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## Phytochemical Screening and *In Vitro* Antibacterial Activities of Leaf Extract *Acacia etbaica* Schweinf Against Multidrug Resistant Enterobacteriaceae Human Pathogens

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### ABSTRACT

*Acacia etbaica* Schweinf belongs to the family *Fabaceae* widely distributed in Africa and various parts of this plant such as bark, leaves, flowers and roots are widely used as a folk medicine for curing of various ailments. This study was aimed to screen the phytoconstituents and evaluate the antibacterial activity of leaf extract of *A. etbaica* against selected multidrug resistant *Enterobacteriaceae* family. Leaves of *A. etbaica* were extracted with petroleum ether, chloroform, acetone, and ethanol by sequential soxhlet extraction. *In vitro* antibacterial activities of *A. etbaica* leaf extracts against selected *Enterobacteriaceae* family gram-positive bacteria such as (*B. subtilis*, *E. faecalis*, *S. aureus*) and gram-negative (*E. coli*, *K. pneumonia*, *V. cholera*) were evaluated by agar well diffusion. The antibacterial potential of acetone and ethanol leaf extracts of *A. etbaica* were determined by 96 well plate broth dilution assay. Among the tested organic leaf extracts, both acetone and ethanolic leaf extract of *A. etbaica* showed a potentially broad spectrum of *in vitro* antibacterial activity against tested multiple drug resistant *Enterobacteriaceae* family gram-positive pathogens such as *B. subtilis*, *E. faecalis*, and *S. aureus* and gram-negative bacterial strains *E. coli*, *K. Pneumonia* and *V. Cholera* with significant MIC values. The significant antibacterial activity of both acetone and ethanolic leaf extracts of *A. etbaica* was due to the subsistence of secondary metabolites phytoconstituents such as flavonoids and phenolic compounds.



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### INTRODUCTION

According to CDC multiple and extensive drug resistant (antibiotic-resistant) clinical bacterial strains causes approximately 2.6 million antibiotic-resistant infections and about 44,000 deaths occurred each year, and the mortality rate of antibiotic resistance is gradually it becomes two-fold each year (CDC-Antibiotic Resistance Threats in the United States, 2019). (Reta et al., 2019) from his cross-sectional study on antibiotic-resistant at tertiary care hospitals at Hawassa University Comprehensive Specialized Hospital in Southern

**Table 1: Phytochemical screening of *Acacia etbaica***

Phytochemicals	Acacia etbaica			
	Petroleum ether	Chloroform	Acetone	Ethanol
Alkaloids	-	-	-	-
Flavonoids	-	+	+++	++
Coumarins	+	+	+	-
Tannins	-	+	+++	++
Terpenoids	+	+	+++	++
Phenolic compounds	-	+	+++	++

NB: (+) Slight intensity; (++) Moderate intensity; (+++) High intensity; (-) Absent

**Table 2: Zone of inhibition (mm) of petroleum ether leaf extract *Acacia etbaica* (PEAE)**

Extract/ Standard	Concentration ( $\mu\text{g/mL}$ )	Zone of inhibition (mm)					
		<i>B.subtilis</i>	<i>S.aureus</i>	<i>E.faecalis</i>	<i>K.pneumoniae</i>	<i>V.cholera</i>	<i>E.coli</i>
AEPE	100	NA	NA	NA	NA	9	10
	250	NA	NA	NA	NA	11	11
	500	NA	NA	10	NA	13	13
Ciproflaxin	20	36	33	36	33	31	26

**Table 3: Zone of inhibition (mm) of chloroform extract *Acacia etbaica* (CHAE)**

Extract/ standard	Concentration ( $\mu\text{g/mL}$ )	Zone of inhibition (mm)					
		<i>B.subtilis</i>	<i>S.aureus</i>	<i>E.faecalis</i>	<i>K.pneumoniae</i>	<i>V.cholera</i>	<i>E.coli</i>
AECH	100	NA	10	10	NA	NA	9
	250	11	11	11	11	11	12
	500	12	12	12	12	12	13
Ciproflaxin	20	36	33	36	33	31	26

**Table 4: Zone of inhibition (mm) of acetone leaf extract *Acacia etbaica* (ACAE)**

Extract/ standard	Concentration ( $\mu\text{g/mL}$ )	Zone of inhibition (mm)					
		<i>B.subtilis</i>	<i>S.aureus</i>	<i>E.faecalis</i>	<i>K.pneumoniae</i>	<i>V.cholerae</i>	<i>E.coli</i>
AEAC	100	12	12	11	11	10	10
	250	14	13	13	13	12	15
	500	16	15	15	15	16	17
Ciproflaxin	20	36	33	36	33	31	26

