Original Research Article

EVALUATION OF SESQUITERPENES IN THE *BARK EXTRACTS* OF *PILIOSTIGMA RETICULATUM* (DL.) HOCHST AND *CLEISTOPHOLIS PATENS* (BENTH.) ENGL & DIELS AND *THEIR ANTIBACTERIAL ACTIVITIES ON SHIGELLA DYSENTERIAE AND STREPTOCOCCUS PYOGENES.*

ABSTRACT

Aim: The study was designed to characterize the sesquiterpenes obtained from the bark extracts of *Piliostigma reticulatum* and *Cleistopholis patens* and subsequently screened the extracts for their antibacterial activities. **Methodology:** Ground forms of the stem bark of *P. reticulatum* and *C. patens* were obtained and extracted with ethyl acetate. The extracts obtained from both plants were screened for antibacterial activities against *Shigella dysenteriae and Streptococcus pyogenes* using the agar well diffusion method. Moreover, fractions obtained from the crude extracts were also assayed for antibacterial efficacy using the disc diffusion method. The chemical components of the extracts were identified using Gas chromatography and mass spectra (GC-MS). **Results:** The result obtained revealed that *P. reticulatum* extract had antibacterial activities on *S. dysenteriae* with zones of inhibition ranging from 6mm - 14mm. While the extract had lesser inhibitory effect against *S. pyogenes* with zones of inhibition of 10mm and 8mm at concentrations of 100 mg/mL and 80 mg/mL respectively. However, *C. patens* was effective against *S. pyogenes* with zones of inhibition of 18mm, 16mm, 14mm, 13mm, and 8mm at concentrations of 100, 60, 40, 20 and 10 mg/mL respectively. Crude extracts

exhibited higher activity compared to purified fractions against test organisms. A number of five (5) Sesquiterpenes (azulenes, alpha and beta pinene, Germacrene D, Limonene, and Farnesol) were identified from both extracts.

Conclusion: The presence of these sesquiterpenes in *P. reticulatum* and *C. patens* could be responsible for the antibacterial activities on the test organisms (*S. dysenteriae and S. pyogenes*) evaluated in this study and this justifies their usage in folkloric medicine. Hence, the extracts obtained from *P. reticulatum* and *C. patens* could be considered as a potential and rich source of antibacterial agent to control infections posed by the test organisms (*S. dysenteriae and S. pyogenes*).

KEYWORDS; Sesquiterpenes, Fatty acids, purification, antibacterial, GC-MS, NMR, *Shigella dysenteriae* and *Streptococcus pyogenes*.

1. INTRODUCTION

Medicinal plants are known to produce phytochemicals that are responsible for their pharmaceutical activities.SesquiterpenesC15 terpenoid is built from their isoprene units andare phytochemicals which are abundant in higher plants and in many living systems [1]. They are essential oils, and they act as irritant when applied topically and when consumed, they irritate the gastrointestinal tract [2]. In nature, sesquiterpenes plays an important role in plant defense, as antibacterial, antiviral, antifungal and insecticides. The biological activity of sesquiterpenes is connected to the presence of α-β- unsaturated γ- lacton ring [3].

Cleistophlis patens is a tree up to 27m high. The infusion of its leaves is used as febrifuge and vermifuge [4]. *Cleistopholis patens* (Benth) Engl and Diels belongs to the family Annonaceae. It is sometimes used as food preservatives [5]. The long narrow leaves held in one plane on slightly drooping branches give this tree a distinctive appearance. The leaves are shiny on their upper surface when fresh. This species can grow to a diameter of 50 cm. In Nigeria, the bark is used to treat typhoid fever and menstrual irregularities[6]. The root bark is used as vermifuge, leaf infusion or decoction is administered against hepatitis, fever, trypanosomiasis, and rheumatic arthritis [5].

Piliostigma reticulatum (DL.) Hochst. (common name; Yoruba: ‘abafin’, Hausa: ‘kalgo’, Igbo: okpoatu’) belongs to the family Leguminosae - Caesalpiniaceae and is found in the savannah region of Nigeria. It is a tree,

occurring up to 30ft in height with an evergreen, dense spreading crown [7]. It is used traditionally in the treatment of diarrhea. Tea from the leaves to treat colds, bark is astringent and used against diarrhoea and dysentery; leaves and bark have haemostatic and antiseptic properties. It is also used to cure ulcers, boils, wounds and syphilitic cancer. Other medical uses are against coughs, bronchitis, malaria, hepato-biliary ailments, hydropsy, sterility, rachitis and kwashiorkor. This study investigates the presence of sesquiterpenes in in the plants (*P*. *reticulatum* and *C. patens*).

2. MATERIALS AND METHODS

2.1 Plant Collection, Preparation and Extraction

The stem bark of both plants were collected from Ibadan, Oyo state, Nigeria. They were washed with tap water, air-dried at room temperature, pulverized into powder with the aid of grinding machine (type N model) and subsequently subjected to extraction procedures using Ethyl acetate as described by Owoyemi and Oladunmoye [8]. The extracts were evaporated to dryness and the percentage yield calculated. The extracts were reconstituted in 30% DMSO before being used to assay for antibacterial activities on test organisms.

2.2 Standardization of Test organisms (*Shigella dysenteriae* and *Streptococcus pyogenes*) for Antibacterial Analysis

A 0.5 McFarland standard was prepared by the addition of 0.5mL of 1% Barium chloride ($Bacl_2$) to 99.5ml of 1% Sulphuric acid (H_2S0_4) solution. The turbidity of the 0.5 McFarland standard was used to calculate bacterial counts in broth culture after 24 hours of incubation at 37^OC in order to obtain a standard bacterial suspension of $1x10^8$ bacterial cells that was used for the antibacterial assay [9,10].

2.1 Antibacterial Activities of Plant (Bark) extracts

The agar well diffusion method described by Perez [11] was employed in evaluating the antibacterial activities of the crude extracts of *P. reticulatum* and *C. patens* extracts against *Shigella dysenteriae* and *Streptococcus pyogenes*, while the purified extracts were evaluated against the test bacteria using the disk diffusion method as described by Zaidan [12]. Sterile Blank discs were impregnated with 0.5ml of the purified extracts and placed on the surface of inoculated agar plate containing the test inoculum and incubated at 37^OC for 24h.

