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Antioxidant and Antimicrobial Activity of the Methanol and Petroleum Ether Extracts of the Stem of *Microdesmis puberula*

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Abstract *Microdesmis puberula* is a plant that is used in traditional medicine for the treatment of diarrhea, stomach-ache, intestinal worms, menstrual complaints, sterility, miscarriage, loss of virility and venereal diseases, treat skin conditions, renal pain, severe headache, erectile dysfunction, and snake bite. In this work, methanol and petroleum ether extracts of the stem of *Microdesmis puberula* were evaluated for phytochemical constituents, antimicrobial activity and antioxidant activity. DPPH scavenging assay and total antioxidant capacity were used for the determination of the antioxidant activity. The agar well diffusion method was used to determine the antimicrobial activities of the extracts against the test organisms, *Klebsiellapneumonia, Bacillussubtilis, Salmonella typhi, Enterococcus faecalis, Neisseria gonorhoeae, Escherichia coli, Pseudomonas aeruginosa, Streptococcus pyrogenes, Staphylococcus aureus* and *Candida albicans.* The broth dilution method was used to determine the minimum inhibitory concentrations (MIC) of the methanol and petroleum ether extracts. The methanol extract exhibited antimicrobial activity against both Gram-positive and Gram-negative test organisms with zones of growth inhibition ranging from 12 to 16 mm in the agar well diffusion test, but the petroleum ether did not exhibit antimicrobial activity as it recorded no zone of growth inhibition. The methanol extract was active against the test organisms with MIC range of 6.25 to 12.5 mg/mL and that of petroleum ether ranged from 50 to 200 mg/mL. The reference drug showed activity between 1.56 to 25 mg/mL. The IC_{50} of the methanol and petroleum ether extract, and the reference drug with regard to the DPPH scavenging activity, were 1.1µg/mL, 1.2 µg/mL and 0.2 µg/mL respectively. Both the methanol and petroleum ether extracts exhibited antimicrobial and antioxidant activity.

Keywords Antioxidant, Antimicrobial, Phytochemical constituents, Total antioxidant capacity, *Microdesmis puberula*

Introduction

Herbal medicines have for centuries been used for the management, cure and relief of symptoms of diseases. Plants are very important source of drugs [1]. Interest in herbal medicines originates from their long use in folk medicines [2].

Leaves, roots and stem of *Microdesmis puberula* have many therapeutic uses. The twigs, leaf and roots are used to treat snakebites [3]. The leaf sap grounded with the twig sap, is usually applied to treat diarrhea [4]. The sap obtained from the leaf and stem or a mixture of them is normally used in the treatment of worms, stomach upset, menstrual problems, infertility, miscarriage among others [5]. The sap obtained from the leaf and stem, or a mixture is also useful for treating skin conditions like burns, eczema, scabies and sores from gonorrhea and circumcision [6]. The ashes obtained from the burnt stems mixed with palm oil and salt is massaged on the hips in limping conditions. It is also used to treat renal pain and severe headache when the ashes of the stem wood are mixed with palm oil [7].

The stem of *M. puberula* is used to treat erectile dysfunction [8]. Stress-associated conditions like pain could also be treated with *M. puberula* [9].

Many human diseases are as a result of infections caused by bacteria [10]. For instance, 7.3% of pregnant women attending antenatal care in Ghana have been identified to have significant bacteriuria [11]. Typhoid fever which is also a bacterial disease is spread through contact with food or water contaminated by fecal matter or sewage [12].

Meningococcal meningitis is also a bacterial disease which causes an inflammation of the lining of the brain and spinal cord. Symptoms include stiff neck, high fever, headaches and vomiting. The bacteria are transmitted from person to person by respiratory droplets and facilitated by close and prolonged contact resulting from crowded living conditions, often with a seasonal distribution. Death occurs in 5–15% of cases, typically within 24-48 hours of onset of symptoms as reported by Ghana Major infectious diseases [13]. Bacterial infections are, therefore, of economic importance to every nation including Ghana. Plants have been found to possess antimicrobial properties [14-17]. The extraction, isolation and identification of a lead compound which will serve as an antimicrobial agent will be of great benefit to Ghana and the world at large.