**Table 5: Zone of inhibition (mm) of ethanol leaf extract *Acacia etbaica* (ACET)**

Extract/ standard	Concen tration ( $\mu\text{g/mL}$ )	Zone of inhibition (mm)					
		<i>B.substilis</i>	<i>S.aureus</i>	<i>E. faecalis</i>	<i>K.pneumon</i>	<i>V.cholerae</i>	<i>E.coli</i>
AEET	100	10	10	10	10	11	NA
	250	12	11	12	12	12	11
	500	14	14	14	14	13	18
Ciproflaxin	20	36	33	36	33	31	26

**Table 6: Minimum Inhibitory Concentration (MIC) of acetone and ethanolic leaf extract of *Acacia etbaica* on Enterobacteriaceae family**

Plant Extract	MIC ( $\mu\text{g/ml}$ ) of acetone and ethanolic leaf extract of <i>Acacia etbaica</i> against three gram positive and three gram negative bacteria					
	<i>B.substilis</i>	<i>E.feacalis</i>	<i>S.aureus</i>	<i>E.coli</i>	<i>K.Pneumonia</i>	<i>V. cholera</i>
AE.AC	0.53	2.58	0.63	1.56	4.12	2.06
AE.ET	1.24	4.12	1.5	3.01	24.87	14.86

Ethiopia, reported that among the gram-positive bacteria isolates *S.aureus* and gram-positive bacteria *Klebsiella spp* are more prevalence, the gram +ve bacterial isolates showed 81.8 % and 81.1 % antibiotic resistance against penicillin G, cotrimoxazole respectively, and gram -ve bacterial isolates showed antibiotic resistance to 85 % and 93.1% to tetracycline and cotrimoxazole respectively.

Diarrheal diseases caused by *E.coli* and *V.cholera* have been an important public health issue of developing countries like Ethiopia leading to high morbidity and 13.6% mortality under-five children (Melese et al., 2019). Enterobacteriaceae family such as several strains of *E.coli*, *Klebsiella*, *Enterobacter* and *Salmonella spp.* showed high drug-resistant against ampicillin, cotrimoxazole and tetracycline respectively and responsible for pathogenesis of nosocomial and community-acquired infections such as urinary, enteric, wound, burn, bloodstream and pneumonia infections (Brenner et al., 2005). For the last two decades, some of the clinically important pathogenic bacterial strains including the Enterobacteriaceae family develop multiple and extensive antibiotic resistance (MDR and XDR) against standard Synthetic, semi-synthetic antibiotics and spreading of resistance among the clinically bacterial strains and gradually standard antibiotics losing their efficacy for treating communicable diseases (Fair and Tor, 2014).

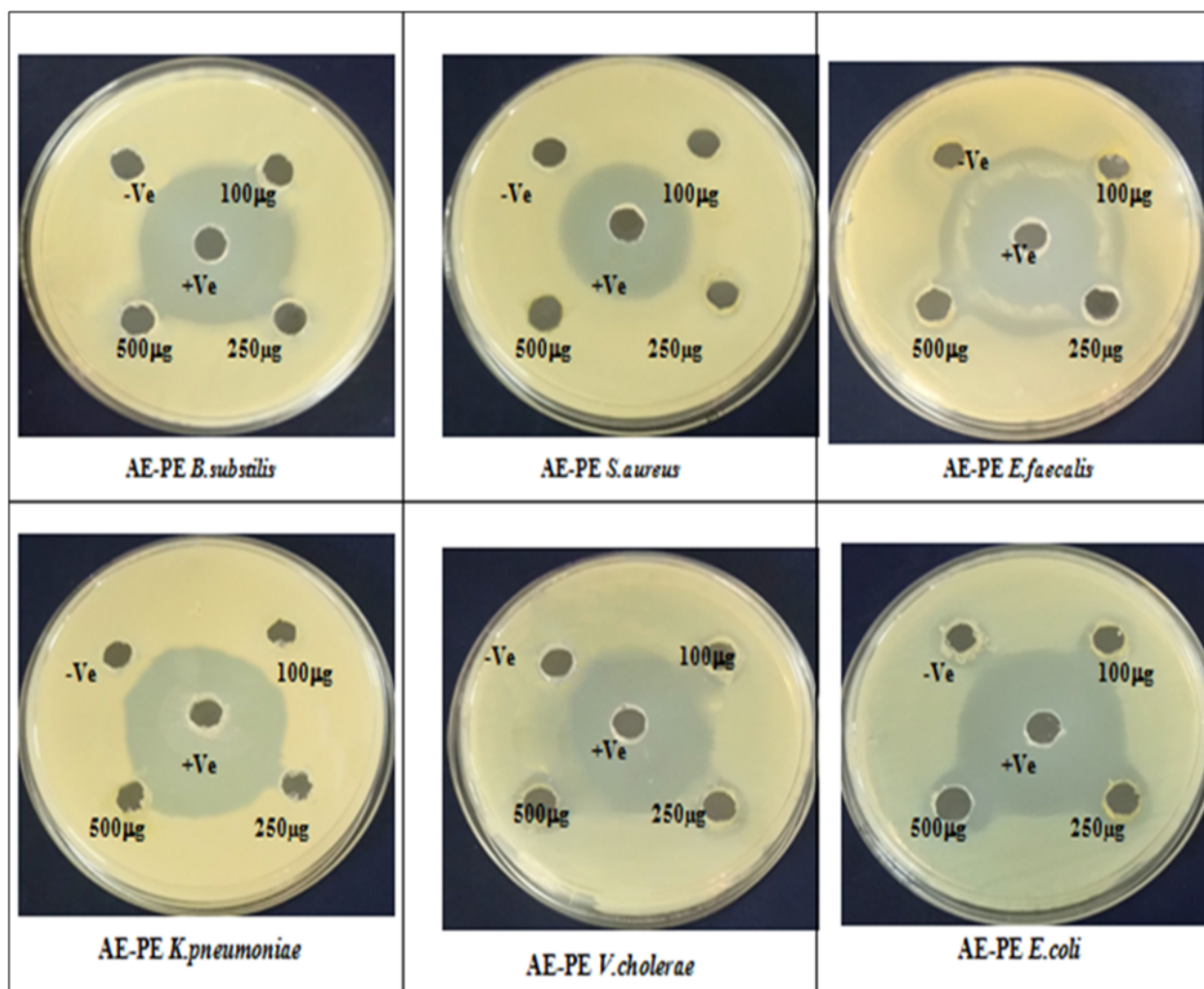
The bacterial strains showed induced antibacterial resistance to antibiotics in the pinnacle of

