

# Biological activities of some *Acacia* spp. (Fabaceae) against new clinical isolates identified by ribosomal RNA gene-based phylogenetic analysis

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**Abstract:** Nowadays, most of the pathogenic bacteria become resistant to antibiotics. Therefore, the pharmaceutical properties of the natural plant extracts have become of interest to researchers as alternative antimicrobial agents. In this study, antibacterial activities of extract gained from *Acacia etbaica*, *Acacia laeta*, *Acacia origena* and *Acacia pycnantha* have been evaluated against isolated pathogenic bacteria (Strains MFM-01, MFM-10 and AH-09) using agar well diffusion methods. The bacterial strains were isolated from infected individuals, and their exact identification was detected on the basis of 16S rRNA gene amplification and sequence determination. Alignment results and the comparison of 16S rRNA gene sequences of the isolates to 16S rRNA gene sequences available in Gen Bank database as well as the phylogenetic analysis confirmed the accurate position of the isolates as *Klebsiella oxytoca* strain MFM-01, *Staphylococcus aureus* strain MFM-10 and *Klebsiella pneumoniae* strain AH-09. Except for cold water, all tested solvents (Chloroform, petroleum ether, methanol, diethyl ether, and acetone) showed variation in their activity against studied bacteria. GC-MS analysis of ethanol extracts showed that four investigated *Acacia* species have different phytochemicals. Eight important pharmaceutical components were found in the legume of *Acacia etbaica*, seven in the legume of *Acacia laeta*, fifteen in the legume of *Acacia origena* and nine in the leaves of *Acacia pycnantha*. A dendrogram was constructed based on chemical composition, revealed that *Acacia laeta* is more closely related to *Acacia etbaica* forming one clade, whereas *Acacia origena* less similar to other species. Our results demonstrated that, investigated plants and chemical compounds present could be used as promising antibacterial agents.

**Keywords:** *Acacia* species, pharmaceutical properties, GC-MS analysis, pathogenic bacteria, 16S rRNA gene sequences, phylogenetic analysis.

## INTRODUCTION

Genus *Acacia* belongs to the family Fabaceae has bipinnate compound leaves with two stipular spines, and corperate pollen grains (Vassal, 1981). It consists of over 1200 species, spreading throughout various regions of Africa, India, Americas, and Asia (Le Houerou, 1980; Carter, 1994; Ali, 1998). Acacias have been cultivated in many countries for various purposes whereas it have a valuable wood that can be used for industries, decorations and as an important sources for gum (Arabian gums), tannins, perfumes, ink, proteins and paint (Seigler, 2003; Arias *et al.*, 2004). It has been reported that several *Acacia* species have been used to prepare disinfectant for microorganisms and for hands washes (Isaacs, 1987; Cock, 2011). Many species of *Acacia* also showed antimicrobial activities, for example, the bark of *Acacia concinna* contains high amount of saponins that behaving as foaming agents (Segelman and Farnsworth, 1969). *Acacia catechu* was found to have antimicrobial activities against some species of pathogenic and non-pathogenic microorganisms (Negi and Dave, 2010). Various solvent extracts obtained from the leaves of *Acacia salicina* 'Lindl.' were tested against infectious bacterial strains

showed that it have a significant antibacterial effect (Boubaker *et al.*, 2012). It was reported that *Acacia aulacocarpa* and *Acacia complanta* extracts have antibacterial effects against a range of bacterial strains (Cock, 2008). Phytochemicals being present in some *Acacia* spp. includes amines and alkaloids, cyanogenic glycosides, cyclitols, fatty acids and seed oils, fluoroacetate, gums, nonprotein amino acids, terpenes, hydrolyzable tannins and flavonoids (Seigler, 2003). *A. tortilis* have  $\alpha$ -cadinol, nerolidol,  $\alpha$ -humulene,  $\gamma$ -cadinene and 2-(E)-octenal with different concentrations (Ogunwande *et al.*, 2008) and *A. nilotica* contains various amino acid like cystine and methionine, etc and many mineral salts like potassium and phosphorus, etc (Rajbir *et al.*, 2008). Therefore, the current study was undertaken to determine the antibacterial activities of *Acacia etbaica* legume, *Acacia laeta* legume, *Acacia origena* legume and *Acacia pycnantha* leaves against three isolated pathogenic bacteria. 16S rRNA gene sequences and phylogenetic analysis were applied for bacterial identification at species level. Bioactive compounds were detected by GC-MS analysis to assess their medicinal potential. Since, there are many disciplines associated with taxonomy to improve the identification, classification and systematic position of plant taxa, so our study also aimed to use chemical compounds for better understanding of the

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phylogenetic relationships among the four species within *Acacia*.

## MATERIALS AND METHODS

### Isolation of pathogenic bacteria

Clinical specimens were collected from patients, isolated on nutrient agar plates and characterized according to the Gram staining. MFM-01 and MFM-10 strains characterized as Gram negative and AH-09 strain as Gram positive are used for further study.

