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A comparative study of antimicrobial activity of the extracts from root, leaf and stem of *Anogeissus leiocarpous* growing in Sudan

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Abstract
Anogeissus leiocarpus plant is widely used in African and Sudanese traditional medicine and is well known for its antimicrobial activities against many pathogenic microorganisms for treating of many diseases. This study was carried out *in vitro* to compare the antibacterial and antifungal activities of alcoholic crude extracts and their petroleum ether, chloroform and ethyl acetate fractions of the leaf, stem and root of *A. leiocarpus* growing in Sudan against five standards bacteria, *Staphylococcus aureus, Escherichia coli, Klebsiella aerogens, Pseudomonas aeruginosa, Salmonella typhi,* and two standards fungi, *Candida albicans and Aspergillus niger*. The bioactive fractions were subjected to TLC and HPLC analysis. The results showed remarkable antibacterial and antifungal activities of *A. leiocarpus* against these seven tested microorganisms. A comparison of RP HPLC-DAD chromatogram of six bioactive fractions clearly represented the presence of different phenolic compounds.

Keywords: *Anogeissus leiocarpus*, Sudan, Extracts, antimicrobial activities, RP HPLC-DAD Analysis, phenolic compounds.

1. Introduction
Anogeissus leiocarpus is an important evergreen tree of the family Combretaceae, widely distributed in Africa [1, 2] and is well known in African and Sudanese traditional medicine for treatment of many diseases such as toothache, diarrhea, respiratory diseases, jaundice, hepatitis, haemorrhoids, headache and as antimalarial, leprotic, laxative and anthelmintic [1, 3, 4, 5, 6, 7, 8], skin diseases and infections, wounds infections, sore feet, boils, cysts, syphilitic and diabetic ulcers [3, 9, 10]. It showed strong antibacterial and antifungal activity against many pathogenic microorganisms [11, 12, 13, 14, 15, 16]. Combretaceae family was reported to have high concentrations of flavonoids, terpenoids, tannins or polyphenolic compounds, which were known for their antimicrobial activity [14, 15, 16, 17]. It was reported to be rich in ellagitannins [18] and a one of the higher plant source of stilbenes [19]. Tannins, polyphenol, flavonoids, steroids, stilbenes, liginan and other metabolites with antimicrobial activity were reported in the genus *Anogeissus* [15, 16, 20, 21, 22, 23, 24, 25, 26]. *A. leiocarpus* bark showed the presence of polyalcohol, terpenoids, traces of alkaloids and derivatives of ellagic acid [17, 27].
This study was carried out to investigate and to compare the antimicrobial activity of the leaf, bark and root extracts of *A. leiocarpus* against seven microorganisms (*Staphylococcus aureus, Escherichia coli, Klebsiella aerogens, Pseudomonas aeruginosa, Salmonella typhi, Candida albicans and Aspergillus niger*) reported as accusative agents and attributed to many diseases, such as toothache, diarrhea, respiratory diseases, and skin diseases and infections [6, 7, 8, 10, 11, 13, 15, 28, 29, 30, 31], traditionally treated by these three parts of the study plant [1, 3, 4, 5, 6, 7, 8].

2. Materials and Methods
2.1. Plant Material Collection and Preparation
The plant samples were taken from healthy old trees. Root, leaf and stem were collected separately from *A. leiocarpus* trees in El Damazeine region in Sudan, and were identified by taxonomist in the department of silviculture, Faculty of Forestry, University of Khartoum, and the voucher specimens were deposited in the Herbarium of the Department of Biochemistry, Commission of Biotechnology and Genetic Engineering, National Centre for Research.
The plant materials were air dried under shade at room temperature. Barks and roots were chipped to small chips using sawmill, then, ground to a coarse powder using electric grinder.

The leaves were ground into powder by using pestle and mortar, and then all samples were stored separately in paper bags.

