



EVALUATION OF ANTIBACTERIAL POTENTIALS AND ANTIOXIDANT PROPERTY OF EXTRACTS OF *LACHNOSTYLIS HIRTA* (L.F.) MUELL. ARG. STEM BARK

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ABSTRACT

Multiple antibiotic resistance among bacterial pathogens is increasing as a global problem. Medicinal plants have been reported to be effective for combating resistant bacterial pathogens. The antibacterial property of extracts of *Lachnostylis hirta* against clinically important bacteria was investigated in this study using macrobroth dilution assay. The phytochemical analyses of the extracts of the plant was determined using standard assay methods and the antioxidant activity was assessed using 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic-acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide (H₂O₂) and ferric reducing power. The minimum inhibitory concentrations (MICs) of the extracts ranged from 0.1953 to 1.5625 mg/ml and 0.1953 to 0.3906 mg/ml for Gram negative and Gram positive bacteria respectively. The extracts were more effective on Gram positive bacteria than Gram negative. The methanolic extract had more phytochemicals than acetone extract except for phenolic. The scavenging activity of the extract was dose dependent. Thought lower than BTH (88.01 %) and Rutin (91.29 %), the highest scavenging potentials of 72.47 and 70.16 % were recorded for acetone and methanolic extracts respectively. At the set significant level, the (ABTS) activity of the extracts differed from the controls significantly. There was a significant difference in the ability of acetone and methanolic extracts to scavenge H₂O₂. The reducing power of acetone extract was lower than the methanolic extract at all the concentrations tested though with no significant difference.

KEYWORDS: antibiotic resistance, bacterial pathogens, *Lachnostylis hirta*, macrobroth dilution assay.

INTRODUCTION

In the recent times high resistance of pathogens to different antibiotics has been reported (Srinivasan *et al.*, 2001; Cavar *et al.*, 2013; Kouadio *et al.*, 2014). Medicinal plants have been used for thousands of years in the treatment of infectious diseases all over the world especially among the low income earners and people living in the rural areas (Duraipandiyam and Ignacimuthu, 2011; Zarai *et al.*, 2013) the recent global event of pathogen resistance. The recent increase in resistance of pathogens has shifted the focus to their use for treatment of infections (Choi *et al.*, 2009). Medicinal plants are relatively safer, cheaper and have been reported to be effectiveness. Their frequent uses pose insignificant threat of pathogen development of resistance (Parekh and Chanda 2008; Patel and Coogan, 2008; Dubey and Padhy, 2012). These attributes make phytomedicine to be of interest in the recent time and be preferred to the conventional chemotherapeutic agents (Loizzo *et al.*, 2010; Castilho *et al.*, 2012; Li *et al.*, 2012; Ye *et al.*, 2013). *Lachnostylis hirta* (L.f) Muell. Arg. is a genus endemic to South Africa especially in the forests in supper abundance. The plant serves as an artifact of regular fires that enter these forests (Webster, 1994). *L. hirta* is a genus of the family *Phyllanthaceae*, native to the southern part of South Africa.

It is a potential foliar that has peculiar stomatal complex with subsidiary cells basically paracytic (Levin, 1986). Though this plant is use for different ailments in the Eastern part of South Africa. Despite the acclaimed folkloric use of the plant as an antimicrobial agent, there is dearth of scientific information about its medicinal uses. This study therefore aimed at investigating the antibacterial potentials and antioxidant property of extracts of *L. hirta* bark.

MATERIALS & METHODS

Plant Materials

Stem bark of *L. hirta* were collected in February, 2012, in Alice Township, Nkokobe Municipality of Eastern Cape of South Africa. The plant was authenticated by Prof. D. Grierson and the voucher (DavMed 2012/2) was submitted to the Giffen Herbarium of the Department of Botany, University of Fort Hare, Alice, South Africa. The fresh plant sample was dried in the oven at 40°C and ground to fine powder. A 50g of ground plant sample was soaked in 500 ml of each of the solvents for 12 h on Stuart Scientific Orbital Shaker (Manchester, UK). The sample was then suction-filtered through Whatman number 1 filter paper and washed with another 200ml solvent. The filtrate was concentrated with Laborata 4000-efficient (Heldoph, Germany). The dried

extract was dissolved in their extracting solvent and topped up with water to make the required concentrations. The reconstituted extracts were filter by 0.45µl pore size membrane filter for sterility.