evolution (Munita and Arias, 2016). Due to the genetic plasticity of bacterial pathogens that trigger neutralization of antibiotic effect, transition of binding receptor site of antibiotics and attainment of genes coded for MDR and XDR (Nikaido, 2009). The emerging of bacterial strains to withstand the effect of antibiotics and undesirable secondary effects associated with synthetic antibacterial agents, microbiologists search for potential antimicrobial substances from various sources of natural sources for combating infectious diseases. In Ethiopia, since long back traditional practicing of medicinal plants has become an integral part of the culture that has been used to treat different human infectious diseases (Jima and Megersa, 2018).

*Acacia etbaica* Schweinf belongs to the family Fabaceae widely distributed in Ethiopia, Eritrea, Kenya, Somalia, Sudan, Tanzania, and Uganda (Ross, 1979).

Various plant parts of *A.etbaica* such as bark, flowers, leaves, pods, seeds, and roots are commonly used by traditional healers to cure various ailments (Kassem et al., 2014). The leaves of *A.etbaica* are applied as a folk remedy to heal swelling by crushing the leaves, mixed with the latex of *Erythrina abyssinica* and rub the paste on the affected part of the swelling area and also used at infected part the body (Teklay et al., 2013).

Hence the current study was intended to evaluate the *in vitro* antibacterial activity organic leaf extracts of *A. etbaica* against the selected multiple



**Figure 1: Zone of inhibition (mm) of petroleum ether leaf extract of *Acacia etbaica* (100, 250, 500 µg/mL) and standard antibiotics Ciprofloxacin (20 µg/mL) against three gram negative and three gram positive bacteria by the Agar well-diffusion method**

drug-resistant *Enterobacteriaceae* family.

## MATERIALS AND METHODS

### Description of study areas

The plant sample was collected from Axum city in the Central Zone of Tigray Ethiopia. The phytochemical analysis and antibacterial potential of leaf extract of *Acacia etbaica* was carried out in the Chemistry Laboratory, Aksum University, Tigray region, Ethiopia.

### Sample collection and authentication

Leaves of *A. etbaica* were collected from Axum city in January 2018 and transported to the Chemistry Laboratory at Aksum University for further processing. The plant specimen was authenticated by Mr. Melaku Wendafrash (M.Sc.) National Herbarium, Department of Biology, Addis Ababa University, Addis Ababa, and it was given the voucher spec-

imen number TT 001.