Natural antioxidants are very efficient in preventing damage caused by oxidative stress [18]. Individuals have in-built antioxidative mechanism and a lot of the biological functions including anti-carcinogenic, and anti-aging and antimutagenic are derived from it [19]. Major studies have shown the roles of reactive oxygen species and other oxidants in causing lots of health problems. This has made scientists appreciate that some diseases can be prevented and managed by antioxidants [20] and a lot more research have been conducted into plant materials for their antioxidant properties [21-26].

Due to its medicinal importance, *M. puberula* has been researched into by scientists to determine the antioxidant and antimicrobial properties of various extracts of the roots [8]. Work done on the stem has concentrated on the anti-stress and analgesic properties of the methanolic extract [9]. However, to the best of our knowledge, no work has been done on the antimicrobial and antioxidant properties of the extracts of the stem. The purpose of this study, therefore, was to determine the antimicrobial and antioxidant properties of the stem of *M. puberula*.

Materials and Methods

Collection and identification of plant materials

The stem of *M. puberula* was collected in September, 2015, at CSIR (Fumesua) in the Kumasi Metropolis in the Ashanti Region of Ghana. It was authenticated in the Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical sciences, Kwame Nkrumah University of Science and Technology, Kumasi, where a voucher of the specimen is lodged. The voucher number is #KNUST/HM/1/2016/L010.

Extraction of bioactive constituents

The fresh stem was collected, shade dried and was later coarsely powdered. 500g of the powdered sample was soaked separately in 2000 ml each of methanol and petroleum ether and extracted using the soxhlet apparatus. The extract obtained were concentrated to dryness using the rotary evaporator. Each of the dried extracts was labeled and stored in a refrigerator, until required for subsequent usage.

Phytochemical screening of extracts

Preliminary phytochemical screening for secondary metabolites was performed using the methods by Trease and Evans [27] and Harborne [28]. The extracts were assayed and analyzed for phytoconstituents such as sterols, Terpenoids, tannins, flavonoids, saponins, cardiac glycoside, coumarins alkaloids and anthraquinones.

Antimicrobial activity determination

Preparation of inoculums

20 mL of nutrient broth each in ten test tubes were each seeded with 0.1 mL of the test organisms and cultured at 37 ^{o}C for 18 hrs in an incubator.

Antimicrobial susceptibility testing

The activity of the extracts against the test organisms was determined according to the method described by Agyare and colleagues [30]. 20 mL of nutrient agar was melted and stabilized at 45ºC for 15 min. The sterile agar was then seeded with 0.1 mL portion of day old cultured organisms in broth. The seeded agar was transferred into sterile Petri-dishes which were then covered and allowed to set. A number 7 cork borer was sterilized using the flame of the Bunsen burner and allowed to cool. In each petri dish, 5 wells were punched out, equidistant from one another using the sterile cork-borer No. 7 (10 mm diameter) and labeled appropriately. A volume of 100 µL of 5%, 10%, 15%, and 20% w/v concentrations of the extracts (methanol and petroleum ether) of *M. puberula* and 100 µg/mL of ciprofloxacin and 10 mg/mL ketoconazole as reference antibacterial and antifungal agents respectively were poured into each well cup. The plates were allowed to stand for 60 minutes on the bench to ensure enough diffusion of the plant extracts into the agar. The Petri-dishes were then incubated for 24 h at 37 ºC. The experiments were performed in triplicate. The zones of growth inhibition were measured and mean zones of inhibition determined. Results were expressed as mean ± standard deviation.

MIC determination by micro-dilution method

The minimum inhibitory concentration (MIC) of the extracts was determined using the micro-dilution method employing 96 well microtitre plates [31]. The plates were initially filled with 100 µL double strength nutrient broth and 10 µL of 24 h organism's suspension. 50 µL volume of plant extract and 40 µL of sterile water were then added to each well to make it up to the required volume of 200 µL and to obtain the required concentrations (200, 100, 50, 25, 12.5, 6.25, 3.1, 1.56 and 0.7813 mg/mL).