The extracts were also allowed to pass through purification procedures using column chromatography; fractions obtained were subjected to spectra analysis using Nuclear Magnetic Resonance (NMR) and Gas Chromatography and Mass spectra (GC-MS).

2.2 Evaluation of the Nuclear Magnetic Resonance (NMR) of purified fractions

The purified sample was placed in an inert solvent (deuterochloroform ($CDCl_3$), deuterium oxide (D_2O), carbon tetrachloride (CCl_4) or deuterated dimethyl sulphoxide (DMSO)] and the solution was placed between the poles of a powerful magnet. The different chemical shifts of the proton according to their molecular environments within the molecule were measured in the NMR apparatus relative to a standard, usually tetramethylsilane (TMS). Chemical shifts were measured in ppm units, where

$\delta = \Delta V X\ 10^6/V_{op}$

ΔV being the difference in absorption frequency of the sample and the reference compound (TMS) in Hertz units and V_{op} in the operating frequency.The intensity of the signals may be integrated to show the number of protons resonating at any one frequency. Each chemical shift value corresponds to a set of protons in a particular environment. The intensity of each signal signifies the number of protons of each type.

2.3. Gas Chromatography and Mass spectra (GC-MS) analysis of purified fractions

Ethyl acetate extracts of Stem bark of *Piliostigmareticulatum* and *Cleistopholis patens* were analyzed with the aid of GC- MS analyzer (Perkin Elmer Gas Chromatography- Mass Spectrum). On Elite-1 column the date was generated. The carrier gas helium (99.999%) was used at flow rate of 1 ml per min in split mode (10:1). 8µ of sample was injected to column at 250^{O}C injector temperature. Temperature of oven starts at 60^{O}C and hold for 6min and then it was raised at rate of 10^{O}C per min to 300^{O}C without holding. Holding was allowed for 6 min at program rate of 5^{O}C per min. Temperature of ion sources was maintained at 240^{O}C. The injector temperature was set at 250^{O}C and detector temperature was set at 260^{O}C.The mass Spectrum of compounds present in samples was obtained by electron ionization at 70eV and detector operates in scan mode 50 to 600Da atomic units. A 0.5 seconds of scan interval and fragments from 50 to 600Da was maintained.

3. RESULTS

3.1 Antibacterial activities of crude extract.

The result of the antibacterial test revealed that*P. reticulatum* exhibited considerably high antibacterial activities against *S. dysenteria*with zones of inhibition of 14, 12, 08and 06mm at concentrations of 100, 60, 40, and 20mg/mLof extracts respectively. *C. patens* had no antibacterial activity against *S. dysenteriae*. *P. reticulatum*

showed a lesser activity against *S.pyogenes* with zones of inhibition of 10mm and 8mm at concentrations of 100mg/ml and 60mg/ml of extract respectively. However,*C. patens* extract had high inhibitory activities on *S. pyogenes* with zones of inhibition ranging from8 to 18mm at different concentrations that ranged from10 mg/mL to 100 mg/mL. (Table 1.)

Table 1. Antibacterial activity of the Ethyl acetate extracts of *P. reticulatum* and *C. patens*

	P. reticulatum (zones of inhibition in mm)					*C. patens* (Zones of inhibition in mm)				
Plants/ Conc (mg/mL)	100	60	40	20	10	100	60	40	20	10
Shigella dysenteriae	14	12	08	06		-	-	-	-	
Strepococcus pyogenes	10	08	-	-	-	18	16	14	13	8

3.2 Antibacterial activity of purified extracts.

Two purified fractions from *P. reticulatum* and three fractions from *C. patens* were subjected to antibacterial analysis and result is presented in Table 2. The result showed a marked difference in the result of the crude extracts and the purified fractions. The extracts of *P.reticulatum*at 100mg/mL had antibacterial activities against *Streptococcus pyogenes* with inhibitory zone of 10mm as compared to the purified fraction ($Pr3_6$ and $Pr5_6$) which had a zone of inhibition of 6 and 4mm respectively. The crude extract was active against *S. dyseteriae* with a zone of inhibition of 14mm while the fractions ($Pr3_6$ and $Pr5_6$) showed zones of inhibition of 12mm and 8mm respectively. The crude extract of *C. patens* was not active against *S. dysenteriae* but had antibacterial activities on *S. pyogenes* with a zone of inhibition of 18mm whereas the purified fractions showed inhibition zones of between 6, 8 and 4mm respectively.

Table 2. Antibacterial activity of purified fractions of *C. patens* and *P. reticulatum* at 100mg/mL

Organisms	Plant extracts / Zones of inhibition in mm at 100mg/mL of extracts						
	Piliostigma reticulatum			*Cleistopholis patens*			
	Crude	Fraction $Pr3_6$	Fraction $Pr5_6$	Crude	Fraction Cp7	Fraction Cp 12	Fraction $Cp12_3$
Shigella dysenteriae	14	12	8	-	-	-	-
Streptococcus pyogenes	10	6	4	18	6	8	4

Legend; - = no activity

3.3 NMR Spectra of purified fractions of *Cleistopholis patens*;

Cp7; Cp7 contains alkanes, amides, alkylether and alcohol overlap at peak 3.545. At peak 3.333, aromatic ketones were observed. Also, at peak 2.978, aromatic ketones and amines were discovered. Thiols, alkylether and amines were present at peak 2.469. Moreover, at peak 2.112, allylic protons and propagylic protons were observed. Epoxides were found at peak1.526 (fig 1).

Cp12: Fraction Cp7 was found to contain at peak 3.490 an alkyl ether, and at peak 2.596, amines were discovered while allylic protons were observed at peak 1.733 (Fig 2).

$Cp12_3$; The fraction $Cp12_3$ was found to contain alkyl esters at peak 3.897 and at peak 2.530,epoxide ether, amines and acetylester thiols were observed (Fig 3).

