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Identification of the Functional Groups, Radical Scavenging Ability and Antimicrobial Properties of the Extract of *Cleistopholis patens*

A. O. Daniels[1*] and D. A. Ibiyemi[2]

[1]Department of Biological Sciences, College of Natural and Applied Sciences, Achievers University, Owo, Ondo State.

[2]Department of Chemical Sciences. College of Natural and Applied Sciences, Achievers University, Owo, Ondo State.

*E-mail: toyosidanny@yahoo.com

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ABSTRACT

Medicinal plants have been evaluated over the years as a source of important biological and pharmacological substances. This work investigated the radical scavenging ability, the functional group present in ethyl acetate extract of stem bark of *Cleistopholis patens* and the antimicrobial activities against bacterial and fungal strains. The radical scavenging ability of the plant was significant although reduced compared to ascorbic acid. In the FTIR spectra recorded, the vibrational assignments, intensities and wave number (cm^{-1}) of dominant peak were obtained from absorption spectra. Probable assignments of the bands were made with respect to the components present in the extracts. By these analysis, functional groups such as amino-acids, amides, amines, carboxylic acid, carbonyl compounds, organic hydrocarbons, halogens, alcohols, phenols, sulfonamides amongst others were detected inthe extract. The antimicrobial effect revealed considerable antibacterial activity against *Proteus vulgaris, Klebsiella aerogenes, and Yersinia enterocolitica*while no antifungal activities were recorded for *Aspergillus niger, Acremoniumcoprophilum* and *Syncephalastrum racemosum*. The presence of the functional groups as revealed by the IR spectra is responsible for the biological activities expressed by the plant extract.

KEYWORDS: Infra-red, radical scavenging ability, antibacterial, antifungal, fungistatic, functional groups, *Cleistopholis patens.*

1. Introduction

Phytochemical are the natural active components of plant materials. They form an integral part of the defense mechanisms against various diseases and stress (Starlin, 2012). Plants act as free radical scavengers as a result of the presence of phytochemicals such as vitamins, terpenoids, phenols, lignins, tannins, flavonoids, quinones, coumarins, alkaloids amongst others (Aiyegoro and Okoh, 2010).

Plants provide new forms of functional components for the development of new therapeutic agents (Starlin, 2012) and scientists are carrying out in-depth study of phytochemical in plants to identify

new sources of therapeutically and industrially valuable compound with medicinal significance, to make judicious use of available natural wealth (Narayanah *et al.*,,2001). *Cleistopholis patens* (Cp) (Annonacae) is a popular medicinal herb used in the treatment of cardiovascular disorders in Nigerian ethnomedicine (Udem*et al.,* 2011). In Nigeria and other parts of African countries, the bark and the leaves of these plants are used in the treatment of typhoid fever and urogenital infections (Adonu *et al*., 2013).

The functional group of plants helps to determine the ability of plant to prevent invasion or invasion of other plant species fromthe regional pool (Levine *et al.,* 2004; Catford*et al*., 2009).The main aim of this study is to evaluate the phytochemical, elemental content of and the functional groups found in the ethyl acetate extracts of *Cleistopholis patens.*

2. Materials and Methods

2.1. Plant Sampling and Preparation

Stem bark of *Cleistopholis patens* was obtained from private garden of a traditional healer at Ibode in Ibadan, Oyo State, Southwest, Nigeria. The plant was identified by the Ekiti State University Herbarium of the Department of Plant Science and Biotechnology, Ekiti State University, Ekiti State. Voucher specimens with voucher numbers WHAE2017/001 was deposited in the herbarium of the University. The plant materials were washed with distilled water, air dried at room temperature, milled into powder and kept in sterile containers until use.

2.2. Preparation of Crude Extracts

Ethyl acetate extract was prepared by dissolving 200gm of powdered plant sample in 500mls of solvent. The solution was allowed to stand for 5 days (120hr) at room temperature of $27\pm1^{o}C$ and then filtered using a sterile muslin cloth and a Whatmann No 1 filter paper as described by Fabricant and Fansworth (2001). The collected filtrate was evaporated to dryness under sterile condition after which the extracts were kept in sterile bottles until ready for use.