ANTIBACTERIAL ACTIVITY

Source and standardization of test bacteria

Bacterial isolates used in this study were collected from the Department of Biochemistry and Microbiology, University of Fort Hare Alice, South Africa. The Isolates include: *Bacillus cereus* ATCC 10702, *Bacillus pumilus* ATCC 14884, *Enterobacter cloaca* ATCC 13047, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 4352, *Klebsiella pneumoniae* ATCC 10031, *Proteus vulgaris* ATCC 6830, *Proteus vulgaris* CSIR 0030, *Pseudomonas aeruginosa* ATCC 19582, *Serratia marcescens* ATCC 9986, *Shigella flexneri* KZN, *Staphylococcus aureus* ATCC 6538 and *Staphylococcus aureus* OK1. Bacterial isolates were The isolates were grown at 37°C in nutrient broth (Biolab No. 2, Wadeville, Gauteng, South Africa) for 18h and diluted to an optical density of 0.1 (0.5 McFarland Standard) at a wavelength of 625nm.

Determination of minimum inhibitory concentration (MIC)

Macrobroth dilution method was used for the determination of minimal inhibitory concentration (MIC) of the extract as described by National Committee for Clinical Laboratory Standards (2005). Mueller Hinton broth was used to prepare different concentrations ranging from 0.0977 to 25 mg/ml by serial dilutions. Each prepared concentration in tubes was inoculated with 100 µl of each of the standardized culture of the test bacteria. Tube containing Mueller Hinton broth without extract was used as negative control. The tubes were incubated aerobically at 37 °C for 18 h. The first tube in the series with no sign of visible growth was taken as the MIC.

Determination of minimum bactericidal concentration (MBC)

A loopful of culture from the first three broth tubes that showed no growth in the MIC tubes were inoculated on sterile Mueller Hinton Agar plates and observed for growth after incubation at 37°C for 24 h. The MBC was taking as the least concentration of the extracts that showed no growth. MIC index (MICI) was calculated as the ratio of MBC and MIC. The result was interpreted as follow: MBC/MIC 2.0 was considered bactericidal, if 2 but 16 it was considered bacteriostatic and lastly if the ratio is 16.0, the extract was considered ineffective as reported by Shanmughapriya *et al.* (2008).

PHYTOCHEMICAL SCREENING OF THE PLANT

Determination of total phenolics content

The total phenolics content of the extract were determined by Folin-Ciocalteu method described by Wolfe *et al.* (2003) with little modification. To 5.0 ml of plant extract with concentration of 0.1 mg/mL, 5.0 mL of 10% Folin-Ciocalteu reagent and 4.0 mL of sodium carbonate (75% w/v) was added. The mixture was vortexed for 15 s and incubated at 40 °C for 30 min for colour appearance. The absorbance was measured at 765 nm using spectrophotometer. Samples of

the extract were evaluated at the final concentration of 0.1 mg/mL. The amount of total phenolic content was expressed as mg/g tannic acid equivalent using the expression obtained from the calibration curve: $Y = 0.1231x$, $R^2 = 0.9742$, where x is the absorbance and Y is the tannic acid equivalent in mg/g.

Determination of total flavonoids content

The total flavonoids were determined using the method of Ordonez *et al.* (2006). A volume of 0.5 mL of 2% $AlCl_3$ ethanol solution was added to 0.5 mL of extract solution. The mixture was incubated for 1 h at room temperature for yellow color appearance; the absorbance was measured at 420 nm. Plant extracts were evaluated at a final concentration of 0.1 mg/mL. Total flavonoids content was calculated as quercetin equivalent (mg/g) using the equation obtained from the curve: $Y = 0.0263x$, $R^2 = 0.9701$, where x is the absorbance and Y is the quercetin equivalent.