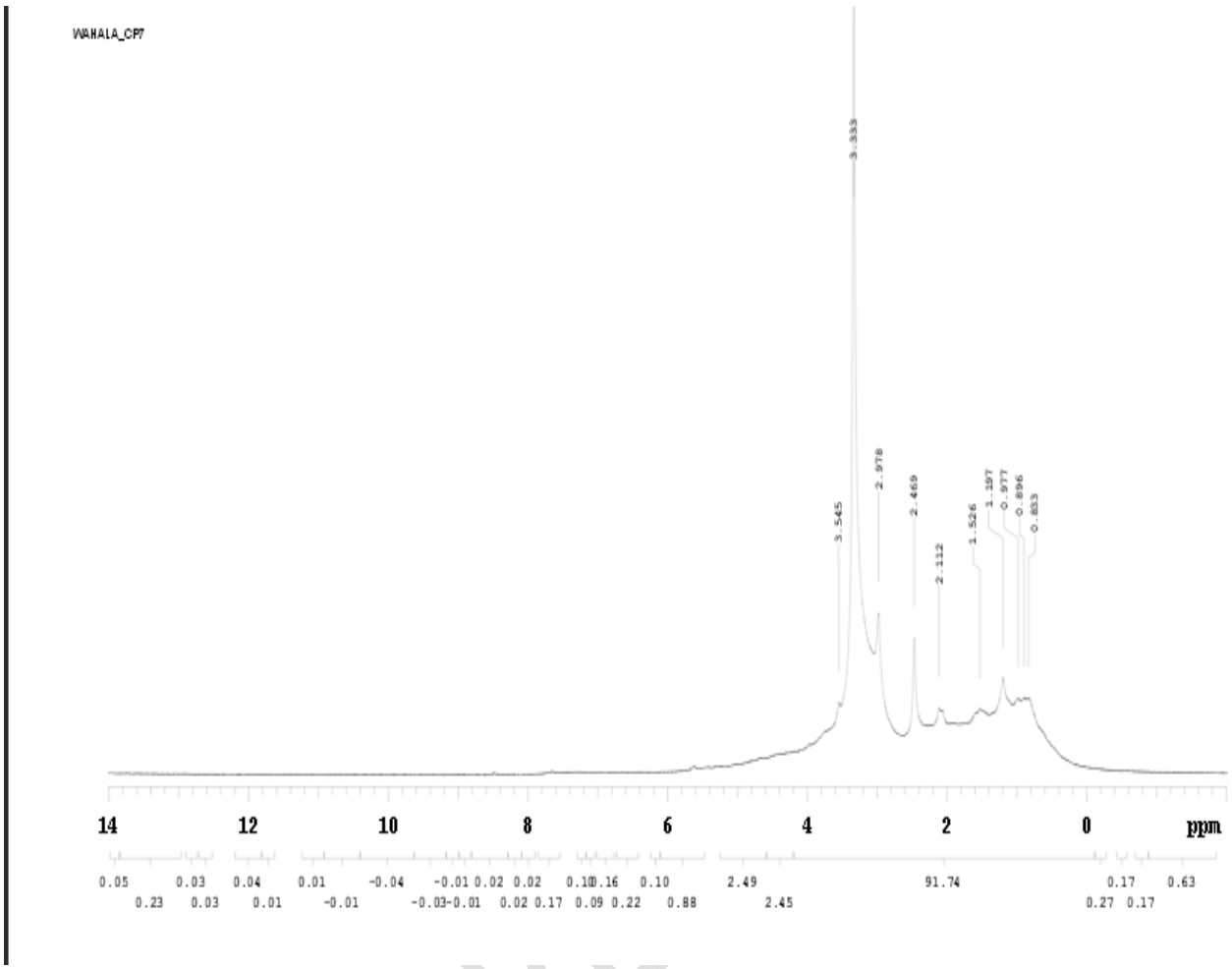


Fig 1. NMR spectra of fraction Cp7 of *Cleistopholis patens*

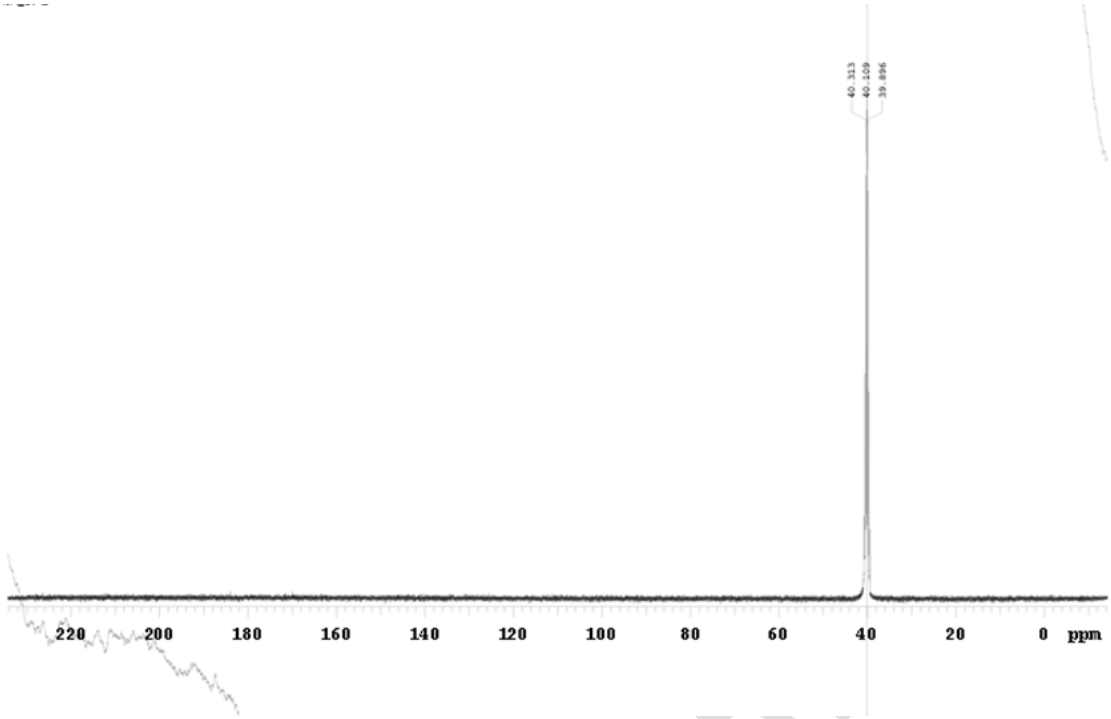


Fig 2. NMR spectra of fraction Cp12 of *C. patens*

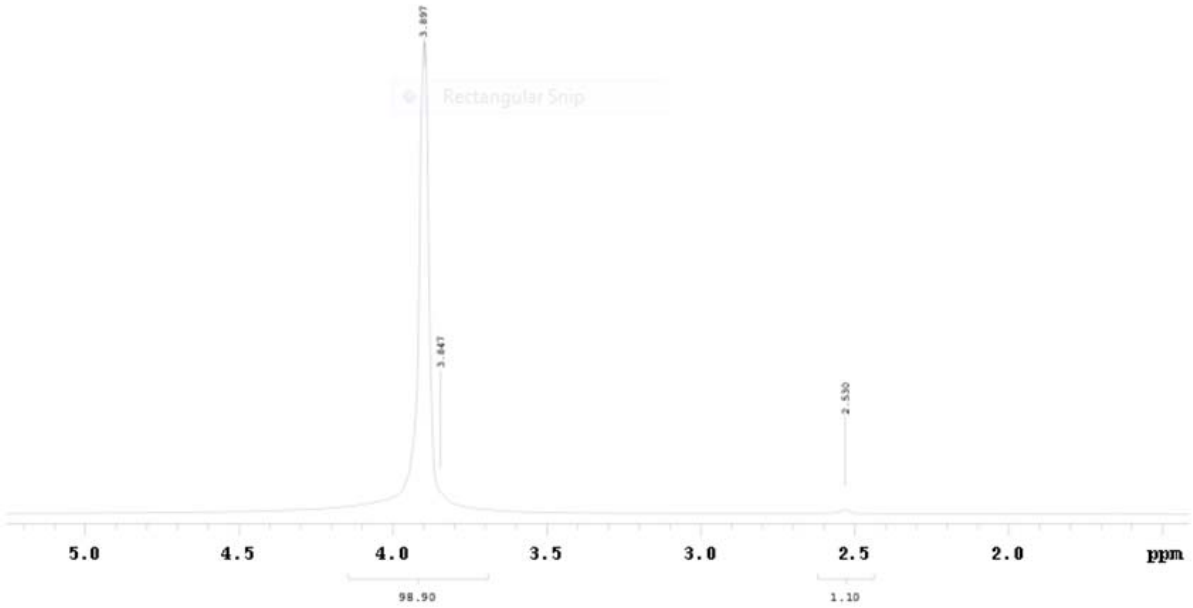


Fig 3. NMR spectra of fraction $Cp12_3$ of *C. patens*

3.4 NMR Spectra of purified fractions of *P. reticulatum*

Pr3$_6$; Aklyl esters and amides were found at peaks 3.457 and 3.379. Peak 2.582 showed the presence of benzylic protons. Alkanes, alcohols and alkyl ethers were found at peak 3.288. Also, Peak 2.472 presented benzyl protons while peak 2.468 presented benzyllic protons (Fig 4).

Pr5$_6$; Fig. 5 presented the proton NMR of fraction Pr5$_6$. The peak 6.780 observed presented vinyl protons; peak 6.509 presented aromatic protons while peak 5.505 presented vinylic protons (Fig 5).

