Combined antibacterial effect of essential oils from three most commonly used Ethiopian traditional medicinal plants on multidrug resistant bacteria

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Abstract

Background: An alarm increase the rate of emerging and re-emerging of multidrug resistant bacteria have been caused great public health concern in the worldwide. They have been resisting for most or majority of currently available and affordable antibiotics and imposed socioeconomic catastrophe at global scale. As a result, there is utmost important to discover new or modify currently available antibiotics. The aim of this study was to evaluate combined antibacterial effect of essential oils obtained from Blepharis cuspidata, Boswellia ogadensis and Thymus schimperi against multidrug resistance (MDR) Escherichia coli, Klebsiella pneumoniae and Methicillin resistant S. aureus.

Methods: Essential oil (EO) was extracted from the aerial part of B. cuspidata, B.ogadensis and T. schimperi by steam distillation and stored in brown bottles at 4 °C. There were mixed in 1:1 ratio and adsorbed to disc and placed on MHA and measured their minimum inhibitory zone seeded with E. coli, K. pneumoniae and MRAS after 18-24 H. minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were measured by broth micro-dilution method. The interaction between EOs was determined by fractional inhibitory concentration index.

Results: The antibacterial potential of mixed oil depends on the doses and type of the EOs and bacteria species. The combined EOs of B.cuspidata and T.schimperi had inhibition zone (39 mm), its MIC and MBC value was 0.39 μl/ml against MRSA. It had inhibition zone (28-35 mm), MIC value 0.39 – 6.25 μl/ml and MBC (0.78 – 12.5 μl/ml) against MDR E. coli and K. pneumoniae. Whereas, combined effects of B. cuspidata and B. ogadensis had MIC values ranges from 0.78 – 6.25 μl/ml for E.coli and K. pneumoniae and 1.56 μl/ml for MRSA. There was strong synergistic effect between the combination of B.cuspidata and T.schimperi. This study revealed that gram negative bacteria were slightly less susceptible than gram positive.

Conclusions: This in vitro study of combined EOs has significant antibacterial effect than using each of them and even it was more potent antibacterial effect on MDR as compare to modern antibiotics. Hence, it can be applied to a pharmaceutical composition as modulator or adjuvant or precursor for synthesis of new antibiotic in future activities.

Keywords: Blepharis cuspidata, Boswellia ogadensis, Thymus schimperi, MRSA
Background

An alarm increase the rate of emerging and re-emerging of multidrug resistant bacteria have been caused serious difficulties in the treatment and continued to be clinical and public health concern in the worldwide [1, 2]. Spreading of methicillin-resistant *S. aureus* [3, 4] and ESBL producing *E. coli* and *K. pneumoniae* become difficult to treat [5, 6]. These bacteria have been continuing to develop resistance for most currently available antibacterial drugs by either mutation or exchange of genetic information [7, 8]. Many resistance mechanisms that emerge and spread in bacteria population are widespread. Recently, discovered factors with major implication for the emergence, dissemination and maintenance of resistance include multidrug efflux, hyper-mutability and plasmid addiction [2, 3, 9]. Such factors have been compromised all or majority of the drugs belonging to a given therapeutic [7, 8]. As a result, it has been initiated for searching a new, better and affordable antibiotic derived from medicinal plant as alternatives or complementary treatment for drug resistance microbial including bacteria [10, 11].

In developing countries, the majority of the population still can’t afford to purchase modern pharmaceutical drugs and continued to use indigenous traditional medicinal plants [12]. Of which, tropical plants are the most valuable source of new bioactive due to their biodiversity coupled with the chemical diversity found within each species [13, 14]. However, higher plants in general and endemic medicinal plants of our country in particular haven’t been screened from the viewpoint of bioactive for phytochemical and pharmacological utilization from a wider perspective [14]. Hence, there is a need to carry out proper research in order to investigate the efficacy and safety of herbal remedies.

With the dearth of novel antibiotics, traditional healers’ extracted Eos from TMP used for treatment of different illness [15]. The chemical composition of essential oils isolated by steam distillation contain varies bioactive compound that exhibited remarkable bacteriostatic and bactericidal activities [14, 16]. It is likely that their mode of action involved several targets in the bacterial cell. Some of them acted on partition in the lipids of the cell membrane resulted in leakage of cell contents [16], inhibited cell cycle (S-phase), inhibited protein synthesis and DNA replication [17, 18]. Therefore, they are purposed as promising antibiotic to overcome the multidrug resistance bacteria.

