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RESEARCH ARTICLE

IN VITRO STUDIES OF ANTIMICROBIAL EVALUATIONS OF PETROLEUM ETHER, CHLOROFORM, ETHYL ACETATE AND METHANOL EXTRACTS OF THE LEAVES OF *PEUCEDANUM WINKLERI* H. WOLFF.

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Abstract

The in vitro antimicrobial activities of crude petroleum-ether, chloroform, ethyl acetate and methanol extracts of the leaves of Peucedanum winkleri H. Wolff, were investigated against some bacterial and fungal pathogens using agar-well difussion method. The extracts exhibited antimicrobial activities with zones of inhibition ranging from 0 – 26 mm, 0 - 25 mm and 0 – 27mm for pet-ether, chloroform and methanol extracts respectively and 0 - 29 mm for ethyl acetate extract. The minimun inhibitory concentration (MIC) for all the four extracts ranged between 6.25 - 12.5 mg/ml. Ethyl acetate extract had the lowest minimun bactericidal/fungicidal concentration (MBC/MFC) value which ranged between 12.5 - 25 mg/ml. Also, the minimun bactericidal/fungicidal concentration (MBC/MFC) of pet-ether and methanol extracts ranged between 25 - 50 mg/ml while that of chloroform extract ranged between 12.5 – 50 mg/ml. The inhibitory effect of these extracts against several bacterial and fungal species is an indication of it broad spectrum antimicrobial potential. This justified the use of the plant in herbal medicine for the treatment of diseases of microbial origin and also introduces the plant as a potential candidate for drug development for the treatment of infectious diseases caused by these pathogens.
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Keywords: *Peucedanum winkleri*, antimicrobial activity, minimum inhibitory concentration (MIC), minimum bactericidal/fungicidal concentration (MBC/MFC).

Introduction

Medicinal plants have always been associated with cultural behaviours and traditional knowledge. The renaissance of interest in plant products has been stimulated by the use of plant extracts in chronic conditions for which conventional drugs is perceived to offer very little specificity in its target (Rhiouani *et al*., 1999). Nearly 80% of the world's population relies on traditional medicines for primary health care, most of which involve the use of plant extracts (Akindele and Adeyemi, 2007a). In developing countries, the World Health Organization (WHO) estimates that about three quarters of the population relies on plant based preparations used in their traditional medicinal system and as the basic needs for human primary health care (Moorthy et al., 2007). It is known that over 30% of all prescription drugs in industrialized countries are from plant origin (Iwu et al., 1999).

Pencedanum winkleri H. Wolff is an annual herb that is widely spread in Asia, Europe and Tropical Africa including Nigeria. In northern part of Nigeria, it is useful among local medicine practitioners for the treatment of typhoid fever, high fever, intestinal disorder and as an analgesic (Madumelu et al., 2013). Phytochemical investigation of the leaves of *P. winkleri*, revealed the presence of carbohydrates, free reducing sugar, cardiac glycoside, saponins,

steroids, flavonoids, alkanoids, tannins and triterpenes (Madumelu et al., 2013). Also, the antimicrobial screening of the leaves of the plant extracted completely with distilled analytical grade methanol by Sigma-Aldrich, showed interesting and relevant antimicrobial properties that were comparable with those of some of the standard drugs used (Madumelu et al., 2013).

Globally, people have been exploring nature particularly plants in search of new drugs with healing powers in view of discovering new drugs that possess potency to combat the menace of drug resistant pathogenic microorganisms, antitumor and anticancer agents. Moreso, the increased incidence of diseases for which there is yet an effective remedy for, has also intensified this quest. In this paper, the antimicrobial properties of crude extracts of the leaves of *P. winkleri* has been studied as part of the exploration for new and novel bio-active compounds.

Materials and Methods

The leaves of *P. winkleri* was collected fresh from Shika village in Zaria, Kaduna state of Nigeria. Plant materials were identified at the herbarium unit of Biological Science Department, Ahmadu Bello University Zaria, Nigeria and a voucher specimen number was deposited in the herbarium.

Preparation of plant extracts

The plant materials were dried at room temperature and then pulverized. The pulverized sample 300 g was packed into a thimble in a soxhlet extractor and extracted exhaustively using 1.5 L of methanol. The resulting extract was concentrated at 40^0C in *vacou* using rotary evaporator and further air dried to a constant weight of 41.8 g. The dried extract was then partitioned using: petroleum ether 60-80^0C, chloroform and ethyl acetate exhaustively and respectively. All solvents used were analytical grade by Sigma-Aldrich, and were all distilled before use. Their respective extracts were concentrated at 40^0C in *vacou* using rotary evaporator and then dried in air until constant masses were achieved and also residual mass for the methanol. All four crude exracts were then subjected to antimicrobial screening.

