) as shown in Table 9. All the solvent extracts showed antibacterial activity compared to DMSO (negative control) which had an inhibition zone of 5.0-8.0 mm (size of formed well). Gram-positive bacteria (Staphylococcus aureus (AT25923), and Streptococcus pyogenes (AT2228) showed higher susceptibility than gram-negative bacteria (Escherichia coli (AT25922), and Klebsiella pneumonia (AT700603)) to all extracts. n-hexane extract represented a lower inhibition zone than the two other extracts. The bacterial strains S. aureus and S. pyogenes were more susceptible to chloroform extract than methanol, and n-hexane of D. Angustifolia leaf extract. Whereas the bacterial strains E. coli was highly susceptible to methanol extract than n-hexane and chloroform extracts. However, all bacterial strains were highly susceptible to the reference chloramphenicol (10µg/well) antibiotic drug except K. pneumonia. The bacterial growth inhibition activity of chloramphenicol, n-hexane, chloroform, and methanol extracts was visible in Figure 6 and Table 8 using the agar well diffusion method.

Table 7. ^{1}H and ^{13}C NMR Chemical shifts values (δ) in ppm for compound DA3

Carbon atom	^{13}C-NMR Experimental	^{13}C-NMR Literature	^{1}H-NMR Experimental	^{1}H-NMR Literature	Nature of Carbon
C-1	37.7	37.2			CH_2
C-2	32.34	31.7			CH_2
C-3	72.3	71.8	3.53m,1H	3.51tdd,1H	CH
C-4	42.64	42.3			CH_2
C-5	141.15	140.7			C=C
C-6	122.14	121.7	5.3-5.4 m(5H)	5.35S,1H	CH
C-7	32.36	31.7			CH_2
C-8	30.1	31.9			CH
C-9	50.6	50.2			CH
C-10	36.9	36.16			C
C-11	20.9	21.12			CH_2
C-12	40.16	39.7			CH_2
C-13	44.36	44.56			C
C-14	57.31	56.8			CH
C-15	25.15	24.4			CH_2
C-16	29.36	28.2			CH_2
C-17	56.4	56.1			CH
C-18	12.4	12.06	0.71s ,3H	1.29d,3H	CH_3
C-19	19.82	19.4	0.99s, 3H	0.74d,3H	CH_3
C-20	40.93	40.5			CH
C-21	21.52	21.09	1.03d,3H	0.91d, 3H	CH_3
C-22	138.75	138.4	5.16dd ,1H	4.98 m, 1H	CH
C-23	129.71	129.34	5.03dd,1H	5.14m, 1H	CH
C-24	51.68	51.26			CH
C-25	31.96	34.01			CH
C-26	21.66	21.12	0.88d,3H	0.84,3H	CH_3
C-27	19.42	18.97	0.87d,3H	0.83 d,3H	CH_3
C-28	25.86	25.4	1.15m,2H	1.15 m,2H	CH_2
C-29	12.63	1 2.3	0.82t,3H	0.80 t,3H	CH_3
(C-1′)	179.4	173.25			C
(C-2′)	34.4	34.72	2.33 (m),2H	2.32m, H-2′	CH_2
(C-3′)	26.1	25.6	1.67m,2H	1.67m,2H	CH_2
(C-4′)	29.7	29.7	1.27- 1.33m,2H	1.29-1.33m,2	CH_2
(C-5′)	29.6	29.6	1.27-1.33m,2H	1.27-1.33m	CH_2
(C-6′)	29.6	29.6	1.27-1.33m,2H	1.27-1.33m	CH_2
(C-7′)	29.5	29.5	1.27-1.33m,2H	1.28-1.33m,2H	CH_2
(C-8′)	29.3	27.8	2.02-2.08 m,2H	2.02-2.08 m, 2H	CH_2
(C-9′)	130.2	130.2	5.3-5.4m,1H	5.3-5.4m,1H	CH
(C-10′)	128.5	1227.9	5.3-5.4m,1H	5.3-5.4m,1H	CH
(C-11′)	25.9	25	2.78t,2H	2.79t,2H	CH_2
(C-12′)	128.3	127.8	5.3-5.4m,1H	5.3-5.4m,1H	CH
(C-13′)	130.4	130.15	5.3-5.4m,1H	5.3-5.4m,1H	CH
(C-14′)	27.6	27.4	2.02-2.08 m,2H	2.02-2.08 m, 2H	CH_2
(C-15′)	14.1	14.1	0.83 m,3H	0.83 m,3H	CH_3