The combined effects of modern antibiotics (ciprofloxacin, ceftazidime and tetracycline) and six phytochemicals (protocatechuic acid, gallic acid, ellagic acid, rutin, berberine and myricetin) showed that it had inhibited the growth of *P. aeruginosa*. This combination of modern antibiotic and natural compounds were revealed more antibacterial effect than single compound [15, 19]. Studies done on *MRSA* and *ESBL* produced *Enterobacteriaceae* showed that EOs was radically reduced their growth [20, 21]. On the other hands, study done on MDR bacteria showed that its growth was inhibited by extracts from clove, jambolan, pomegranate and thyme [22]. Likewise, in-vitro interaction between the tested antimicrobial and eleven EOs showed promising effect against drug resistance *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and clinically isolated strains [22–24].

The combined plant extract exhibited more antibacterial effect on the MDR bacteria than any of the individual plant extracts [23]. As a result of different phytochemicals such as coumarins, flavonoids, phenolic, alkaloids saponins, tannins, terpenoids, quinones, anthraquinones, cardiac glycosides and others are found in each plant in different concentration [24, 25]. Many studied showed that combination of selected phytochemicals and antibiotics was resulted in foliate biosynthesis inhibitors, DNA/protein synthesis inhibitors and cell permeability/cell wall inhibitors [10, 18, 19]. Many studies showed that Eos also inhibit macromolecules (DNA, RNA, protein and polysaccharide) synthesis in pathogen bacteria [15, 24]. Synergistic effects of essential oils can provide effective therapy against multidrug resistant bacteria [23]. These synergistic combinations represent a largely untapped source of novel pharmaceutical products with novel and multiple mechanisms of action that could overcome pathogenic microbial resistant [19, 22–25]. With this notion, we purposed to evaluate combined antibacterial effect of essential oils from *B. cuspidata*, *B. ogadensis* and *T. schimperi* against MDR bacteria. This study could serve as a baseline data to investigate new bioactive from essential oil and find out the scientific rationale for the combined effects of untapped traditional medicinal plants used by different societies.

Methods

In vitro experimental study was employed to evaluate combined antibacterial effect of essential oils obtained from *B. cuspidata*, *B. ogadensis* and *T. schimperi* against clinical isolated MDR gram negative (*E.coli* and *K. pneumoniae*) and *MRSA* and their reference strains.

Medicinal plants selection criteria

In this study, plant selection was on the basis of knowledge of herbalist lived in Bale zone. Those herbalists were used for treating various skin diseases, urinary tract infection sexual transmitted infection, hypertension, tumorcical, sexual impotence and others. Dawe Kechen and Dawe Serare are found in Bale zone, south east Ethiopia. They are the most remote area with no infrastructure (transport and power supplies). Until this field work, there is no hospital; even one health center with
no full functioning the health activity in each district. As a result, the community imposed to use medicinal plants for treatment of various diseases. For instance, *V. schimperi* (Qorsa finchaanii), *C. myricoides* (Handaraafa) and *Z. scabra* was used to treat cancer, tumor, urinary tract infection and gonorrhea. Whereas, *B. cuspidate* (Qoree waraantii), *B. ogadensis* and *B. edulis* (Suree Lukkuu) used for treatment of kidney, liver cirrhosis, hepatitis, skin diseases, cancer and diabetes.

**Plant collection and preparation**

Essential oils obtained from aerial parts of *T. schimperi*, *B. cuspidate* and *B. ogadensis* were evaluated for their antibacterial effect on multidrug resistant bacteria. *T. schimperi* was collected from Dawe Kechen. *B. cuspidate* and *B. ogadensis* were collected from Dawe Serare. Authentication of each plant sample was carried out in the Department of Biology, Faculty of Natural and Computational Science, Addis Ababa University by Dr. Melaku Wondafrash. Those identified plant samples were deposited at the National Herbarium with voucher number *Thymus schimperi* (E-25/07), *Blepharis cuspidate* (E-11/07) and *Boswellia ogadensis* (E-09/07).