For the reference antibacterial drug, ciprofloxacin, 50µg was dissolved in 10 ml of water with organisms and concentrations (50, 25, 12.5, 6.25, 3.125 1.56 and 0.78 mg/mL) were obtained.

After that the plates were incubated for 24 h at a temperature of 37 ºC. The MIC was determined as the lowest concentration of test sample that inhibited growth, which was indicated by the absence of purple colouration upon the addition of 10 µL of 125 mg/mL MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). The experiment was carried out in triplicates.

Antioxidant activity tests

Antioxidant activity tests were done using DPPH free radicals scavenging activity assays and total antioxidant capacity (TAC) assay.

DPPH Free radical scavenging activity assay

The free radical scavenging activity of all the extracts was determined using the method described by Shen and colleagues [32].

Preparation of DPPH solution

0.1 mM of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) solution was prepared by dissolving 1.9 mg of DPPH in methanol to prepare 100 ml of solution. The solution was kept in darkness for 30 min.

The procedure

1 ml of DPPH solution was added to 3 ml of the solution of plant extract in methanol at different concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.56 µg/mL. It was then agitated and allowed to set for 30 min at room temperature. The absorbance was then measured at 517 nm using UV-VIS spectrophotometer. Ascorbic acid was employed as the reference drug. A solution prepared by dissolving 0.002g of DPPH radical in methanol to form 100 ml of solution was employed as the control.

The ability of scavenging the DPPH radical (% inhibition) was calculated using the formula.

$$\frac{Ao - A}{Ao} X\ 100\%$$

Where, A_0 is the absorbance of control, A is the absorbance of test solution at varying concentrations.

The test was done in triplicates and the mean was calculated. The half maximal inhibitory concentration (IC_{50}) of methanol and petroleum ether extract and the reference drug were then obtained from a dose-response curve.

Total antioxidant capacity assay (TAC)

The determination of TAC was carried out using a slight modification of the method described by Prieto *et al*. [33].

Preparation of buffer solution

To 109 mL of 28 mM sodium phosphate, 10 mL of 0.6 M sulfuric acid and 10 mL of 4 mMammomiummolybdate were mixed together in a beaker.

The procedure

1 mL of the plant extract in methanol was placed in each test tube. 3mL each of the ascorbic acid solutions of concentrations (50, 25, 12.5 6.25, 3.125, 1.567, 0.78, 0.39 µg/mL) were put in test tubes and 3 mL of the buffer solution was also added to the ascorbic acid. 0.3 mL methanol served as blank. All the tubes were incubated at 95 ^{0}C for 90 min.

UV –VIS spectrophotometer was used to measure the optical density at 695 nm after the tubes were cooled to room temperature. Ascorbic acid was used as the reference antioxidant drug. The total antioxidant capacity was expressed as ascorbic acid equivalents AAE.

Results and Discussion

Extraction of plant material

The active principles were extracted using methanol and petroleum ether separately on the pulverised stem. The yields of the extract were calculated as percetnages. The yields of extracts were 20.4 % for methanol and 2.2 % for petroleum ether. The percentage yield of extracts was higher in methanol than in petroleum ether. The degree of extraction depends on the polarity of the solvents, and that a better yield would be achieved from the solvent which is more polar [34]. This is because polar solvents are capable of dissolving the important therapeutic drug constituents which are to be extracted [35].

Phytochemical Screening

The methanol extract showed the presence of saponins, flavonoids, alkaloids and cardiac glycoside. The petroleum ether extract also showed the presence of saponin. The results are presented in Table 1

Table 1: Phytochemical constituents of powdered stem and extracts of *M. puberula*

Phytochemical	Powdered sample	MMP	PEMP
Saponins	+	+	+
Coumarins	-	-	-
Flavonoids	+	+	-
Cardiac glycoside	+	+	-
Alkaloids	+	+	-
Tannin	-	-	-
Anthraquinones	-	-	-
Sterols	-	-	-
Terpenoids	-	-	-

Key: (+) = presence of secondary metabolite; (-) = absence of secondary metabolite

MMP – Methanol extract of *M. puberula*; PEMP - Petroleum ether extract of *M. puberula*

The phytochemical tests indicated the presence of saponins, flavonoids alkaloids and cardiac glycoside. Flavonoids, saponins and cardiac glycosides have been reported to be present in the stem extract of the plant [9].