Determination of total flavonols content

The total flavonols content were determined using the method of Kumaran and Karunakaran (2007). Two milliliter (2.0 mL) of the sample was mixed with 2.0 mL of $AlCl_3$ prepared in ethanol and 3.0 mL of 50 g/L sodium acetate solution were added. The mixture was incubated at 20 °C for 2.5 h after which the absorption was read at 440 nm using spectrophotometer. Plant extracts were evaluated at a final concentration of 0.1 mg/mL. Total flavonoids contents were calculated as quercetin (mg/g) using the following equation based on the calibration curve $Y = 0.0263x$, $R^2 = 0.9824$, where x is the absorbance and Y is the quercetin equivalent.

Determination of proanthocyanidins content

The total proanthocyanidin were determined using the procedure reported by Sun *et al.* (1998). A volume of 0.5 mL of 0.1 mg/mL of extract solution was mixed with 3.0 mL of 4% vanillin-methanol solution and 1.5 mL hydrochloric acid, the mixture was allowed to stand for 15 min at room temperature, the absorbance was measured at 500 nm. Total proanthocyanidin contents were expressed as catechin (mg/g) using the following equation of the curve: $Y = 0.5902x$, $R^2 = 0.9714$, where x is the absorbance and Y is the catechin equivalent.

ANTIOXIDANT SCREENING

ABTS radical scavenging activity

Modified method of Ye *et al.* (2013) was used to determine the ability of the extracts to scavenge free radicals generated by the ABTS. Twenty milliliter of 14 mM ABTS 2,2-Azino-bis (3-ethylbenzo -thiazoline- 6- sulfonic acid diammonium salt (ABTS) and 20 ml of a 4.9 mM potassium peroxodisulphate ($K_2S_2O_8$) was prepared by dissolution in water and incubation in the dark at room temperature for 16h. The absorbance of the solution was adjusted by adding 80% (v/v) ethanol to obtain an optical density of 0.70 at 734 nm. A 0.2mL of the tested concentrations of each of the extracts was added to 2 mL of ABTS⁺ solution and vortexed. The mixture was kept at room temperature for 6 min and the absorbance was measured at 734nm. The relative antioxidants potentials o the extracts were compared to that of BHT and Rutin which are used as standards. Ethanol

instead of the extract was used as blank. The scavenging effect was measured as follows:

$$\text{Scavenging effect (\%)} = 100 \times [1 - (A_s/A_c)]$$

Determination of diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

According to Hajlaoui *et al.* (2010) fifty microlitres of various concentrations of each of the extracts of *L. hirta* was added to DPPH (prepared by dissolving 40 mg DPPH in 100 mL methanol) and the mixtures were left for 30 min at room temperature in the dark. Absolute methanol was used as control and the absorbance of the mixture was read against methanol used as a blank at 517 nm by UV-VIS 3000PC spectrophotometer (Optima Scientific). Butylated hydroxytoluene (BHT) and Rutin were used as the positive controls and all tests were carried out in triplicate and the antiradical activity of the extracts was expressed as follows:

$$\text{DPPH scavenging effect (\%)} = 100 \times [1 - (A_s/A_c)]$$

Where A_c is the absorbance of the control, and A_s is the absorbance of the sample.

Determination of H₂O₂ inhibition activity

The ability of extracts of *L. hirta* leaf to scavenge hydrogen peroxide was determined according to Gulcin *et al.* (2003). A 1.0 ml of different concentrations of the extract was added to a 0.6 ml of hydrogen peroxide (20mM) prepared in phosphate buffer saline (pH 7.4). The absorbance of the hydrogen peroxide at 230 nm was determined after 10 min at room temperature against a blank solution containing phosphate buffer solution alone. For back ground subtraction a separate blank sample was used while BHT and ascorbic acid were used as positive controls. The percentage scavenging of hydrogen peroxide of the samples was calculated as follows:

$$\text{H}_2\text{O}_2 \text{ inhibition capacity (\%)} = 100 \times [1 - (A_s/A_c)]$$

Where A_c is the absorbance of the control, and A_s is the absorbance of the sample.

