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Screening of phytochemicals and *in vitro* evaluation of antibacterial and antioxidant activities of leaves, pods and bark extracts of *Acacia nilotica* (L.) Del.

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ABSTRACT

The objective of this study was to determine the phytochemical content, antibacterial activity and antioxidant activity of leaves, bark and pods of *Acacia nilotica*. The different extracts of acacia were evaluated for total phenolic, flavonoid and protein contents, antibacterial (agar well diffusion and broth dilution methods) and antioxidant (DPPH; 1,1-diphenyl-2-picrylhydrazyl assay) activities. The characterization and identification of phenolic compounds was carried out by Liquid Chromatography–tandem Mass Spectrometry analysis. The MS^2 fragmentation pattern showed the presence of galloylated catechins and gallocatechin derivatives in tested extracts. The results indicated that all parts of the plant, but especially leaves, were effective in inhibiting the growth of antibiotic resistant strains of *Escherichia coli* and *Salmonella species* obtained from clinical and food isolates. The leaves were found to be rich in total phenolic content, proteins and high antioxidant activity as compared to pods and bark. The presence of functional groups of active compounds was confirmed by Fourier transform infrared spectroscopy (FTIR) analysis of plant extracts. It was concluded that all tested parts of *A. nilotica* had antibacterial and antioxidant activities. These properties might be due to the presence of high total phenolic content, proteins and/or flavonoids. Hence the extracts of leaves, bark and pods of *A. nilotica* represent a potential source of antibacterial and antioxidant compounds that may be used in food, agriculture and/or pharmaceutical products.

1. Introduction

Acacia nilotica (L.) Del. is a medicinal plant belonging to the family Mimosaceae. It is a medium size tree locally named as "Babul" or "Kikar" and is widely distributed in tropical and subtropical regions. In traditional medicine, it is believed that *A. nilotica* is rich in nutrients and contains therapeutic compounds that are capable of prevention, mitigation and treatment of various infectious diseases and pathological conditions. Ayurvedic medicine practices suggest the use of leaves, bark and pods of *A. nilotica* against cancer, cough, diarrhea, fever, small pox, piles and menstrual problems (Ambasta, 1992). The plant is rich in polyphenolic compounds, in which catechins are hypothesized to possess antioxidant and anti-inflammatory activities (Maldini et al., 2011). The bark and pods were reported to have inhibitory effect against hepatitis C virus (Hussein et al., 2000). In aerial parts of the plant a variety of phenolic compounds were identified, with a wide range of biological activities (Singh et al., 2008). The plant *A. nilotica* is reported to have antibacterial effects against pathogenic microorganisms such as *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* (Oladosu et al., 2013). In recent years researchers have tried to isolate strong, nontoxic antioxidants from edible plants to prevent autoxidation and lipid peroxidation with the aim to replace synthetic antioxidants (Tamuly et al., 2015). Plant extracts containing high amounts of bioactive compounds especially antioxidants, have the potential of being used in food, agriculture, nutraceuticals, cosmetics

Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; FTIR, (Fourier transform infrared spectroscopy); LC-ESI-IT–MS, Liquid chromatography-Electrospray ionization-Ion trap–Mass spectrometer; TIC, (Total ion chromatogram); MIC, Minimum inhibitory concentration; MBC, Minimum bactericidal concentration.

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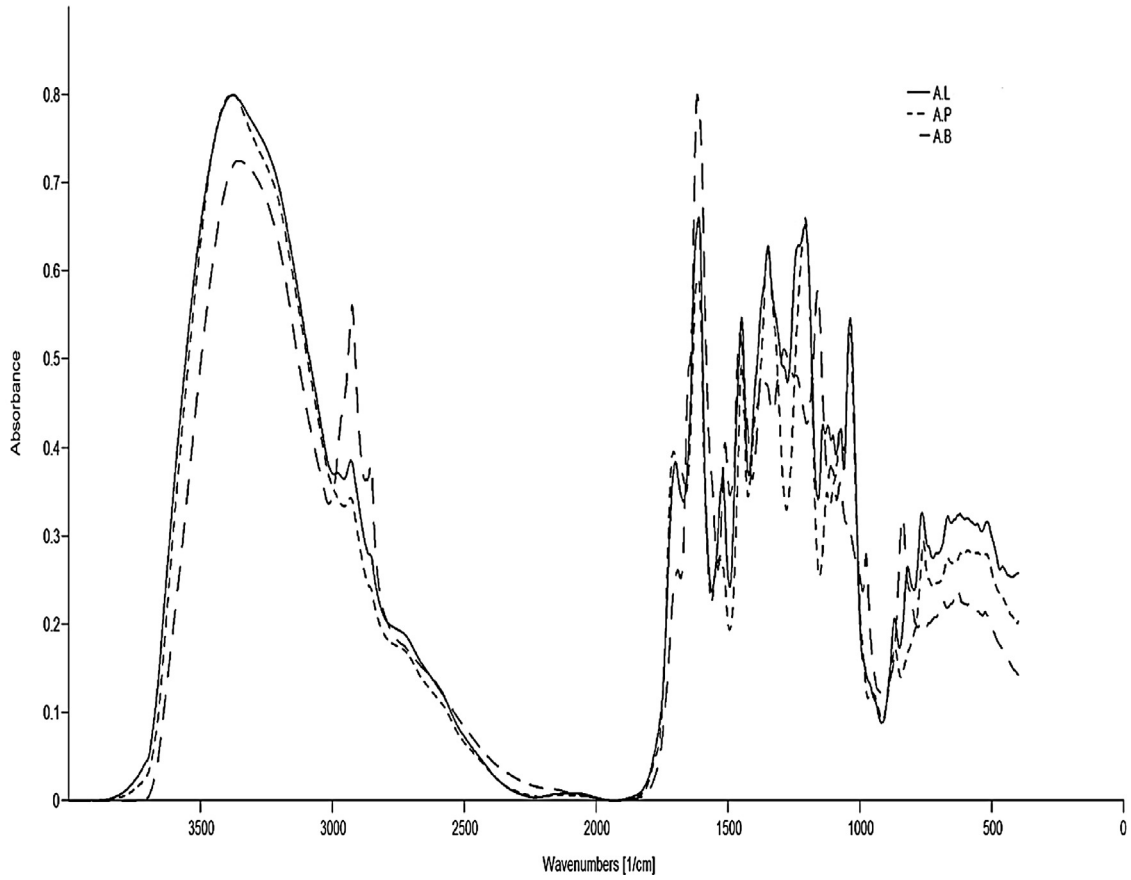

Fig. 1. FTIR spectrum o f acacia leaves (AL), pods (AP) and bark (AB) extracts.

and pharmaceutical products (Tuncel and Yılmaz, 2015). The plant phytochemicals and their products are generally nontoxic and contain many medicinal benefits. Antioxidant activities of plants are commonly correlated with their polyphenolic compounds content (Singh and Kumari, 2015).