2.3. Sterilization of Crude Extract

A five gram portion of the crude extract was reconstituted in 50% dimethyl sulfoxide, the solution wassterilized by passing them through milipore membrane filter with pore size 0.45µm. The filtrates was then stored in sterile bottles until use.

2.4. Collection and Maintenance of Microorganisms

The test bacteria and fungi were obtained from the Department of Microbiology, Obafemi Awolowo University, Ile-ife, Osun State, Nigeria. The organisms were collected and maintained by regular subculturing on nutrient and potato dextrose agar slants respectively. The test organisms were subjected to biochemical test to confirm the authenticity of the organisms.

2.5. Standardization of Inoculum

One percent (1%) solution of Barium chloride was prepared by dissolving 0.5g of dehydrated barium chloride ($BaCl_2.H_2O$) in 50ml of distilled water. A 0.5ml aliquot of barium chloride solution was added to 99.5ml of the sulphuric acid solution and mixed together. The solution was transferred into a capped tube of the same type used for both the control and the test inocula. The solution was kept under refrigeration of $+4^{O}C$ (Cheesebrough, 2000).

2.6. Antibacterial Test

Test organisms were suspended in Nutrient broth and incubated for 4 hours to obtain a concentration corresponding to McFarlands constant (0.5 X 10^{8}cfu/ml). The inoculum was standardized with the prepared barium sulphate as described above. Sterile Petri dishes were inoculated by the pour plate method. One ml (1ml) of the test inoculum was pipetted aseptically into each Petri dish and about 20 ml of sterilized nutrient agar was poured into the inoculated Petri dish.

The agar plates were allowed to set. Wells of 6mm diameter were made over the agar plates equidistant from each other using sterile cork borer and 0.5ml of each plant extracts of different concentrations as prepared by the serial dilution were added to the

wells using a micro-pippette. The extracts were allowed to diffuse into the agar for about 20 min after which the plates were incubated for 24 h at 37ºC. Thereafter, the diameter of inhibition zones formed around each well was measured in mm and recorded. The experiment was carried out in triplicates and the average values recorded.

2.7. Phytochemical Screening of Plant Sample

Active plants were screened qualitatively for phytochemicals using the methods of Odebiyi and Sofowora (1993), Trease and Evans (2002) and Banso and Ngbede, (2006).

2.7.1 Test for Alkaloids

A 0.2g amount of plant extract was acidified with 1% hydrochloric acid (HCl) for 2 min and was then treated with a few drops of Dragendorff's reagent in a test tube. The formation of white precipitate indicates the presence of alkaloids.

2.7.2. Test for Aaponins

Sterile distilled water was used to dissolve 0.2g of plant extract. A 2ml amount of the solution was placed in different test tubes and was shaken vigorously for a few minutes. Frothing which persists on warming was taken as an evidence of the presence of saponin.

2.7.3. Test for Tannins (Gelatin Test)

To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

2.7.4. Test for Flavonoids (Shinoda's Tests)

Plant extract was dissolved in 2ml of dilute NaOH. A yellow solution that turns faint or colourless on addition of a few drops of hydrochloric acid and a change in colour while standing indicates the presence of flavonoids.

2.7.5. Test for Cardiac Glycosides (Liberman's Test)

The Liberman's test was used to determine the presence of cardiac glycosides. A 5g amount of plant extract was dissolved in 20ml of acetic anhydride and cooled with ice. Concentrated H_2SO_4 was then carefully added. A colour change from violet to blue and then to green indicated the presence of a steroidal nucleus (a glycone portion of the cardiac glycoside).

2.7.6. Test for Steroids (Salkowski Test)

A 0.5g portion of plant extract was dissolved in 2ml of chloroform and 0.2ml of concentrated H_2SO_4 was carefully added to form a layer. A reddish –brown colour ring at the interface between the layers indicated the deoxy- sugar characteristic of cadenolides which indicated the presence of steroids.

2.8. Determination of Mineral Content of Plants Samples

Mineral was estimated by the used of an Atomic Absorption Spectrophotometer. The sample solutions in the sample bottles were analyzed for the concentration of the individual elements. Each element has specific cathode discharge lamp and this lamp was used to determine a particular element. Discharge lamp emits radiation at a wavelength specific for each element being assayed. This specificity can be obtained only from a pure sample of the element that is excited electrically to produce an arc spectrum on that element.