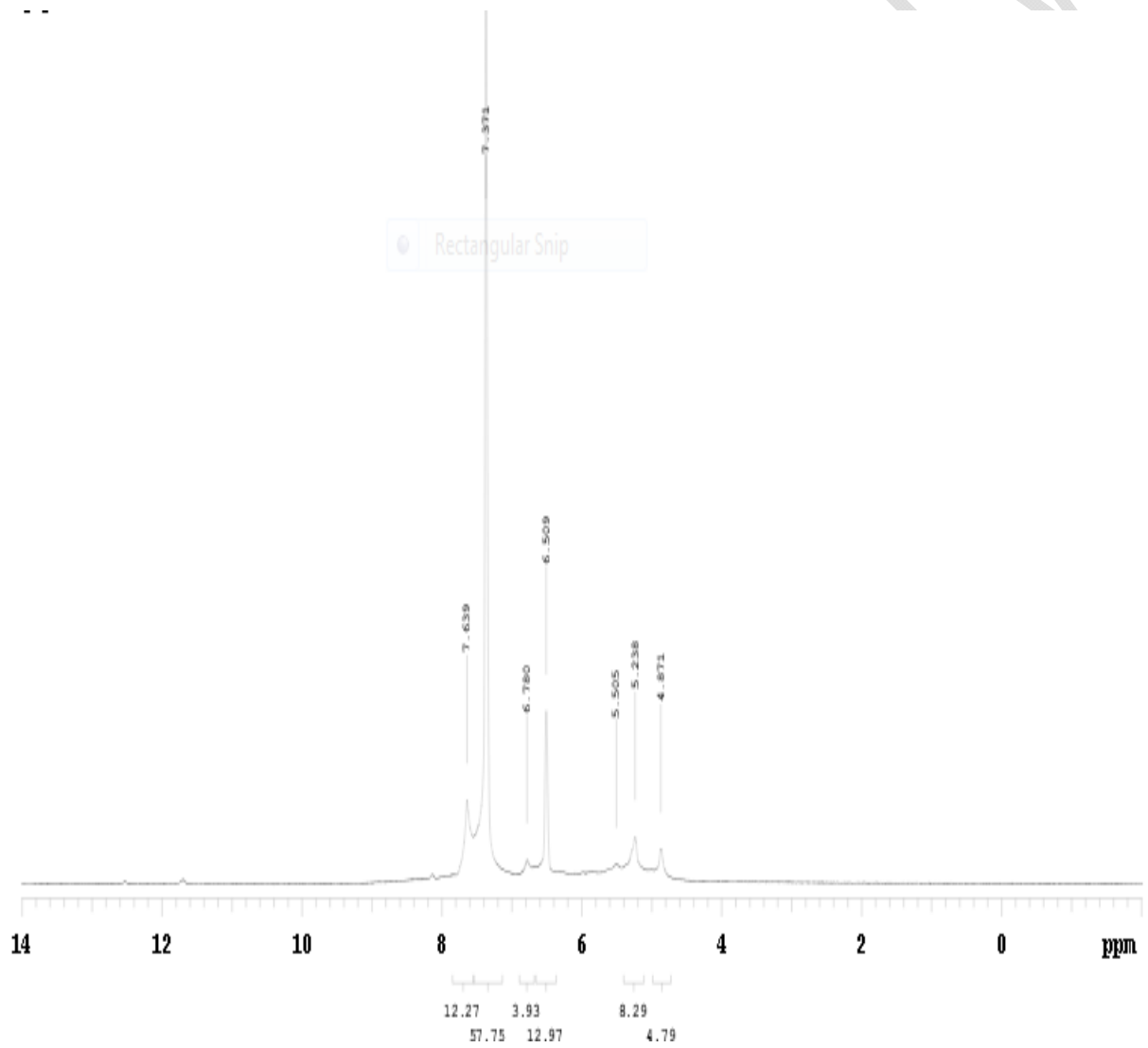


Fig 4. NMR spectra of fraction Pr3$_6$ of *P.reticulatum*

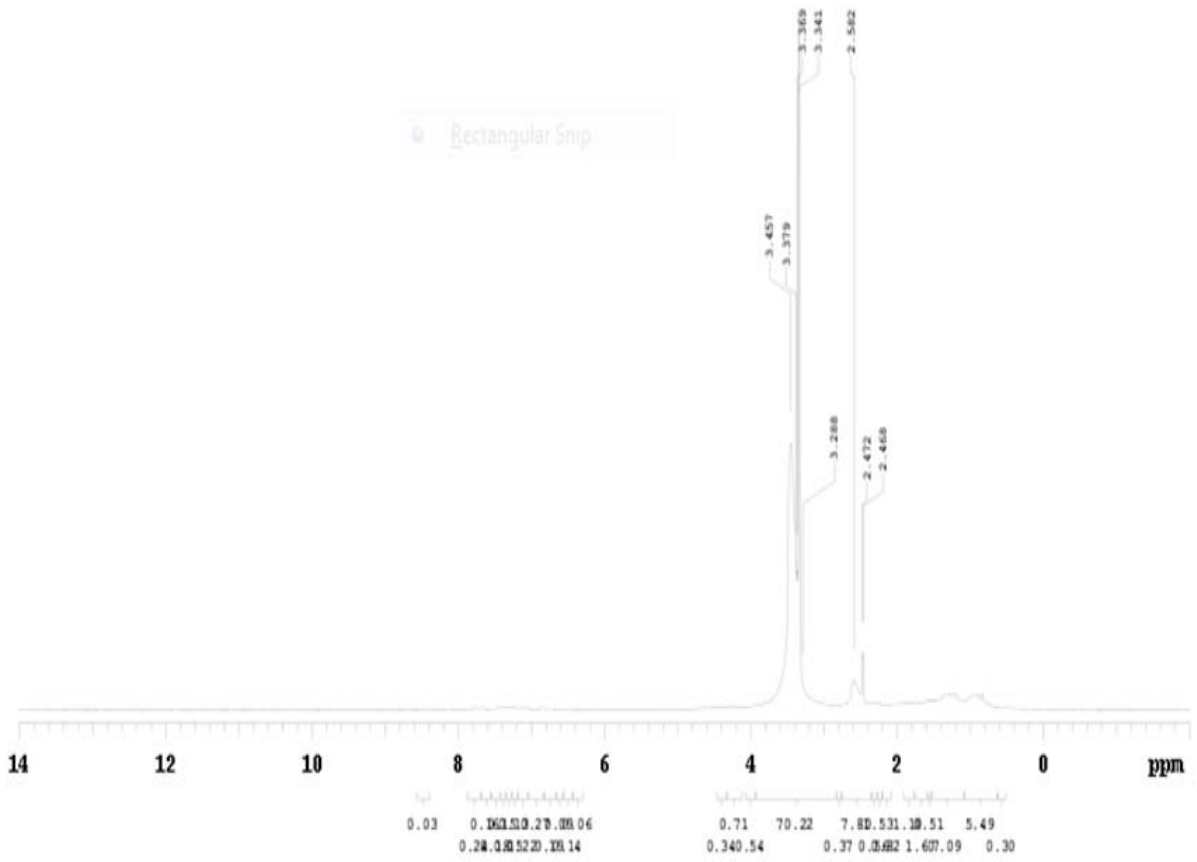


Fig 5. NMR spectra fraction of Pr5$_6$ of ***P. reticulatum***

3.5 GC-MS Spectra;

Five sesquiterpenes were identified in fraction Cp7 of *Cleistopholis patens* fraction as presented in figure 6 and Table 3 respectively. The compounds include: 2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl- also known as farnesol, which is the most abundant sesquiterpene accounting for 37.54% of all sesquiterpenes in fraction Cp7 of *Cleistopholis patens*. The