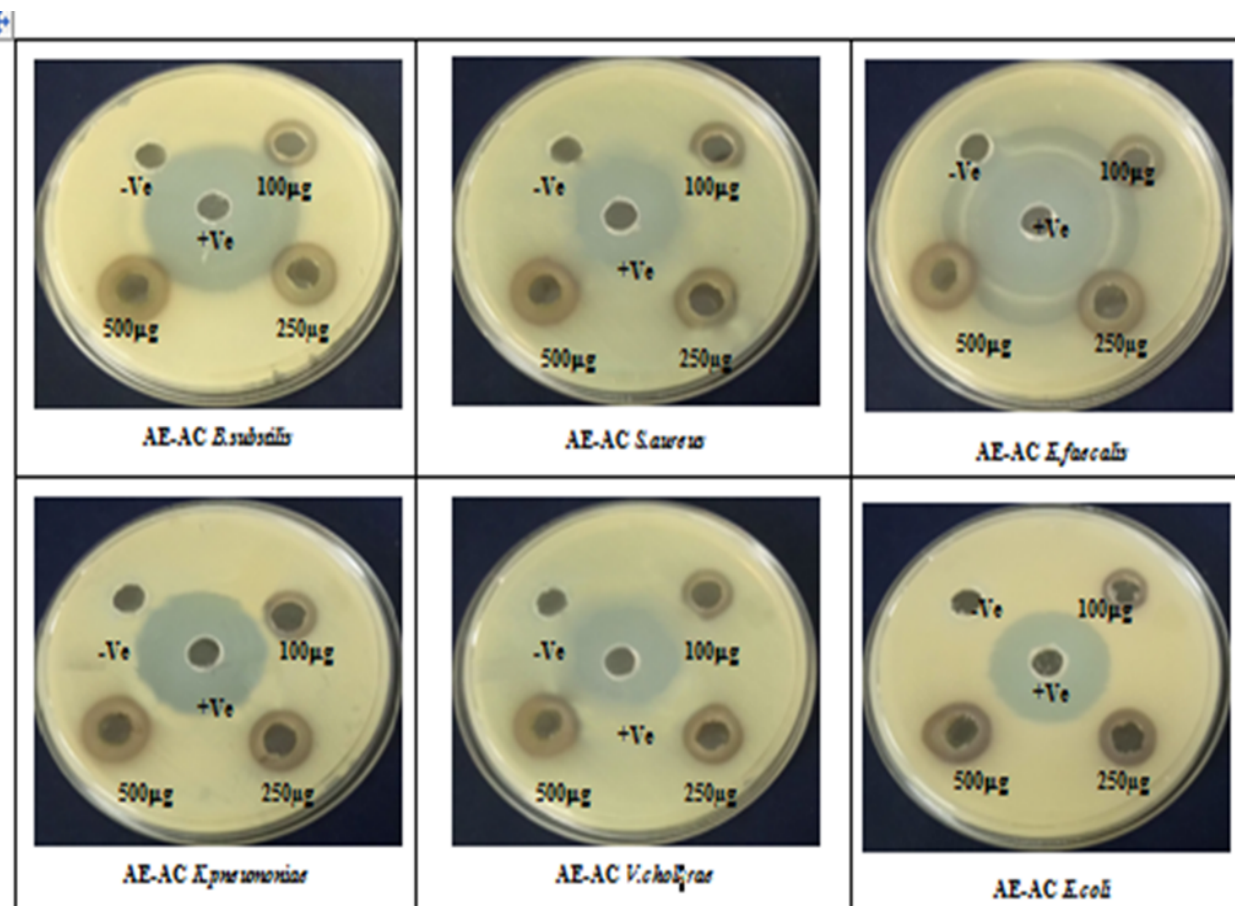
### Preparation of Plant extract

The leaves of *A. etbaica* were properly washed with distilled water, dried in shade at room temperature for two weeks days, crushed into fine powder by using an electrical grinder. 100 grams of plant powder was taken and subjected to sequential extraction by Soxhlet apparatus using 500ml of petroleum ether, chloroform, acetone & ethanol, and these organic extracts were concentrated at 35-40°C under reduced pressure and vacuum using a rotary evaporator (Das *et al.*, 2010). The concentrated plant extracts were then put in an airtight container and stored at 4° C before use in phytochemical and antibacterial activities.

### Phytochemical screening of organic leaf extract

The phytochemical screening of *A. etbaica* leaf organic extracts were conducted to determine the presence of phytoconstituents like alkaloids,





**Figure 2: Zone of inhibition (mm) of chloroform leaf extract of *Acacia etbaica* (100, 250, 500 µg/mL) and standard antibiotics Ciprofloxacin (20 µg/mL) against three gram negative and three gram positive bacteria by the Agar well-diffusion method**

flavonoids, tannins, coumarins, terpenoids, and phenols (Harborne, 1998).

#### Bacterial Strains

The bacterial strains such as three Gram-positive *Bacillus subtilis* (ATCC3915) *Enterococcus faecalis* (ATCC29212), *Staphylococcus aureus* (ATCC25923) and three Gram-negative bacteria *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae*, (ATCC700603) *Vibrio cholera*. (ATCC 39315) were procured from Ethiopian Health and Nutrition Research Institute (EHNRI) Clinical Bacteriology laboratory, Addis Ababa, Ethiopia.