2.9. Radical Scavenging Ability of Plant

The crude extract of (ethyl acetate) of the plants was screened for DPPH radical Scavenging activity. DPPH radical scavenging activity was measured according to the method of Ayoola *et al*. (2006). Solution of the extract was prepared by dissolving 1g of dry extract in 10 ml of methanol. An aliquot of 1ml of DPPH solution in methanol and 1ml of plant extract in methanol at various concentrations (20, 40, 80 and 100ppm) were mixed and incubated at 25°C for 30 min. and absorbance of the test mixture was read at 517 nm using a spectrophotometer (T 70 UV-VIS Spectrometer, PG Instruments Ltd.) against a DPPH control containing only 1 ml of methanol in place of the extract. The DPPH solution in methanol was prepared daily before the absorbance measurements. DPPH is a purple coloured stable free radical. When reduced it gives the yellow

coloured Diphenyl picryl hydrazine. All experiments were performed in triplicates and the results were averaged. Percent inhibition was calculated using the following expression:

$$\%\ Inhibition = (1 - (A\ DPPH + sample - A\ sample + distilled\ water)\ X\ 100$$

$DPPH$

2.10. Evaluation of the Total Antioxidant Ability Using FRAP Assay.

The determination of the total antioxidant activity (FRAP assay) in the extract is a modified method of Benzie and Strain (Okudu *et al.,* 1994). The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3 \cdot 6H_2O$. The temperature of the solution was raised to 37 °C before use. Plant extracts (150 µL) were allowed to react with 2850µl of the FRAP solutionfor 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593nm. The standard curve was linear between 200 and 100 µM $FeSO_4$. Results are expressed in M Fe (II)/g dry mass and compared with that of BHT, ascorbic acid.

2.11. Purification and Fractionation of Plant Extract Using Column Chromatography

2.11.1. Column Chromatography Procedure

The crude extract of *Cleistopholis patens* (1.5 g) each was dissolved in petroleum ether. A 90gm amount of 60-120 mesh silica gel was used to pack the column by wet packing. Silica gel 60g was used as the stationary phase while varying solvent combinations of increasing polarity were used as the mobile phase (Cosa *et al.,* 2006). The wet packing method was used in preparing the silica gel column. Elution of the extract was done with solvent systems of gradually increasing polarity using Petroleum ether, chloroform, ethyl acetate and methanol. A measured volume (100ml) of each solvent combination was collected gradually with a 10ml syringe and sprayed uniformly by the sides of the glass into the column each time. The eluted fractions were collected in aliquots of 100 ml in conical flasks. The fractions were allowed to run through a short column and the fractions were collected, and spotting of fractions and thin layer chromatography was carried out to ascertain the purity of the compounds

The values of the extra

2.12. Functional Group Determination Using FTIR

All spectra were obtained with the aid of OMNI sample attenuated total reflectance accessory on an ASCO FTIR spectrophotometer. (FTIR 4600). Purified extract of the stem bark of *Cleistopholis patens* was suspended in methanol were encapsulated and placed directly on the germanium piece of the infrared spectrometer with constant pressure applied. Data of infrared absorbance were collected over the wave number ranged from 4000/cm to 650/cm. The reference spectra were acquired from the cleaned blank crystal before the presentation of each sample replicate. All spectra were collected with a resolution of 4.0-1.0 cm and to improve the signal-to-noise ratio. The FTIR spectra of all samples were analyzed on the basis of peak values in the region of infra-red radiations. Samples were run in triplicates.

3.0. Result and Discussion

3.1. Antibacterial Activity of Plant Extract

The antibacterial activity of the plant extract is depicted in Table 1. The extract was effective against *Proteus vulgaris, Klebsiella aerogenes*, and *Yersinia enterocolitica* with zones of inhibition ranging from10mm at 20mg/ml to 22mm at 100mg/ml, against *Proteus vulgaris,* 8mm at 10mg/ml to 18mg/ml against *Klebsiella aerogenes* and from 4 mm at 10mg/ml to 16mm at 100mg/ml against *Yersinia.*

Table 1: Antibacterial activity of the plant extract

Test org/ Zones of inhibition (mm)	100	60	40	20	10
Proteus vulgaris	22	18	14	10	-
Klebsiella aerogenes,	18	14	12	10	8
Yersinia enterocolitica	16	14	10	6	4