The absence of flavonoids, cardiac glycosides and alkaloids in the petroleum ether extracts might be due to the fact that petroleum ether was unable to dissolve them. Methanol extract, on the other hand, showed the presence of these phyto-constituents which could be due to the high polarity of the methanol. Studies show that, solvents which have different solubility levels have different ability to extract different phyto-constituents and this depends on the solubility or polarity of the solvent [36]. Methanol may have a higher solubility for several phyto-constituents.

Antimicrobial activity

Zone of growth inhibition (Agar well diffusion)

The methanol extract exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria, and fungi organisms with zones of growth inhibition ranging from 12 to 16 mm in the agar well diffusion test but the petroleum ether extract did not exhibit antimicrobial activity at the concentration used. The methanol extract showed activity against *K. pneumonia, B. subtilis, S. typhi, E. faecalis*and the fungus *C. albicans.* The results are shown in Table 2.

Table 2: Mean zones of growth inhibition of methanol and petroleum ether extract of stem *of M. puberula*at various concentrations against test organisms

Organisms	Zone of growth inhibition							
	Methanol extract(µg/mL)				Petroleum ether extract (µg/mL)			
	200	150	100	50	200	150	100	50
K. pneumonia	14.3±0.7	12.3±0.8	NA	NA	NA	NA	NA	NA
N. gonorhoeae	NA	NA	NA	NA	NA	NA	NA	NA
S. typhi	14.3±0.3	NA	NA	NA	NA	NA	NA	NA
B. subtilis	14.3±0.3	13 ±0.0	NA	NA	NA	NA	NA	NA
S. aureus	NA	NA	NA	NA	NA	NA	NA	NA
S. pyrogenes	NA	NA	NA	NA	NA	NA	NA	NA
E. faecalis	14.7±0.3	NA	NA	NA	NA	NA	NA	NA
P. aeruginosa	NA	NA	NA	NA	NA	NA	NA	NA
E.coli	NA	NA	NA	NA	NA	NA	NA	NA
C. albicans	16.3±0.3	NA	NA	NA	NA	NA	NA	NA

Legend: NA - not active

Petroleum ether extract not being active against any of the ten organisms may indicate that the active principles from the plant extracts could not diffuse into the agar medium and cause damage to the test organisms [30, 31]. It could also mean that diffusion was poor or the active principles are not active against the organisms.

The reference drugs, ciprofloxacin and ketoconazole used respectively against the bacteria and fungus, recorded various zones of inhibitions against the organisms. Ciprofloxacin was active against all the bacteria with zones of inhibition ranging from 14.3 ± 0.7 to 29.0 ± 1.0 mm. It showed the least activity against *S. typhi* and the highest activity against *S. pyogenes* and *B. subtilis*. Ketoconazole was active against the fungus C. albicans. The results are presented in table 3.

Table 3: Mean zone of growth inhibition of reference drugs against test organisms

Test Organism	Zone of growth inhibition (mm)			Mean + standard deviation (mm)
K. pneumonia	14	14	15	14.7 ± 0.3
N. gonorhoeae	19	22	20	20.3 ± 0.9
S.typhi	28	31	28	29.0 ± 1.0
B. subtilis	14	15	14	14.3 ± 0.3
S. aureus	17	16	16	16.3 ± 0.3
S. pyrogenes	15	13	15	14.3 ± 0.7
E. faecalis	20	20	20	20 ± 0.0
P. aeruginosa	22	25	24	23.7 ± 0.9
E. Coli	19	19	23	20.3 ± 1.3
C. albicans	16	16	17	14.3 ± 0.3

Minimum inhibitory concentration (MIC) of extracts and reference drugs against test organisms