### 16S rRNA gene amplification and sequence determination

The Genomic DNA from isolated bacteria was extracted according to the method described by (Hesham, 2014) and the 16S rRNA gene was amplified. Gene amplification was done with universal primers: forward 27F (5-AGAGTTTGATCCTGGCTCAG-3) and reverse 1492R (5-CGGCTACCTTGTTACGACTT-3) (Lane, 1991). Polymerase chain reaction (PCR) was performed in 50 $\mu$ l as a final volume containing GoTaq green master mix (Promega, Madison, WI, USA), 1 $\mu$ l DNA sample and 1 $\mu$ l of each primer (at a concentration of 0.5mM). The steps of PCR program were as the following: one step denaturation for 5min at 95°C, followed by 36 cycles of 94°C denaturation for 1 min, 55°C annealing for 1 min, 1.5min extension at 72°C, and a final extension step of 7 min at 72°C. 5 $\mu$ l of PCR products were then analyzed using 1.5% 0.5 $\times$ TBE agarose gel electrophoresis. Ethidium bromide was used for gel staining and photograph was taken under UV light. The correct size of the product was purified and sequenced in both directions (ABI automated sequencer, Macrogen Company, Korea).

### Sequence alignment and phylogenetic analysis

The sequences of 16S rRNA gene obtained from the isolates were aligned and compared with the known 16S rRNA gene sequences in Genbank database using the BLAST search at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine the closest available database sequences. To detect the exact taxonomic position of the isolates, phylogenetic trees were constructed with MEGA program version 4.0 (Hesham *et al.*, 2012).

### Plant samples collection

*Acacia etbaica* legume, *Acacia laeta* legume, *Acacia origena* legume and *Acacia pycnantha* leaves were collected during February, 2013 from Abha area, Aseer region, Saudi Arabia. The voucher specimen of samples have been stored in the herbarium of Biology Dept., Faculty of Science, King Khalid University, Kingdom of Saudi Arabia. Plant materials were washed thoroughly using tap water followed by sterilized distilled water (DW). Fifteen grams from each part was crushed directly by grinder (Thomas Wiley laboratory mill, model 4) for 15 minutes and the solution samples were filtered through

2-layered muslin cloth. Other fifteen-gram from each part was dried in shade at room temperature and then grinded to be a powder using sterilized pestle and mortar. According to Girish and Satish (2008), the resultant filtrates and powder was further subjected to the extraction protocols.

### Solvent extraction

Either filtrates or shade dried fine powdered 1g from each parts was subjected to fresh extraction by adding 20 ml from water, chloroform, petroleum ether, methanol diethyl ether, and acetone. All sample were kept in rotary shaker at 99 rpm at 24°C for 48 hours. The resultant extract was filtered and placed in incubator at 39°C until the solvent was completely evaporated. All samples were weighed, dissolved in sterile dimethyl sulfoxide (DMSO) and subjected for the antibacterial activity test (Alamri and Moustafa, 2012).

### Screening of antibacterial activities

Antibacterial activities of various solvent extracts of *Acacia etbaica* legume, *Acacia laeta* legume, *Acacia origena* legume and *Acacia pycnantha* leaves were estimated using agar well diffusion method (Nostro *et al.*, 2000). Mueller-Hinton Agar (Merck) media were prepared according to the manufacturer's instruction. Plates were incubated overnight at room temperature to solidify and ensure sterility before use. 0.1ml from various bacterial strain was introduced to every plate and 6mm well was cut from agar media using sterilized cup-borer. 0.1ml of each extract was poured in the well and all Petri dishes were incubated at 37°C for 24 hours. Dimethyl sulfoxide (DMSO) was used as negative control while cefoxitin -30mcg was used as positive control. The experiment was performed in triplicate and the antibacterial activity of each extract was expressed in terms of the mean of diameter of zone of inhibition (in cm). One way (ANOVA) was applied to detect significance values among various treatments.

### Chemical constituent analysis using GC-MS

For GC-MS analysis, a Clarus 500 Perkin elmer (Auto system XL) Gas Chromatograph machine and coupled to a mass detector Turbo mass gold Perkin Elmer Turbomass was used. The working procedures as followings: helium gas (99.999%) as a carrier gas at a constant flow rate and injector temperature at 200°C as described previously by (Ezhilan BP and Neelamegam, 2012; Moustafa *et al.*, 2013). Interpretation unknown chromatograph resulting from GC-MS were compared with the spectrum of the known components saved in the database of National Institute Standard and Technology (NIST). The presence or absence of chemical compounds were converted into a "1" and "0" in different. Squared Euclidean distance and Jaccard's coefficient for binary data (similarity) were used to calculate the distances among studied *Acacia* spp. and to generate the dendrogram gained clustering following Ward's method (Sneath and Sokal 1973).