next most abundant is Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,4.alpha.,7.alpha) accounting for 3.23% followed by alpha.-Pinene .Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl accounting for 1.65% of the total fraction. This is followed by 1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [s-(E,E) which accounts for 1.53% of the total fraction and finally Cyclohexene, 3-methyl-6- (1-methyl ethyl diene)- which accounts for 0.22% of the total fraction.

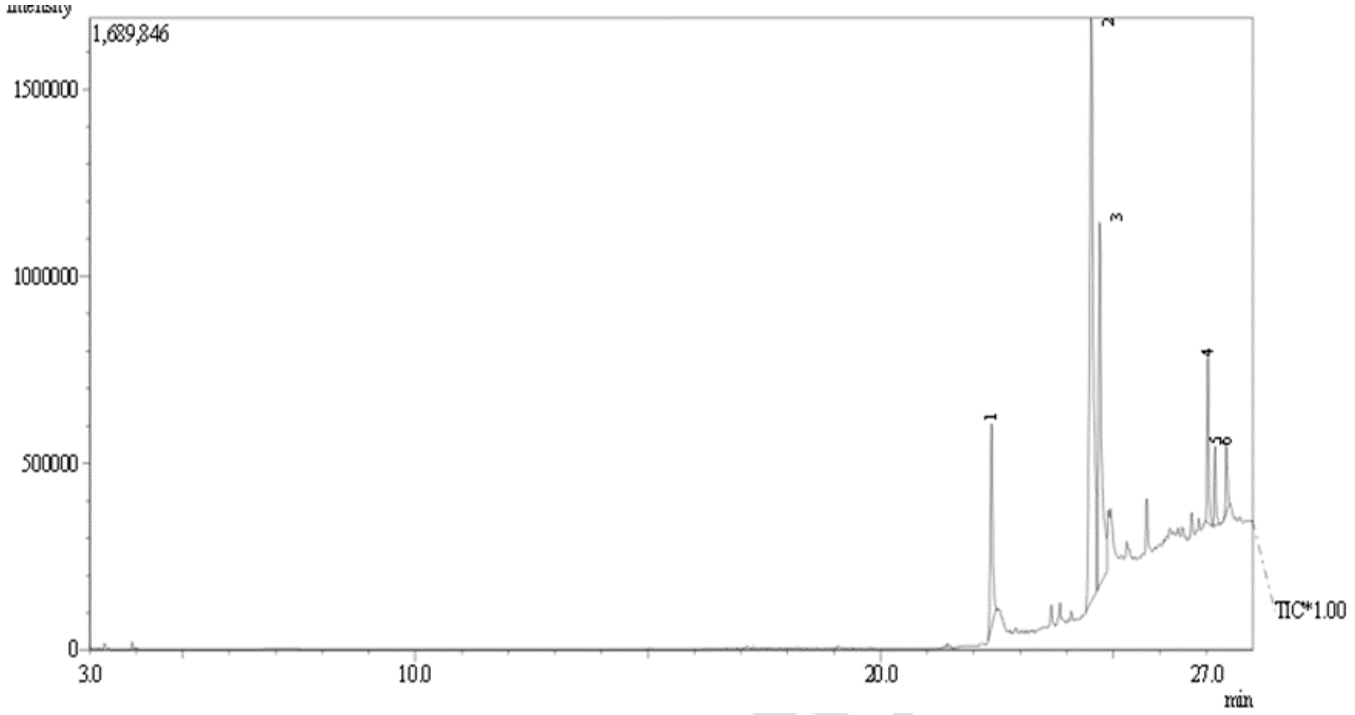
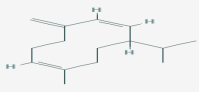
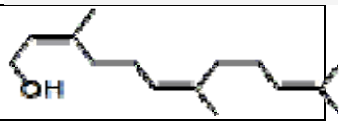