Reducing power assay

The reducing power of the extracts of *L. hirta* was determined as described by Yildirim *et al.* (2001). A 1.0 ml of the plant extract was added to 2.5 ml of 0.2 M phosphate buffer (pH 6.60) and 2.5 ml of 1.0 % potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] and mixed. The mixture was incubated in a water bath at 50°C for 20 min. A 2.5 ml of trichloroacetic acid solution (10% w/v) was added and the mixture was centrifuged at 3,000 rpm for 10 min. One ml of the supernatant was mixed with 3 ml of FeCl_3 (0.1% w/v) and absorbance was measured at 700 nm. The ascorbic acid (Vit C) and BTH were used as controls.

Statistical analysis

Statistical analysis was carried out with paired student t-test using Statistical Package for Social Sciences (SPSS version 17). The data was expressed as the mean and an alpha level of 0.05 and $P < 0.05$ was considered to be statistically significant.

RESULTS

The extracts of the plant was screened against 13 clinically important bacteria (9 Gram negative and 4 Gram positive). The extracts inhibited the growth of the test bacteria at varying degrees. The MICs of the extracts ranged from 0.1953 to 1.5625 mg/ml and 0.1953 to 0.3906 mg/ml for Gram negative and Gram positive bacteria respectively (Table 1). The extracts were more effective on Gram positive bacteria than Gram negative. Except in few cases the minimum concentrations of the extracts needed to inhibit the pathogens were lower than the concentrations needed to kill them. The result of the MBC/MIC (MIC index) showed that acetone extracts exerted bactericidal effects on the five Gram negative bacteria and one Gram positive bacterium while methanolic extract showed bactericidal effects on four Gram negative and three Gram positive bacteria. Methanolic extract was not effective against *Ent. cloaca* ATCC 13047 and *K. pneumoniae* ATCC 10031. The Phytochemicals in the extracts were expressed in term of the standard chemicals.

TABLE 1: The antibacterial activity of the extracts of *L. hirta* stem back extracts against bacteria pathogens

Test organisms	Gram Reaction	Acetone Extract			Methanolic Extract		
		MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
<i>E. coli</i> ATCC 25922	-ve	1.5625	1.5625	1.0*	0.1953	1.5625	8.0**
<i>Ent. cloaca</i> ATCC 13047	-ve	0.1953	0.7812	4.0**	0.7812	12.500	16.0***
<i>K. pneumoniae</i> ATCC 4352	-ve	0.3906	0.7812	2.0*	0.7812	1.5625	2.0*
<i>K. pneumoniae</i> ATCC 10031	-ve	0.7812	3.1250	4.0**	0.1953	3.1250	16.0***
<i>Pr. vulgaris</i> ATCC 6830	-ve	0.3906	1.5625	4.0**	1.5625	1.5625	1.0*
<i>Pr. vulgaris</i> CSIR 0030	-ve	1.5625	3.1250	2.0*	1.5625	3.1250	2.0*
<i>Ps. aeruginosa</i> ATCC 19582	-ve	0.7812	6.2500	8.0**	0.7812	6.2500	8.0**
<i>Ser. marcescens</i> ATCC 9986	-ve	0.7812	1.5625	2.0*	1.5625	3.1250	2.0*
<i>Shig. flexneri</i> KZN	-ve	1.5625	1.5625	1.0*	0.3906	1.5625	4.0**
<i>B. cereus</i> ATCC 10702	+ve	0.3906	1.5625	4.0**	0.3906	0.7812	2.0*
<i>B. pumilus</i> ATCC 14884	+ve	0.3906	0.7812	2.0*	0.3906	0.7812	2.0*
<i>S. aureus</i> ATCC 6538	+ve	0.3906	1.5625	4.0**	0.3906	0.7812	2.0*
<i>S. aureus</i> OK1	+ve	0.1953	0.7812	4.0**	0.1953	0.7812	4.0**