3.2. Antifungal Activity of Plant Extract

The extract did not show any antifungal activity (Table 2). The reason might be that the fungal strains used were not susceptible to the extract based on the extraction solvent. Many author found the ethanol extract to have more functionality than other solvent extracts. Okechukwu *et al.*, (2015) recorded activity of the ethanolic leaf extract of *C. patens* against Candida *spp*, Liu *et al.*, (1990) reported significant activity against *Candida albicans, Aspergillus fumigatus*, and *Cryptococcus neoformans.* On the other hand, Adonu *et al*., (2013) recorded considerable antibacterial effect of the plant against *E. coli* and *Pseudomonas aeruginosa.* This conforms to the activity of the plant against the bacteria used in this work. The general biological activity recorded of this plant as observed in the results justify its use in traditional medicine against microbial diseases (Adonu *et al*., 2013).

Table 2: Antifungal activity of the plant extract

Test org/ Zones of inhibition (mm)	100	60	40	20	10
Aspergillus niger	-	-	-	-	-
Acremonium coprophilum	-	-	-	-	-
Syncephalastrum racemosum	-	-	-	-	-

Legends; - means no activity

3.3. Phytochemical Content of Plant

This study has revealed the presence of phytochemicals considered as active medicinal chemical constituents. Important medicinal phytochemicals such as tannin, flavonoids, steroids, alkaloids and glycosides were present in the samples. *Cleistopholis patens* contain glycosides, steroids, phenol, tanins and saponins (Table 1). Phytochemicals in plants are known to be responsible for antimicrobial activities (Cowan, 1999) antioxidant (Maquid , 2017).

Phytochemical screening of plants has revealed the presence of numerous chemicals including alkaloids, tannins, flavonoids, steroids, glycosides, saponins. Many plant extracts and phytochemicals show antioxidant/free radical scavenging properties (Larson 1988; Nair *et al*. 2007; Parekh and Chanda 2007). Secondary metabolites of plants serve as defense mechanisms against predation by many microorganisms, insects and herbivores (Lutterodt *et al.* 1999; Marjorie, 1999). This view was supported by Ahmadian, (2000), who reported that tanins and flavonoids of medicinal origin were found to possess significant pharmacological activities: antidiarrheal, analgesic, and anti-inflammatory amongst others in the animal body system.

2.4. Elemental Component of Plant

Mineral components of C. patens are Na (24.03%), K (30.14%), Ca (32.31%), Mg (26.09%), Zn (28.09%), Fe (6.70%), Cu (0.03%) while lead (Pb) was not detected (Table 2). Minerals and trace metals are required by humans in trace amounts to remain healthy and maintain good health and excess of it might be toxic. Potassium content in *C. patens* was particularly high as shown in Table 3. This is makes this plant a cheap source of this important mineral. Sodium, also very high is part of the resting membranes potentials of a cell (i.e the difference in

electric charge between the inside and outside of a cell (Dosumu *et al.*, 2012). Sodium also allows for nerve cell fuctions; without which the brain would not be able to send messages and the muscles would not move. *Cleistopholis patens* has a high concentration of Sodium. Iron (Fe) Copper (Cu) and Zinc (Zn) are needed by man for biochemical functions in the system. Fe and Cu are present in the enzyme cytochrome oxidase involved in energy metabolism. These metals ions are trace elements and are therefore required in minute quantities. Consumption of these minerals through the plant materials readily supply required microelements (Marles, 2017).

Calcium is a major constituent of the bone and the blood concentration of calcium has to stay relatively constant for the muscle and heart to function. It is a coordinator among inorganic elements. If excess amounts of K, Mg or Na are present in the body, Ca is capable of assuming a corrective role. Magnesium is an activator of many enzymes systems and maintains the electrical potential in nerves (Golam-Akond *et al*., 2011).

Both sodium and potassium are required to maintain osmotic balance of the body fluid, pH of the body, regulation of muscle and nerve irritability, control of glucose absorption and enhance normal retention of protein during growth (Achinewhu, 1986). This result correlates with the findings of Adeyanju *et al*., (2010), Musa *et al.*, (2008) and Ojewale (2013). Consequently, the presence of these minerals makes the plant to be a potential source of nutritional supplement as well as nutraceuticals that can positively serve as a model for modern drugs.