B–Stigmasteryl (9Z, 12Z) - pentadeca-9, 12-dienoate

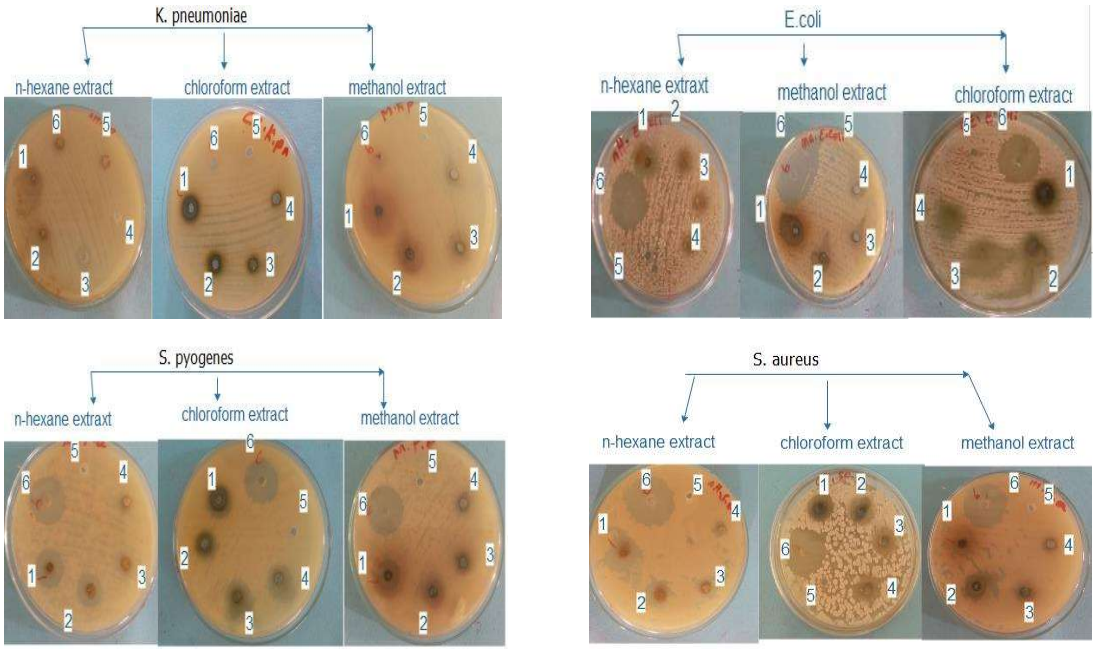
Figure 5: Proposed structure of compound DA3

Table 8: Inhibition activity of leave extracts of *D. Angustifolia* against bacterial species

SN	Extracts and compounds	Conc. (mg/ml)	Mean zone of inhibition			
			Gram-positive bacteria		Gram-negative bacteria	
			S. aureus	*S. pyogenes*	*E. coli*	*K. pneumonia*
1	n-Hexane	200	22	17.5	14.5	10.5
		100	18	15	12	8.75
		50	15	13.5	10	6.5
		25	12.	11	8	6
2	Chloroform	200	23	20.5	16.5	12
		100	20.5	18.25	14	9.75
		50	18	16	12	8.25
		25	16.5	12	9.5	8
3	Methanol	200	21.5	19	18.5	12 .5
		100	20	17.5	15.35	10.5
		50	18.75	15	13	9
		25	16.5	13	10	7
4	Chloramphenicol		30	28	24.5	8
5	DMSO		8	8	8	5

Table 9 showed the antibacterial activity of different solvent extracts of *D. Angustifolia* leaves against S. aureus, and E. coli, and was found to display inhibition zones within the range between 6 and 23 mm. The highest zone of inhibition was found in the chloroform extract (23,20.5, 16.5, and 12 mm) followed by methanol extracts (21.5, 19, 18.5, and 12.5 mm) while there was a lower zone of inhibition in the n-hexane extract (22, 17.5, 14.5 and 10.5

mm) against S. aureus, S. pyogenes, E. coli and K. pneumonia respectively. The reference chloramphenicol (10µg/well) gave the highest inhibition zone compared to all extracts against all bacterial strains (S. aureus, S. pyogenes, and E. coli, except K. pneumoniae).



Key: 1=200mg/mL concentrations, 2= 100mg/mL concentrations, 3=50mg/mL concentrations, 4=25 mg/mL concentrations, 5 is DMSO and 6 is chloramphenicol in each extract

Figure 2: Antibacterial activities of solvent extracts

This study also showed that chloroform extract was relatively more effective than methanol extract against S. aureus and E. coli bacterial strains. While the methanol extract was to be relatively more effective than chloroform and n-hexane extracts against bacteria. So, the crude chloroform extract of the D. Angustifolia plant was selected as the best candidate for column chromatography to isolate bioactive compounds. Because the inhibition zone of chloroform extract was the highest value in the three bacteria species except in bacterial strains except K. pneumonia (Table 9).

Antioxidant Investigation test

DPPH Radical Scavenging Assay

The scavenging of DPPH radicals by antioxidants is due to their hydrogen or electron-donating ability. In an alcoholic solution, DPPH gives a strong absorption band at 517 nm. When the odd electron becomes paired off in the presence of a scavenger, the absorption reduces and the DPPH solution is decolorized as the color changes from deep violet to light yellow (Perigo, 2016).The percentage of DPPH radical scavenging activities of the n-hexane, chloroform, and methanol extracts of D. angustifolia left at different concentrations are shown in Figure 7, and Table 9.