2.2. Preparations of the Extracts

The plant materials were extracted by maceration overnight in 80% alcohol and the alcoholic extracts were fractionated (liquid/liquid) in sequence using solvents with increasing polarities petroleum (PE), chloroform ($CHCl_3$) and ethyl acetate (EtOAc). The solvents were evaporated by using a rotary evaporator under reduced pressure and the extracts were concentrated to dryness at room temperature.

2.3. Preparation of Microbial Suspensions

One ml aliquots of a 24 hours broth culture of test organisms were aseptically distributed into sabouraud dextrose agar (for fungi) or nutrient agar (for bacteria) and incubated at 25 °C for 4 days. The microbial growth was harvested and washed with sterile normal saline and finally suspended in 100 ml of normal saline and the suspension was stored in the refrigerator.

2.4. Antimicrobial Activity Test

The antimicrobial activity of *A. leiocarpus* leaf, bark and root alcoholic extracts and their petroleum ether, chloroform and ethyl acetate fractions against was assessed against five bacteria and two fungi (*Pseudomonas aeruginosa*, *Klebsiella aerogens*, *Escherichia coli, Salmonella typhi, Staphylococcus aureus*, *Aspergillus niger* and *Candida albicans*), using agar diffusion method [13, 32] with minor modifications.

Twenty ml of aliquots inoculated sabouraud dextrose (for fungi) or nutrient (for bacteria) agar were distributed into sterile Petri dishes. The agar was left to set and in each of these plates 4 cups (10 mm in diameter) were cut using bore tool 10 mm in diameter. Alternative cups were filled with 0.1 ml from different extracts and fractions using automatic micropipette. The extracts were allowed to diffuse at room temperature for two hours, and then the plates were incubated at 25 °C for 24 hours.

In another set of experiment, the solvent used for extract dissolution was added as negative controls by filling the cups with it instead of extracts; also positive controls of organisms in a media were added. The test was carried out in triplicate

2.5. Determination of the Minimum Inhibitory Concentrations (MIC)

MIC values were determined according to a modified method of the serial dilution [10, 15]. It was determined by incorporating serial amounts (10, 5, 2.5, 1.25, 0.62, 0.31 mg/ml) of the extracts solutions into sets of test tubes containing culture media. Using a micropipette, 0.01 ml of the standard test bacterial and fungal suspension was added to each of the test tubes. All inoculated tubes were incubated at 37 °C for 24 h. At the end of 24 h incubation growth (turbidity in broth) was observed.

3. Results and Discussions

The results of the *in vitro* antimicrobial activity of the extracts and fractions of leaf, bark and root of *A. leiocarpus* against five standards pathogenic bacteria, *Staphylococcus aureus*, *Escherichia coli, Klebsiella aerogens*, *Pseudomonas aeruginosa* and *Salmonella typhi*) and two standards pathogenic fungi, (*Candida albicans* and *Aspergillus niger*), the causative agents of toothache, diarrhea, respiratory tract disease, skin diseases and wound infections were shown in Table 1 and Figures 1 and 2.

Table: 1 Antimicrobial activity of *A. leiocarpus* extracts against five standard pathogenic bacteria and two standard pathogenic fungi

Plant parts	Extracts	Inhibitory zones diameters (IZD) for tested organisms (mm)							Inhibition in percentage (%)						
		Sall	Ps	Kell	Ec	Sa	Ca	As	Sall	Ps	Kell	Ec	Sa	Ca	As
Leaves	Methanol	14	18	11	11	22	12	12	–	68%	–	–	74%	–	–
	Pet. ether	–	–	–	–	13	11	–	–	–	–	–	–	–	–
	Chloroform	14	14	14	12	12	15	14	–	–	–	–	–	60%	–
	Ethylacetate	22	24	13	15	27	23	24	74%	78%	–	60%	84%	76%	78%
Bark	Methanol	20	12	11	12	24	12	13	70%	–	–	–	–	–	–
	Pet. ether	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Chloroform	15	14	15	17	18	13	14	60%	–	60%	64%	68%	–	–
	Ethylacetate	22	14	14	13	22	17	16	74%	–	–	–	74%	64%	62%
Roots	Methanol	13	19	19	13	25	11	13	–	70%	70%	–	80%	–	–
	Pet. ether	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Chloroform	12	13	14	12	14	12	14	–	–	–	–	–	–	–
	Ethylacetate	15	15	21	14	22	14	15	60%	60%	72%	–	74%	–	–