*= bactericidal effect, **= Bacteriostatic effects, ***= ineffective

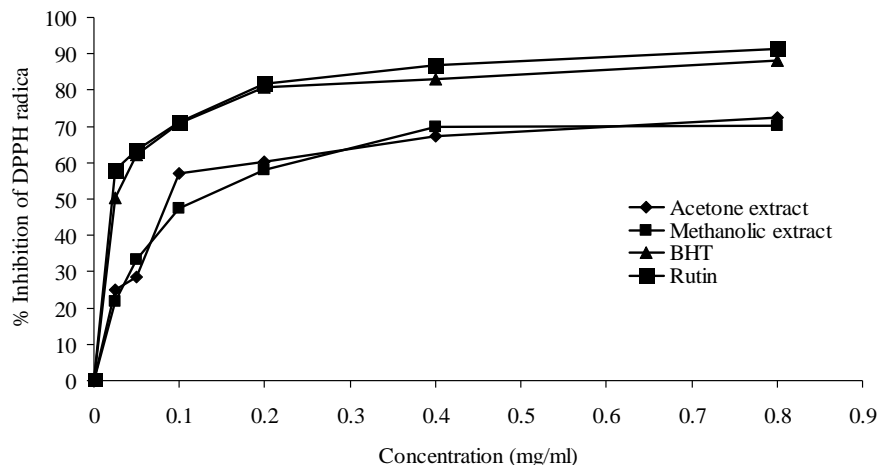


FIGURE 1: DPPH radical scavenging potential of acetone and methanolic extracts of *L. hirta*

The methanolic extract was better than the acetone extract except in phenolic. The phenolic component of the acetone extract was 6.35 mg/g while that of the methanolic was 4.80 mg/g. The amount of flavonoids in the methanolic extract (6.43 mg/ml) doubles that which is in the acetone extracts (3.09 mg/ml). The scavenging activity of the extract was dose dependent; the effect was higher at higher concentrations of the extracts. The highest activity was noticed at 0.8 mg/ml with scavenging potentials of 72.47 and 70.16 % for acetone and methanolic extracts respectively (Figure 1). The activity of the extracts were lower than those of BHT (88.01 %) and Rutin (91.29 %). There is no

significance difference between the DPPH scavenging activities of the extracts but they differ significantly from the two controls. As shown in Figure 2, the ABTS activity was also noticed to be dose dependent. At the highest concentration tested the activity of BHT was 20.60 and 14.17 % higher than acetone and methanolic extracts respectively while Rutin was 13.86 and 6.88 % higher than acetone and methanolic extracts respectively. There is a significance difference between the ABTS scavenging activities of the extracts with methanolic extract performing better than the acetone extract. Compared with the controls the extracts differ significantly at the set significant level.

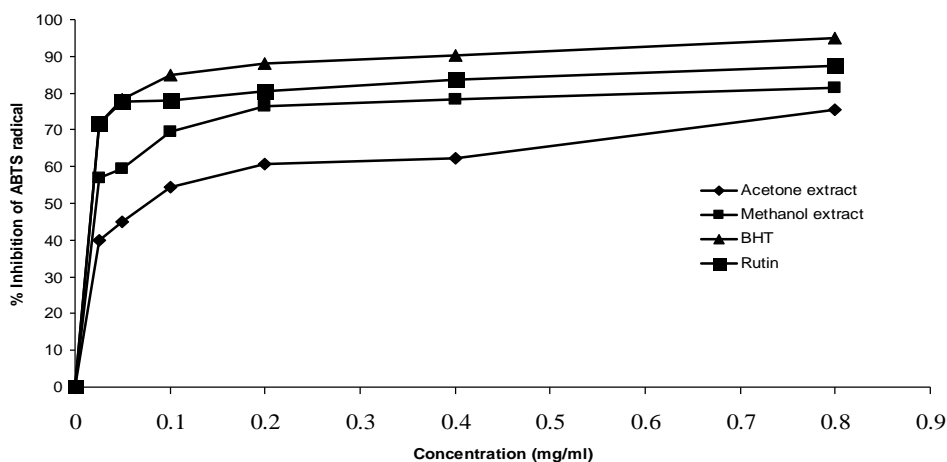


FIGURE 2: ABTS radical scavenging potential of acetone and methanolic extracts of *L. hirta*