Table 9. Absorbance of standard ascorbic acid, solvent extracts, and % Radical scavenging activities of *D. angustifolia*

Conc. (mg/L)	**Absorbance (λ_{max} =517nm)**				**DPPH % inhibition**			
	Ascorbic acid	n-hexane	chloroform	methanol	Ascorbic acid	n-hexane	chloroform	methanol
6.25	0.093	0.625	0.519	0.479	90.58	36.7	47.42	51.47
12.5	0.09	0.576	0.447	0.441	90.90	41.64	54.70	55.32
25	0.087	0.412	0.346	0.315	91.25	58.26	64.94	68.10
50	0.083	0.321	0.305	0.257	91.60	67.45	69.12	74.00
100	0.067	0.223	0.254	0.213	93.21	77.4	74.27	78.42
200	0.053	0.205	0.201	0.170	94.63	79.23	79.64	82.78
Control	0.987							

Table 9 showed the absorbance of DPPH free radical was decreased by the addition of crude n-hexane, chloroform, and methanol extracts as its concentration increased. Due to the presence of a higher amount of phenolic in chloroform, methanol and flavonoids in n-hexane and methanol extract play a great role in decreasing the absorbance of DPPH free radical by transfer of proton or the greater value absorbance of DPPH free radical, they should be confirmed that the lower the scavenging activity of the plant extract. The lowest absorbance of DPPH free radical was found in the extract of methanol followed by chloroform extract, while the highest absorbance was obtained in n-hexane extract in all concentrations studied. As shown electron to DPPH radical (scavenging its free radicals), and DPPH radical became quenched. Therefore, its absorbance was decreased if the solvent extract constituents of concentration increased from lower to higher. This evidence was the violet color of DPPH was completely fading into yellow as its antioxidant-like property molecules (extracts) concentration increased(Kiren *et al*., 2014).

The percentage of inhibition was observed in all the antioxidant models that free radicals were scavenged by the all-crude extracts in a concentration-dependent manner up to the given concentration. The data were compared to those obtained with the reference compound L-ascorbic acid. The entire investigation of crude showed moderate to high radical scavenging activities compared with the absorbance of the control (0.987). The DPPH radical scavenging activities at 200µg/mL were 82.78%, 79.23%, 79.64%, and 94.63% for methanol, n-hexane, chloroform, and ascorbic acid respectively. From the result view methanol extracts have comparable antioxidant effects which could be attributed to their hydrogen-donating ability. The others of n-hexane and chloroform show moderate antioxidant tendency when compared to methanol.

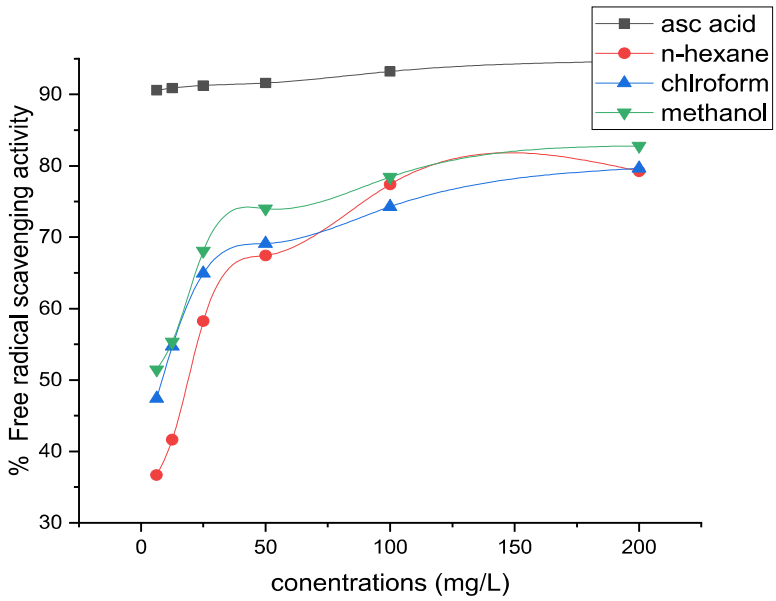


Figure 3: Percentage of free radical scavenging activity of the standard and the solvent extracts.

CONCLUSIONS

In this study, the phytochemical screening tests showed that extracts of leaves of the plants were rich in flavonoids, saponins, phenols, tannins, terpenoids, steroids, etc., and Chromatographic isolation of the crude extracts compound coded as DA3 has characterized as β–Stigmasteryl (9Z, 12Z) - pentadeca-9,12-dienoate. The results revealed that the highest zone of inhibition was found in the chloroform extract (23, 20.5, 16.5, and 12 mm) against S. aureus, S. pyogenes, and E. coli and K. pneumoniae respectively. The reference chloramphenicol (10µg/well) gave the highest inhibition zone compared to all extracts against all bacterial strains (S. aureus, S. pyogenes, and E. coli, except K. pneumoniae). The radical scavenging activities at 200 µg/mL of crude extract were promising with percentage activities of methanol (82.78%) being the strongest inhibition values and attributed to its hydrogen-donating ability compared to the standard.

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