Plants also contain secondary metabolites in addition to minerals and primary metabolites that are responsible for antioxidant and antibacterial potentials (Akinmoladun et al., 2007). The use of such secondary metabolites alone or in combination with antibiotics against drug resistant pathogens can be an alternative approach to overcome the escalating issues of drug resistant infections (Sibanda and Okoh, 2007).

The medicinal values of plants and natural extracts have now been considered as alternative therapy, especially against various infectious diseases in both developed and developing countries. The use of medicinal plants as an alternative therapy against infectious diseases might be best exemplified in the field of antimalarial therapy, where the recommended first-line active drug compound (*i.e.*, artemisinins) was originally derived from *Artemisia annua*. Food-borne pathogens are a major cause of infectious diseases outbreaks in both developing and developed countries (Pires et al., 2012). *Salmonella* and *E. coli* are few of the main food-borne pathogens responsible for food poisoning and subsequent enteric infections (Costa et al., 2012). In South East Asia, there is an absence of official *Salmonella* surveillance, but it is estimated that up to 22.8 million cases of Salmonellosis occur annually with 37,600 deaths (Majowicz et al., 2010). Antibacterial drug resistance is an evolutionary phenomenon, resulting from repeated exposure of antibiotics in human or agriculture (Walsh, 2000). *Salmonella* and *S. aureus* isolated from retail poultry meat were found resistant to various commercially available antibiotics (Akbar and Anal, 2013). Thus food contaminated with drug resistant bacteria is a major threat to public health (Akbar and Anal, 2014). Therefore, it is essential to explore new potent antibacterial compounds from natural sources to overcome the problem of antibacterial resistance. The objective of this study was to determine the phytochemical profile, total phenolic, flavonoid and protein contents as well as antioxidant and antibacterial activities of leaves, pods and bark extracts of *A. nilotica* against multidrug resistant enteric pathogens.

2. Materials and methods

2.1. Preparation of crude plant extracts and microbial sample collection

The leaves, pods and bark of the *A. nilotica* plant (wild) were collected from Lahore, Pakistan. The plant was identified and voucher

Table 1
Phytoconstituents present in *Acacia nilotica*

Phytochemicals	*Acacia nilotica* parts		
	Leaves	Pods	Bark
Saponins	+	+	+
Tannins and Phenols	+	+	+
Alkaloids	+	+	–
Glycosides	+	+	–
Anthraquinones	–	–	–
Flavonoids	+	+	+
Proteins	+	+	+
Phenols	+	+	+
Anthocyanins	–	–	–

Where (+) means available and (–) means not available.

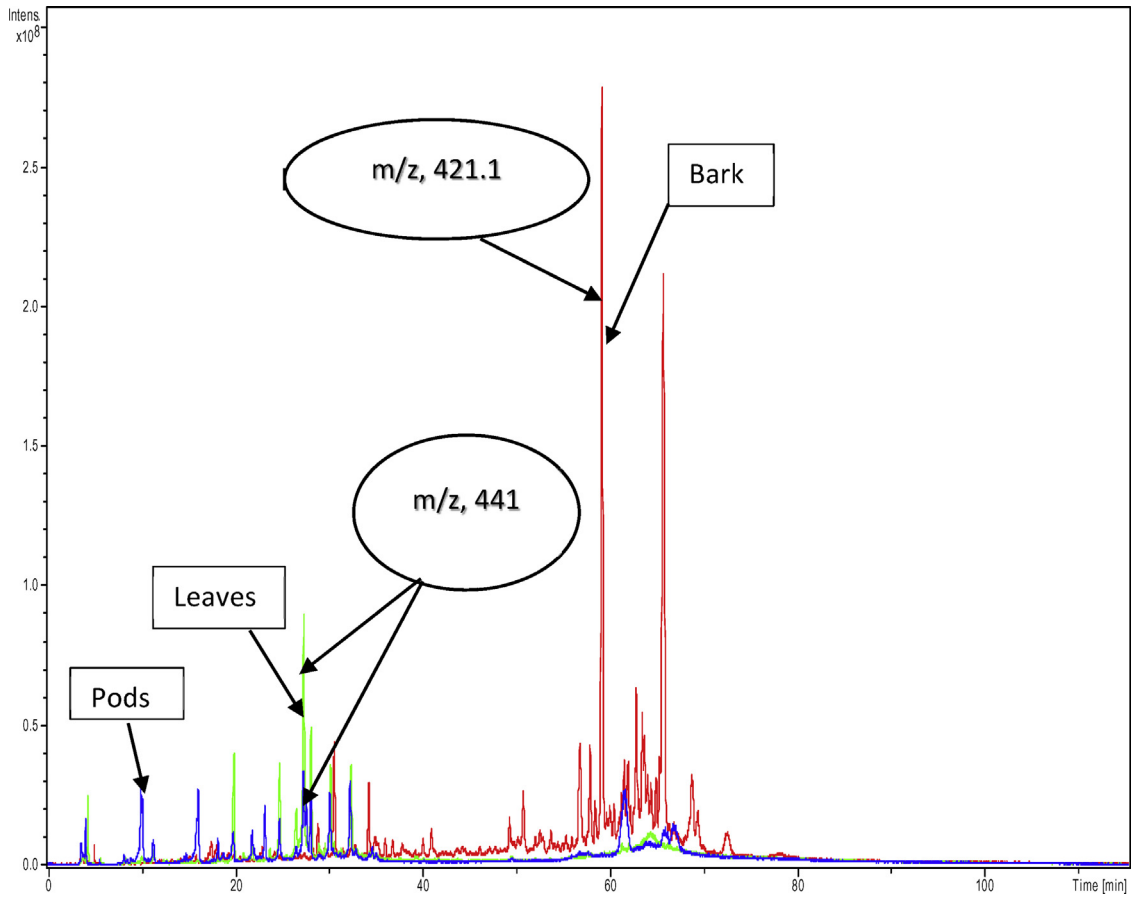

Fig. 2. LC–MS total ion chromatogram (TIC) of 80% ethanol extract of bark (red), leaves (green) and pods (blue). (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this article.)

specimen was deposited to Botany Society Government College of Science, Lahore, Pakistan. The selected plant parts were washed thoroughly under running tap water to remove the surface dirt, followed by rinsing with sterilized distilled water. The plant samples were dried under shade in an open air for 48 h. The dried samples were grounded by means of a mechanical grinder (Philips Co., Ltd., China) and finally into finely divided powder by pestle and mortar. The extraction from these dried parts of *A. nilotica* was conducted following the method as described by Adwan et al. (2010) with slight modifications. Powdered plant samples (30 g) were placed in 250 ml of ethanol (80%, v/v) in conical flasks and placed on shaking incubator (Gallenkamp, UK) for 48 h at 200 rpm. The extracts were filtered and concentrated by means of a rotary evaporator (Büchi rotavapor R-144, Switzerland) followed by lyophilization for 24 h in a freeze dryer (Scanvac Cool Safe 55-4, Denmark). The freeze dried extracts were stored at 4 °C until further use and reconstituted in analytical grade methanol to prepare stock solutions (1 mg/ml) for total phenolic, flavonoid and DPPH assay. For antibacterial activity stock solutions (100 mg/ml) of each extract were prepared in analytical grade dimethyl sulfoxide (DMSO).