The scavenging activity of methanolic extract was higher than the acetone extract and the standard (BHT) in all the concentrations tested. There is a significant difference between acetone and methanolic extracts ability to scavenge H_2O_2 as shown in Figure 3. There is also a significant difference between acetone extract and BHT and methanolic extract and Rutin. Comparing acetone extract with Rutin and methanolic extract with BHT there is no significant

difference. The reducing power of acetone extract was lower than the methanolic extract at all the concentration tested though with no significant difference. There is a significant difference between the test (extracts) and the controls. The reducing powers of the control (BHT and Vit. C) were higher than those of acetone and methanolic extracts of *L. hirta* as shown in Figure 4.

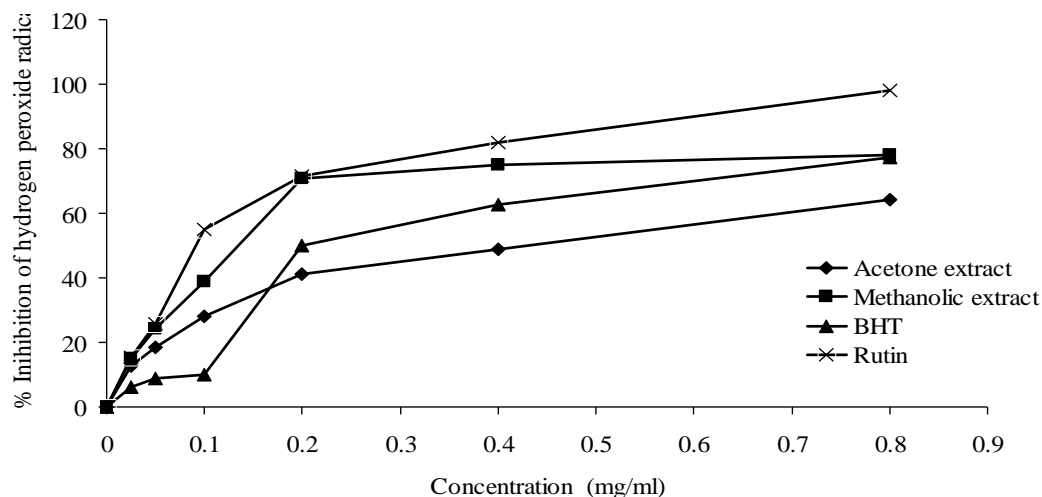


FIGURE 3: Hydrogen peroxide scavenging potential of acetone and methanolic extracts of *L. hirta*