Sall = *Salmonella typhi* Ps = *Pseudomonas aeruginosa* Ec = *Escherichia coli*
Kell = *Klebsiella aerogens* Sa = *Staphylococcus aureus*
Ca = *Candida albicans* As = *Aspergillus niger*

The Antibacterial Activity results (Table 1 and Figure 1), showed that, all tested bacteria were found to be sensitive towards the extracts and fractions of the plant. The highest antibacterial activity was found to be in the ethyl acetate fraction of the leaves against *Staphylococcus aureus* (27 mm).

The ethyl acetate fraction of the leaves was active against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella*

typhi and, *E. coli* at 27 mm, 24 mm, 22 mm, 15 mm, respectively. On the other hand, the ethyl acetate fraction of the root was found to be active against *S. aureus*, *K. aerogens P. aeruginosa* and *S. typhi* at 22 mm, 21 mm, 15 mm, 15 mm, respectively. However, among the bark, the chloroform and the ethyl acetate fractions both appeared to posses high activity among the most tested organisms, the ethyl acetate fraction possessed activity against *S. aureus* and *S. typhi* of 22 mm, 22 mm respectively, while, chloroform fraction possessed activity against *S. aureus*, *E. coli*, *K. aerogens* and *S. typhi* of 18mm, 17 mm, 15, 15 respectively.

Nearly, the all most of the tested extracts were possessed potent antibacterial activity against *S. aureus*, a clinically recognized human bacterial pathogen causing dental caries and periodontal disease [28], justified the traditional use of *A. leiocarpus* leaf decoction for toothache and the usage of the bark and root as chewing sticks for teeth cleaning and oral hygiene. These results demonstrate the presence of antibacterial principles in the plant chewing sticks may account for the lower incidence of dental caries among the chewing stick users and may contribute to the reported anticaries effect of chewing sticks.

S. aureus is also responsible for the respiratory infections [29], so, the potent activity of the extracts against this pathogen also justified the traditional use of *A. leiocarpus* for respiratory tract disease. It also reported to be a one of the commonest bacterial agents associated in the skin and wounds infections [10, 13, 30], so the results against this organism justified the traditional use of *A. leiocarpus* for the skin infection and wounds infection.

From the results it appeared that, there was an excellent susceptibility of *Escherichia coli, Salmonella typhi* and *Klebsiella aerogens* organisms to the extracts whereas, these bacteria were reported to be as agents associated with diarrhea [31], the results clearly demonstrated the anti-diarrhea activity of *A. leiocarpus*, in agreement with the traditionally remedy of diarrhea with *A. leiocarpus* decoctions.

The susceptibility of *Pseudomonas aeruginosa*, an organism reported to be responsible for opportunistic infections, such as respiratory tract inflammations [15], and a one of the commonest bacterial agents in the skin infections [10, 30], to the extracts of *A. leiocarpus*, justified the traditional use of *A. leiocarpus* for the respiratory tract infection and as a remedy for skin disease.