Fig 6: GC-MS Spectra of fraction Cp7 of *Cleistopholis patens*.

Table 3: Sesquiterpenes identified in fractions CP_7 of *C. patens* using GC-MS

S/N	RT	Name of compound	Chemical formula	Molecular weight	Percentage concnetration	Nature of compound	Chemical structure
1	4.992	Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl (alpha.-Pinene.)	$C_{10}H_{16}$	136	1.65	sesquiterpene	
2	11.663	Cyclohexene, 3-methyl-6-(1-methylethyldiene)-Limonene	$C_{10}H_{16}$	136	0.22	sesquiterpene	
3	14.895	1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,4.alpha.,7.alpha) Azulene,	$C_{15}H_{24}$	204	3.23	sesquiterpene	
5	15.140	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [s-(E,E Germacrene D	$C_{15}H_{24}$	204	1.53	sesquiterpene	
5	24.924	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-(Farnesol)	$C_{15}H_{26}O$	222	37.54	sesquiterpene	

3.5.1 GCMS of Cp12 extracts of *C. patens*

The extract Cp12 contains the following compounds as shown in Fig 7 and Table 4 respectively. Three sesquiterpenes were identified. The most abundant is 1,2,3,4,5,6,7,8- octahydro-1,4-dimethyl-7-(1-methylethenyl) and accounts for 4.86% of the total sesquiterpenes in the fraction.1,6-cyclodecadiene,1-methyl-5-methylene-8-(1-methylethyl) and Naphtalene, 1,2,3,4,4a, 5,6,8a-octahydro-7methyl-4-methylene-1-(1methylethyl) have the same quantity of 2,26% but Naphtalene, 1,2,3,4,4a, 5,6,8a-octahydro-7methyl-4-methylene-1-(1methylethyl) is a cyclic sesquiterpene.

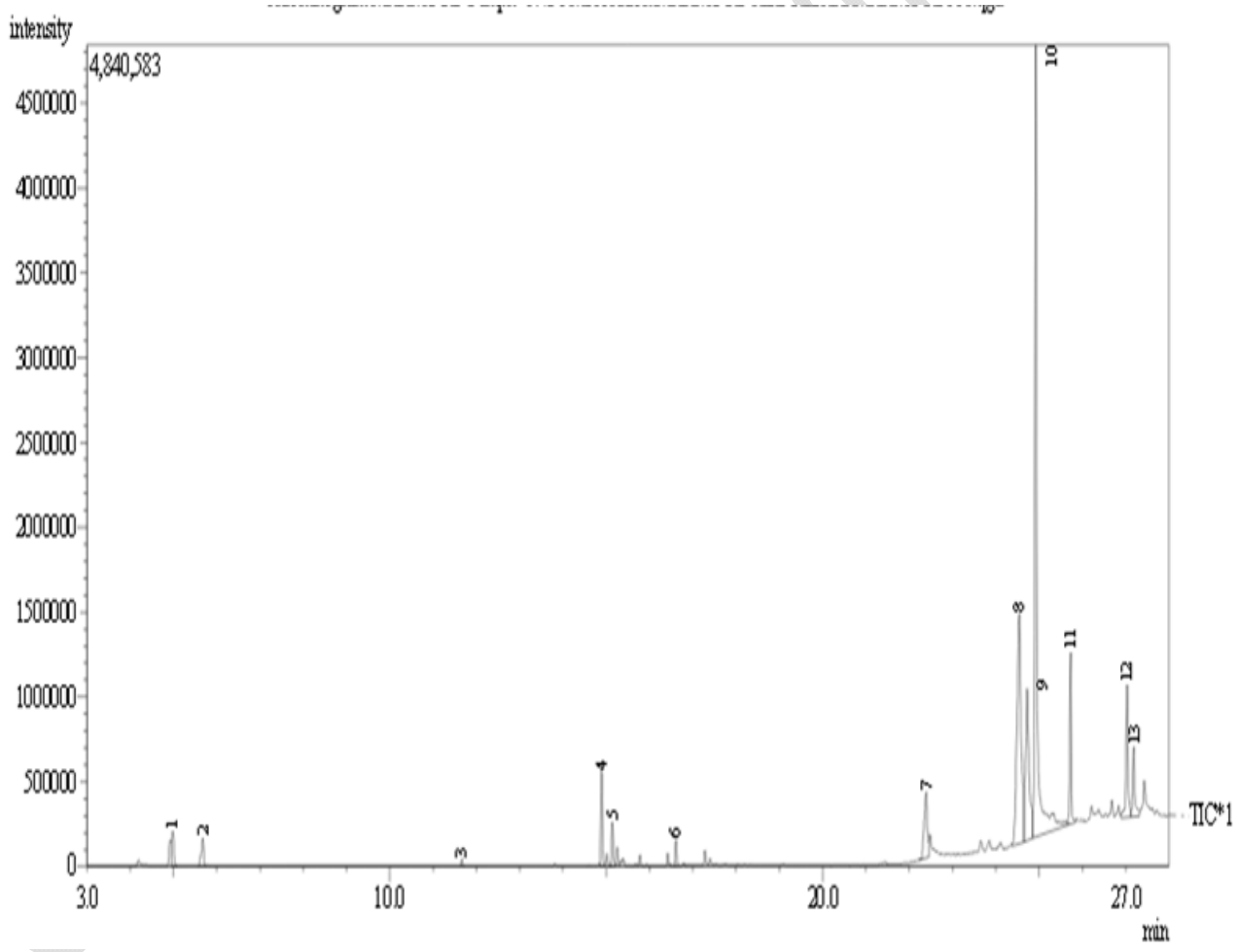
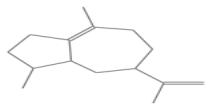
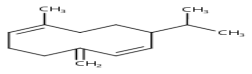
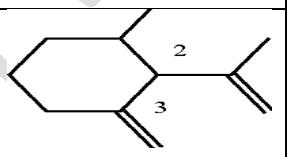