**Extraction of essential oils**

Health and well grown fresh leaves of each plant was collected and cut into small pieces. Plant materials were washed thoroughly under running tap water. Then, it’s subjected to steam distillation using AMIO-37/04 model for 4 h. Essential oils were extracting from aerial parts of *T. schimperi*, *B. cuspidate* and *B. ogadensis* as guideline described by WHO on quality of herbal medicine. The purified essential oils were stored in brown colored bottle vials at 4 °C until used [9, 11, 26, 27].

**Culture media**

Nutrient agar, MacConkey, Muller Hinton agar (MHA), Muller Hinton Broth (MHB), blood agar (BA), manitol salt agar (MSA), chocolate agar and biochemical reagents were obtained from Department of Medical Microbiology, Immunology and Parasitology, CHS, AAU and Tikur Anbessa Specialized Hospital (TASH), Bacteriology Unit.

**Test organisms**

The reference strains of *S. aureus* (ATCC25923), *E.coli* (ATCC25922), *K. pneumoniae* (ATCC700603) were obtained from TASH and Ethiopian Public Health Institution and their multidrug resistant strains isolated from different samples of patient’s attending TASH according to CLSI guideline [26].

**Modern antibiotics**

Modern antibiotics such as tetracycline (30 μg), ciprofloxacin (5 μg), gentamycin (10 μg), cephalotaxon (30 μg), chloramphenicol (30 μg), cefotaxime (5 μg), cefazidime (10 μg), ceftriaxone (30 μg), amikacin (30 μg), cefuroxime (5 μg), ceftriaxone (30 μg), cefoxitin (30 μg), cloxicillin (5 μg), augmentin (30 μg) were used for testing MDR bacteria according to CLSI guideline [26].

**Screening for multidrug resistant bacteria**

Multidrug resistance gram negative and *S. aureus* were isolated from clinical specimen such as CSF, urine, wound and blood. Triplicate of each MRSA and MDR gram negative bacteria were isolated from clinical specimen. All bacterial cultures were first grown on 5% blood agar plates at 37 °C for 18 to 24 h prior to inoculation onto the MHA. Few colonies (3 to 5) of similar morphology of the respective bacteria were transferred with a sterile inoculating loop to a liquid medium until adequate growth of turbidity with McFarland of 0.5. Then the bacterial suspension was streaked on MHA plates using a sterile swab in such a way as to ensure thorough coverage of the plates and a uniform thick lawn of growth following incubation. The susceptibilities of clinical isolates were tested by using the MHA contains a range of antimicrobial agents. Dilutions of overnight broth cultures were inoculated onto antibiotic containing plates to yield final inoculums of approximately 10⁶ CFU per spot according to CLSI for MRSA and *E.coli* and *K. pneumoniae* [26, 28].

**Screening for gram negative**

Selected multidrug resistant gram negative such as *K. pneumoniae* and *E. coli* were screened for their resistant for more than two different classes of antibiotics following disk diffusion method as CLSI guideline and WHO recommendation [26].

**Screening for methicillin resistance Staphylococcus aureus**

In this study, cefoxitin was used as marker of meca/mecC mediated by methicillin resistant *S. aureus* and drug of choice for disk diffusion method as recommended by CLSI guideline [26]. This strain was selected based on antibiotic profile mentioned (Table 1).

**Determination of MIC and MBC values**

After preliminary screening of essential oils, those revealed potent antimicrobial effect were further tested to determine MIC and MBC for MDR gram negative bacteria (*K. pneumoniae* and *E. coli*) and MRSA. It was determined by MHB broth micro-dilution method. Each 96-well micro-titer plate was liquated with 50 μL of MHB; 10th well (sterility control) was added with 100 μL of MHB. And the 9th well (growth control) was added
with MHB with 5% DMSO. 50 μL of essential oils initially dissolved in 5% DMSO was added into the first well. A serial 2-fold dilution was performed by transferring 50 μL of the suspension to the subsequent wells up till the 8th well; this procedure was performed by modifying Wiegand protocol. 0.5 McFarland broth inoculum was diluted in the ratio of 1:100 and added into 1st-8th well in achieving the final inoculums size at 5 × 10^5 CFU per ml [27].

