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Drug Discovery

To Cite:
Shabanda IS, Haruna Y, Usman AA. Antibacterial activity and partial characterization of antibacterial agent from *combretum glutinosum* leaf extracts against microbes isolated from dental caries. *Drug Discovery* 2024; 18: e4dd1967
doi: https://doi.org/10.54905/disssi.v18i41.e4dd1967

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Peer-Review History
Received: 06 November 2023
Reviewed & Revised: 10/November/2023 to 20/January/2024
Accepted: 24 January 2024
Published: 27 January 2024

Peer-Review Model
External peer-review was done through double-blind method.

Drug Discovery
pISSN 2278–540X; eISSN 2278–5396

DISCOVERY SCIENTIFIC SOCIETY

Antibacterial activity and partial characterization of antibacterial agent from *combretum glutinosum* leaf extracts against microbes isolated from dental caries

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ABSTRACT

Medicinal plant synthesizes phytochemicals through secondary metabolism; these metabolites can be utilized for several pharmacological approaches, and also serves as precursors for the synthesis of valuable drugs. Therefore, there is need to detect the phytochemical and antibacterial properties of *Combretum glatinusum* plant in view of providing a scientific explanation of its medicinal properties. *Combretum glatinusum* leaves was extracted using methanol, ethanol and water. Qualitative phytochemical and FTIR analysis were conducted using standard laboratory procedures, while agar well diffusion and serial dilution methods was adopted for zone of inhibition and minimum inhibitory concentrations respectively. In qualitative analysis, *Combretum glatinusum* leaves revealed the presence of alkaloids, flavonoids, saponins, tannins terpenoids and glycosides in methanol extracts. In contrast, ethanol extracts revealed the presence of alkaloids, flavonoids, saponins, tannins terpenoids, steroids and glycosides. Similarly, aqueous extract revealed the presence of alkaloids, flavonoids, saponins, tannins, terpenoids, steroids and glycosides. A dose-dependent increases in zone of inhibitions were observed in *Combretum glatinusum* leaves extracts at all concentrations. The activity was comparable (P>0.05) with standard drugs at some concentrations of the extracts. Against *S. aureus, C. glutinosum* leaves water extract revealed a MIC of 12.5mg/ml, methanol extract revealed a MIC of 100mg/ml, ethanol extract also revealed an MIC of 100mg/ml. Against *K. pneumonia, C. glutinosum* Leaves water extract revealed a MIC of 50mg/ml, methanol extract revealed an MIC of 100mg/ml and ethanol extract revealed an MIC of 100mg/ml. Against *S. aureus, C. glutinosum* leaves water extract revealed a MBC of 100mg/ml, methanol and ethanol extract revealed a MBC >200mg/ml, respectively. While against *K. pneumonia, C. glutinosum* leaves water extract revealed an MBC of 25mg/ml, methanol and ethanol extract revealed an MBC of 200mg/ml respectively and FTIR analysis revealed the presence of numerous functional groups. Hence, this study

validates the medicinal properties of *C. glutinusum* plant and further supports it use in traditional medicine.

Keywords: Phytochemicals, Antibacterial, *Combretum glatinusum*, Leaves, Dental caries, Methanol, Ethanol, Water

1. INTRODUCTION

Medicinal plant synthesizes phytochemicals through secondary metabolism; these metabolite can be used for several pharmacological approaches, they also serves as precursors for the synthesis of valuable drugs (Mendoza and Silva, 2018; Alagbe et al., 2023). These secondary metabolites from medicinal plants have been documented to be effective in curing numerous diseases. Plants have been utilized in traditional medicines for over decades; however, there are little scientific data to validate their efficacy. Human beings depend on plants for the treatment of numerous diseases since ancient time (Atanasov et al., 2015; Abdulhamid et al., 2023). Recent advancements have significantly enhanced our understanding of this process, encompassing various aspects such as microbiology, saliva, tooth composition, tooth structure, diffusion mechanisms, demineralization kinetics, the phenomenon of re-mineralization, and the factors contributing to tooth decay (Pani, 2022).