Fig. 7. GC-MS of fraction Cp12 from *Cleistopholis patens*

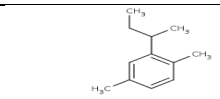
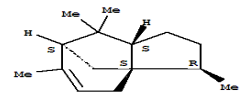
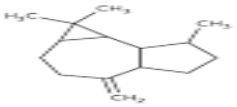
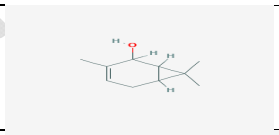
Table 4. Sesquiterpenes identified in fraction CP_{12} of *C. patens* using GC-MS

S/N	RT	Name of compound	Chemical formula	Molecular weight	% Conc	Nature of compound	Molecular structure
1	14.892	1,2,3,4,5,6,7,8- octahydro-1,4-dimethyl-7-(1-methylethenyl) (Azulene,) α- Guiene	$C_{15}H_{24}$	204	4.86	Sesquiterpene	
2	15.142	1,6-cyclodecadiene,1-methyl-5-methylene-8-(1-methylethyl). (Garmacrene D)	$C_{15}H_{24}$	204	2.26	sesquiterpenoid	
3	15.142	Naphtalene, 1,2,3,4,4a, 5,6,8a-octahydro-7methyl-4-methylene-1-(1methylethyl) (Azulene)	$C_{15}H_{24}$	204	2.26	Cyclic Sesquitwerpene	

3.5.2 Compounds identified in fraction $CP12_3$ of *C. patens*

From the fraction $Cp12_3$ of *C. patens*, Five (5) sesquiterpenes were identified including Farnesol isomer which accounts for 57.625 of the total fraction, Benzene, 1,4- dimethyl- which accounts for 10.01% of the total fraction.1H-3a,7-Methanoazulene and Aromadendrene both account for 3.07% of the total fraction while trans-3(10)-Caren-2-ol occurred in minute quantity of 0.99% of the total fraction.

Table 5: Sesquiterpenes identified in fractions $CP12_3$ of *C. paten* by GC-MS

S/N	RT	Name of compound	Chemical formula	Molecular weight	% Conc	Nature of compound	Molecular Structure
1	4.233	Benzene, 1,4-dimethyl-	C_8H_{10}	106	10.01	Sesquiterpenes	
2	15.083	1H-3a,7-Methanoazulene (Azulene)	$C_{15}H_{26}$	:206	3.07	Sesquiterpene	 t
3	15.083	Aromadendrene (Azulene)	$C_{15}H_{24}$	204	3.07	Sequiterpene	
4	16.800	trans-3(10)-Caren-2-ol (carenol)	$C_{10}H_{16}O$	166	0.99	Sesquiterpene	
5	:25.125	Farnesol isomer a (Farnesol)	$C_{15}H_{26}O$	222	57.62	Sesquiterpenoid	

3.5.3 Compounds identified in fraction $Pr3_6$ of *P. reticulatum*

Sesquiterpenes identified in fraction $Pr3_6$ of *P. reticulatum* are listed in table 6 and figure 8. The most abundant sesquiterpene is 2,6,10-Dodecatrien-1-ol, with a concentration of 91.37% followed by 2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (Z,E)- with a concentration of 5.99%. Fraction $Pr5_6$ had no sesquiterpene components.

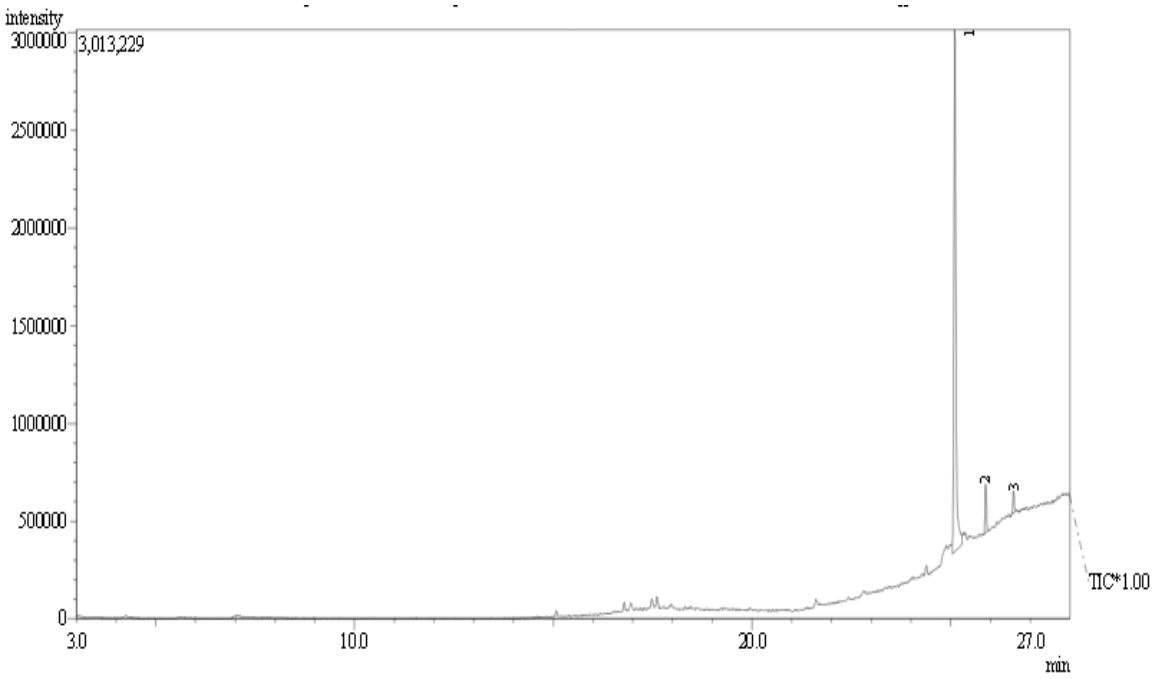
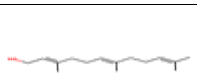
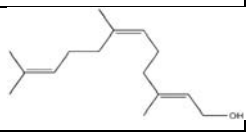