**Table 1:** Antibacterial activity of *Acacia* species extracts against *Staphylococcus aureus* strain MFM-10

Solvents and controls	Mean diameter of zone of inhibition (cm) ± SE at concentration of 0.5 g/ml.							
	<i>Acacia origena</i> legumes		<i>Acacia pycnantha</i> Leaves		<i>Acacia etabica</i> legumes		<i>Acacia laeta</i> legumes	
	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh
Positive control	2.47±0.13	2.47±0.13	2.47±0.13	2.47±0.13	2.47±0.13	2.47±0.13	2.47±0.13	2.47±0.13
Methanol	1.02±0.09**	1.29±0.61	1.02±0.71*	1.29±0.08*	0.73±0.02**	1.00±0.01	0.86±0.05	1.13±0.02*
Chloroform	1.67±0.04*	1.96±0.23*	1.67±0.22	1.98±0.36**	0.83±0.21	1.12±0.03*	1.33±0.18	1.62±0.03
Petroleum ether	1.46±0.02	1.77±0.41	1.46±0.23*	1.75±0.22	0.86±0.11**	1.15±0.11	1.03±0.21**	1.32±0.04
Acetone	1.44±0.01*	1.85±0.12**	1.56±0.15	1.76±0.07*	0.76±0.07	1.05±0.09*	1.33±0.12	1.61±0.08**
Diethyl ether	1.77±0.03	1.93±0.18	1.53±0.02*	2.10±0.05**	0.73±0.01*	0.96±0.11	1.03±0.11*	1.26±0.02
Hot water	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cold Water	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

**Table 2:** Antibacterial activity of *Acacia* species extracts against *Klebsiella pneumoniae* strain AH-09

Solvents and controls	Mean diameter of zone of inhibition (cm) ± SE at concentration of 0.5 g/ml.							
	<i>Acacia origena</i> legumes		<i>Acacia pycnantha</i> Leaves		<i>Acacia etabica</i> legumes		<i>Acacia laeta</i> legumes	
	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh
Positive control	1.99±0.02	1.99±0.02	1.99±0.02	1.99±0.02	1.99±0.02	1.99±0.02	1.99±0.02	1.99±0.02
Methanol	1.23±0.01	1.46±0.12	1.34±0.01**	1.78±0.12	1.34±0.02**	1.78±0.14**	1.33±0.17	1.78±0.21*
Chloroform	1.26±0.01**	1.46±0.09	1.36±0.21	1.36±0.14	1.36±0.14**	1.36±0.09*	1.36±0.19*	1.36±0.11
Petroleum ether	1.39±0.09*	1.66±0.07	1.46±0.31*	1.94±0.09	1.46±0.21**	1.77±0.04	1.46±0.09	1.89±0.01**
Acetone	1.45±0.11*	1.50±0.09	1.53±0.14	2.24±0.19	1.89±0.63	2.11±0.11**	1.77±0.07**	1.87±0.09
Diethyl ether	1.69±0.31	1.83±0.01	1.24±0.12*	1.67±0.13	1.23±0.23*	1.77±0.23	1.25±0.11	1.78±0.41**
Hot water	0.00	0.00	0.00	0.00	0.00	0.76±0.01	0.00	0.00
Cold Water	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

**Table 3:** Antibacterial activity of *Acacia* species extracts against *Klebsiella oxytoca* strain MFM

Solvents and controls	Mean diameter of zone of inhibition (cm) ± SE at concentration of 0.5 g/ml.							
	<i>Acacia origena</i> legumes		<i>Acacia pycnantha</i> Leaves		<i>Acacia etabica</i> legumes		<i>Acacia laeta</i> legumes	
	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh
Positive control	2.88±0.04	2.88±0.05	2.77±0.06	2.77±0.07	2.88±0.02	2.84±0.03	2.90±0.02	2.91±0.03
Methanol	1.66±0.21*	1.99±0.24	0.99±0.01**	1.19±0.01	1.65±0.12*	1.99±0.21	1.33±0.21*	1.53±0.04*
Chloroform	1.99±0.23	2.71±0.28**	1.36±0.09	1.67±0.61*	0.77±0.32	0.99±0.03	1.26±0.33	1.57±0.01
Petroleum ether	1.26±0.01**	1.59±0.02	1.53±0.21	1.85±0.13	1.36±0.12**	1.67±0.16	1.46±0.21	1.79±0.06**
Acetone	1.86±0.01	2.19±0.11*	1.44±0.12	1.73±0.18	1.53±0.41	1.85±0.12	1.33±0.05	1.66±0.08
Diethyl ether	1.99±0.03	2.43±0.23	1.43±0.49	1.72±0.12	1.49±0.09	1.73±0.41*	1.30±0.01	1.59±0.18*
Hot water	0.00	0.00	0.00	0.00	0.00	1.00±0.03	0.00	0.00
Cold Water	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Negative control, dimethyl sulfoxide; Positive control, Cefoxitin-30mcg. \*p<0.05, \*\*p<0.01, represent significant difference compared with positive control.