Dental caries, also known as tooth decay, is often described as the presence of cavities in the teeth rather than a comprehensive disease process (Pitts et al., 2017). Nevertheless, it has been established for more than a century that dental decay is the result of bacterial fermentation of food, leading to the production of acids that dissolve the mineral content of the teeth (Pitts et al., 2017). A significant number of these phytochemicals are synthesized as a plant defense mechanism in response to biotic attack or environmental stresses (Vaughan et al., 2018). The antibacterial effects of phytochemicals have been documented, and the biotic are killed by several different mechanism. Several of these phytochemicals serve as a defense mechanism against damaging environmental conditions (such as free radicals, salinity, and temperature), pathogenic microorganisms (such as bacteria, viruses, and fungi), and insect pests by plants (Chowdhary et al., 2021). The biological activity of these secondary metabolites is then transcribed to numerous positive pharmacologies in treating various bacterial-related human diseases (Wink, 2015).

Combretum glutinosum a shrub species of the genus Combretum, found in the Sahel belt in Senegal, Burkina Faso, Ghana, Mali, Gambia, Niger, Nigeria and Cameroon, and some parts of Sudan (Alowanou et al., 2020). It is known as Kantakara in Hausa, rat in Wolof and jambakatan kè in Maninka. Its other names are *Combretum cordofanum* Engl. & Diels, C. passargei Engl. & Diels, C. leonense Engl. & Diels. In traditional medicine, the plant's bark, leaves and roots are used for treating various ailments such as influenza, rheumatism, impotence and syphilis. It is also used in treating stomach pain and malaria. It is also used by different cultures in managing tooth infections. A decoction of the leaves is used as a traditional analgesic. Although, there is limited pharmacological evidences regarding the medicinal uses of this plant (Dupont et al., 2002). Therefore, there is need to detect and quantify the phytochemical properties of *Combretum glatinusum* plant to provide scientific explanation on its medicinal properties.

2. MATERIALS AND METHODS

Plant Samples Collection and Identification

Combretum glatinusum leaf was collected from Katanga Village, Jega Local Government, Kebbi State the plant sample was authenticated by a Taxonomist from Plant Science and Biotechnology Department, Kebbi State University of Science and Technology, Aleiro. Kebbi State, Nigeria. A voucher specimen (KSUSTA/PSB/H/VOUCHER NO: 185C) is deposited in the same herbarium.

Plant Preparation and Extraction

The fresh leaves and stembark of *Combretum glatinusum* were rinsed in sterile distilled water and shed-dried for a week. The dried samples were pulverized, using mortar and pestle, into fine coarse form. One hundred grams (100g) each of the powdered plant samples was weighed and soaked in 3.5 liters of methanol, ethanol and water for 72hours. The extract was filtered using muslin cloth and then filtrated through a Whatman filter paper No. 1 and concentrated using rotary evaporator set at 45°C (Harborne, 1973). The concentrated extracts were transferred into an open container and allowed to stay until it dried. The percentage yield was determined using the expression as follows

$$\text{Percentage yield} = \frac{\text{weight of extract}}{\text{weight of ground plant material}} \times \frac{100}{1}$$

Qualitative Phytochemical Screening of Extracts

The Phytochemical screening for determination of alkaloids, cardiac glycosides, flavonoids, tannins, saponins, terpenoids, phenols, glycosides and anthocyanins in extract were carried out according to the methods described by Harbone, (Sofowora, 1993; Trease and Evans, 1989; Sani et al., 2018).

Antibacterial Studies

Collection of Bacterial

S.aureus and *K. pneumoniae* was isolated from tooth of an infected patient suffering from severe toothache from Federal Medical Centre Birnin Kebbi, Kebbi State, Nigeria. The bacteria were screened using selective and differential media.

Preparation of Inoculums

After the sub-culturing, the sub-cultures were inoculated on fresh nutrient agar plates using sterile cotton swabs at 370C for 24hrs. The pure cultured microbes on the nutrient agar plates were used as the inoculums.

Identification of Bactria

The bacteria species were identified via Morphological characteristics, gram staining and biochemical screening following the protocol established by Cheesbrough, (Ericsson, 1960).

Antibiotic Sensitivity Test

Nutrient agar media is poured into 100mm petri dishes and allowed to solidify. Bacterial inoculum is prepared by diluting the agar culture to match the 0.5 McFarland turbidity standards. A sterilized swap was used to collect the culture; excess culture was removed by gently pressing the swap against the surface of the tube. The swap was then streaked across the nutrient agar plates to form a bacterial lawn, in other to achieved a uniform growth the swap was streaked in the agar plate in one direction, rotated at 120°C it was streaked again rotated at another 1200. Flame sterilized forceps were used to pick and gently pressed antibiotic disc into the plates and were then incubated at 35°C overnight. The antibacterial activity was interpreted by a clear zone around a disc, which is measured in mm with a ruler (Ericsson, 1960).

