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Evaluation of the antimicrobial activity of the crude root extracts of *Allamanda cathartica* L (Apocynaceae)

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Abstract
This work was conducted in other to obtain preliminary information on the antimicrobial potential of the petroleum ether, ethyl acetate and methanol crude root extracts of *Allamanda cathartica* (Apocynaceae). Each of the root extracts was screened *in vitro* for antimicrobial activity against *Staphylococcus aureus, klebsiella pneumoniae, Streptococcus pneumoniae, Escherichia coli* and *Candida albicans*, by agar well diffusion method, at the concentrations of 50mg/ml, 100mg/ml and 150mg/ml. The ethyl acetate extract was the only extract active against all the bacteria used in the study it was also the only extract active against *Escherichia coli* producing mean inhibition zone diameters of 10±1.05mm, 14±2.0mm and 16±2.0mm for concentrations of 50mg/ml, 100mg/ml and 150mg/ml respectively. It was also the most active against *S. pneumoniae* and *K. Pneumoniae*. The methanol extract had the greatest activity of all the extracts against *S. aureus* with mean inhibition zone diameters of 16±1.35mm, 18±0.0mm and 17±1.41mm at 50mg/ml, 100mg/ml and 150mg/ml respectively. The Minimum Inhibitory Concentration of the ethyl acetate extract (being the most active) was determined against *S. aureus*, *E. coli*, *K. pneumonia* and *S. pneumoniae* and it was found to be 12.5mg/ml, 25mg/ml, 50mg/ml and 50mg/ml respectively. The extracts were not active against *candida albicans*. Conclusively, the ethyl acetate and methanol extracts of *Allamanda cathartica* showed good antibacterial activities and can serve as a lead to the development of new antibacterial agents.

Keywords: *Allamanda cathartica*, antimicrobial assay, root extract

1. Introduction
With increase of resistance to conventional antibiotic by microorganisms, there is need to sort for alternative source of antibiotic. The plant kingdom has presented very good alternative source. It is estimated that there are 250,000 to 500,000 species of plants on earth [1]. Traditional healers, since time immemorial, have depended on a good number of these plants species for the prevention and cure of infections. In particular, the antimicrobial activity of plant oils and extracts has formed the bases of many applications, including raw and processed food preservation, pharmaceuticals alternative medicine and natural therapies [2, 3]. Plants are rich in a wide variety of secondary metabolities such as tannins, terpenoids, Alkaloids and flavonoids. which have been found *in vitro* to have antimicrobial activity [4], This is a clear pointer that phytochemical could find their way into the world's almost empty antimictobial arsenal or become "lead" for development of new anti-microbial agents. The tragedy of anti-microbial resistance is such that there is a need for the development of new antimicrobial agents and a reduction on the rate of prescribing and misuse of anti-microbial agents.
Allamanda cathartica (Apocynacaea) also called golden trumpet is a medicinal plant. Many herbalists have use the leaves, roots, flowers or stem bark for the treatment of various fevers, jaundice, gastrointestinal disorders and malaria (Iwu, *et al*, 1993 and Etukudo, 2003) [5, 6]. Its larvicidal activities against *ancylostoma* species have also been reported by (Santos *et al*,) [7]. Also Rajamanickam and Sivasubraniam, 2013 [8] showed that the leaf extracts have antimicrobial activity, while Hema and Krishnaveni 2014 [9] showed the antimicrobial activity of the flower.
The need to develop newer drugs of plant origin forms the basis of this study. Thus this study evaluates the *in vitro* antimicrobial activity of the root extracts of *Allamand cathartica* against Escherichia *coli, Staphylococcus aureus, Streptococcus pneumomiae* and *candida albicans* using methanol, petroleum ether and ethyl acetate as the solvent

2. Materials and Methods

2.1 Instruments and Glass ware used

Measuring cylinder, beakers, desiccator, water bath (Techmel and Techmel, USA), weighing balance rotatory evaporator (Labscience, England) petndishes, macerating bottles, test tubes, pipette, incubator, autoclave, evaporating dish.

2.2 Reagents and solvents Used

5% ferric chloride, 2% hydrochloric acid solution, concentrated hydrochloric acid solution, 01% sodium hydroxide, dilute hydrochloric acid, chloroform, ammonia solution, 70% ethanol, ethyl acetate (JHD, Chin
a) methanol(JHD, China) and petroleum ether (JHD, China) kedde's reagent, glacial acetic acid, concentrated sulphuric acid, anhydrous acetic anhydride, molisch reagent, Fehling's solution, hagers reagent, dragendoff's reagent and mayer's reagent, 10% sulphuric acid.