### Gen bank accession number

The sequences of 16S rRNA gene of isolated strains MFM-01, AH-09 and MFM-10 have been deposited in the EMBL, DDBJ and Gen Bank nucleotide sequence databases under Accession Numbers KF234423, KF234424 and KF234425 respectively.

### RESULTS

#### 16S rRNA gene sequences and phylogenetic analysis for genetic identification

The Genomic DNA from isolated bacteria (MFM-01, AH-09 and MFM-10) was extracted and 27F and 1492R as universal primers were used for the PCR amplification

**Table 4:** GC-MS analysis of ethanol extract of *Acacia etbaica* legume, *Acacia laeta* legume, *Acacia origena* legume and *Acacia pycnantha* leaves.

No.	Compounds	Chemical Formula	A. <i>etbaica</i>	A. <i>laeta</i>	A. <i>origena</i>	A. <i>pycnantha</i>
1	2-Propanone, 1,3-dihydroxy-	C <sub>3</sub> H <sub>6</sub> O <sub>3</sub>	+	-	-	-
2	Glycerin	C <sub>3</sub> H <sub>8</sub> O <sub>3</sub>	+	-	-	-
3	Sucrose	C <sub>12</sub> H <sub>22</sub> O <sub>11</sub>	+	-	-	-
4	Methyl α-D-mannopyranoside	C <sub>7</sub> H <sub>14</sub> O <sub>6</sub>	+	+	-	-
5	Methyl β-D-mannopyranoside	C <sub>7</sub> H <sub>14</sub> O <sub>6</sub>	+	+	-	+
6	N-hexadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	+	+	+	+
7	9,12-Octadecadienoic acid, methyl ester	C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>	+	-	-	-
8	Eicosane	C <sub>20</sub> H <sub>42</sub>	+	-	-	-
9	2,5-furandione, dihydro-3-methylene-	C <sub>5</sub> H <sub>4</sub> O <sub>3</sub>	-	+	-	-
10	9-octadecenoic acid	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	-	+	+	+
11	Gamolenic Acid	C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>	-	+	+	-
12	Octadecanoic acid	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>	-	+	+	+
13	2,2'-bioxirane	C <sub>4</sub> H <sub>6</sub> O <sub>2</sub>	-	-	+	-
14	Acetic anhydride	C <sub>4</sub> H <sub>6</sub> O <sub>3</sub>	-	-	+	-
15	Acetic acid	C <sub>2</sub> H <sub>4</sub> O <sub>2</sub>	-	-	+	-
16	2-propanone, 1,3-dihydroxy-	C <sub>3</sub> H <sub>6</sub> O <sub>3</sub>	-	-	+	-
17	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	C <sub>6</sub> H <sub>8</sub> O <sub>4</sub>	-	-	+	-
18	1,3-Dioxolane-4-methanol	C <sub>4</sub> H <sub>8</sub> O <sub>3</sub>	-	-	+	-
19	2-furancarboxaldehyde 5-(hydroxymethyl)-	C <sub>6</sub> H <sub>6</sub> O <sub>3</sub>	-	-	+	-
20	1,2,3-propanetriol, monoacetate	C <sub>5</sub> H <sub>10</sub> O <sub>4</sub>	-	-	+	-
21	1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	C <sub>4</sub> H <sub>9</sub> NO <sub>5</sub>	-	-	+	-
22	Tetrahydro-4H-pyran-4-ol	C <sub>4</sub> H <sub>9</sub> NO <sub>5</sub>	-	-	+	-
23	Methyl hexofuranoside	C <sub>7</sub> H <sub>14</sub> O <sub>6</sub>	-	-	+	-
24	Isopentyl nitrite	C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub>	-	-	-	+
25	1-octadecyne	C <sub>18</sub> H <sub>34</sub>	-	-	-	+
26	1-Hexadecene	C <sub>16</sub> H <sub>32</sub>	-	-	-	+
27	Phytol	C <sub>20</sub> H <sub>40</sub> O	-	-	-	+
28	9,12,15-octadecatrienoic acid, (Z,Z,Z)-	C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>	-	-	-	+

and sequencing of the 16S rRNA gene fragment. The alignment and comparison of the 16S rRNA gene sequences of the strains MFM-01, AH-09 and MFM-10 to the published 16S rRNA gene sequences in Gen bank database by BLAST search were determined. The sequences results of the 16S rRNA of the strains MFM-01, AH-09 and MFM-10 were showed high homologous to *Klebsiella oxytoca*, *Klebsiella pneumoniae* and *Staphylococcus aureus* with similarities ranged from 99 to 100%. To assert the position of each strain in phylogeny, a number of sequences representative some *Klebsiella* and *Staphylococcus* species were selected from Gen bank database for construction of the phylogenetic trees. The tree results indicated that strain MFM-01 and *K. oxytoca* shared a one cluster (fig. 1). Therefore, the strain MFM-01 was identified as *K. oxytoca*. The results revealed that the strain AH-09 and *K. pneumoniae* were in the same clade cluster (fig. 2). Therefore, the strain AH-09 was identified as *K. pneumoniae*. For the strain MFM-10, phylogenetic analysis confirmed its taxonomic position as *S. aureus* (fig. 3).