E. coli and *S. enterica* subsp. *enterica* were isolated from fish and poultry meat, respectively, in the Bioprocess Technology laboratory at the Asian Institute of Technology Bangkok, Thailand. Other human clinical isolates, namely *E. coli* and *S. typhimurium* were acquired from the clinical laboratory of Thammasat Hospital, Pathumthani, Thailand.

Table 2
Assignment of FTIR bands to functional groups present in *Acacia nilotica* extracts.

Range (cm^{-1})	Groups and class of compound	Assignment and remarks	Leaves (cm^{-1})	Pods (cm^{-1})	Bark (cm^{-1})
3450–3250	—OH In alcohols and phenols	O—H Stretch	3376	3379	3351
3000–2850	C—H In alkanes	C—H Stretch	2982, 2930	2931	2926
2936–2913	CH_3—, CH_2— In aliphatic compounds	CH_3—, CH_2— Anti-symmetric stretch	2930	2931	2926
1724–1700	C=O In carboxylic acids, ketones	C=O Stretch	1700	1707	None
1650–1580	N—H, 1° Amines	N—H Bend	1611	1614	1617
1600–1520	NH_3^+ In NH4OH	NH_3 Deformation	1520	1534	None
1618–1498	Benzene ring in aromatic compounds	C=C Aromatic ring stretch	1611, 1520,	1614, 1534	1617, 1512
1550–1475	N—O Nitro compounds	N—O Asymmetric stretch	1520	1534	1512
1360–1290	N—O Nitro compounds	N—O Symmetric stretch	1349	1351	None
1300–1150	Alkyl halides	C—H wag (—CH_2X)	1209	1208	1288, 1244, 1161
1095–1074	NH_2 in NH_4OH; C—OH In secondary alcohols	NH_2 Groups in-plane rocking vibrations; C—O stretch	1076	None	1076
1040–1030	C—O—C In aliphatic ethers, Si—O in silicates	C—O—C Anti-symmetric stretch, Si—O stretch	1037	1038	None
900–700	=CH In aromatic hydrocarbons	=C-H Out-of-plane bending	765, 819, 869	759, 868	773, 839
700–610	Alkynes	—C≡C—H: C—H Bend	621, 667	618, 667	631, 684

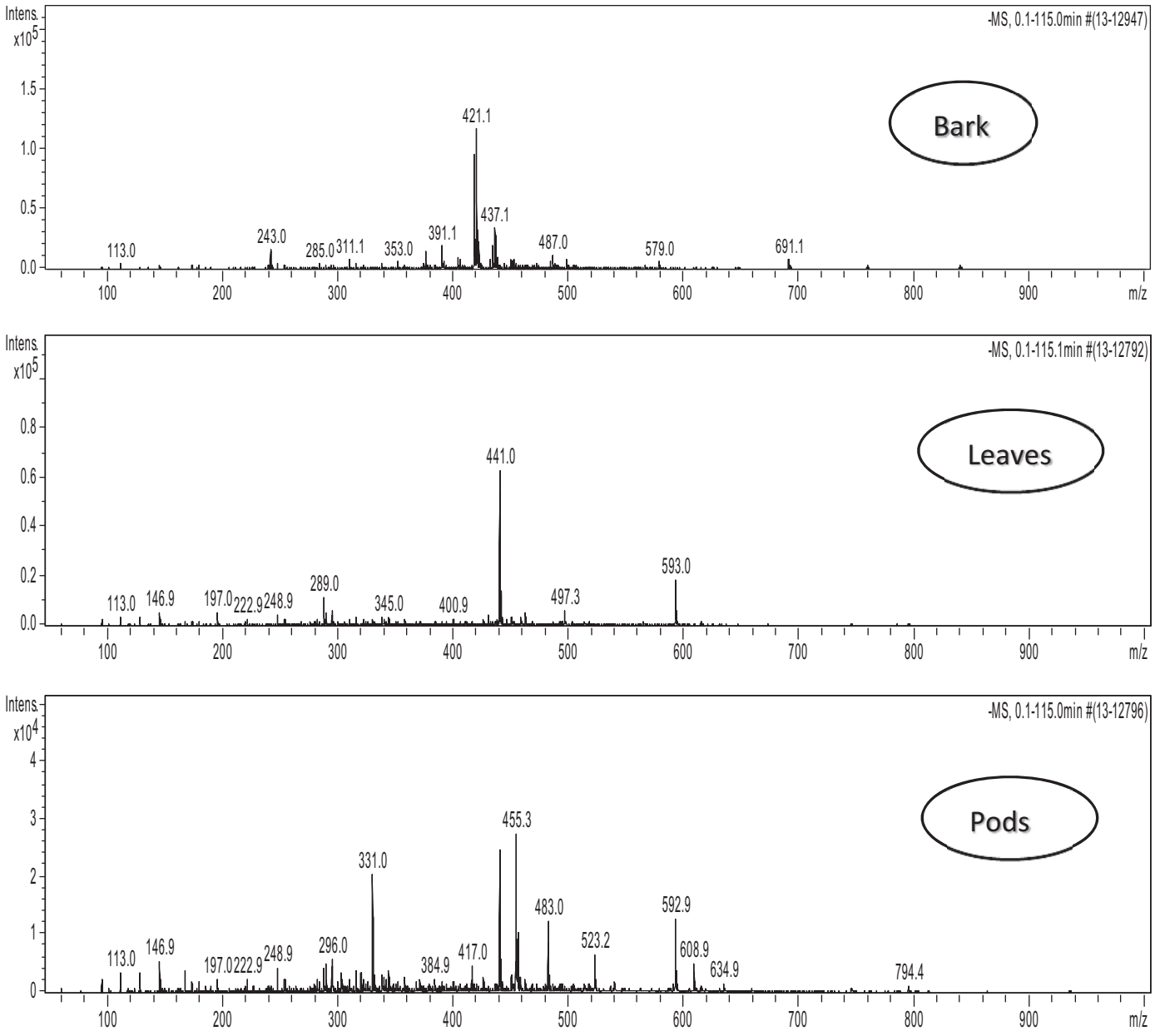

Fig. 3. ESI-MS (negative ion mode) finger prints of bark, leaves and pods extract.

2.2. Phytoconstituents analyzed

The preliminary screening for presence of major phytoconstituents was done by treating leaves, pods and bark extracts with chemical tests to identify the presence of saponins (Frothing test), tannins and phenols (Ferric chloride test), alkaloids (Hager's test), glycosides, anthraquinones (Borntrager's test), flavonoids (Shinoda test), proteins (Ninhydrin test) and anthocyanins (Doughari and Ioryue, 2009; Alhakmani et al., 2013).