Determination of Anti-bacterial Activity

Antibacterial activity of the plant extracts was conducted using agar well diffusion method (ditch method) as reported by (Russell and Hugo, 1994).

Determination of Minimum Inhibitory Concentration (MIC)

MIC of plant extracts were determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The tubes dilution method was used. The lowest concentration (highest dilution) of extract preventing microbial growth is considered minimal inhibitory concentration (MIC) (Fatope et al., 1993).

Minimum Bactericidal Concentration

Minimum bactericidal concentration was carried out by inoculating sample from the MIC tubes showing no bacterial growth on nutrient agar plates and was incubated at 370C for 24hrs. The plates were then observed for the presence or absence of microbial growth. The least concentration of extract showing no bacterial growth was considered the MBC (Fatope et al., 1993).

Data analysis

The data collected were expressed mean ± SD and further subjected to one way analysis of variance (ANOVA) and statistical difference between the means was separated using New Duncan's Multiple Range Test. With the aid of a statistical package (IBM SPSS Statistics 20). $P < 0.05$ is considered significant.

3. RESULTS AND DISCUSSION

Results

Qualitative Phytochemicals Constituents of Combretum glatinusum Leaves

The qualitative phytochemical constituents of *Combretum glatinusum* leaves extracted with methanol, ethanol and water are presented in (Table 1). The results revealed the presence of flavonoids, alkaloids, saponins, tannins, terpenoids and glycosides in methanol extracts. While ethanol extracts revealed the presence of alkaloids, flavonoids, saponin, tannins terpenoids, steroids and glycosides. And similarly, aqueous extract also revealed the presences of alkaloids, flavonoids, saponins, tannins, terpenoids, steroids and glycosides.

Table 1 Qualitative Phytochemicals Constituents of *Combretum glatinusum* Leaf

LEAF			
Phytochemicals	**ME**	**EE**	**WE**
Alkaloids	+	+	+
Flavonoids	+	+	+
Saponins	+	+	+
Tannins	+	+	+
Terpenoids	+	+	+
Steroids	-	+	+
Glycoside	+	+	+

Key: AE = Ethanol extract, ME = Methanol extract, WE = Water extract, + = Positive, - = Negative

Biochemical and Morphological Characteristics of Isolated Bacteria

The biochemical and morphological characteristics of the test bacteria are presented in (Table 2). The results showed that *S. aureus* is gram-positive, cooci shaped, positive to catalase test, and its colony type is small (0.05). However, the result also showed that *Klebsiella pneumoniae* is gram negative, rod shaped, positive to catalase and citrate test and its colony type is small (0.5-1μm).

Table 2 Morphological Characteristics and Biochemical Test for Isolated Bacteria

Colony Type	Color	Gram stain	Cell Shape	Oxidase	Catalase	Indole	Citrate	Bacteria
Small (0.05mm)	Pink	G+	Cocci	-	+	ND	ND	*S. aureus*
Small (0.5-1μm)	Red	G-	Rod	-	+	-	+	*Klebsiella pneumoniae*

ND= Not Detected

Antibiotic Sensitivity Profile of the Bacterial Strains

Table 3 present antibiotic sensitivity profiles of Staphylococcus aureus and Klebsiella pneumoniae against different antibiotic drugs. A clear zone around the discs reveals diameter of inhibition zones, which were measured in mm and translated to the categories of susceptible (+) or resistant (-). The values are translated according to the latest published Clinical and Laboratory Standards Institute (CSLI) guidelines. It was shown that, Staphylococcus aureus was sensitive to Ciprofloxacin (CPX), Erythromycin (E) and levofloxacin (LEV). Meanwhile Klebsiella pneumonia shows sensitivity to Amoxacilin (AM), Ciprofloxacin (CPX), Ceftriaxone (CTX), Nitofuruntoin (NFT) and Augmentin (AT).

Table 3 Drug Sensitivity Profile of the Test Bacteria

Bacterium	Antibiotics									
	LEV	AM	CPX	E	APX	AMP	CH	CTX	AT	NFT
Staphylococcus aureus	+	-	+	+	-	-	-	-	-	-
Klebsiella pneumonia	-	+	+	-	-	-	-	+	+	+

LEV=levofloxacin, AM=Amoxiciline, CPX=Ciprofloxacin, E=Erythromycine, APX=Ampiclox, AMP=Ampiciline, CH=Chloramphenicol, CTX= Ceftriaxone, AT=Augmentin, and NFT= Key: += susceptible, - = resistant.