#### Cultures of Bacteria

All the selected bacterial strains were cultured on nutrient agar plates and incubated for 24 hours at 37°C. The cultured bacterial strains were inoculated into Mueller-Hinton Broth and incubated at 37°C for 24 h before use.

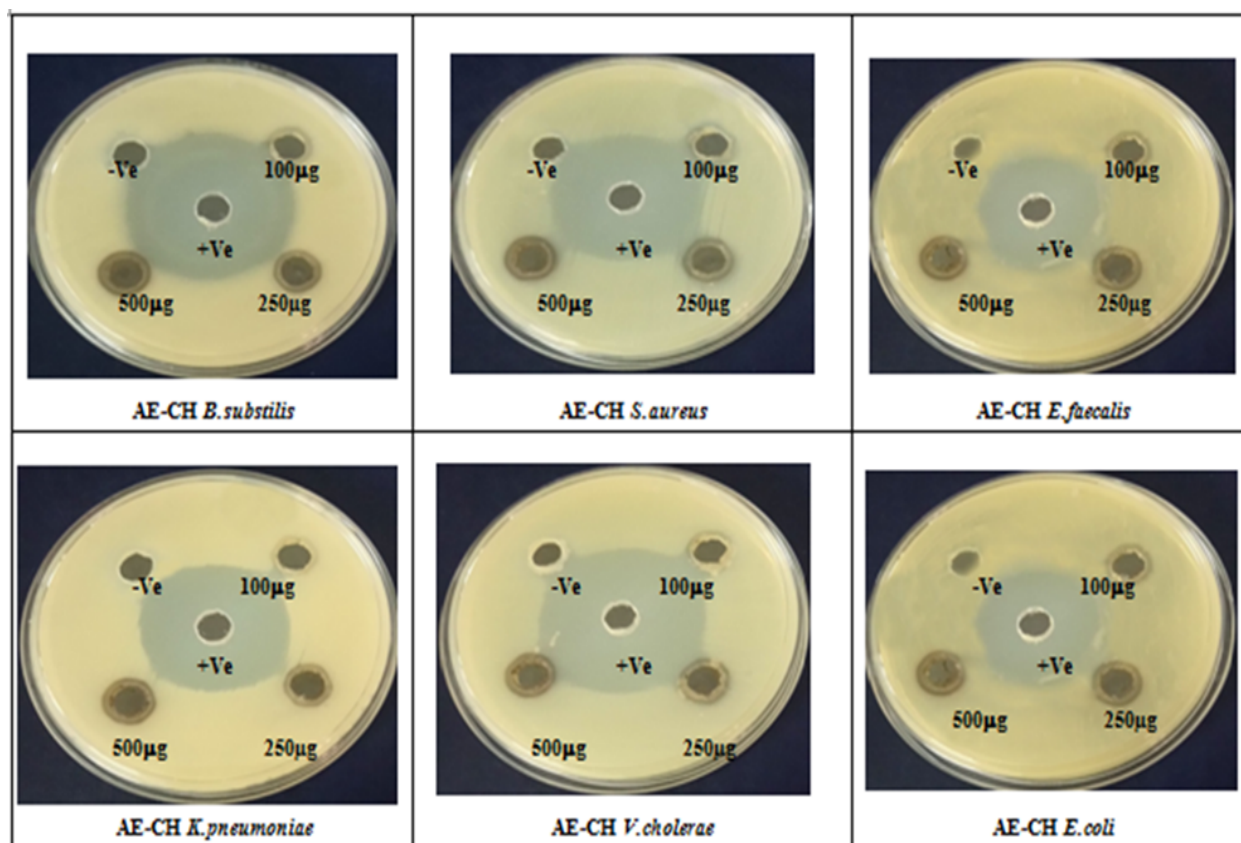
#### Standardization of inoculate

$1 \times 10^8$  CFU/ml of the selected bacterial strains were used to test antibacterial activity of plant

extracts by using McFarland turbidity standard (Thirumurugan, 2010).

#### Antibacterial Activity (Agar Well-diffusion Assay)

The antibacterial activities of the crude leaf extracts of *A. etbaica* were determined by agar well diffusion method (Bauer et al., 1966). 50 µl inoculums of tested bacterial strains were inoculated on the surface of solidified Mueller-Hinton Agar (MHA), by using a sterile cotton swab. The agar wells of 6-mm diameter were made using a sterile cork borer. About 100 µl of 10% solution (1 mg/ml) of each organic leaf extracts of *A. etbaica* in Dimethyl sulphoxide (DMSO) was dispensed in separate agar well with the help of a micropipette. The agar dishes were pre-incubated at 37°C for 2 hours to allow the uniform diffusion of organic leaf extracts of *A. etbaica* into the agar. After pre-incubation, the plates were incubated at 37°C for 24 hours. The dissolution of the organic leaf extracts of *A. etbaica* was facilitated with the addition of 10% (v/v) DMSO which not affected the growth of microorganisms (as shown by our control experiments).