Table 4 gives the results for the minimum inhibitory concentrations of the methanol and petroleum ether extracts. The methanol extract demonstrated a better antibacterial and antifungal activities against the test organisms than the petroleum ether extract. The methanol extract recorded MIC values ranging from 6.25 to12.5 µg/mL for the tested

organism whereas the petroleum ether extract recorded values between 50 to 200 µg/mL. The methanol stem extract of *M. puberula* was moderately active against *K. pneumonia, N. gonorhoeae, B. subtilis, S. aureasand C. albicans* but it was potent against *S. tyhi, S. pyrogenes, E. faecalis, P. aeruginosa and E. coli.* However, the methanol extract showed higher activity against *K. pneumonia, B. subtilis, S. aureus, E. faecalis*and *E. coli* than ciprofloxacin, the standard antibacterial drug. The petroleum ether extract was not active against the fungus *C. albicans.* The methanol extract, however, has similar antifungal activity to that of ketoconazole, the standard drug.

Table 4: Minimum inhibitory concentrations (MIC) of extracts and reference drugs against test organisms

Test organisms	**Minimum Inhibitory Concentration (µg/mL)**			
	Methanol	Petroleum ether	Ciprofloxacin	Ketoconazole
K. pneumonia	12.5	200	25	NT
N. gonorrhoeae	12.5	50	1.56	NT
S. typhi	6.25	200	12.5	NT
B. subtilis	12.5	50	25	NT
S. aureus	12.5	200	25	NT
S. pyogenes	6.25	200	1.56	NT
E. faecalis	6.25	200	25	NT
P. aeruginosa	6.25	200	3.13	NT
E. coli	6.25	200	25	NT
C. albicans	12.5	NA	NT	12.5

Legend: NT- not tested; NA- not active

The MIC for the reference drug, ciprofloxacin showed activity between 1.56 to 25 mg/mL.The high MIC values of the petroleum ether extract indicates that the extract has little potency or the organisms are capable of developing antibiotic resistance, whilst the low MIC values of the methanol extract indicates the efficacy of the extract [37]. Extracts with minimum inhibitory concentrations less than 8 mg/mL are considered to possess potent antibacterial and antifungal activities [38]. The antimicrobial activity of the methanol extract might therefore be recognized as moderate.

The presence of flavonoids and alkaloids in the extract might be responsible for the antimicrobial activities because both have antimicrobial properties. Studies have revealed that flavonoids possess antimicrobial activity [39]. The show of antimicrobial activity against both gram positive and gram negative bacteria indicates the presence of broad spectrum antibiotic compounds [40].

It could be seen that the MIC varied from one microorganism to another, indicating that the extracts are more potent against some of the organism than others. The methanol extracts showed higher potency against *S. subtilis, S. aureus, E. faecalis* and *E. coli* than the standard drug ciprofloxacin.

Antioxidant Capacity Determination

Reactive oxygen species (ROS) and free radicals are oxidants that are constantly generated within organisms with dire consequences if not held in check. In order for organisms to counteract oxidants and prevent their damaging biological molecules such as DNA, lipids and proteins, organisms that consume oxygen are endowed with well-integrated antioxidant systems, which include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, and macromolecules, such as albumin, ceruloplasmin, and ferritin, and an array of small molecules, such as ascorbic acid, α-tocopherol, β-carotene, and reduced glutathione [18, 19, 26].

Animal and plant tissues possess many different antioxidant components [18], and it is relatively difficult to measure each antioxidant component separately. Hence methods have been developed in recent years to evaluate the total antioxidant capacity of biological samples [32, 33].

Total antioxidant capacity (TAC)

The total antioxidant capacity of the reference drugs and the extracts were determined using the method described by Prieto *et al.* [33]. TAC was expressed as ascorbic acid equivalents. Figures 1 and 2 give the results of the TAC for the methanol and petroleum ether extracts respectively.