Antibacterial Effect of C. glutinosum Leaves Solvents Extracts against Staphylococcus aureus

The antibacterial effect of *C. glutinosum* leaves solvent extracts against *Staphylococcus aureus* is presented in (Table 4). At 30mg/ml there were no significant (P>0.05) differences in zone of inhibitions of *C. glutinosum* leaves water and methanol extract compared to the standard drug (levofloxacin), in contrast the zone of inhibition of *C. glutinosum* leaves ethanol extract is significantly (P<0.05) lower compared to standard drug (levofloxacin). However, at 60mg/ml, only the zone of inhibition of *C. glutinosum* leaves water extract was not significantly (P>0.05) different compared to compared to the standard drug (levofloxacin), still the zone of inhibitions of both *C. glutinosum* leaves methanol and ethanol extract were significantly (P>0.05) lower compared to the standard drug (levofloxacin). At 90mg/ml there were no significant (P>0.05) differences in zone of inhibitions of *C. glutinosum* leaves water and methanol extract compared to the standard drug (levofloxacin), in contrast the zone of inhibition of *C. glutinosum* leaves ethanol extract is significantly (P<0.05) lower compared to the standard drug (levofloxacin).

Table 4 Antibacterial Effect of *C. glutinosum* Leaves Solvents Extracts against *Staphylococcus aureus* using Ethanol as Vehicle

Zone of Inhibition (mm)			
Solvent Extract	**30mg/ml**	**60mg/ml**	**90mg/ml**
Water	21.00±1.00b	22.33±0.58c	23.67±1.15b
Methanol	21.00±1.00b	19.67±0.58b	21.67±1.52ab
Ethanol	14.00±1.00a	15.33±0.57a	21.33±0.57a
Levofloxacin	19.67±0.58b	22.00±1.00c	23.67±0.57b

Results are presented as mean ± SD (n=3).

Values with different superscripts were significantly different along the column at (*P*>0.05).

Antibacterial Effect of C. glutinosum Leaves Solvents Extracts against Klebsiella pneumonia using Ethanol as Vehicle

The antibacterial effect of C. glutinosum leaves solvent extracts against Klebsiella pneumonia is present in (Table 5). At 30mg/ml there were no significant (P>0.05) differences in zone of inhibitions of C. glutinosum leaves water extract compared to the standard medication (nitrofurantoin), in contrast the zone of inhibition of C. glutinosum leaves methanol and ethanol extract are significantly (P<0.05) lower compared to a standard medication (nitrofurantoin). Similarly, at 60mg/ml there were no significant (P>0.05) differences in zone of inhibitions of C. glutinosum leaves water extract compared to a standard medication (nitrofurantoin), in contrast the zone of inhibition of C. glutinosum leaves methanol and ethanol extract are significantly (P<0.05) lower compared to a standard medication (nitrofurantoin). However, at 90mg/ml there were no significant (P>0.05) differences in zone of inhibitions of C. glutinosum leaves ethanol extract compared to a standard medication (nitrofurantoin), in contrast the zone of inhibition of C. glutinosum leaves water and methanol extract are significantly (P<0.05) lower compared to a standard medication (nitrofurantoin).

Table 5 Antibacterial Effect of *C. glutinosum* Leaves Solvents Extracts against *Klebsiella pneumonia*

Zone of Inhibition (mm)			
Plant Extract	**30mg/ml**	**60mg/ml**	**90mg/ml**
Water	21.33±0.58b	22.00±1.00c	23.33±0.58b
Methanol	15.33±0.58a	16.00±1.00a	21.00±1.00a
Ethanol	14.33±0.58a	20.33±0.58b	27.67±0.57c

Nitofuruntoin	21.67±0.58b	22.33±0.58c	27.33±0.57c

Values are presented as mean ± SD (n=3).

Values with different superscripts were significantly different along the column at (P>0.05).

Minimum Inhibitory Concentration of C. glutinosum Leaves Solvents Extracts against Staphylococcus aureus

The minimum inhibitory concentration of C. glutinosum leaves solvent extracts against Staphylococcus aureus is present in (Table 6). C. glutinosum Leaves water extract revealed an MIC of 50mg/ml, methanol extract revealed an MIC of 100mg/ml and ethanol extract revealed an MIC of 100mg/ml.