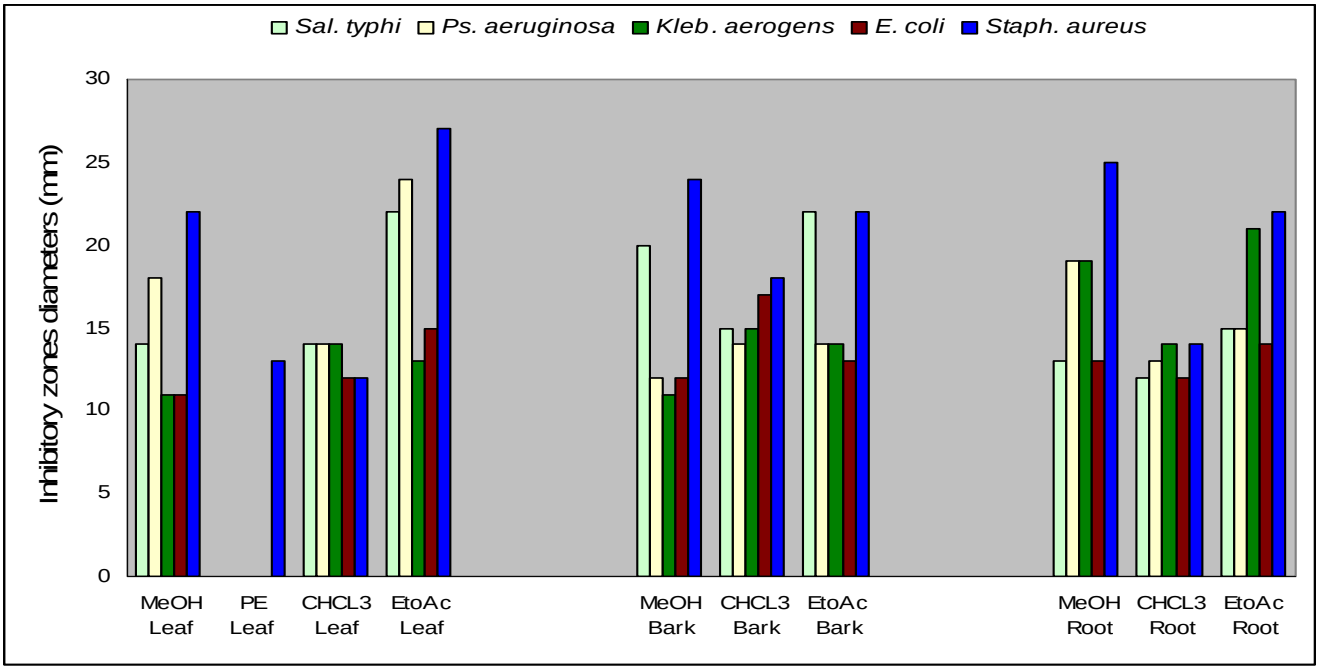


Fig: 1 Antibacterial activity of *A. leiocarpus* extracts against five standard bacteria

The antifungal activity results showed that, two fungi were found to be sensitive towards the extracts and fractions of the plant. Similarly as in the antibacterial results, the ethyl acetate fractions showed the antifungal activity higher than the others against the two tested fungi, also the highest antifungal activity was found in the ethyl acetate fraction of the leaves (*Aspergillus Niger*, 24mm).

The ethyl acetate fractions of the leaves, bark and root showed activity against *Candida albicans* and *Aspergillus Niger* of 23&24 mm, 17&16 mm, 14&15 mm respectively. The susceptibility of *Candida albicans*, the principal causative agent of candidiasis and a one of ring worm causing agents [6, 8, 10, 11, 29], and the susceptibility of *Aspergillus niger* a one causative agent of respiratory tract infection and superficial fungal infections of skin [7, 6, 8, 10] to the extracts of *A. leiocarpous* justified the traditional use of the plant for the treatment of respiratory tract infection and skin infection.