2.3 Collection of plant material

The roots of *Allamada cathartica* were collected in the month of July from Choba Campus, University of Port Harcourt, rivers state, Nigeria. The plant was identified by Dr. Oladele of Department of Forestry, University of Port Harcourt. It is at the herbarium of the Department of Pharmacognosy and Phytotherapy of, University of Port Harcourt with the voucher number UPH0290

2.4 Media Used: Nutrient Agar (Tilan Brotech limited India), mueller hinton agar (titan biotech lmited India) and potato dextrose agar (Titan biotch limited India). They were all supplied by titan biotech limited England. Media were prepared according to manufacturer's specifications and sterilized by autoclaving at 121 ^{0}C for fifteen minutes.

2.5 Test organisms

The test organisms were clinical isolates of *Klebsciella pneumoniae, Escherichia coli, Streptococcus pneumoniae, Staphytococcus aureus* and *Candida albicans.*
Two gram negative organisms, *(E. coli and K. pneumoniae)* and two gram positive organisms, (*S. pneumonia* and *S. aureus)* with a fungus, (*Candida albicans).*

2.6 Instruments and Glass ware used

Measuring cylinder, beakers, desiccator, water bath (Techmel and Techmel, USA), weighing balance rotatory evaporator (Labscience, England) petndishes, macerating bottles, test tubes, pipette, incubator, autoclave, evaporating dish.

2.7 Sample Preparations

The roots of the plant were obtained, cleaned and carefully sorted out to ensure the absence of impurities such as sands, leaves, pulp and stone particles. They were allowed to shade dry in room temperature to reduce the moisture contents. The dried roots were then pulverized using a mechanical blender.

2.8 Extraction

150g, 150g and 600g of the pulverized root were weighed and introduced into three separate macerating bottles. The pulverized root was then covered with one liter of ethyl acetate, methanol and about 2 liters of petroleum ether respectively. This was macerated for 48 hours after which it was filtered. The filtrate obtained was then concentrated using a rotary evaporator after which it was placed in an evaporating dish and concentrated to dryness in a water bath at 40 ^{0}C. The obtained crude extracts were stored in a desecrator the percentage yields of the extracts were determined as:

$$\% \text{ yield} = \frac{weight\ of\ crude\ extract}{weight\ of\ dry\ sample} \text{x} \frac{100}{1}$$

2.9 Phytochemical Screening

The presence or absence of alkaloids, tannins saponins flavonoids, anthraquinones, cardiac glycosides, terpenes, and carbohydrates were determined according to the methods of (Trease and Evans, 1989 and sofowora, 1998, 2008) [10-12].

2.10 *In vitro* antimicrobial assay

2.10.1 Standardization of Test Organisms

A sterile wire loop was used to inoculate the test organisms into a universal bottle containing 10ml of sterile normal saline. The turbidity of the culture was adjusted to 0.5 McFarland standards (equivalent to approximately 1.5 x 10^8 cfu/ml, (Okore 2005) [13].

2.11 Preparation of Stock Solutions of Crude Extract

0.5g each, of the crude extracts was constituted into 10ml of ethanol to obtain 50mg/ml. one gram (1g) each of the crude extracts was dissolved in 10mls of ethanol to obtain 100mg/ml.
1.5g each of the crude extracts was dissolved in 10mls of ethanol to obtain 150mg/ml.

2.12 Antimicrobial Susceptibility Testing

2.12.1 Agar well Diffusion Assay

Mueller Hinton agar medium was prepared and distributed into suitable containers. It was sterilized in an autoclave at 121 ^{0}C for 15 minutes. The sterile medium was cooled at 45 ^{0}C. 0.1ml of standardized inoculums (0.5m McFarland standard) was introduced into each 20ml of the prepared and sterilized Mueller hinton agar. It was then poured aseptically into sterile petridishes. The plates were then allowed to stand in a horizontal position until the agar solidified.
Discs of agar were removed from the agar layer with the aid of a cork borer of 6mm diameter in order to produce wells on the agar plates. The various concentration of the plant extract (50mg/ml, 100mg/ml and 150mg/ml) was then introduced into the wells using a micro pipette. This was carried out in triplicates for each concentration.
The plates were then incubated at 37 ^{0}C for 24hrs after which they were observed for zones of inhibition.
The same methodology was used for the anti-fungal assay. A potato dextrose agar was used in place of Mueller Hinton agar and Incubation was done at 25 ^{0}C for about 6days days.

2.12.2 Antibiotic Sensitivity Testing

In vitro susceptibility of the microbes was determined using Bauer-disc. Diffusion technique (Bauer *et al*, 1996). Half ml (0.5ml) of the organisms was inoculated into 20mls of Mueller Hilton agar swirled and poured aseptically into sterile petri-dishes and allowed to set. Commercial discs containing anti-biotics were aseptically placed on the surface of the agar plate and were incubated for 24hours at 37 ^{0}C. Zones of inhibition after incubation were measured in millimeters.