2. Radical Scavenging Activity of Plant

Antioxidants are reductants, and inactivators of oxidants (Siddhuraju and Becker, 2007). The antioxidant potential of *C. patens* was investigated in this work. The ferric reducing ability of the ethyl acetate extract of the plant showed significant FRAP activity as presented in Fig. 1. FRAP assay was used by several authors for the assessment of antioxidant activity of various food product samples (Halvorsen *et al.*, 2006; Pellegrini *et al.*, 2003). Figure 1 shows the antioxidant activity of the ethyl acetate extract of *C. patens.* At 100mg /ml of extract, the extract was able to mop up radical oxygen to 40%. This ability is visibly low compared to that reducing ability of Ascorbic acid. Literature search has shown a very few evidence of the scavenging ability of this plant Ayoola *et al*, (2019) in their work showed that the anti -oxidant potential of the methanol leaf extract of the plant was moderate which is in line with the observation recorded in this work.

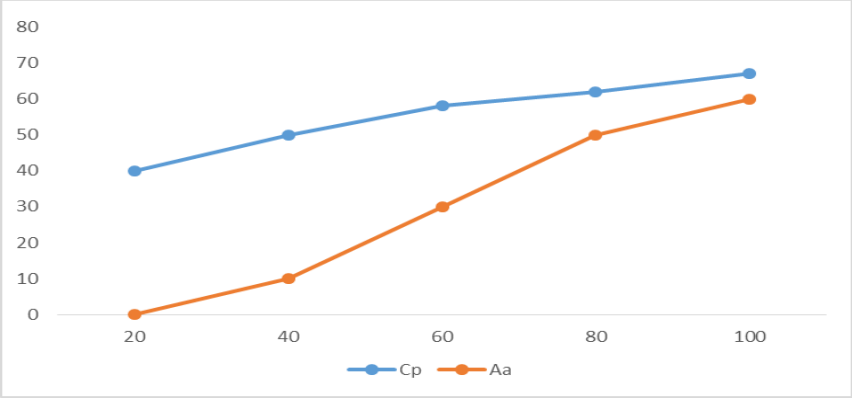

Fig. 1: Radical scavenging ability of the ethyl acetate extract of the stem bark of *C. patens* using ascorbic acid as standard. (Cp is *C. patens* and Aa is ascorbic acid).

3. FTIR (Fourier Transform Infra-Red) Spectrophotometer Results of Plant Extract Fractions.

The FTIR spectra were used to identify the functional groups of the active components in the plant samples based on the peak values in the region of infrared radiations. The FTIR has been widely used both for quantitative and qualitative analysis of plant chemicals (Smith, 2011).

4. FTIR Spectra of Fraction Cp 7

Samples from *C. patens*, (Cp 7) have the following functional groups as presented in Fig 2, 15 peaks were observed. At the far end, the peaks observed at 447.50cm^{-1}, 802.41 and 856.42 are likely to contain halogens which are hidden at the fingerprint region. The peak observed at 949.01cm^{-1} has a strong intensity with a double bond carbon compound (C=C), a bending intensity suspected to be a mono-substituted alkene.

At the peak observed in the 1033.88cm^{-1} position is strong stretching double bond sulfoxide (S=O). At the peak observed at position 11.41 cm^{-1}, strong stretching single bond carbon-oxygen (C-O) group of a tertiary alcohol was observed. Similarly, at position 1327.07cm^{-1}, a strong stretching S=O group of sulfone was observed while at position 1365.65cm$^{-1,}$ a strong stretching S=O of sulfonate was also observed. At position 1435.09cm-1 of the spectra, a medium intensity bending C-H of an alkane with a methyl group attached to it. At position 1465.98cm-1, a medium intensity bending alkene (C-H) with a methylene group attached to it was observed. Also, at the position 1589.40cm-1 position on the spectra, a medium intensity bending amine group (N-H-) was observed. Position 1681.88cm-1 revealed the presence of a medium intensity stretching imine/oxime compound with a double bond (C=N). In the same vein, position 2360.95cm-1 of the spectra revealed the presence of a strong intensity stretching carbon dioxide (O=C=O). At position 2962.76cm-A medium stretching alkene (C-H) was observed. At position 3394.83, a strong broad stretching alcohol (O-H0) was detected.