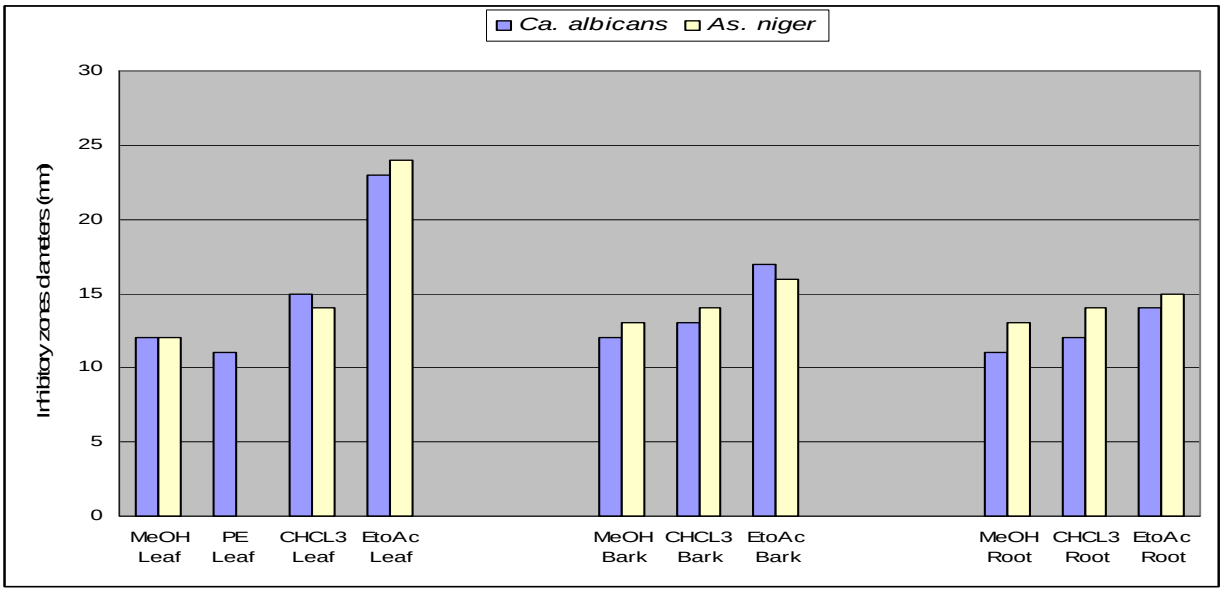


Fig: 2 Antifungal activity of *A. leiocarpus* extracts against two standards fungi

The minimum inhibitory concentration (MIC) results of *A. leiocarpus* extracts against the tested bacteria and fungi were shown in Tables 2 and 3. The MIC range against bacteria was found to be 10 – 2.5 mg/ml, and against fungi was found to be 10 – 5 mg/ml. The results of MIC values showed that, the extracts with low activity had high MIC, while, the extracts with high activity had low MIC in agreement with the results reported in current literature [13, 33, 34].

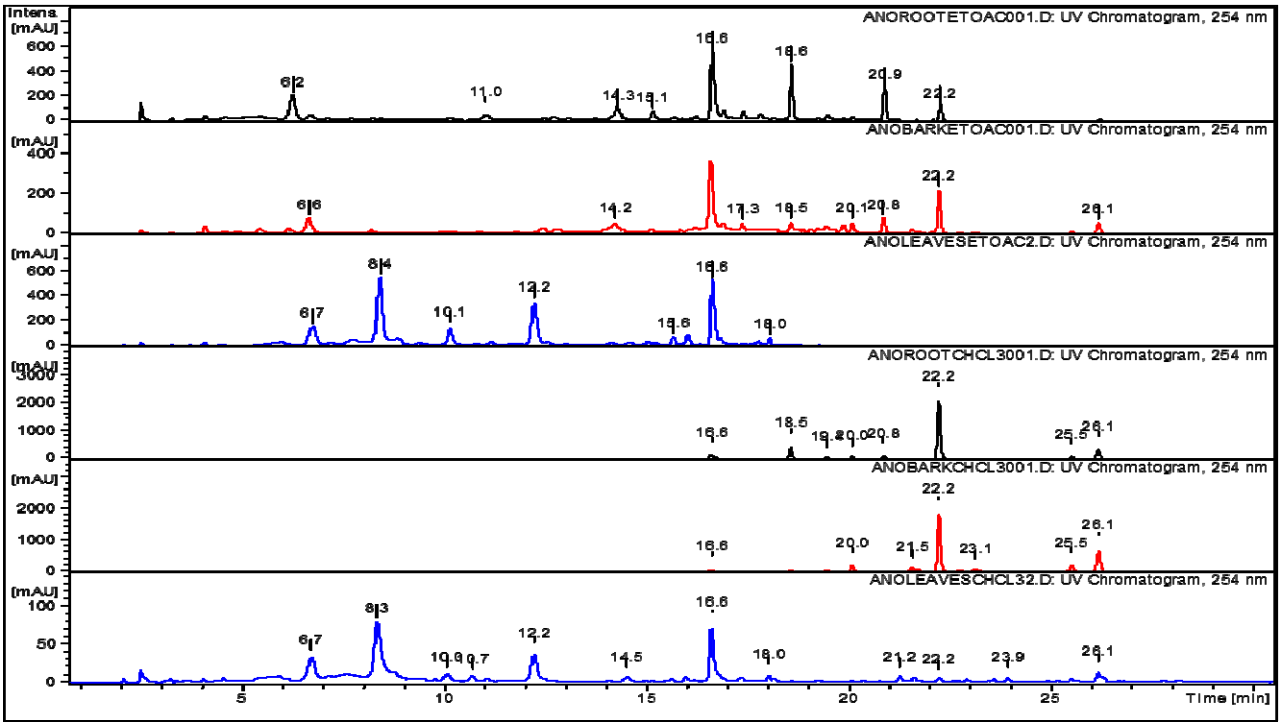
Table 2: Minimum inhibitory concentration of *A. leiocarpus* extracts against five tested bacteria