**Figure 3: Zone of inhibition (mm) of acetone leaf extract of *Acacia etbaica* (100, 250, 500 µg/mL) and standard antibiotics Ciprofloxacin (20 µg/mL) against three gram negative and three gram positive bacteria by the Agar well-diffusion method**

Besides Ciprofloxacin (20 µg) was used a positive control for selected bacterial strains. The antibacterial activity was determined by measuring the inhibition zone diameter in millimeters in millimetre-scale after incubation (Lalitha, 2015).

#### **Minimum inhibition concentration of effective plant extracts (MIC)**

The acetone and ethanol leaf extract of *A. etbaica* were dissolved in 10% DMSO. The initial test concentrations were serially diluted in a 96 well plate and inoculated with 5 µl of  $10^8$  CFU ml<sup>-1</sup> bacterial suspensions which showed highly susceptible against the tested plant extract. The 96 well plates were incubated for 24 hours at 37 °C for bacterial growth. The culture intensity of each well was read at 600 nm and compared with the untreated controls. The MIC of the selected extract was determined as the lowest concentration of the extract inhibiting the visual growth of the test cultures (Wikler, 2009).

## **RESULTS AND DISCUSSION**

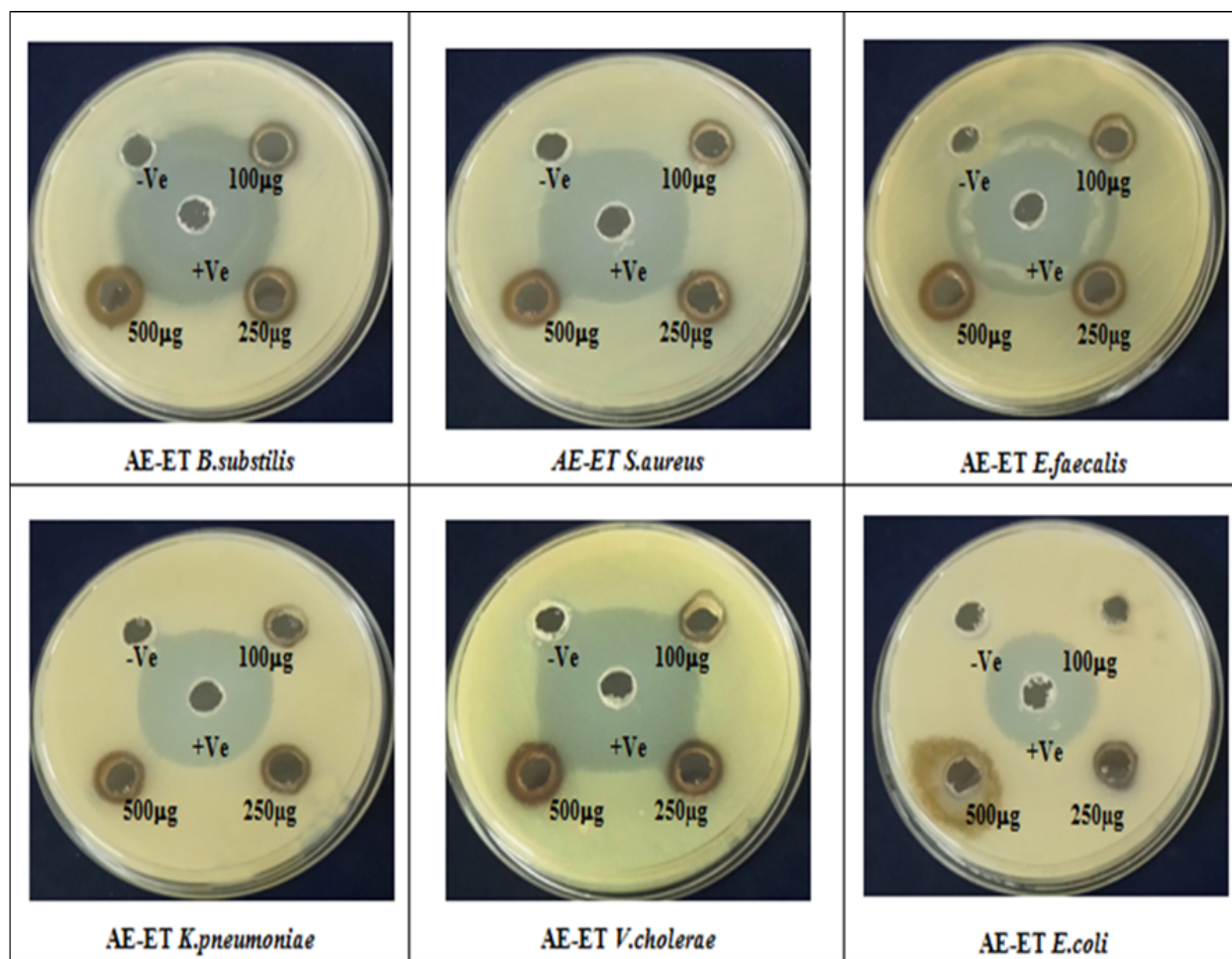
### **Preliminary phytochemical screening**