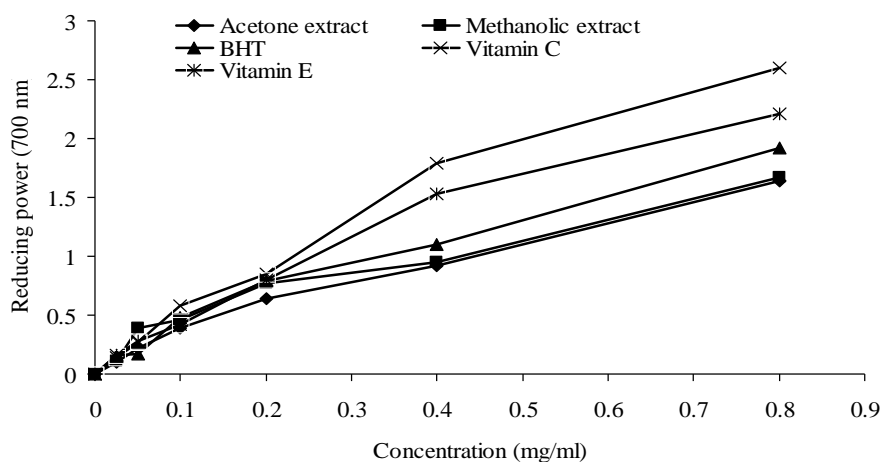


FIGURE 4: Reducing power of acetone and methanolic extracts of *L. hirta*

DISCUSSION

The extracts of *L. hirta* screened possessed antimicrobial activity. The extracts have broad antibacterial properties. They were able to inhibit the growth of both Gram negative and Gram positive bacteria. The activity of *L. hirta* against enteropathogens appears to be particularly interesting. The extracts were able to inhibit *Staphylococcus aureus* strains at concentrations 0.3906 mg/ml. *Staphylococcus aureus* has been identified as commonly associated with both community and hospital acquired infections (Datta *et al.*, 2011). The obtained results indicate a difference in antimicrobial activity the extracts of *L. hirta*. Gram positive pathogens are more susceptible to the extracts than Gram negative bacteria. This is in agreement with earlier observations of other researchers who reported Gram negative bacteria to be more resistant to plant extracts than Gram positive bacteria (Ali *et al.*, 2001; Jigna and Sumitra, 2006; Afolayan and Ashafa, 2009; Sofidiya *et al.*, 2009; Olajuyigbe and Afolayan, 2011). The reason for difference in susceptibility may be considered to be the differences in the cell walls of the two groups of bacteria. Phenolic compounds have been reported to be good bactericidal. Van der Watt and Pretorius (2001), Panizzi *et al.* (2002),

Chanwitheesuk *et al.* (2007) reported high positive correlation between antimicrobial activity of plant species and their phenolic compounds. Better performance of acetone extracts on the test bacteria could be due to the high content of phenolic in the extract. Phytochemical composition of extracts of *L. hirta* was shown on Table 2. The two extracts contain high phenolic and flavonoid compounds which must have contributed highly to their antibacterial and antioxidant properties. Though there are many compounds in higher plants that possess bioactivity (Larson, 1988), the solvents used in the extraction affects the quality of the phytochemicals as noticed in this study, this observation has earlier been reported by Arabshahi-Delouee and Urooj (2007). Antioxidant properties of the extracts tested in this study were concentration dependant. The antioxidant potentials of the extracts were due to the presence of different phytochemical present in them this has earlier been previously stated by other researchers (Bouaziz *et al.*, 2004; Kuti and Konuru, 2004). Strong antioxidant properties have been attributed to different phytochemical compounds in the extracts and this observation has been reported. Phenolic compounds possess a high potential to scavenge radicals can be explained by their ability to donate a hydrogen atom from their phenolic hydroxyl groups (Sawa

et al., 1999). The extracts could serve as electron donor due to their high reducing power and according to Yen and Wu (1993) this ability serves as a significant indicator of potential antioxidant activity. Flavonoids have high radical-

scavenging activity also been reported that most powerful antioxidants are to be found among the flavonoids and they are 20 folds higher than Vit. C and 50 times the potency of Vit. E (Sawa *et al.*, 1999; Martini *et al.*, 2004).

TABLE 2: Phytochemical constituents of *L. hirta* stem bark extracts

Phytochemicals	Extract	
	Acetone	Methanolic
Phenolic (mg/g)*	6.35±1.05	4.80±2.29
Flavonoids (mg/g)**	3.09±0.11	6.43±2.64
Flavonols (mg/g)**	4.61±1.25	4.98±0.27
Proanthocyanidin (mg/g)***	5.26±1.52	5.32±1.62

Data are expressed as means ± SD of triplicate determinations; *Expressed as mg tannic acid/g of dry plant materials, **Expressed as mg quercetin/g of dry plant materials and ***Expressed as mg catechin/g of dry plant materials

Several researchers have reported relatively higher antioxidant capacity of methanolic extracts (Sosulski *et al.*, 1982; Zielinsk & Kozłowska, 2000) compared to other solvents. The methanolic extract of *L. hirta* showed better radical scavenging ability this report is similar to the reports of Tepe *et al.* (2005) and Albayrak *et al.*, (2010) that reported methanolic extracts to be better than other extracts in biological activity. Our present data suggest that methanolic extract was better in term of antioxidant property but antibacterial property of acetone extract was better. We noticed that extracting solvents affects the antioxidant and antimicrobial potentials of extracts. Extracts of *L. hirta* possesses bioactive compounds however; isolation and characterization of these active compounds in the extracts with their action mechanisms are still open to investigation.

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Conflicts of interest statement

We declared no conflict of interest.

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