2.3. FTIR Analysis of extracts

A FT-IR spectrometer (Perkin Elmer, USA) with a middle range infrared light source (4000–400 cm^{-1} wavenumbers) was used to analyze the chemical finger-printing of the extracts from various parts of the plant. The plant extracts were ground with potassium bromide (KBr) to a fine powder, placed under high pressure with compression dye until formation of a pellet and then examined.

2.4. LC-ESI–MS of Acacia nilotica extracts

The qualitative analysis of leaves, pods and bark extracts of plant was carried out by liquid chromatography-Electrospray ionization-Ion trap–Mass spectrometer (LC-ESI-IT–MS) by using a LC system (Hitachi, Japan) consisting of LC pump and vacuum degasser. The data acquisition and qualitative analysis were performed using Hystar 3.1 and Quantanalysis (Bruker Daltonics, USA). The crude extracts were separated on Hypersil BDS (150 mm × 4.6 mm I.D. 5 μm) column protected by a Hypersil Gold C18 guard column (10 mm × 2.1 mm I.D. 5 μm) using a gradient elution at constant flow rate of 400 μl/min by using a mobile phase A represented by water acidified with formic acid (0.1%) and mobile phase B represented by acetonitrile acidified with formic acid (0.1%). The

Table 3
LC-ESI-MSMS analysis of leaves pods and bark extracts of *Acacia nilotica*.

Sample	MS (*m/z*)	MS/MS fragments (*m/z*)	Tentative identification
Leaves	441	289, 245	Epicatechin-5-gallate
	289	245, 205, 179	Epicatechin
	197	169, 125	Ethyl gallate
	169	125	Gallic acid
	431	341, 311	Vitexin
	417	348.9, 197	L-Arabinose
	341	272.9, 178.9	Caffeic acid hexose
Pods	441	289, 245	Epicatechin-5-gallate
	289	245, 205, 179	Epicatechin
	457	305	Digallocatechin-5-gallate
	320.9	252.8, 169	m-Digallic acid
	168.9	125	Gallic acid
	483	414.8, 331.1, 276.9	Dialloyal glucose
	331.1	271, 211, 193, 169, 125	Monogalloyal glucose
	341.1	272.8, 179, 160.9	Caffeic acid hexose
	285.1	216.9, 131	Kaempferol
Bark	421.1	299.1	Magniferin
	317	248.9, 180.9, 113	Myricetin
	285.1	241, 216.9, 150.9	Kaempferol
	303	284.8, 259, 234.9, 125	Taxifolin

gradient elution was: 0–15 min, from 0% to 25% B; 15–25 min from 25% to 70% of B; 25–35 min, from 70% to 80% of B; 35–45 min, from 80% to 30% of B; 45–50 min, from 30% to 25% of B and 50–60 min, from 25% to 10% of B. An Esquire 4000 ion trap mass spectrometer (Bruker Daltonics, USA) with an Electro Spray Ionization Source (ESI) interface operated in the negative mode was used for LC–MS/MS analysis. The ESI drying gas temperature was maintained at 365 °C, flow rate of 9 L/min, ESI capillary +3000 nA and nebulizer at 40 psi. The qualitative analysis was performed using total ion chromatograms (TIC) for mass range 50–1000 *m/z*. Data were processed using DataAnalysis (Bruker Daltonics, USA). The LC–MS/MS method was optimized for skimmer 1 (−23.7 V), skimmer 2 (−6.0 V), capillary exit (−69.2 V), octopole (−2.31 V), lens 1 (5.0 V) and lens 2 (60 V).

2.5. Quantification of phenolic compounds by HPLC/DAD

The quantification of phenolic compounds of acacia leaves, pods and bark extracts was carried out by high performance liquid chromatography/diode array detection (HPLC/DAD) using gallic acid, catechin, tannic acid, isoquercetin, quercetin, hydroquinine, eriodictyol, apigenin, kaempferol and rutin as standards, acquired from Sigma–Aldrich (USA). The samples and standards were dissolved in ethanol (HPLC grade) and filtered by 0.22 μm sterile Millex filters (Millipore, Bedford, U.K.) before injection. Aliquots of 20 μl were injected into the HPLC/DAD system.

The analysis was carried out by using an Agilent HPLC 1100 system (Agilent, Germany) composed of auto sampler and diode array detector. The separation was performed on LiChroCART RP-18 column (150 × 4.6 mm, 5 μm, Purospher STAR Merk, USA). A binary solvent system was used as mobile phase consisting of (A) acetonitrile and (B) 10 mM ammonium formate buffer pH 4 adjusted with formic acid. Separation was carried out by gradient elution: 0–5 min, B 100%; 5–10 min, A 0–20%; 10–20 min, A 20% constant; 20–60 min, A 20–40%. The column temperature was set at 40 °C with a solvent flow rate of 1 ml/min. The diode array detector was monitored at 270, 330, 350 and 370 nm. The phenolic compounds in each sample were identified by comparing with pure standards of each identified compound using retention time and absorbance spectra. The quantification of phenolic compounds was performed based on peak area comparison (external standard method).

2.6. Determination of total phenolic and total flavonoid content

The total phenolic content of plant extracts was determined by Folin–Ciocalteu reagent (Sigma–Aldrich, Switzerland) following the method of Ainsworth and Gillespie (2007) with slight modifications. Stock solutions (1 mg/ml) of dried crude plant extracts were diluted with deionized water to prepare 100 μg/ml. For each plant extract, 0.5 ml (100 μg/ml) was mixed with 2 ml of freshly prepared Folin–Ciocalteu reagent (1:10 diluted with de-ionized water) and further neutralized with 4 ml of sodium carbonate solution (7.5% w/v). The reaction mixture was incubated at room temperature for 30 min and subjected to shaking intermittently. The absorbance was then measured at 765 nm using UV–visible spectrophotometer (UNICAM UV/Vis Spectrophotometer, UK). The results were expressed as gallic acid equivalents (GAE) per gram of sample.

The total flavonoid content was determined by colorimetric method as described by Chang et al. (2002); with slight modifications. The plant extract, 0.5 ml (1 mg/ml) was mixed with ethanol (95% v/v, 1.5 ml), aluminum chloride hexahydrate (10% w/v, 0.1 ml), potassium acetate (1 M, 0.1 ml) and deionized water (2.8 ml). The reaction mixture was incubated for 40 min at 25 °C and absorbance was measured at 415 nm against blank. The total flavonoid content was determined by using quercetin (Sigma–Aldrich, USA) as standard. The results were expressed as quercetin equivalents (QE) per gram of sample. Each experiment was conducted in triplicates.

2.7. Determination of DPPH radical scavenging activity

The antioxidant activity of plant extracts was determined in terms of DPPH (1, 1-diphenyl-2-picryl hydrazyl) radical-scavenging activity following the method as described by Cuendet et al. (1997); with slight modifications. Different concentrations (1000 μg/ml, 500 μg/ml, 250 μg/ml and 125 μg/ml) of each extract

Table 4
Retention times, UV visible spectra and contents of individual phenolic compounds (mg/kg of dried extract) by HPLC/DAD.