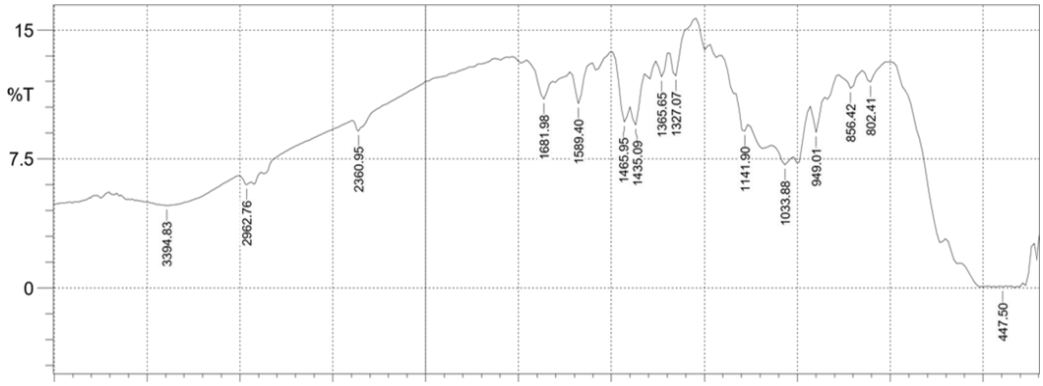

Fig. 2: FTIR of fraction Cp7 of *Cleistopholis paten*

FTIR Spectra of Fraction Cp12

Thirteen peaks were observed in these spectra of this fraction as presented in fig 3. Position 802.41cm^{-1} presented a strong stretching Akyl/arylhalides (C-Cl). This I hidden in the fingerprint region. Position 856.42 cm-^{1} is also hidden in the fingerprint region. Position 949.01cm-^{1} has a strong bending alkene group (C-H). Position 1033.88 cm-^{1} has a strong stretching sulfoxide (S=O). Similarly, position 1327.07cm-^{1} contains a medium bending phenol group (O-H) while position 1365.65 presented a medium bending alkane with a methyl group (C-H). In the same manner, position 1435.09cm-^{1} contains medium bending alkane with a methyl group attached to it (C-H). Position 1543.10cm-^{1} shows the presence of a strong stretching Nitro compound (N-O).

Position 1589.40cm-^{1} contains a medium intensity bending amine (N-H). While 1681.98cm^{-1} presents a medium intensity stretching amine /oxime (C-N).

Position 2962.76 on the spectra shows a medium intensity stretching alkene (C-H) and position 3379.40 contains a medium intensity stretching aliphatic primary amine (NH).

FTIR Spectra of Fraction Cp12$_3$;

The spectra of fraction Cp12$_3$ contains the following functional group as presented in fig 4. Position 648.10cm-^{1} presents a strong stretching halo compound (C-Br). While position 1026.16cm^{-1} presents a strong stretching vinyl compound (C-O). Positon 1111.03cm-^{1} presents a strong stretching secondary alcohol(C-O) and position1219.05 shows the presence of a strong intensity stretching alkyl/ aryl ether (C-O). At peak 1411.94cm-1, a strong stretching sulphate was observed (S=O). While a peak 1573.79cm^{-1}, a strong bending amide (N-H) was observed. A broad stretching acid was noticed at peak 2530.69cm-^{1} while a medium intensity stretching aldehyde (C-H) was obtained at position 2831.60cm^{-1}. In the same manner, a medium intensity, weak, broad and stretching amine salt was observed at the 2947.33cm-^{1} position on the spectra. Position 3333.10cm-^{1} revealed a strong, sharp, stretching alkyne (C-H).