Fig. 8. GC-MS of fraction Pr3$_6$ from *Piliostigma reticulatum*

Table 6; Sesquiterpenes identified in fraction Pr 3$_6$ of *P. reticulatum* by GC-MS

S/N	RT	Name of compound	Chemical formula	Molecular weight	% conc.	Nature of compound	Molecular structure
1	25.103	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (E,E)- (Farnesol)	$C_{15}H_{26}O$	222	91.37	Sesquiterpene	
2	25.883	2,6,10-Dodecatrien-1-ol,3,7,11-trimethyl-, (Z,E)- (Farnesol)	$C_{15}H_{26}O$	222	5.99	Sesquiterpene	

4. DISCUSSION

Findings from this study reveals the antibacterial activities of P. reticulatum and C. patens bark extracts on test pathogens. *P. reticulatum* extract was effective against *Shigella dysentariae* which is responsible for multidrug resistant Shigellosis and dysentery. *S. dysentariae* is known to be resistant to third generation cephalosporins, and fluoroquinones [13]. However, the extracts obtained from the two plants evaluated in this study; *P.reticulatum* and *C. Patens* exhibited antibacterial activities against *Streptococcus pyogenes* which is implicated

in sepsis, Strept throat, toxic shock syndrome, glomerulonephritis amongst others causing about 600million infections annually [14]. This organism is resistant mainly to macrolides and tetracyclines [15]. The antibacterial activities of the crude and purified fractions suggest a synergistic relationship between the components of the individual plants which is evidenced in the higher antibacterial activity of the crude extract (Table 1).

P. reticulatum is obviously a broad spectrum antibacterial agent having activities against both Gram positive and Gram negative bacteria whereas *C. patens* is effective only against Gram- positive *Streptococcus pyogenes*. The broad spectrum status of *P. reticulatum* makes it a better specimen as a pharmaceutic as compared with *C. patens.* Okechukwu [16] in their study suggest *C. patens* to possess more antifungal activities especially on candidasis than antibacterial, this could be the reason behind the narrow antibacterial spectrum of *C. patens* and this corroborates the findings of this study. However, *P. reticulatum* is known to be active against a broad range of bacteria, especially those implicated in enteric infections. It is also used as antiplasmodic and are usually prescribed for gastrointestinal diseases [17]. Zerbo [10] also documented the antibacterial, anti-inflammatory and antioxidant activities of the plant extracts.

Monoterpenes and sesquiterpenes are usually the main group of compounds found in essential oils. In addition, phenylpropanoids are also very frequent. Moreover, some essential oils may also contain fatty acids and their esters and more rarely nitrogen and sulfur derivatives [18,19]. The two plants are rich in sesquiterpenes, on the qualitative basis, the major sesquiterpenes are the α and β pinene, azulene, sativen, cubene and β- ocimen. Boyom [4] in their work discovered that essential oils extracted from the stem bark of *C. patens* was found to contain terpenoids (97%) and sesquiterpenes (93%). *P. reticulatum* has also been shown by researchers to be abundant in sesquiterpenes [20] and this is evidenced in this study. Sesquiterpenes account for the highest quantity of essential oils found in the plants extracts used in this study. Sesquiterpenes are known to confer antimicrobial activities, most especially; antifungal [21], antioxidant [20], anti-inflammatory [22] bacteriacidal [23] and antitumor activities [24]. The root bark of *C. Patens* essential oil was shown by Watermann and Mohammad (1985) in their work to contain two sesquiterpenes and five alkaloids. Quattara [25] however discovered various sesquiterpenes in *C. patens*. The biological activities of isolated sesquiterpenes that include: α-pinene and (+)-β-pinene found in *C. patens* were found to possess antifungal activities against *Candida albicans* [26] and anti-inflammatory effects in human chondrocytes exhibiting potential antiosteoarthritic activity [27]. Beneficial features

of Guamarene in clinical practices are its anti-inflammatory, epithelializing, antioxidant, antiseptic, antifungal, antitumoral, antiulcer and immune modulator properties. Anti-inflammatory effect suppresses by inhibition of lipid peroxidation COX-2. It is used in conjunctival injuries, skin damage resulting from UV exposure, atopic dermatitis, gingival, mucosal diseases of mouth and after oral surgery due to its epithelializing effect.

Farnesol is a natural 15-carbon organic compound which is an acyclic sesquiterpene alcohol. Farnesol has been suggested to function as a chemopreven-tive and anti-tumor agent [28]. Recently, farnesol was described as a quorum-sensing molecule with possible antimicrobial properties [29]. Antibacterial effect of germacrene D, has been reported previously [30]. The presence of these sesquiterpenes in *P. reticulatum* and *C. patens* coupled with their corresponding biological activities could be responsible for the antibacterial activities on the test organisms (*S. dysenteriae and S. pyogenes*) evaluated in this study. This findings justifies their usage in traditional medicine in the treatment of various microbial infections including dysentary and sepsis.

5. CONCLUSION

Findings from this study revealed the presence of therapeutically potent antibacterial sesquiterpenes in copious quantities in the leaf extracts of *P. reticulatum* and *C. patens* which were active against pathogenic bacteria (*S. dysenteriae and S. pyogenes*). The result of the crude and purified extracts showed a strong synergistic activity in the components of each plants. These plants with their rich storage of biologically active sesquiterpenes could be considered as lead candidates in drug discovery for therapeutic purposes especially against *S. dysenteriae* and *S. pyogenes*.

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