Phenolic compound		Leaves		Pods		Bark	
	HPLC-DAD λ_{max}(nm)	rt (min)	Concentration (mg/kg)	rt (min)	Concentration (mg/kg)	rt (min)	Concentration (mg/kg)
Gallic acid	270	6.9	87502 ± 151.1	6.9	139458 ± 191.9	6.9	544 ± 11.6
Catechin	270	12.5	82588 ± 171.3	12.5	6369 ± 29.2	12.5	18501 ± 71.1
Tannic acid	270		–	12.7	6874 ± 31.9	12.7	1459 ± 11.4
Rutin	270, 350	15.6	6856 ± 15.4	15.4	4026 ± 17.8	15.3	28 ± 1.2
Iso-quercetin	270, 350	16.5	9725 ± 41.6	16.5	824 ± 3.8	16.7	7479 ± 119.5
Hydroquinine	270		–		–	23.1	812 ± 2.7
Eriodictyol	270		–		–	31.5	108 ± 0.95
Quercetin	270, 350	33.9	1637 ± 11.6	34.0	592 ± 5.7	34.1	1069 ± 17.3
Apigenin	270, 350	42.2	103 ± 1.7		–	42.2	180 ± 2.2
Kaempferol	270, 350	43.4	114 ± 2.1		–	43.8	637 ± 4.9

The results were expressed as Mean ± S.D. for 3 replicates, rt is retention time, and (–) indicates below limit of detection.

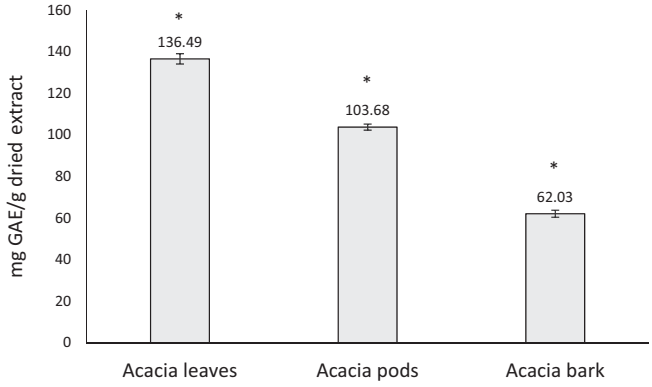

Fig. 4. Total phenolic content expressed as mg of GAE/g of dried extract in *Acacia nilotica* (leaves, pods and bark). Significant ($p < 0.05$) differences between groups are indicated by *. Numbers (above graphs) represent the calculated means and bars represent the estimated standard deviation of the mean.

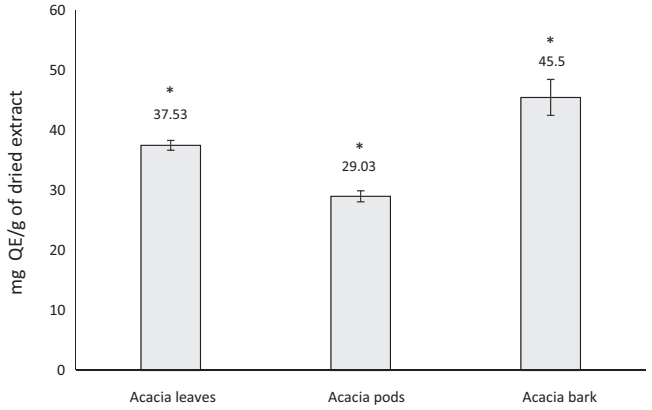

Fig. 5. Total flavonoid content expressed as mg of QE/g of dried extract in *Acacia nilotica* (leaves, pods and bark). Significant ($p < 0.05$) differences between groups are indicated by *. Numbers (above graphs) represent the calculated means and bars represent the estimated standard deviation of the mean.

were prepared from stock solution. Ascorbic acid was used as a positive control. Fifty microliters from each concentration of samples were added to 5 ml of freshly prepared 40 ppm methanol solution of DPPH (Sigma–Aldrich, USA) and the reaction mixture was kept at 25 °C for 30 min at a dark place. After incubation the absorbance was read against blank at 517 nm. The methanol was used as blank and DPPH solution without plant extract was used as control. The assay was carried out in triplicates for each extract and percentage inhibition was determined by the following equation:

$$\text{DPPH\%inhibition} = \frac{(\text{AC} - \text{AS})}{\text{AC}} \times 100$$

where AC is the Absorbance Control and AS is the Absorbance test sample.

The IC_{50} values representing the concentration of sample required to scavenge 50% of the DPPH free radical, were calculated by implementing $1/Y^2$-weighted non-linear regression; log (inhibitor concentration) *vs.* normalized response model with a variable slope (Chen et al., 2013). Data analysis was performed using GraphPad Prism® version 6.01 (San Diego, US).

2.8. Quantification of protein content

The Bradford (1976) assay was used to quantify the amount of protein in each crude plant materials. Briefly, ground plant materials (0.5 g) were soaked in 10 ml of 0.5, 1 and 1.5 N (Normality) sodium hydroxide separately for an hour, followed by ultra-sonication at 45 °C for 30 min. The samples were then subjected to centrifugation at 5000 rpm for 20 min and supernatant was collected. The supernatant (100 μl) was add to Coomassie Brilliant Blue dye (Bio-Rad, USA) solution (5 ml diluted 1:4 with de-ionized water) and the optical density was measured at 595 nm after 15 min of incubation at room temperature. The concentration of protein in each plant extract was determined by using Bovine Serum Albumin (BSA) (Sigma–Aldrich, USA) as standard.

Table 5
Estimated IC_{50} values of *Acacia nilotica* (leaves, pods and bark) and L-ascorbic acid (standard).

Parts of *Acacia nilotica*	IC_{50} (μg/ml)	Hillslope
Leaves	360 (285–455)	1.09 (0.78–1.41)
Pods	422 (348–511)	1.28 (1.01–1.54)
Bark	1410 (767–2592)	0.94 (0.62–1.27)
(L-ascorbic acid)	305.6 (259–360)	1.29 (0.99–1.58)

Parameters presented as mean estimates (95% confidence intervals).