Plant part	Extract	Tested organism	MIC
Leaves	Methanol	*Salmonella typhi*	10mg/ml
		Escherichia coli	5mg/ml
		Staphylococcus aureus	2.5mg/ml
	Ethyl acetate	*Salmonella typhi*	5mg/ml
		Pseudomonas aeruginosa	10mg/ml
		Escherichia coli	5mg/ml
		Staphylococcus aureus	2.5mg/ml
Bark	Methanol	*Salmonella typhi*	10mg/ml
		Staphylococcus aureus	2.5mg/ml
	Chloroform	*Salmonella typhi*	10mg/ml
		Escherichia coli	5mg/ml
		Klebsiella aerogens	10mg/ml
		Staphylococcus aureus	5mg/ml
	Ethyl acetate	*Salmonella typhi*	10mg/ml
		Pseudomonas aeruginosa	10mg/ml
		Klebsiella aerogens	10mg/ml
		Staphylococcus aureus	2.5mg/ml
Root	Methanol	*Klebsiella aerogens*	10mg/ml
		Staphylococcus aureus	2.5mg/ml
	Ethyl acetate	*Salmonella typhi*	10mg/ml
		Escherichia coli	5mg/ml
		Klebsiella aerogens	10mg/ml
		Staphylococcus aureus	2.5mg/ml

Table 3: Minimum inhibitory concentration of *A. leiocarpus* extracts against two tested fungi