Bacterial cell viability and MIC values were determined by observing the turbidity. The lowest concentrations of essential oils with clear suspension were considered as the MIC values. The lowest concentrations of essential oils in the post-incubation suspensions which did not harbor any bacterial growth upon spotting on MHA after overnight incubation at 37 °C were considered as the MBC values. Test was performed in triplicate alongside antibiotics ciprofloxacin (5 μg) for gram negative and cefoxitin (30 μg) for MRSA as positive control respectively [5, 9, 26, 27].

Fractional inhibitory concentration index
In vitro drug interaction was determined by the checkerboard method as described elsewhere and the results were analyzed with the FIC index. Growth control wells containing medium were included in each plate. Each test was performed in triplicate. The concentration of antibiotics needed to inhibit growth was recorded. The following formula was used to calculate FIC: MIC of drug in combination FIC/ MIC of drug alone = The FIC index (ΣFIC) calculated as the sum of each FIC, was interpreted as follows: synergy is defined as a FIC index of ≤0.5. Antagonism is defined as a FIC index of ≥2. An indifferent/additive effect is defined as a FIC index of 0.5 < X < 2 or a micro dilution decrease of 1 dilution in the MIC of one drug or no change in the MIC of either of the drugs [24].

Statistical analysis
Statistical data were reading values of inhibition zones (in diameter) and concentration values (MIC &MBC) analyzed using SPSS, version 21 according to CLSI. Each experiment values are expressed as mean ± S.D. Statistical significance was determined by student's t-test. Values with p < 0.05 were considered significant.

Results
Antibacterial effect of modern antibiotics
The present study revealed that tested multidrug resistant strains isolated from clinical samples were resisted for the majority of currently available and affordable antibiotics in developing countries. The clinical isolates, those referred to as methicillin resistant S. aureus (MRSA) were selected on the basis of their resistance to methicillin. This gram positive bacterium was resisted not only cefoxitin but also resisted for amikacin, amoxicine, ampicillin and cefotaxime (Table 1). On the other hand, gram negative bacteria (E. coli and K. pneumoniae) was unpredictably resisted for third generation cephalosporine and most commonly used penicillin classes (Table 1). Such resistant strains had developed genes or gene products that enable them to resist for tested antibiotic. Unless and otherwise, new drugs or modify currently available antibiotic will be revolutionized, emerged multidrug resistant bacteria impose potentially large health and socioeconomic burden on societies and worries future provision of health care services.

Antibacterial effect of essential oils
This study revealed that essential oils extracting from B. cuspidata, B. ogadensis and T. schimper were demonstrated antibacterial effect against tested bacteria on their 10 μl/disc (Table 2). They had appreciable antibacterial effect not only against reference strains of S. aureus (ATCC25923), E. coli (ATCC25922) and K. pneumoniae (ATCC70603) but also on multidrug resistant strains isolated from clinical specimens. Their effectiveness varied with concentration, type of the essential oils and the type of bacteria species. Overall, all essential oils had overriding antibacterial effect against gram positive and gram negative bacteria.

Essential oil extracted from B. cuspidata had comparably elicited high antibacterial effect than others. It had 22 mm and 25 mm inhibition zone in diameter against

Table 1 Antibiotics resistant profile of multidrug resistant bacteria at AAU, 2018

| Modern drugs | Drug susceptibility test for multidrug bacteria strains |
|--------------|--------------------------------------------------------|
|              | S.1 | S.2 | S.3 | E.1 | E.2 | E.3 | K.1 | K.2 | K.3 |
| Ampicillin   | R   | R   | R   | N   | R   | R   | N   | I   | R   |
| Augmentin    | R   | I   | S   | R   | R   | R   | R   | R   | R   |
| Chloramphenicol | R   | R   | R   | S   | S   | S   | R   | R   | R   |
| Erythromycin | R   | R   | S   | R   | S   | S   | R   | I   | N   |
| Gentamicin   | N   | N   | N   | R   | R   | I   | R   | S   | N   |
| Ceftriaxone  | N   | N   | N   | R   | R   | R   | R   | R   | R   |
| Cefazidine   | I   | R   | R   | N   | N   | N   | R   | R   | R   |
| Amoxicillin  | R   | R   | R   | R   | R   | R   | R   | R   | R   |
| Cefiroxime   | R   | R   | R   | S   | R   | R   | N   | R   | R   |
| Cefoxitin    | R   | R   | R   | R   | R   | N   | R   | R   | R   |
| Ciprofloxacin| S   | S   | S   | S   | S   | S   | S   | S   |
| Norfloxacin  | N   | N   | N   | S   | S   | S   | S   |
| Amikacin     | R   | I   | R   | R   | R   | I   | R   | R   | S   |