2.9. Antimicrobial activity

The agar well diffusion method was used to determine the antibacterial effects of plant extracts by method as described by Taye et al. (2011) with slight modifications. Each inoculum was adjusted to 0.5 McFarland standard and spread with the help of sterilized cotton swab on the surface of Muller Hilton Agar (MHA)

Table 6
Antibacterial effect of *Acacia nilotica* against clinical and food isolates of *E. coli* and *Salmonella.*

Concentration	Parts of *Acacia nilotica*	Diameter of zone of inhibition against pathogens			
		E1	E2	S1	S2
(mg/ml)		(mm)	(mm)	(mm)	(mm)
25	Leaves	20.0 ± 1.0^a	16.33 ± 1.52^a	17.0 ± 1.0^a	14 ± 1.73^a
	Pods	17.7 ± 3.05^a	11.33 ± 0.58^b	13.7 ± 0.58^b	12.7 ± 1.15^a
	Barks	7.7 ± 0.58^b	8.7 ± 0.58^c	7.7 ± 1.15^c	7.7 ± 0.58^b
50	Leaves	21.7 ± 1.52^a	18.33 ± 0.58^a	19.33 ± 1.52^a	18.7 ± 1.52^a
	Pods	18.3 ± 3.21^a	$14.7 \pm .52^b$	17.33 ± 1.52^a	16.33 ± 0.58^b
	Barks	13.7 ± 1.52^b	12.0 ± 1.0^c	11.0 ± 2.64^b	11.0 ± 1.0^c
100	Leaves	25.0 ± 1.0^a	23.0 ± 1.0^a	24.33 ± 0.58^a	22.7 ± 0.58^a
	Pods	22.0 ± 1.0^b	21.7 ± 1.52^a	21.0 ± 1.0^b	18.0 ± 1.0^b
	Barks	18.0 ± 1.0^c	16.0 ± 1.0^b	16.3 ± 0.58^c	13.7 ± 1.52^c
30 μg/ml	Amikacin (positive control)	20.4 ± 1.51	23.8 ± 1.73	23.4 ± 2.14	23.5 ± 1.53

The results were expressed as mean ± S.D, based on 3 replicates. Different superscript letters (a–c) within a column indicate statistically significant ($p < 0.05$) differences between means at same concentration within a group of leaves, pods and bark. E1 = *E. coli* and S1 = *Salmonella typhimurium* isolated from clinical samples, E2 = *E. coli* isolated from fish, S2 = *Salmonella enterica* isolated from poultry meat.

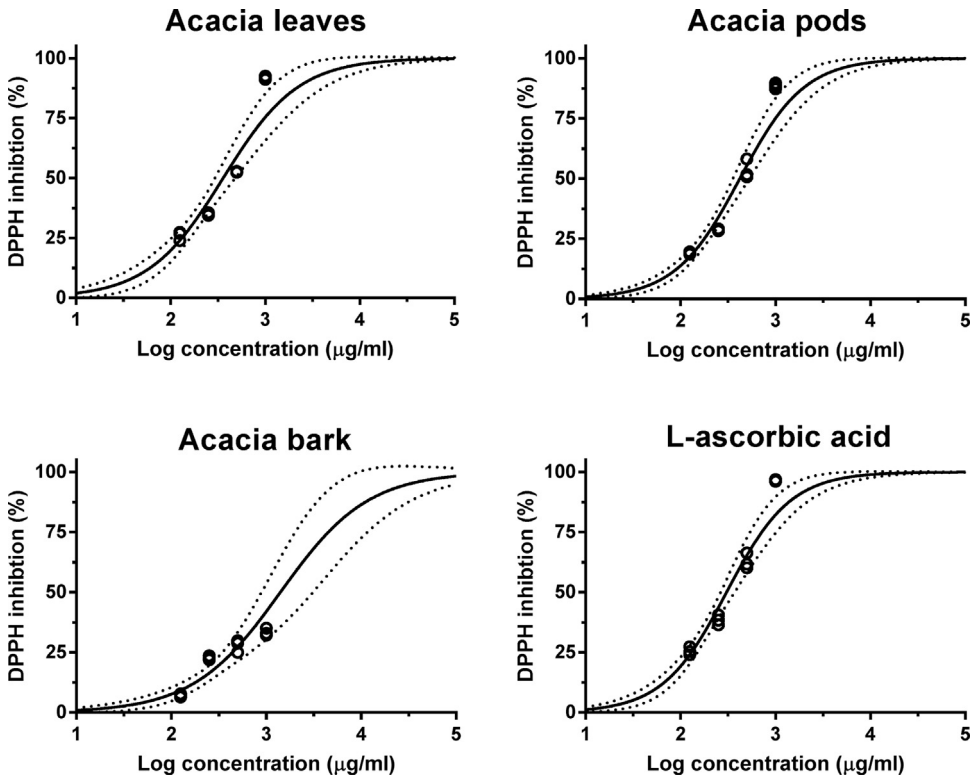

Fig. 6. Estimated DPPH inhibition of *Acacia nilotica* (leaves, pods and bark) and L-ascorbic acid (standard) at different concentrations, using non linear regression. Open circles represent observations, the solid lines represent the estimated means curves, and the broken lines represent the 95% confidence intervals of the mean estimates.

(Himedia, India) plates. Wells of 6 mm in diameter were made on each plate with the help of sterilized cork-borer and 100 μl of different concentrations (25, 50 and 100 mg/ml) of each extract were introduced into the agar wells by using micropipette. Amikacin (30 μg/ml) was used as a positive control whereas dimethyl sulfoxide (DMSO) was used as negative control. The plates were incubated for 24 h at 37 °C. The results were interpreted by measuring the diameter of clear zone around the wells. All experiments were conducted in triplicates.

2.10. Determination of minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC)

MIC and MBC were determined according to method described by Kubo et al. (2004) with minor modification. Stock solution of each extract was prepared in DMSO. Two-fold serial dilutions of extracts were filtered through 0.45 μm Millipore filters and prepared in sterile nutrient broth to obtain the concentrations 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 mg/ml. Each inoculum was adjusted to 0.5 McFarland standard and then diluted to 1:100 for broth dilution method. The standardized inoculum was introduced in each concentration of extract. The test tubes containing solvent blank in nutrient broth along with standardized inoculum were used as a growth control and test tubes containing broth without inoculum and extract were used as negative control. The test tubes were incubated for 24 h at 37 °C in the incubator and the lowest concentration which had no visible growth after 24 h incubation was considered as the MIC. MBC was determined by sub-culturing all concentrations that had no detectable growth. 100 μl from each dilution was inoculated on the surface of freshly prepared nutrient agar plates and incubated for 24 h. The minimum concentration that had no visible growth on agar plates after 24 h incubation was considered as the MBC. Each experiment was conducted in triplicates.

2.11. Statistical analysis

All experiments were carried out in triplicates and results are expressed as mean values with standard deviation (±SD) of three replicates. One-way analysis of variance (ANOVA) and Duncan's multiple range tests were carried out to determine significant group differences ($p < 0.05$) between means by using SPSS statistical software package (SPSS, version 16.0).

3. Results and discussion

3.1. Phytoconstituents analyzed

The leaves and pods of *A. nilotica* contained saponins, tannins, phenols, alkaloids, glycosides, flavonoids and proteins. Whereas alkaloids and glycosides were not found in the barks as shown in Table 1.