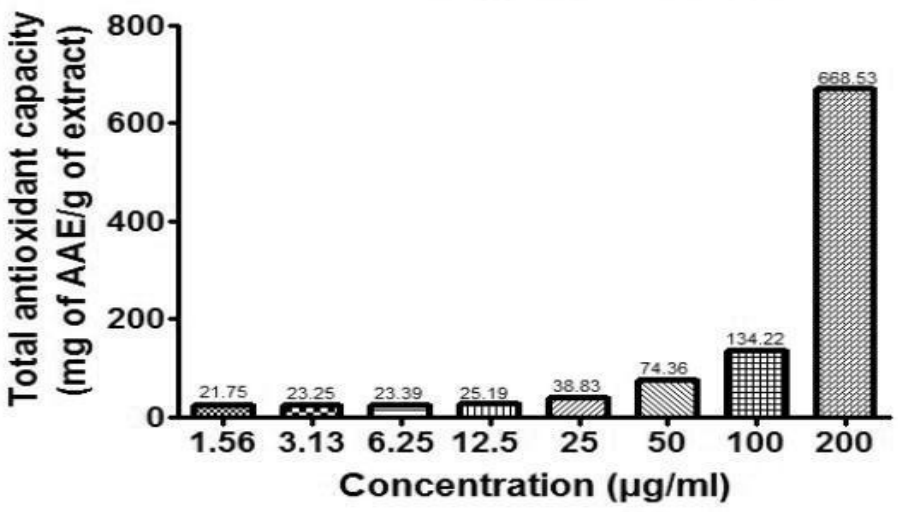


Figure 1: Total Antioxidant Capacity (TAC) of methanol extract of M. puberula

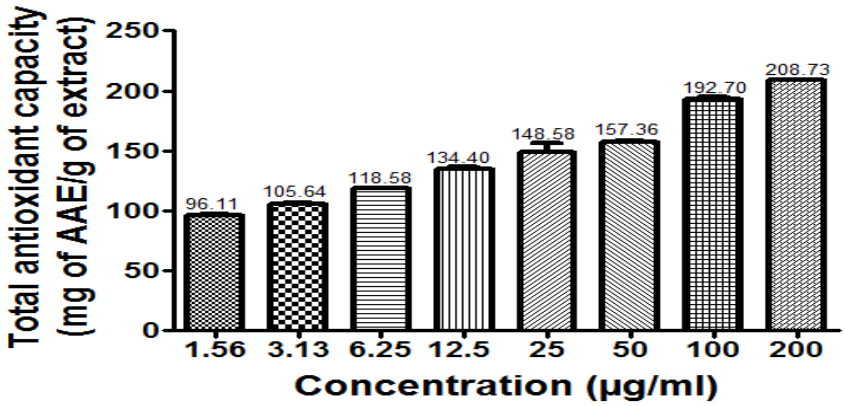


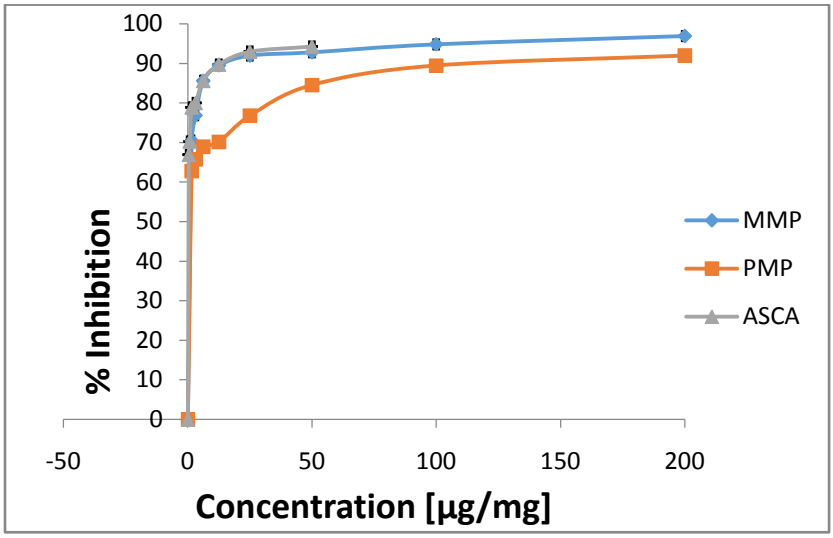
Figure 2: Total Antioxidant Capacity (TAC) of petroleum ether extract of M. puberula

TAC increased with increasing concentration of extract. Higher total antioxidant capacity was exhibited by the methanol extract than the petroleum ether extract at all concentrations. At the least concentration of 1.56 µg/mL, methanol recorded TAC of 21.75 mg AAE/g whereas petroleum ether extract recorded 96.11 mg AAE/g. This means that the methanol extract is four times more potent as an antioxidant than the petroleum ether extract. At the highest concentration of 200 µg/mL, methanol extract exhibited a TAC of 668.53 mg AAE/g with the petroleum ether recording exhibited 208.73 mg AAE/g. This means that methanol extract is three times more potent as an antioxidant than the petroleum ether extract. This indicates that the methanol extract has a higher capacity to mop up oxidants than the petroleum ether extract.