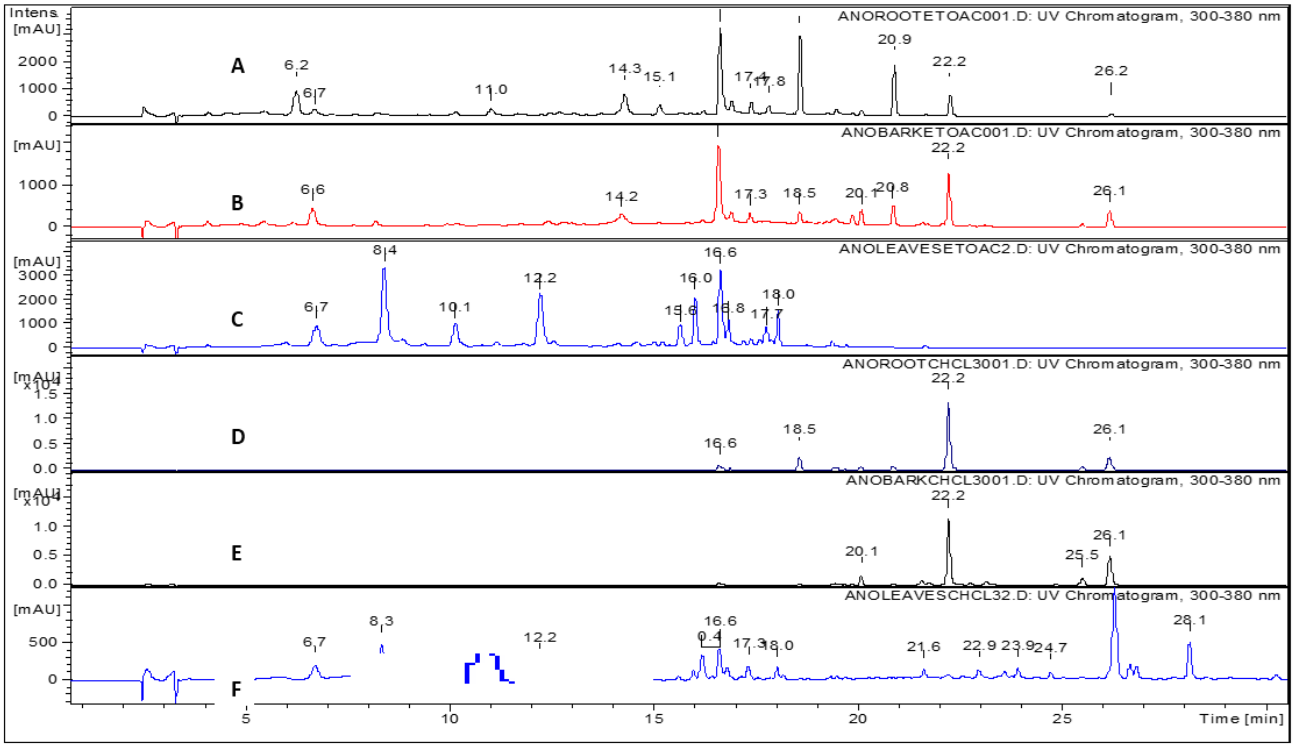
Plant part	Extract	Tested organism	MIC
Leave	Chloroform	*Candida albicans*	10mg/ml
	Ethyl acetate	*Candida albicans*	5mg/ml
		Aspergillus niger	5mg/ml
Bark	Ethyl acetate	*Candida albicans*	5mg/ml
		Aspergillus niger	5mg/ml
Root	Ethyl acetate	*Candida albicans*	10mg/ml
		Aspergillus niger	5mg/ml

A comparison results of RP HPLC-DAD chromatogram of six bioactive fractions of the three studied parts of A. *leiocarpous* namely, ethyl acetate root (A), ethyl acetate bark (B), ethyl acetate leave (C), chloroform root (D), chloroform bark (E) and chloroform leaf at λ_{max} 254 nm and 300-380 nm are presented in Figures 3 and 4. These UV range enabled the detection of phenolic metabolites [35] and the chromatograms clearly represented the presence of many similar phenolic compounds well known for their antibacterial and antifungal activity [36].



A. EtOAc root B. EtOAc bark C. EtOAc leaves
D. $CHCl_3$ root E. $CHCl_3$ bark D. $CHCl_3$ leaves

Fig: 3 RP-HPLC-DAD chromatograms of the ethyl acetate and chloroform phases of the *A. leiocarpus* leaf, bark and root recorded at λ_{max} 254 nm



A. EtOAc root B. EtOAc bark C. EtOAc leaf
D. $CHCl_3$ root E. $CHCl_3$ bark F. $CHCl_3$ leaf

Fig: 4 RP-HPLC-DAD chromatograms of the ethyl acetate and chloroform phases of the *A. leiocarpus* leaf, bark and root recorded at λ_{max} 300-380nm

4. Conclusion

In conclusion the results of the *in vitro* susceptibility of five bacteria, *Staphylococcus aureus, Escherichia coli, Klebsiella aerogens, Pseudomonas aeruginosa, Salmonella typhi,* and two fungi, *Candida albicans and Aspergillus niger* to the *A. leiocarpus* leaf, bark and root extracts showed the potent antibacterial and antifungal activities of the extracts against these microorganisms. The results confirmed the antimicrobial activity of *A. leiocarpus* and justifying its traditional uses as a medicinal plant for treatment of many diseases caused by these organisms such as toothache, skin infections, acute respiratory tract infections, fever, cough, stomach pains and diarrhea.

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