3.2. FTIR characterization of extracts

The FTIR spectrum was used to identify the functional groups corresponding to the each of the active components present in plant extracts based on the peak values in the region of infrared radiation. The FTIR spectrums of freeze dried extracts and assignment of bands to functional groups are shown in Fig. 1 and Table 2, respectively. The functional groups were assigned to its corresponding peaks in accordance with D'Angelo and Zodrow (2011). The C=O in carboxylic acid and ketones (1724–1700 cm^{-1}), NH_3^+ in NH_4OH due to NH_3 deformation (1600–1520 cm^{-1}), N—O nitro compounds due to N—O symmetric stretch (1360–1290 cm^{-1}) and C—O—C in aliphatic ethers, Si—O in silicates (1040–1030 cm^{-1}) group frequencies were present in leaves and pods extracts but absent in the bark. Whereas NH_2 in NH_4OH; C—OH in secondary alcohols (1095–1074 cm^{-1}) group frequencies due to NH_2 groups in-plane

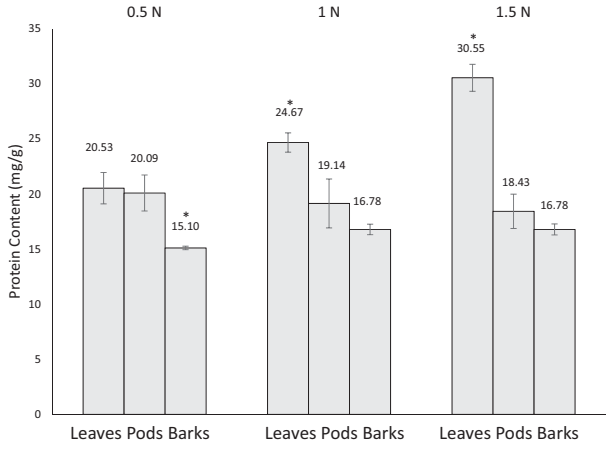

Fig. 7. Protein content (mg/g) in various crude plant parts after treatment with 0.5, 1 and 1.5 N sodium hydroxide. Significant ($p < 0.05$) differences between groups are indicated by *. Numbers (above graphs) represent the calculated means and bars represent the estimated standard deviation of the mean.

rocking vibrations; C—O stretch were present in the leaves and bark extracts only. The FTIR results confirmed the presence of alcohols, phenols, alkanes, carboxylic acids, ketones, aromatic compounds, amines, secondary alcohols, alkynes and alkyl halides in the plant extracts. The leaves were found rich in functional groups which support the higher efficacy of leaves extracts over the bark and pods.

3.3. *LC-ESI–MS of* Acacia nilotica *extracts*

The LC-ESI-IT–MS/MS analysis was performed for qualitative analysis of leaves, pods and bark extracts of plant as shown in Table 3. The total ion chromatogram (TIC) and ESI-MS (negative ion mode) finger prints of leaves, pods and bark extracts are shown in Figs. 2 and 3. The obtained *m/z* values suggested the presence of phenolic compounds in all the tested extracts belonging to the classes of gallic acid, catechin and gallocatechin derivatives. The precursor ions obtained in all extracts were tentatively identified based on MS^2 spectrum data, literature and Riken tandem mass spectral database (ReSpect) (Sawada et al., 2012; Koolen et al., 2013; Sánchez-Rabaneda et al., 2003; Mihailović et al., 2015). The leaves were found to be abundant in epicatechin (*m/z*, 289), epicatechin-5- gallate (*m/z*, 441), ethyl gallate (*m/z*, 197), gallic acid (*m/z*, 169), vitexin (*m/z*, 431), L-arabinoside (*m/z*, 417) and caffeic acid hexose (*m/z*, 341). The pods exhibited similar components; epicatechin-5-gallate (*m/z*, 441), m-digallic acid (*m/z*, 321), epicatechin (*m/z*, 289), digallocatechin-5-gallate (*m/z*, 457), gallic acid (*m/z*, 169), dialloyal glucose (*m/z*, 483), caffeic acid hexose (*m/z*, 341) and kaempferol (*m/z*, 285). The bark extract showed maximum relative abundance of ions at *m/z*, 421 that was suspected to be magniferin (Ramirez et al., 2013). The other suspected compounds from bark extracts were taxifolin (*m/z*, 303), myricetin (*m/z*, 317) and kaempferol (*m/z*, 285).

3.4. *Quantification of phenolic compounds by HPLC/DAD*

The standards of phenolic compounds were evaluated by HPLC using a visible diode array detector, monitoring at 270, 330, 350 and 370 nm. The described method showed good separation of standard compounds. Absolute quantification of phenolic compounds by using the available standards was carried out as shown in Table 4. The leaves were found rich in gallic acid (87,502 ± 151.1 mg/kg), catechin (82,588 ± 171.3 mg/kg), isoquercetin (9725 ± 41.6 mg/kg), rutin (6856 ± 15.4 mg/kg) and quercetin (1637 ± 11.6 mg/kg), whereas, apigenin and kaempferol were present in minute quantities. The pods were rich in gallic acid (139,458 ± 191.9 mg/kg), tannic acid (6874 ± 31.9 mg/kg), catechin (6369 ± 29.2 mg/kg) and rutin (4026 ± 17.8 mg/kg), whereas quercetin and isoquercetin were found in minute quantities. The bark extract contained all the tested standards but only catechin (18,501 ± 71.1 mg/kg), isoquercetin (7479 ± 119.5 mg/kg), tannic acid (1459 ± 11.4 mg/kg) and quercetin (1069 ± 17.3 mg/kg) were present in considerable quantities.

Table 7
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of *Acacia nilotica* extracts.

Microbial Strains	MIC			MBC		
	Leaves (mg/ml)	Pods (mg/ml)	Bark (mg/ml)	Leaves (mg/ml)	Pods (mg/ml)	Bark (mg/ml)
E1	3.12	3.12	6.25	6.25	12.5	12.5
E2	3.12	6.25	6.25	3.12	12.5	12.5
S1	1.56	3.12	3.12	3.12	6.25	6.25
S2	1.56	3.12	3.12	3.12	6.25	6.25

The results were expressed as means of triplicates. E1 = *E.coli* and S1 = *Salmonella typhimurium* were isolated from clinical samples, E2 = *E. coli* isolated from fish, S2 = *Salmonella enterica* isolated from poultry meat.

3.5. *Total phenolic and flavonoid contents*

The total phenolic contents were expressed as mg of GAE/g of dried extract. The total phenolic contents in leaves, pods and bark were 136.49 ± 2.49, 103.68 ± 1.46 and 62.03 ± 1.69 mg GAE/g of extract, respectively, as shown in Fig. 4. The higher total phenolic content in leaves support its higher antioxidant potential as compared to pods and bark. The ethanol was used as a solvent for extraction of phenolic compounds preferably because it served as a preservative and less toxic as compared to other organic solvents (Tung et al., 2007). Moreover, 80% ethanol was considered favorable for extraction of total phenolic content (Wong et al., 2015).

The total flavonoid contents for each extract were determined by using a quercetin standard calibration curve. The total flavonoid contents in leaves, pods and bark were 37.53 ± 0.82, 29.03 ± 0.92 and 45.5 ± 2.99 mg QE/g, respectively, as shown in Fig. 5. The result indicated that bark was rich in flavonoids as compared to leaves and pods.