Test organisms

Microbial strains of pathogens tested includes *Staphylococcus aureus, Methicillin resistant Staphylococcus aureus (MRSA), Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Klebsiella pneumoniae, Streptococcus pyogenes, Corynebacterium ulcerans, Proteus mirabilis, Candida albicans Candida krusei and Candida tropicalis.* Pure isolates of these microorganisms were obtained from the Department of Medical microbiology Ahmadu Bello University Teaching Hospital Zaria, Nigeria. All the isolates were checked for purity and maintained in slants of Nutrient agar for the bacteria and in slants of Sabouraud dextrose agar for the fungi.

Media used

Mueller Hinton Agar was the medium used as the growth medium for the microbes.

Antibacterial/fungal activity

The antibacterial/fungal activities of the extracts was determined using some pathogenic microorganisms. 0.5 g of the extract was weighed and dissolved in 10 ml of DMSO to obtain a concentration of 50 mg/ml. This was the initial concentration of the extract used to check the antibacterial/fungal activities of the extracts. Mueller Hinton Agar was prepared according to the manufacturer's instructions, sterillized at 121^0C for 15 mins, poured into sterile petridishes and was allowed to cool and solidify. Diffusion method was used for the screening of the extracts. The sterillized medium was then seeded with 0.1 ml of the standard inoculum of the test microbe, the inoculum was spread evenly over the surface of the medium by the use of a sterile swab. Well was then bored at the centre of each inoculated medium using a sterile 6 mm diameter standard cork borer. Approximately 0.1 ml of the crude extract at 50 mg/ml was then introduced into the well on the inoculated medium. The inoculated medium was incubated at 37°C for 24 h for the bacteria and at 30^0C for 1-7 days for the fungi. Controls were set up in parallel using the solvents that were used to reconstitute the extracts. Each plate was observed for zones of inhibition of growth. The zones were measured with a transparent ruler and the results were recorded in millimetres. The effects were compared with those of some standard drugs namely: cefuroxine, sparfloxacin and erythromycin at a concentration of 40, 40 and 50 µg/ml respectively for bacterial and fluconazole at a concentration of 50 µg/ml for fungi.

Minimum inhibitory concentration (MIC)

The estimation of MIC of the crude extracts was carried out using the broth dilution method. Mueller

Hinton broth was prepared, 10 ml was dispensed into test tubes and was sterilized at 121^{o}C for 15 mins, the broth was allowed to cool. MC-farland's turbity standard scale number 0.5 was prepared to give turbid solution. Normal saline was prepared, 10 ml was dispensed into sterile test tube and the test microbe was inoculated and incubated at 37^{o}C for 6 h. Dilution of the test microbes was done in the normal saline until the turbidity marched that of MC-farland's scale by visual comparison. At this point, the test microbes has a concentration of about $1.5x10^{8}$ cfu/ml. Two-fold serial dilution of the extract in the broth was made to obtain concentrations of 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.125 mg/ml. The initial concentration was obtained by dissolving 0.5 g of the extract in 10 ml of the sterile broth. Having obtained the different concentrations of the extract in the sterile broth, 0.1 ml of the test microbe in the normal saline was then inoculated into the different concentrations of the extract in the broth, the broth was incubated at 37^{o}C for 24 h, after which each test tube of the broth was observed for turbidity (growth). The lowest concentration of the extract in the broth, which shows no turbidity was recorded as the MIC.

Minimum bacteriocidal/fungicidal concentration (MBC/MFC)

The MBC/MFC were carried out to check whether the test microbes were killed or only their growth was inhibited. Mueller Hinton agar was prepared and sterilized at 121^{o}C for 15 mins, the medium was poured into sterile petridishes and were allowed to cool and solidify. The contents of the MIC in the serial dilution were then subcultured onto the prepared medium, incubation was made at 37^{o}C for 24 h, after which each plate was observed for colony growth. The lowest concentration of the extracts without a colony growth was recorded as the MBC/MFC.

Results

The results of the antibacterial/fungal test, MIC and MBC/MFC of the extracts are presented in table 1 - 4.