Table 6 Minimum Inhibitory Concentration of *C. glutinosum* Leaves Solvents Extracts against *Staphylococcus aureus*

Conc. in mg/ml										
Solvents	**200**	**100**	**50**	**25**	**12.5**	**6.25**	**3.126**	**1.5625**	**0.78125**	**0.3900025**
Water	-	-	-	+	+	+	+	+	+	+
Methanol	-	-	+	+	+	+	+	+	+	+
Ethanol	-	-	+	+	+	+	+	+	+	+
Levofloxacin	-	-	-	-	+	+	+	+	+	+

Minimum Inhibitory Concentration of C. glutinosum Leaves Solvents Extracts against Klebsiella pneumonia

The minimum inhibitory concentration of *C. glutinosum* leaves solvents extracts against *Klebsiella pneumonia* is present in (Table 7). *C. glutinosum* Leaves water extract revealed an MIC of 12.5mg/ml, methanol extract revealed an MIC of 100mg/ml and ethanol extract also revealed an MIC of 100mg/ml.

Table 7 Minimum Inhibitory Concentration of *C. glutinosum* Leaves Solvents Extracts against *Klebsiella pneumonia*

Conc. in mg/ml										
Solvents	**200**	**100**	**50**	**25**	**12.5**	**6.25**	**3.126**	**1.5625**	**0.78125**	**0.3900025**
Water	-	-	-	-	-	+	+	+	+	+
Methanol	-	-	+	+	+	+	+	+	+	+
Ethanol	-	-	+	+	+	+	+	+	+	+
Nitrofurantoin	-	-	-	-	-	-	+	+	+	+

Minimum Bactericidal Concentration of C. glutinosum Leaves and Stembark against S. aureus and K. pneumonia

The minimum bactericidal concentration of *C. glutinosum* solvent extracts against *S. aureus* and *K. pneumonia* is present in (Table 8). Against *S. aureus C. glutinosum* leaves, water extract revealed an MBC of 100mg/ml and methanol, and ethanol extract revealed an MBC >200mg/ml, respectively. While against *K. pneumonia, C. glutinosum* leaves water extract revealed an MBC of 25mg/ml and methanol and ethanol extract revealed an MBC of 200mg/ml, respectively.

Table 8 Minimum Bactericidal Concentration of C. glutinosum Leaves against S. aureus and K. pneumonia

Conc. in mg/ml		
Solvents Extract	***S. aureus***	***K. pneumonia***
Water	100mg/ml	25mg/ml
Methanol	>200mg/ml	200mg/ml
Ethanol	>200mg/ml	200mg/ml

FTIR analysis of C. glutinusum Leaves and Barks

The FT-IR spectra of the C. glutinusum leaves and barks, shown in Table 9, and represent the characteristic frequencies of the functional groups associated with the compounds. The spectra of the leaves exhibits characteristics peaks at 3323.0 cm-1 for O-H, stretching

vibration, 2851.4 cm-1 is for C-H streching vibration, 1630.7 cm-1 is for C=O stretching vibration, 1176.0 cm-1is for C-OH and 1043.7 is for C-OR stretching vibration.While, the bark spectra of the C. glutinusum, exhibit characteristics peaks at 3369.5 cm-1 for O-H stretching vibration, 1634.4cm-1 is for C=O stretching vibration, 1183.4 cm-1 for C-N stretching vibration, and 1051.1 for C-OH stretching vibration.

Table 9 Summary of FTIR result

Samples	Peak Absorption Frequency (cm-1)	Intensity	Functional groups	Classes
Leaf	3323.0	Strong & Broad	O-H	Alcohol
	2851.4	Medium	C-H	Alkane
	1630.7	Medium	C=O	Carbonyl
	1176.0	Medium	C-OH	Alcohol
	1043.7	Medium	C-OR	Ether
Bark	3369.5	Strong & Broad	O-H	Alcohol
	1634.4	Strong	C=O	Carbonyl
	1183.4	Medium	C-N	Amines
	1051.1	Medium	C-OH	Alcohol

4. DISCUSSION

Phytochemicals are secondarily derived chemical compounds originating from plants; generally, they are synthesized by plants to protect them against foreign microbes. Phytochemicals are also synthesized by plant to prevent insects and other animals from consuming them (Velu et al., 2018). The phytochemical composition of *Combretum glatinusum* indicated the presence of Terpenoids, glycosides, Steroids, Tannins, Saponins, Alkaloids, and Flavonoids. These Phytochemicals are documented to have bactericidal activities when isolated and are the active ingredients for the anti-microbial activities of many plants (Barbieri et al., 2017). Other than the antioxidant properties of flavonoid, it also protects against platelet aggregation, microorganisms, hepatotoxins, viruses, tumors, ulcers, and free radicals (Akbari et al., 2022).