#### Antibacterial activity of plant extract

The prepared extracts were tested for antibacterial activity against the isolated strains MFM-01 and MFM-10 as a Gram negative and AH-09 as a Gram-positive bacteria. Data indicated that organic extract from *Acacia etbaica* legume, *Acacia laeta* legume, *Acacia origena* legume and *Acacia pycnantha* leaves showed significant anti-bacterial activities than water extracts (tables 1, 2 and 3). Aqueous extract from *Acacia etbaica* fresh legume was found to be very low in inhibition of various bacterial strains showed inhibition zone (0.76-1.00) cm against *K. pneumoniae* strain AH-09 and *K. oxytoca* strain MFM-01 (table 2 and table 3). Extract gained from fresh plant materials were found effective in producing inhibition zone against tested bacteria than the dry one (tables 1, 2 and 3). Results from the current study (table 1) indicated that extract gained from *Acacia etbaica* legume, *Acacia laeta* legume, *Acacia origena* legume and *Acacia pycnantha* leaves have varied antibacterial activity against *S. aureus* strain MFM-10. Among the four plants tested, the diethyl ether extracts of *Acacia pycnantha* fresh leaves showed highest antibacterial activity with inhibition zone of 2.1cm at 0.5g/ml.

Chloroform extract of *Acacia pycnantha* fresh leaves and *Acacia origina* fresh legume showed potent antibacterial activity with inhibition activity of 1.98cm and 1.96cm, respectively. Petroleum, ether and acetone extract of *Acacia pycnantha* fresh leaves and *Acacia origina* fresh legume exhibited moderate antibacterial activity with respective means between 1.85 to 1.75cm. Extract gained from fresh legume of *Acacia laeta* have a slightly lower antibacterial activity with zone of inhibition ranging from 1.62cm to 1.1cm and the least activity observed in extract of *Acacia etabica* with inhibition zone between 1.03 and 0.87cm. Results in table 2 indicate that acetone extracts of *Acacia pycnantha* fresh leaves and *Acacia etabica* fresh legume showed highest antibacterial activity with zone of inhibition of 2.2cm and 2.1cm respectively against *K. pneumoniae* strain AH-09. Methanol, petroleum ether, acetone and diethyl ether extract of *Acacia etabica* fresh legume, *Acacia laeta* fresh legume, *Acacia origina* fresh legume and *Acacia pycnantha* fresh leaves showed moderate antibacterial activity with zone of inhibition ranging from 1.94cm and 1.36cm respectively. However, table 3 indicate that chloroform and diethyl ether extracts of *Acacia pycnantha* fresh leaves showed highest inhibition diameters of 2.71cm and 2.45cm respectively, followed by those prepared in acetone extract with a zone of inhibition 2.19cm against *K. oxytoca* strain MFM-01. Methanol, chloroform, petroleum ether, acetone and diethyl ether extract from *Acacia etabica* legume, *Acacia laeta* legume, *Acacia origina* legume and *Acacia pycnantha* leaves displayed a broad range of antibacterial activity with a zone of inhibition between 1.90cm to 0.7cm.

**Table 5:** Jaccard's similarity coefficient among 4 *Acacia* spp. based on phytocomponents

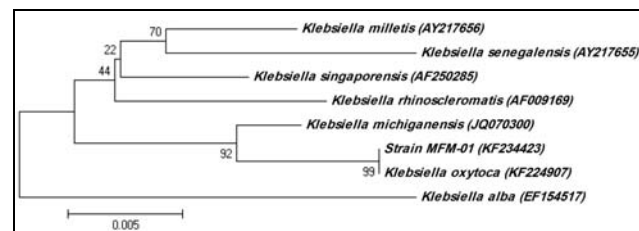
	<i>A. etbaica</i>	<i>A. laeta</i>	<i>A. origina</i>
<i>A. etbaica</i>	100		
<i>A. laeta</i>	33.33	100	
<i>A. origina</i>	4.545	21.05	100
<i>A. pycnantha</i>	13.33	30.77	14.29