DPPH scavenging assay

The method described by Shen *et al*. [32] was used to determine the DPPH scavenging activity. The scavenging activity was studied at eight concentrations (200µg, 100µg, 50µg, 25µg, 12.5µg, 6.25µg, 3.13 µg and 1.56 µg) for the extracts and (50 µg, 25 µg, 12.5 µg, 6.25 µg, 3.13 µg, 1.56 µg, 0.78 µg and 0.39 µg) for ascorbic acid. Generally, the percent inhibition increased with increasing concentration of extract and ascorbic acid. Ascorbic acid recorded the

highest percent inhibition at concentrations less than 100 µg/mg, but the methanol extract recorded percent inhibition values similar to the ascorbic acid above this concentration. This means that the methanol extract and the standard drug possess similar antioxidant capacity above 100 µg/mg concentration. The petroleum ether extract recorded the least percent inhibition at all concentrations. The petroleum ether extract showed the least ability to scavenge free radicals with the methanol extract showing a higher ability to scavenge free radicals.



M M P- methanol extract of *M. puberula*; P M P- petroleum ether extract of *M. puberula*;
A S C A - Ascorbic acid

Figure 3: DPPH scavenging activity of methanol extract, petroleum ether extract and ascorbic acid

Table 5: The half maximal inhibitory concentration (IC_{50}) of methanol extract, petroleum ether extract and the reference drug

Compound	IC_{50} (µg/mL)
Methanol extract	1.2
Petroleum ether extract	1.1
Ascorbic acid	0.2

The methanolic and petroleum ether extracts showed antioxidant activity with IC_{50} values of 1.2 µg/mL and 1.1 µg/mL respectively. The half maximal inhibitory concentration (IC_{50}) is the concentration of the extract or standard drug that is able to scavenge or inhibit 50% of the oxidants. The lower the IC_{50} value, the better the free radical scavenging activity [41]. The IC_{50} value for ascorbic acid used as a positive control was 0.2 µg/mL. The results indicate a high antioxidant activity for both methanol and petroleum ether crude extract of *M. puberula*. The antioxidant properties of the two extracts are, however, about one fifth of that of the reference drug.

The antioxidant capacity of the methanol extract may partly be from the flavonoids it contains. Flavonoids are low molecular weight polyphenolic compounds that are abundant in nature. Many flavonoids, such as kaempherol, quercetin, luteolin, myricetin, eridictyol, and catechin, have been shown to have antioxidant, anti-inflammatory, antiallergic, anticancer, and antihemorrhagic properties [42, 43]. The alkaloid and saponincontent of the methanol extracts may also play a role in its antioxidant activities since some both saponins and alkaloids have been shown to exhibit antioxidant properties [44, 45]. The antioxidant property of the petroleum ether extract is due to the presence of saponins [45], the only phytoconstituent that was identified to be present in it.

Conclusion

The methanol and petroleum ether extracts of the stem of *M. puberula* showed the presence of saponins, flavonoids, alkaloids and cardiac glycosides. The extracts of *M. puberula* exhibited antimicrobial activities against the test organisms. The MIC values obtained for the methanol extract against both Gram-positive and Gram-negative

bacteria indicates the stem of *M. puberula* might be useful in herbal medicine for healing diseases caused by bacteria and the *C. albicans* fungus. *M. puberula* is therefore a rich source of antimicrobial agents. The free radical scavenging activity of the extracts of the stem of *M. puberula*, as shown by the total antioxidant capacity and IC_{50} values, indicate that the stem of *M. puberula* is a rich source of natural antioxidants.

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