In the synthesis of recent antibiotics plants, based phytochemicals (secondary metabolites), such as terpenoids, alkaloids, tannins, and flavonoids, played vital pharmacological activities and are potentially used as starting material in pharmaceutical processes (Elshafie et al., 2023). Secondary metabolites such as steroids, alkaloids, phenolic, lignans, carbohydrates and glycosides have been documented with several medicinal applications including, anti-allergenic, antitumor, antimicrobial, anti-inflammatory, anti-diabetes and antioxidant activities (Alamgir, 2018). In the present study, *Combretum glatinusum* leaf extracts revealed numerous Phytoconstituents, this might be the reason for pharmacological activity of the plant. Reviews suggest that Alkaloids have several biological functions, such as forming hydrogen bonds with enzymes, receptors, and proteins.

These are due to the presence of functional groups, a proton-accepting nitrogen atom, and with several proton-donating amine hydrogen atoms, attributing to their pharmacological properties (Hussain et al., 2018). Saponins have several of antimicrobial mechanisms due to the alkaloids on them. Their presence is a potential threat to bacterial survival (Zaynab et al., 2021). Tannins are compounds that interact with proteins to form a stable waste insoluble complex (Fraga-Corral et al., 2020). Since bacterial cells are comprised of proteins, tannins are active detoxifying agent by engulfing the protein compounds and, therefore stop their growth. Flavonoids also have an OH group attached to phenols, which are found to be effective bactericidal agents; their, activities are probably due to their ability to complex with intracellular and soluble proteins and to complex with bacterial cell walls (Górniak et al., 2019).

The presence of phenolic hydroxyl groups having high protein binding affinity may halt microbial enzymes and simultaneously increase affinity to cytoplasmic membranes, thus increasing the antibacterial activity (Kurek-Górecka et al., 2013). Through the deactivation of enzyme activity, phenols result to cells enzymes pop off crash and also cell lysis (Parusnath et al., 2023). In the present study, the antibacterial activity observed from *Combretum glatinusum* leaf extracts might be attributed to the presence of this phytochemical. The absorption frequencies that corresponded to the intensity of the spectra suggest that, the leaves and bark exhibited

a majority of detected functional groups. The leaf spectra displayed a strong peak at 3323.0 cm-1, while the bark spectra showed a peak at 3369.5 cm-1, which corresponded to the O-H (stretching) functional groups found in alcohols.

Additionally, the leaf spectra revealed peaks at 1630.7 cm-1 and 1634.4 cm-1, while the bark spectra displayed peaks at the same frequencies, indicating the presence of C=O (Stretching) functional groups in Carboxylic. Furthermore, the leaf spectra exhibited a medium peak at 1043.7 cm-1, and the bark spectra showed a peak at 1051.1 cm-1, these indicate the presence of primary amines through the C-N (stretching) functional group. A medium peak at 2851.4 cm-1 in the leaf spectra confirmed the presence of the C-H (stretching) group in alkanes. Finally, peaks at 1176.0 cm-1 and 1183.4 cm-1 in the leaf and bark spectra indicated the presence of the C-O (stretching) functional group in alcohols and ethers. Similar absorption peaks within the same range were also observed (Cheng and Cheng, 2014).

5. CONCLUSION

The present study revealed the presence of several pharmacologically active phytochemicals in methanol, ethanol and water extracts. The study also revealed outstanding antibacterial properties of this plant against *S. aureus* and *K. pneumonia*. Hence, this study validates the Antibacterial properties of *C. glutinusum* plant and further supports its use in traditional medicine.

Acknowledgement

Special thanks to everyone who contributed in conducting this research and writing this manuscript.

Author Contributions

Ibrahim Sani Shabanda: Designed the experiment and supervised the bench work; Yusuf Haruna: Carried out data analysis; Aishatu Abubakar Usman: Conduct the bench work and wrote the manuscript.

Ethical approval

The study was approved by the Ethics Committee of. Kebbi State University of Science and Technology, Aliero, Kebbi State, Nigeria. (Ethical approval code KSUSTA 5232).

Informed consent

Written & Oral informed consent was obtained from participant included in the study.

Conflicts of interests

The authors declare that there are no conflicts of interests.

Funding

The study has not received any external funding.

Data and materials availability

All data associated with this study are present in the paper.

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