#### Phytocomponents of different extracts and cluster analysis

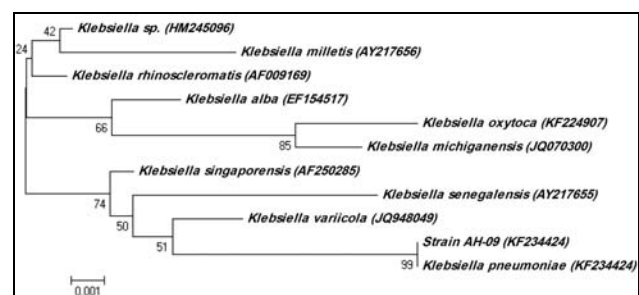
GC-MS analysis obtained from ethanol extract of *Acacia etbaica* legume, *Acacia laeta* legume, *Acacia origina* legume and *Acacia pycnantha* leaves showed the presence of phytochemical constituents (fig. 4). Characterization, identification, absence, presence and molecular formula in each *Acacia* species are shown in (table 4). Results showed that in the ethanol extracts eight compounds were identified in *Acacia etbaica* and seven compounds in *Acacia laeta* legumes. While fifteen compounds present in the extracts of the *Acacia origina* legume and nine in the extract of the *Acacia pycnantha* leaves.

Resulted dendrogram based on chemical composition grouped the 4 accession numbers into three clusters considered as A, B and C (fig. 5). One group consisted of

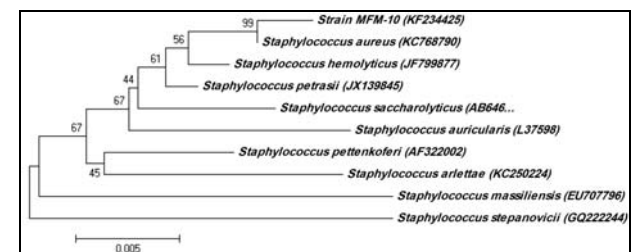
very closely related chemical compounds from *Acacia etbaica* and *A. laeta* followed by *A. pycnantha*, respectively while *A. origina* have less similarity with other species. Jaccard's similarity coefficients ranged from 4.545 (between *A. etbaica* and *A. origina*) to 33.33 (between *A. laeta* and *A. etbaica*) (table 5).



**Fig. 1:** Phylogenetic analysis of 16S rRNA gene of strain MFM-01 and other related *Klebsiella* spp. By Neighbor-Joining method. Numbers at the nodes indicate bootstrap support (%) based on 100 replicates. The scale bar indicates 0.005 nucleotide substitutions per nucleotide position. Gen bank accession numbers are given in parentheses.



**Fig. 2:** Phylogenetic analysis of 16S rRNA gene of strain AH-09 and other related *Klebsiella* spp. By Neighbor-Joining method. Numbers at the nodes indicate bootstrap support (%) based on 100 replicates. The scale bar indicates 0.001 nucleotide substitutions per nucleotide position. GenBank accession numbers are given in parentheses.



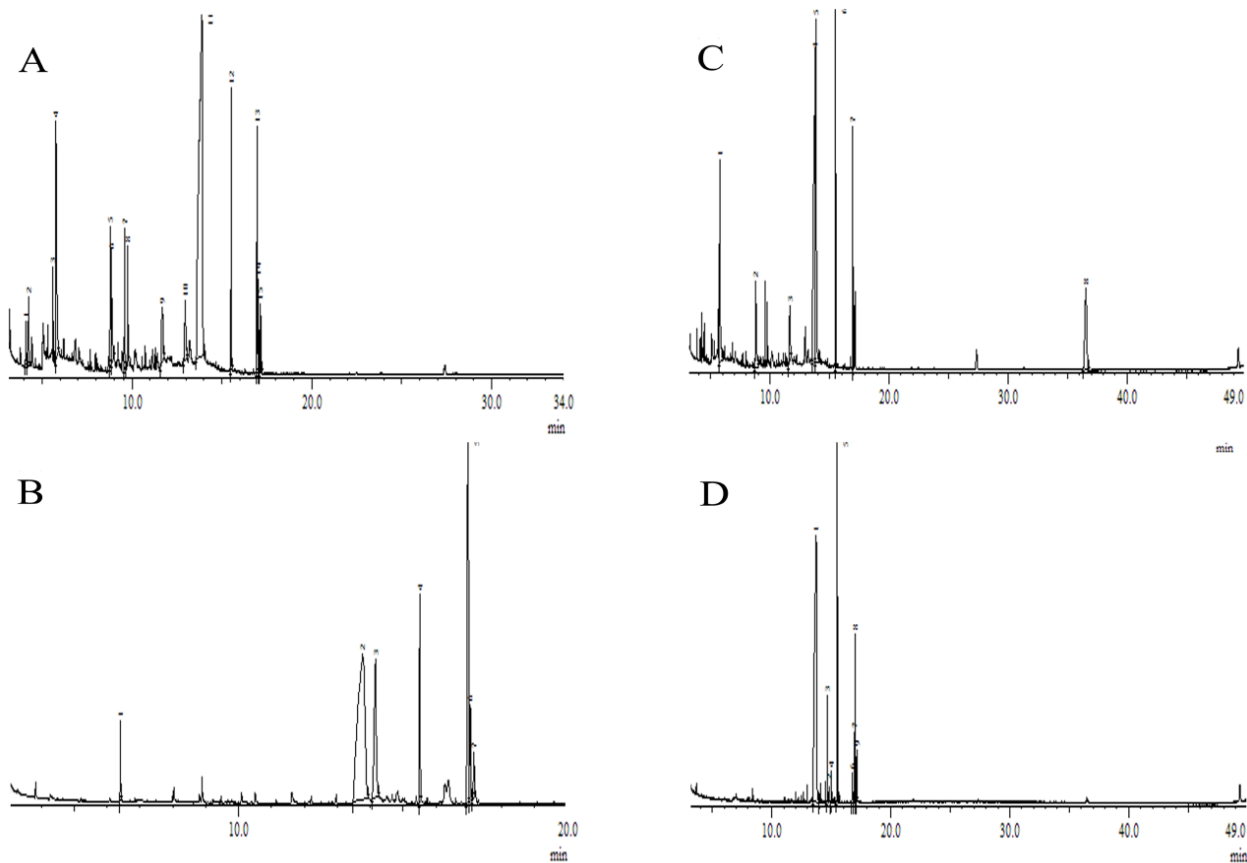
**Fig. 3:** Phylogenetic analysis of 16S rRNA gene of isolate MFM-10 and other related *Staphylococcus* spp. By Neighbor-Joining method. Numbers at the nodes indicate bootstrap support (%) based on 100 replicates. The scale bar indicates 0.005 nucleotide substitutions per nucleotide position. Gen bank accession numbers are given in parentheses.