Discussion

All four extracts tested, showed varying degrees of antibacterial activities against the test bacterial species. The antibacterial activities of pet-ether, chloroform, ethyl acetate and methanol extracts shows relevant and comparable activities with those of the standard drugs (Cefuroxine, Sparfloxacin and Erythromycin) used. The zones of inhibition of the ethyl acetate extract was between 0 - 29 mm and was found to be more effective than pet-ether, chloroform and methanol extracts with zones of inhibition which ranged between 0 - 26 mm, 0 – 25 mm and 0 – 27 mm respectively. Also, the four extracts were resistant to *Streptococcus pyogenes, Corynebacterium, Proteus mirabilis* and *Pseudomonas aeruginosa* (Table 1). Similarly, the four extracts showed relevant antimycotic activities against two out of the three tested fungal isolates (Table 2). The minimun inhibitory concentration (MIC) of all four extracts ranged between 6.25 - 12.5 mg/ml (Table 3). From Table 4, ethyl acetate extract had the lowest minimun bactericidal/fungicidal concentration *(*MBC/MFC) value which ranged from 12.5 – 25 mg/ml implying a higher concentration of the active components. Furthermore, the minimun bactericidal/fungicidal concentration (MBC/MFC) value for pet-ether and methanol extracts ranged between 25 - 50 mg/ml, while that of chloroform extract ranged between 12.5 – 50 mg/ml.

The inhibitory effect of these extracts of the leaves of *P. winkleri* against several bacterial and fungal species is an indication of broad spectrum antimicrobial potential and could be atributed to the presence of biologically active secondary metabolites (carbohydrates, free reducing sugar, cardiac glycoside, saponins, steroids, flavonoids, alkanoids, tannins and triterpenes) reported to be present in the plant (Madumelu et al., 2013). This justified the use of the plant in herbal medicine for the treatment of diseases of microbial origin and also introduces the plant as a potential candidate for drug development for the treatment of infectious diseases caused by these pathogens.

Conclusion

The ethyl acetate extract, showed higher activity against both bacterial and fungal isolates. The ability of the four extracts of the leaves of *P. Winkleri* to inhibit the growth of several bacterial and fungal species is an indication of its broad spectrum anti-microbial potential which makes the plant a candidate for bioprospecting for antibiotic and antifungal drugs. It is concluded that the leaves of *P. Winkleri* could be a potential source of active antimicrobial agents. Isolation and characterization of active compounds from the ethyl acetate extract, is on-going.

Table 1. Antibacterial activity profile of crude petroleum-ether, chloroform, ethyl acetate and methanol extracts of the leaves of *P. Winkleri*.

	Zone of inhibition (mm)						
Microorganism	Pet-ether (50 mg/ml)	Chloroform (50mg/ml)	Ethyl acetate (50 mg/ml)	Methanol (50 mg/ml)	Cefuroxine (40 µg/ml)	Sparfloxacin (40 µg/ml)	Erythromycin (50 µg/ml)
S. aureus	21	20	24	21	22	36	0
MRSA	21	20	27	21	30	34	0
S. pyogenes	0	0	0	0	30	30	24
C. ulcerans	0	0	0	0	0	32	0
E. coli	26	25	29	25	30	35	0
S. typhi	24	23	26	25	0	30	0
P. mirabilis	0	0	0	0	0	27	29
P. aeruginosa	0	0	0	0	0	39	22
K.pneumoniae	25	25	29	27	40	47	32

Table 2. Antifungal activity profile of crude petroleum-ether, chloroform, ethyl acetate and methanol extracts of the leaves of *P. Winkleri*.

	Zone of inhibition (mm)				
Microorganism	Crude Pet-ether 50 mg/ml	Crude chloroform 50 mg/ml	Crude ethyl acetate 50 mg/ml	Crude methanol 50 mg/ml	Fluconazole 50 µg/ml
C. albicans	22	20	24	24	32
C. krusei	0	0	0	0	34
C. tropicalis	21	20	23	21	29

Table 3. The MIC of crude petroleum-ether, chloroform, ethyl acetate and methanol extracts of the leaves of *P. Winkleri.*

Microorganism	pet-ether (mg/ml)	chloroform (mg/ml)	ethyl acetate (mg/ml)	methanol (mg/ml)
S. aureus	12.5	12.5	12.5	12.5
MRSA	12.5	12.5	6.25	12.5
E. coli	6.25	6.25	6.25	12.5
S. typhi	12.5	12.5	6.25	6.25
K. pneumoniae	6.25	6.25	6.25	6.25
C. albicans	12.5	12.5	12.5	12.5
C. tropicalis	12.5	12.5	12.5	12.5

Table 4. The MBC/MFC of crude petroleum-ether, chloroform, ethyl acetate and methanol extracst of the leaves of *P. Winkleri.*

Microorganism	pet-ether (mg/ml)	chloroform (mg/ml)	ethyl acetate (mg/ml)	methanol (mg/ml)
S. aureus	50	50	25	50
MRSA	50	25	25	50
E. coli	25	25	12.5	25
S. typhi	25	50	25	25
K. pneumoniae	25	12.5	12.5	25
C. albicans	50	50	25	50
C. tropicalis	50	50	25	50

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