2.12.3 Determination of Minimum Inhibitory Concentration (MIC)

Nutrient agar medium was prepared and distributed into suitable container. It was sterilized in an autoclave set at 121

^{0}C for 15 minutes. The sterile medium was cooled at 45 ^{0}C. 1ml of the different concentration of the crude extract (6.25mg/ml, 12mg/ml, 25mg/ml 50mg/ml and 100mg/ml) was introduced into 20ml of the sterile nutrient agar medium. It was then poured aseptically into sterile petri-dishes. The plates were then allowed to stand in a horizontal position until the agar solidified.

With the aid of a sterile swab, the organisms *(E. coli, S. pneumoniae, K. pneumoniae, S. aureus*) was streaked on the surface of the agar. The plates were then incubated at 37 ^{0}C for 24 hours after which they were observed for growth.

3. Results

Table 1: Percentage yield of the crude root extracts of *Allamanda Cathartica*

Extract	Percentage yield%
Petroleum ether	0.63
Ethyl acetate	3.03
Methanol	8.85

Result for Phytochemical screening

The result for the Phytochemical screening showed the presence of Cardiac Glycoside; Carbohydrate, Flavonoids, Phenolics, Terpenoids and steroids

Table 2: the result of *in vitro* antimicrobial activity of the crude root extracts of Allamanda cathartica

Organism	Conc. mg/ml	Methanol extract Mean IZD (mm)	Ethyl acetate extract mean IZD (mm)	Petroleum ether extract mean IZD (mm)
E. coli	50	Nil	10±1.05	Nil
	100	Nil	14±2.00	Nil
	150	Nil	16±2.00	Nil
S. aureus	50	16±1.35	13±1.35	Nil
	100	18±0.00	14±1.41	18±2.00
	150	17±1.41	17±1.41	17±0.00
K. pneumonia	50	Nil	10±1.05	Nil
	100	13±1.00	11±1.16	Nil
	150	17±3.31	17±1.53	Nil
S. pneumonia	50	11±1.41	16±1.41	Nil
	100	14±1.87	17±0.00	Nil
	150	18±1.87	20±0.00	Nil
C. albicans	50	Nil	Nil	Nil
	100	Nil	Nil	Nil
	150	Nil	Nil	Nil

Nil = no activity, IZD = Inhibition Zone Diameter.

Table 2 above showed the result of the *in vitro* antimicrobial activity of the crude root extracts of *Allamanda cathartica* of different solvents. The result showed that ethyl acetate has activity on all the bacteria used in this work. It gave the highest activity against S. pneumonia with IZD of 20±00mm. the activities of the other extracts of methanol and petroleum ether were not on all the bacteria used. None of the extracts had action on the C. albicans, thus none of them showed antifungal property.

Table 3: The result of Standard Antibiotic sensitivity testing for the organisms

Gram negative organisms			Gram positive organisms		
Antibiotics	***E. coli* IZD (mm)**	***K. pneumoniae* IZD (mm)**	**Antibiotic**	***S. pneumoniae* IZD (mm)**	***S. aureus* IZD (mm)**
Ofloxacin	7	11	Ciprofloxacin	Nil	10
Pefloxacin	12	13	Norfloxacin	Nil	Nil
Ciprofloxcin	15	8	Gentamycin	Nil	Nil
Augmentin	13	Nil	Amoxicillin	Nil	Nil
Gentamycin	Nil	Nil	Streptomycin	11	13
Streptomycin	12	5	Rfampicin	16	15
Cephelexin	Nil	3	Erythromycin	11	Nil
Nalidixic acid	Nil	5	Chloramphenicol	Nil	Nil
Cotrimoxazole	10	11	Ampicillin/ Cloxacillin	Nil	Nil
Ampicilin	14	Nil	Levofloxacin	13	12

Nil = No activity, IZD = Inhibition zone diameter

From the table 3 above on the standard antibiotic sensitivity testing for the organisms, some of the antibiotics are sensitive while others are not. Those that are nil have no activity while those that are sensitive are represented with their inhibition zone diameters.

Table 4: The minimum inhibitory concentration of the ethylacetate root extract of *Allamanda cathartica*

Organism	Concentration (mg/ml)				
	6.25	12.5	25	50	100
E.coli	+	+	-	-	-
S. aureus	+	-	-	-	-
K. pneumoniae	+	+	+	-	-
S. pneumoniae	+	+	+	-	-

Key = + growth - = no growth

From table 4 above, the minimum inhibitory concentration of ethyl acetate extract on the test organisms are as follows; E. coli = 25mg/ml, S. aureus = 12.5mg/ml, K. pneumonia = 50mg/ml and S. pneumonia = 50mg/ml

4. Discussion and Conclusion

4.1 Discussion

A good number of plant materials have proven to have promising anti-microbial activity. This work evaluates the anti-microbial activity of the petroleum ether, ethylacetate and methanol root extracts of *Allamanda cathartica* against *E.coli K. pneumounae, S.pneumoniae and S. aureus*as

From the results obtained, the percentage yield of the crude root extacts of *Allamanda Cathartica* were 0.63%, 3.03% and 8.85% with petroleum ether, ethylacetate and methanol, respectively. The different in the percentage extracts may be due to the different in the polarity index of the solvents used.