## DISCUSSION

Molecular identification based on 16S rRNA gene sequences and phylogenetic analysis confirmed the taxonomic position of the strain MFM-01 as *K. oxytoca* (fig. 1), strain AH-09 as *K. pneumoniae* (fig. 2) and as MFM-10 as *S. aureus* (fig. 3). Molecular methods based upon 16S rRNA gene sequences and phylogenetic analysis have become the “golden index” for bacterial identification (Shen and Feng, 2004; Rani *et al.*, 2008). In addition to, the comparisons on the basis of 16S rRNA gene sequences are among the most powerful tools for the identification of microorganisms (Wu *et al.*, 2006; Hesham *et al.*, 2012). Recently, PCR amplification of 16S rDNA and sequencing provide a valuable and reliable tools of identification of pathogenic bacteria (Jenkins *et al.*, 2012; Böhme *et al.*, 2013).

The secondary products present in the plant materials plays a crucial rule in considering the plant have a beneficial medicinal effect. There are a particular plant species, consistent with the concept that the combination of secondary metabolites in a specific plant is taxonomically distinct (Parekh *et al.*, 2005). Therefore, it is necessary to examine traditional medicine that is from plant origin as a first step for further leading to drug

development. Ethno botanical examination is one of the common methods employed in choosing the plant for further study. Considering these, four *Acacia* species were selected based on traditional knowledge in order to screen their antibacterial potency against *K. oxytoca* strain MFM-01, *S. aureus* strain MFM-10 and *K. pneumoniae* strain AH-09. The results show that the organic extracts from the *Acacia etbaica* legume, *Acacia laeta* legume, *Acacia origena* legume and *Acacia pycnantha* leaves possess antimicrobial activities against the tested pathogens at 0.5g/ml. The hot water extract of *A. etbaica* fresh legume possess minimum antibacterial activity against *K. oxytoca* strain and *K. pneumoniae* strain AH-09, while cold water extract showed no activity at all. This confirms the results from previous studies indicated that water is not a suitable solvent to extract antibacterial compounds compared to methanol, ethanol ethyl acetate, methanol, n hexane and butanol (Karaman *et al.*, 2003; Parekh *et al.*, 2005; Parekh and Chanda, *et al.*, 2006; Majhenic *et al.*, 2007; Malu *et al.*, 2009; Qaralleh *et al.*, 2009; Bakht *et al.*, 2011). The result of each plant extract varied from that of the other against same pathogen and against different pathogens. The antibacterial activity gained from chloroform extract of *Acacia origena* fresh legume showed the highest activity against *K. oxytoca* strain MFM-01 and *S. aureus* strain MFM-10, while the

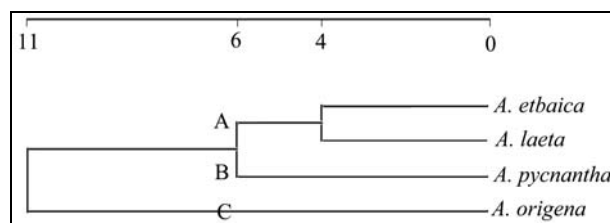


**Fig. 4:** Typical GC–MS chromatogram for ethanol extract of *Acacia origena* legume (A); *Acacia laeta* legume (B); *Acacia etbaica* legume (c) and *Acacia pycnantha* leaves (D).