Previous reports indicated the presence of various phenolic compounds in Acacia species such as ellagic acid, gallic acid, leucocyanadin, isoquercitin, rutin, catechin, m-catechol and their derivatives (Singh et al., 2007). These polyphenolic compounds are strong antioxidants and are responsible for various biological activities like antidiabetic, antiaging, anticancer and prevention of cardiac diseases (Dixon et al., 2005). A strong relationship between total phenolic content and antioxidant activity has been reported by Dorman et al. (2003). The antioxidant activities of polyphenolic compounds are because of their redox properties which are mainly responsible for neutralizing and absorbing free radicals (Itagaki et al., 2009). The total phenolic content in ethanol extracts of leaves in the current study was found higher than that previously reported by Gowri et al. (2011). Kalaivani and Mathew (2010) reported total phenolic and flavonoid contents in *A. nilotica* leaves, determined by using different extraction solvents (petroleum ether, benzene, dichloromethane, chloroform, ethanol and water). In the current study, total phenolic content of leaves was lower than that reported for ethanol and chloroform extracts but higher than all other solvent extracts reported, whereas the total flavonoid content in the current study was higher than all solvent extracts of leaves determined by them.

The total phenolic and flavonoid contents of leaves and pods extracts in the presented study were higher than that previously

reported by Abdel-Farid et al. (2014). In the present study, the extracted amount of total phenolic and flavonoid contents in barks were found to be less than that in *Acacia confusa* bark (Tung et al., 2007). The reported variation in the phenolic and flavonoid contents in plants could be explained by the fact that presence of phenolic compounds is affected by plant species, maturity, growing conditions, soil conditions and post-harvest treatment (Jeffery et al., 2003).

3.6. DPPH radical scavenging activity

There is considerable variation in antioxidant potential among the various parts of *Acacia* (Table 5 and Fig. 6). The highest DPPH inhibition (91.98 ± 0.73%) was found by leaves extract at 1000 μg/ml, whereas it was 88.49 ± 1.22% and 33.37 ± 1.53% for pods and bark, respectively. The positive control, L-ascorbic acid, showed similar DPPH inhibition (96.50 ± 0.36% at 1000 μg/ml) as acacia leaves. DPPH inhibition of ethanol extracts of acacia leaves (91.98 ± 0.73%) in the current study was very close to methanol leaves extract (94.62%), reported by Al-Fatimi et al. (2007).

The corresponding IC_{50} values are reported in Table 5, where a lower IC_{50} value would represent a higher antioxidant potential. The smallest IC_{50} value of 360 μg/ml was found for leaves extract, whereas, the highest IC_{50} value of 1410 μg/ml was found for bark extract. The standard L-ascorbic acid had a IC_{50} value of 306 μg/ml.

DPPH is a nitrogen centered stable violet colored free radical that is converted to yellow color due to reduction by hydrogen or electron donating ability of antioxidants present in tested extracts. All selected plant parts in this study were capable of scavenging DPPH free radicals. The DPPH radical scavenging activity decreased in order among the plant parts as leaves > pods > bark. This was supported further by the total phenolic content, indicating a positive trend between antioxidant activity and the amount of phenolic compound present in extracts.

3.7. Quantification of protein content

The results for estimation of protein content in the crude *A. nilotica* (leaves, pods and bark) by Bradford assay are shown in Fig. 7. Maximum protein content was 30.5 ± 1.23 mg/g for leaves after treatment with 1.5 N (Normality) sodium hydroxide. The protein content was found to be only 16.78 ± 0.5 mg/g after treatment with 1 and 1.5 N sodium hydroxide from acacia bark. In pods there was no considerable change in protein content (20.09 ± 1.63, 19.14 ± 2.22 and 18.43 ± 1.56 mg/g with 0.5, 1 and 1.5 N sodium hydroxide, respectively) with the change in concentration of sodium hydroxide. It was concluded that the leaves of acacia contained significantly ($p < 0.05$) higher protein content than the pods and bark, when treated with 1 and 1.5 N sodium hydroxide.

3.8. Antimicrobial activity

All selected parts of Acacia plant were found to be effective against the selected pathogens. The lowest tested concentration (25 mg/ml) of all parts of Acacia inhibited the growth of both clinical and food isolates of *E. coli* and *Salmonella*. The maximum diameter of zone of inhibition was 25.0 ± 1.0 mm and 24.33 ± 0.58 mm against E1 and S1 (*E. coli* and *Salmonella* obtained from clinical source), respectively, by leaves extract at a concentration of 100 mg/ml as shown in Table 6. The antibacterial effects of Acacia pods were comparable to leaves and maximum zones of inhibition at a concentration of 100 mg/ml were 22.0 ± 1.0 mm and 21.0 ± 1.0 mm against E1 and S1, respectively. Whereas the maximum diameter of zone of inhibition by bark extract was 18.0 ± 1.0 mm and 16.33 ± 0.58 mm against E1 and S1, respectively. The results demonstrated that all selected pathogens were susceptible to all tested parts of plant. This indicated the strong antibacterial potential of *A. nilotica* against the antibiotic resistant pathogens tested here. The antibacterial results of current study are in accordance with literature, where extracts of *A. nilotica* were effective against pathogenic bacteria including *E. coli* and *Salmonella* (Sharma et al., 2014; Khan et al., 2009; Okoro et al., 2014).

3.9. MIC and MBC of Acacia nilotica extracts

MIC and MBC of Acacia leaves were in the range of 1.56–3.12 mg/ml and 3.12–6.25 mg/ml, respectively, against all tested bacterial strain as shown in Table 7. The leaves showed the lowest MIC and MBC values as compared to pods and bark extracts. The MIC values of acacia leaves and bark extracts against multidrug resistant *E. coli* were lower in the current study than previously reported by Sharma et al. (2014) for *E. coli* causing otitis infection. The leaves extract showed lower MIC value against *S. typhimurium* than previously reported by Okoro et al. (2014) for *S. typhi,* the reported difference might be due to different strain of *Salmonella*.

All parts of plant were found to be effective even against the clinical isolates of *E. coli* and *Salmonella typhimurium* that were resistant to various commercially available antibiotics. Therefore, acacia can be an alternative approach to treat resistant pathogens either in the form of its purified extract or in combination with commercially available antibiotics. However, more research is needed to validate these findings in animal models and in human clinical trials. We examined the antibacterial activity of ethanol extracts of leaves, pods and bark of *A. nilotica* against drug sensitive and multi drug resistant *E. coli* and *Salmonella* sp. obtained from clinical and food sources. It was found that all extracts showed antibacterial activity against tested pathogens.

4. Conclusion

This study demonstrated that extracts of leaves, pods and bark of *Acacia nilotica* showed high DPPH radical scavenging activity (antioxidant activity), which could be due to high phenolic and flavonoid content. All extracts of the plant showed antibacterial activity against antibiotic resistant and sensitive pathogens (*E. coli* and *Salmonella*). However, leaves had the lowest MIC and MBC values against the tested pathogens as compared to pods and bark. Therefore, the extracts of leaves, bark and pods can be used as a natural source of antioxidant and antibacterial compounds. However, further research is needed to identify the individual active components responsible for antioxidant and antibacterial activities and their development for food and pharmaceutical applications.

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