The phytochemical screening test conducted on the pulverized root, showed the presence of cardiac glycosides carbohydrate, flavonoids, phenols and triterpenoids and steroids

Plants generally owe their antimicrobial properties to the presence of various secondary metabolites like phenolics, coumarins, tannins, quinones, terpenoids, flavonoids, lecitins and polypeptides etc.

Hence, *Allamanda cathartica* roots could owe its antimicrobial activity to the presence of flavonoids as proposed by Ya *et al*, 1988, [14] and Phenols as proposed by Manson and Wasserman 1987 [15] or any other of the other phytochemicals.

For the anti-microbial *in vitro* assay carried out on the crude extracts, the ethyl acetate extract was the only extract active against *Escherichia coli.* It gave inhibition zone diameter IZD of 10 ±1.05mm, 14±2.0mm and 16±2.0mm at concentrations of 50mg/ml, 100mg/ml and 150mg/ml concentrations respectively. The ethyl acetate root extract showed the greatest activity against *K. pneumoniae*. Having gave the inhibition zone diameters of 10±1.53mm, 11±1.16mm and 17±1.53mm at concentrations of 50mg/ml, 100mg and 150mg/ml respectively. It was also the most active against S. Pneumoniae having gave the inhibition zones diameters of 16±1.41mm, 17± 0.0mm and 20±0.0mm at 50mg/ml, 100mg/ml and 150 mg/ml concentrations respectively. It was second to the methanol root extract in terms of inhibitory effectiveness against *S. aureus.* The methanol root extract showed inhibition zone diameter of 16±1.35, 18±0.00 and 17±1.41 at concentrations of 50mg/ml 100mg/ml and 150mg/ml respectively against *S. aureus*. On the other hand,Ethyl acetate root extract showed inhibition zone diameters of 13±1.35mm, 14±1.41mm and 17±1.41mm against *S. aureus* at concentrations of 50mg/ml, 100mg/ml and 150mg/ml respectively. The petroleum ether extract was only active against *S. aureus* at concentrations of 100mg/ml and 150mg/ml showing inhibition zones diameters of 18±2.0mm and 17±0.0mm respectively.

The methanol and ethyl acetate root extracts were the only extracts active against S. pneumoniae. The ethyl acetate extract showed inhibition zone diameters of 16 ±1.41mm, 17± 0.0mm and 20±0.0mm at concentrations of 50mg/ml, 100mg/ml and 150 mg/ml respectively. The methanol root extract produced inhibition zone diameters of 11±1.41mm, 14±1.87mm and 18±1.87mm at concentrations of 50mg/ml, 150mg/ml and 150mg/ml respectively.

From the above, it could be inferred that the ethyl acetate extract may have a broad spectrum of activity while the methanol root extract could be used for treating infections due to gram positive organisms.

Rajamanickam *et al* reported that the petroleum ether leaf extract of Allamanda *cathartica* had the greatest antimicrobial activity of all the solvent extracts employed for the study [8]. This was not the case with the root as the ethyl acetate root extract showed the greatest activity. According to the reports of Rajamanickam *et al*, only the petroleum ether extract of the leaves of *A. cathartica* was found active against K. Pneumoniae, but both the methanol and ethyl acetate root extract showed activity against this organism. This could be attributed to variations in the secondary metabolites present in each part of same plant.

The ethyl acetate extract having the most activity against the selected clinical isolates and also having the broadest spectrum of activity, its minimum inhibitory concentration was determined against *S. aureus, E. coli, K. pneumoniae,* and *S. pneumoniae* and it was found to be 12.5mg/ml, 25mg/ml, 50mg/ml and 50mg/ml respectively.

The methanol, ethyl acetate and petroleum ether root extracts of *Allamanda cathartica* showed no antifungal activity having failed to inhibit the growth of *candida albicans.*

4.2 Conclusions

Results obtained from this study showed that the ethyl acetate crude root extract of *Allamanda cathartica* showed the broadest spectrum of activity of all the three root extracts having being able to inhibit the growth of the gram negative and gram positive organisms used in this study. The methanol extract also showed strong antibacterial activity. Thus the ethyl acetate and methanol root extracts of Allamanda cathartica could serve as a source of new antibiotics or lead to the development of new antibiotics. On the other hand none of the extract could be use as antifungal agent.

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