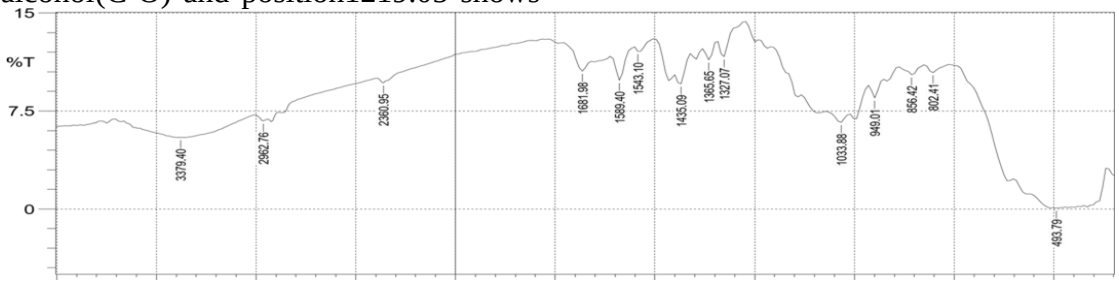

Fig 3: FTIR of fraction Cp12 of *Cleistopholis patens*

5. Cp 12$_3$

The spectrum for fraction Cp12$_3$ is presented in Fig 4. Twelve (12) peaks were observed in the spectrum. The peak observed at 3333.10cm-1 showed primary amines. The peak at 2497.33cm-1 is an alkane with a Sp3 C-H stretch. An aldehyde with a C-H is likely to be found at the 2831.6cm-1, While at peak 2530.69cm-1, a very broad stretch of an acid (thiol) with a very weak bond is likely to be present. Peak observed at 2345.52 is likely to have an overlap of C-H stretch. Peak at 1666.55cm-1 is likely to be a saturated amide while the peak at 1442 has an Sp3 C-H bend (alkanes)

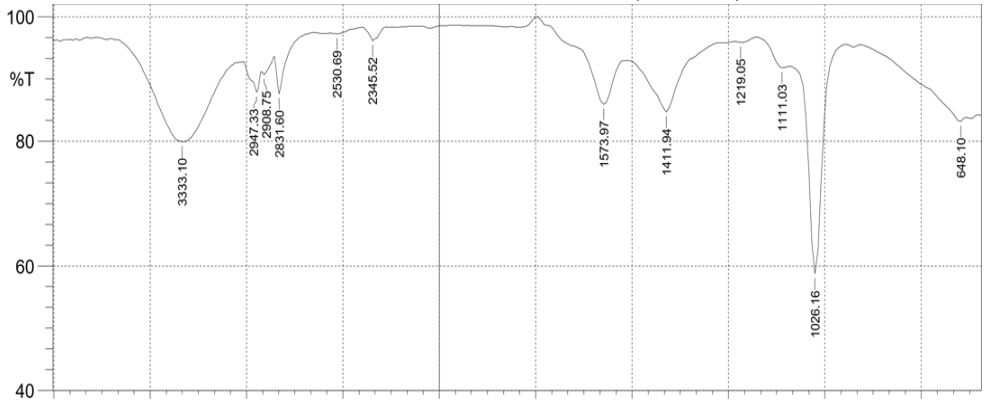

Fig 4: FTIR of fraction Cp12$_3$ of *Cleistopholis patens*

FTIR analysis carried out on the purified fractions of ethyl acetate extract of the stem bark of *Cleistopholis patens* revealed the presence of several compounds based on the peaks obtained in the spectra. Studies have shown the ethanolic extract of *C. patens* to contain an alkaloid; isomoschatoline (Atti *et al.,* 2004). Muruganantham *et al*. (2009) carried out the FTIR and EDS spectral analysis of plant parts like leaf, stem, and root of the medicinal plants*Eclipta alba* and reported the presence of

characteristic functional groups of carboxylic acids, amines, amides, sulphur derivatives, polysaccharides, nitrates, chlorates, and carbohydrate that are responsible for various medicinal properties of both herbal plants. Thangarajan *et al*. (2012), while analyzing the ethanolic extracts of *Ichnocarpus frutescens*, by FTIR, revealed functional group components of amino acids, amides, amines, carboxylic acid, carbonyl compounds, organic hydrocarbons and halogens. Pednekar and Raman (2013) analyzed the methanolic leaf extract of *Ampelocissus latifolia* by FTIR and reported that the transition metal carbonyl compounds and aliphatic fluoro compounds were only present in the extract. Little information is however available on the IR spectra analysis of *C.patens.* This work has therefore provided information on the functional groups present in *C. patens* so as to close the lacuna that has been established as a result of the paucity of information in this aspect.

6. Conclusion

C. patens used in the traditional folkloric treatment of diseases is a good candidate in drug production and pharmacopoeia. The functional groups identified are probably responsible for its biological activities since the compounds represented by the functional groups identified have been confirmed to be responsible for antimicrobial and medicinal activities.

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