diethyl ether extracts gained from the same plant showed the highest activity against *K. pneumoniae* strain AH-09. On the other hand, some bacteria show resistance against one type of extract, while other types of extract of the same plant show the best effect against the same bacteria. The antibacterial activity gained from diethyl ether extracts of *Acacia pycnantha* fresh leaves showed the highest activity against *S. aureus* strain MFM-10 and methanol extract gained from *Acacia pycnantha* dry leaves showed the lowest activity against the same pathogen. The antibacterial activity gained from acetone extracts of *Acacia pycnantha* fresh leaves showed the highest activity against *K. pneumoniae* strain AH-09 and methanol extract gained from *Acacia pycnantha* dry leaves showed the lowest activity against the same pathogen. In addition, the antibacterial activity gained from petroleum ether extracts of *Acacia pycnantha* fresh leaves showed the highest activity against *K. oxytoca* strain MFM-01 and methanol extract gained from *Acacia pycnantha* dry leaves showed the lowest activity against the same pathogen. Various susceptibility between gram-positive (*K. oxytoca* strain MFM-01 and *K. pneumoniae* strain AH-09) and Gram-negative bacteria (*S. aureus* strain MFM-10) to various extracts could be attributed to types of bacterial cell components (Nikaido and Varra, 1985). In addition, it is noted that the extraction capacity of active constituents to yield strong antibacterial activity from these *Acacia* species differ from solvent to solvent against various pathogens. For example, the chloroform and diethyl ether extract of *Acacia origena* fresh legume, *Acacia etbaica* fresh legume, *Acacia laeta* fresh legume, and *Acacia pycnantha* fresh leaves possess the highest solubility/extractability for various plant metabolites against *S. aureus* strain MFM-10. The diethyl ether extract of *Acacia origena* fresh legume and acetone extract of *Acacia etbaica* fresh legume, *Acacia laeta* fresh legume, and *Acacia pycnantha* fresh leaves possess the highest solubility/extractability for various plant metabolites against *K. pneumoniae* strain AH-09. In addition, the chloroform extract of *Acacia origena* legume, petroleum ether extract of *Acacia pycnantha* leaves and *Acacia laeta* legume and methanol extract of *Acacia etbaica* possess the highest solubility/extractability for various plant metabolites against *K. oxytoca* strain MFM-01. Thus, the polar (methanol and acetone) and non-polar (chloroform, diethyl ether, petroleum ether) solvents used in this study play an important role in determining biologically active compounds from plant material as antibacterial. In agreement with the previous reports referred to the capacity of polar and non-polar solvents to yield a great number of bioactive molecules (Wojdylo *et al.*, 2007; Ruikar *et al.*, 2009; Tiwari *et al.*, 2011; Sujatha and Suresh, 2013).

The phytochemistry assay indicates that *Acacia origena* legume has broad-spectrum of bioactive compound, the

total amounts were about two-times more as compared to those from *Acacia etbaica*, *Acacia laeta* and *Acacia pycnantha*. The presence of broad spectrum of bioactive compounds in the *Acacia origena* legume justifying, at least in part, higher antibacterial than other species investigated. Almost all identified compounds by GC-MS in the extracts of investigated *Acacia* spp. are basically biologically important, for example, *n*-hexadecanoic acid, octadecanoic acid possesses antioxidant and antimicrobial principles (Tamokou *et al.*, 2012). The compound 2-furancarboxaldehyde, 5-(hydroxymethyl) also shows antimicrobial activity, which have many application in pesticides, pharmaceutical and cosmetics (Rigal and Gaset, 1983). On the basis of 28 chemical content cluster analysis showed that *A. laeta* and *A. pycnantha* had good similarity, followed by *Acacia etbaica*, respectively; and *Acacia origena* had no resemblance with others.



**Fig. 5:** Dendrogram showing intra genetic relationships of investigated *Acacia* species based on chemical compositions

Distance matrix variation indicates the presence of genetic variation among the tested *Acacia* species that reflected on chemical constituents. So, the combining several of molecular patterns such as proteins, isoenzymes with a chemical approach will allow a perfect discrimination between all species of *Acacia* genus in the future.

## CONCLUSIONS

Molecular identification based on 16S rRNA gene sequences and phylogenetic analysis confirmed the taxonomic positions of the pathogenic isolated Gram negative and Gram-positive bacteria at species levels. In the light of the fact that microorganisms are becoming resistant against the drugs in use, the chloroform, petroleum ether, methanol diethyl ether and acetone extracts from *Acacia etbaica*, *Acacia laeta*, *Acacia origena* and *Acacia pycnantha* possess significant inhibitory effect against tested pathogens that may be of great use for the development of pharmaceutical drugs.

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