As shown in Table 1, the phytochemical analysis of

organic leaf extracts of *A. etbaica*, revealed that the acetone leaf extracts of *A. etbaica*, confirmed the presence of a maximum number of phytoconstituents like flavonoids, tannins, phenolic compounds terpenoids, and alkaloids were absent. The ethanolic leaf extracts of *A. etbaica* showed the presence of a significant number of phytoconstituents like flavonoids, tannins, phenolic compounds terpenoids and the absence of both alkaloids and coumarins. The current results were in agreement with previous reports of (Saleh et al., 2014).

### ***In vitro* antibacterial activity of crude leaf extract of *A. etbaica* against Enterobacteriaceae family**

The *in vitro* antibacterial activity of organic leaf extract of *A. etbaica* at various concentrations (100, 250, and 500 µg/mL) and standard antibiotic ciprofloxacin (20 µg/mL) were shown in the (Tables 2, 3, 4, 5 and 6). The *A. etbaica* organic leaf extract showed the zone of inhibition (*in vitro* antibacterial activity) ranging from 10 mm to 18 mm diameter (diameters which included the 6 mm of agar wells) with the significant results were shown by both acetone and ethanolic leaf extract of *A. etbaica* against both selected bacterial strains of



**Figure 4: Zone of inhibition (mm) of ethanol leaf extract of *Acacia etbaica* (100, 250, 500 µg/mL) and standard antibiotics Ciprofloxacin (20 µg/mL) against three gram negative and three gram positive bacteria by the Agar well-diffusion method**

#### *Enterobacteriaceae* family.

The antibacterial activity of petroleum ether leaf extract of *A. etbaica*, against the tested bacteria as shown in the Table 2 and Figure 1, exhibited insignificant zone of inhibition produced against the gram-positive *E. faecalis* (10 mm) at 500 µg/ ml and also showed same results with the gram-negative bacteria *V. cholerae* (9- 13 mm) and *E. coli* (10- 13 mm) at a concentration range between 100 µg/ ml -500 µg/ mL.

The antibacterial activity of chloroform leaf extract of *A. etbaica* as shown in Table 3 and Figure 2 exhibited prominent zone of inhibition produced against, tested gram-positive *B. subtilis* (11-12 mm), *S. aureus* (10-12 mm), and *E. faecalis* (10-12 mm) and the gram-negative bacteria *K. pneumoniae* (11-14 mm), *V. cholera* (18- 20 mm) and *E. coli* (16- 24 mm) at a concentration range between 100 µg/ ml -500 µg/ ml.

The antibacterial activity of acetone leaf extract *A. etbaica*, as shown in Table 4 and Figure 3, exhib-

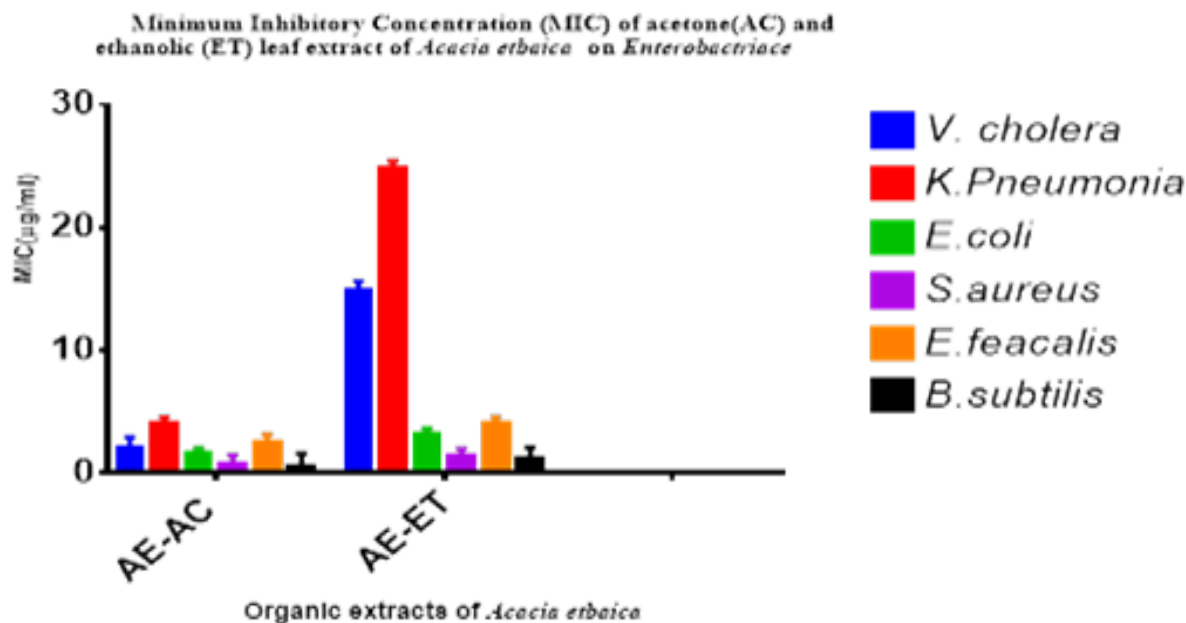
ited a significant broad spectrum of the zone of inhibition against the tested gram-negative bacteria *E. coli* (10- 17 mm), *V. cholera* (10-16 mm) and *K. pneumonia* (10- 17 mm) and the gram-positive bacteria *B. subtilis* (12- 16 mm), *S. aureus* (12- 15 mm) and *E. faecali* (11- 15 mm) at a concentration range between 100-500 µg/ mL. Therefore acetone leaf extract *A. etbaica* showed an effective broad spectrum of *in vitro* antibacterial activity against tested both gram-positive and gram-negative bacteria.

The antibacterial activity of ethanolic leaf extracts *A. etbaica* as shown in Table 5 and Figure 4 had shown a broad spectrum of the highest zone of inhibition against gram-negative bacteria *E. coli* (11-18 mm), *V. cholera* (11-13 mm), *K. pneumoniae* (10-14 mm) and gram-positive bacteria *B. subtilis* (10-13 mm), *S. aureus* (10- 14 mm) and *E. faecali* (10-14 mm) at 500 µg/ ml at a concentration range between 250 µg/ ml -500 µg/ ml.

#### **Minimal inhibitory concentration (MIC)**

The Minimum Inhibitory Concentration (MIC) of the





**Figure 5: Minimum Inhibitory Concentration (MIC) of acetone and ethanolic leaf extract of *Acacia etbaica* against *Enterobacteriaceae* in µg/ml**

effective acetone and ethanolic leaf extract of *A. etbaica* (AE-AC and AE-ET) at various concentrations were assessed by 96 well plate broth microdilution methods with a concentration range from 1.25 µg /ml to 100 µg /ml. Only the tested bacteria, which were showed highly susceptible to the selected plant extracts was taken to determining the MIC. The maximal zones of inhibition and MIC values for tested bacterial strains, which were sensitive to the leaf extract of *A. etbaica* , were in the range of 10-18 mm and lowest MIC values of 0.532 µg/ml against tested gram +ve bacteria. As showed in Table 5, the acetone leaf extract of *A. etbaica* was highly susceptible against tested *B.subtilis*, *S.aureus*, *E. feacalis*, *V. cholea*, *K. pneumonia*, and *E.coli* with the significant MIC value 0.532, 0.625, 2.5, 2.06, 4.12 and 15.60 µg/ml respectively.

As showed in the Figure 5, the ethanolic leaf extract of *A. etbaica* was highly susceptible to *B.subtilis*, *S.aureus*, *E. feacalis*, *E.coli* , *V. cholea*, and *K. pneumonia*, with the significant MIC values of 1.24, 1.5, 4.12, 3.008, 14.86 and 24.87. Among the tested organic, leaf extract of *A. etbaica* both acetone leaf and ethanolic leaf extract of *A.etbaica* shown strong broad-spectrum antibacterial activity against tested

*Enterobacteriaceae* family in the range of 11- 18 mm with less MIC values of between 0.532 to 24.87 µg /ml.

## CONCLUSIONS

Both the acetone and ethanolic leaf extract of *Acacia etbaica* exhibited a broad spectrum of promising *in vitro* antibacterial activities against tested MDR *Enterobacteriaceae* family of both selected bacterial strains among the other tested extracts. The qualitative phytochemical analysis of the acetone and ethanolic leaf extract of *A etbaica* shown the presence of the maximum number of phyto-constituents like flavonoids, tannins, terpenoids, and polyphenolic components. The high phenolic and flavonoid content present in both acetone and ethanolic leaf extract of *A.etbaica* accompanied by producing a significant zone of inhibition against all tested MDR *Enterobacteriaceae* family. The current studies demonstrated that the broad-spectrum antibacterial activity of acetone and ethanolic leaf extracts of *Acacia etbaica* may be helpful for the isolation of novel potent antibacterial agents against both hospital-acquired and communicable infectious bacterial pathogens without any side effects.



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## Conflict of Interest

The authors have confirm no conflict of interest.

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