S S. aureus, E E. coli, K K. pneumoniae, N Not done, R Resistance (≤ 16 mm IZ), S Sensitive (≥21 mm IZ) I = Intermediate (17-20 mm IZ) according to CLSI guideline [26].
MSSA and MRSA respectively. Whereas, MDR E. coli and K. pneumoniae had 19 mm and 24 mm inhibition zone in diameter at their 20 μl/disc respectively. It had MIC value (1.56 μl/ml) and MBC (3.12 μl/ml) against MRSA and MIC values ranging from 3.12 to 12.5 μl/ml against tested multidrug resistant gram negative bacteria (Table 2).

Essential oil extracted from T. schimper had moderate antibacterial effect on tested Enterobactericeae. It had 17 mm and 24 mm inhibition zone in diameter against E. coli and K. pneumoniae respectively (Table 2). It had also appreciable on both MSSA and MRSA (24 mm) inhibition zone in diameter. On the other hand, B. ogadensis had 19 mm inhibition zone in diameter and MIC value (3.12 μl/ml) and MBC (6.25 μl/ml) against MRSA. Moreover, it had MIC value ranging 3.12–6.25 μl/ml and MBC 3.12–12.5 μl/ml for tested reference and MDR E. coli and K. pneumoniae.

Combined antibacterial effect of essential oils
In our study, the combined essential oils were showed strong inhibitory action against all reference strains and multidrug resistant bacteria at 5 μl/disc (Table 3). Of which, the combined essential oil obtained from B. schimper + B. cuspidata (1:1 ratio) showed significant inhibition against MSSA and MRSA (≥21 mm IZ) and E. coli and K. pneumoniae (≥21 mm IZ).

### Table 2: Inhibition zone (mm) of essential oils against MDR bacteria at AAU, 2018

| Medicinal plant | μl/disc | Gram positive | Gram negative |   |   |
|----------------|---------|---------------|---------------|---|---|
|                |         | MSSA          | MRSA          | E. coli | K. pneumoniae |
|                |         | R | S | R | S |
| T. schimper    | 100     | 16 ± 0.9      | 16 ± 0.4      | 11 ± 0.4 | 10 ± 0.7 | 17 ± 0.4 | 17 ± 1.2 |
|                | 100     | 21 ± 0.6*     | 21 ± 0.3      | 17 ± 0.4 | 13 ± 0.1 | 24 ± 0.2* | 24 ± 1.0 |
| B. cuspidata   | 100     | 19 ± 0.3      | 19 ± 0.2      | 15 ± 1.3 | 11 ± 0.3 | 19 ± 0.3 | 18 ± 0.2 |
|                | 100     | 22 ± 0.6*     | 25 ± 0.1      | 19 ± 0.8 | 15 ± 0.1 | 23 ± 0.3* | 23 ± 0.9 |
| B. ogadensis   | 100     | 13 ± 0.8      | 12 ± 0.1      | 9 ± 0.2 | 8 ± 0.7 | 17 ± 0.9 | 17 ± 0.3 |
|                | 100     | 19 ± 0.8      | 17 ± 0.3      | 14 ± 0.2 | 14 ± 0.2 | 20 ± 0.6 | 21 ± 0.9 |
| Positive control |        |              |              | Cefoxitin |             |             |             |
|                |         | S | R | – | – | – | – |
|                |         | – | – | R | S | R | S |
| Negative control (5% DMSO) | N | N | N | N | N | N | N |

The values represent mean ± standard deviation, N NO inhibition zone, R Resistant (≤16 mm IZ), S Susceptible (≥21 mm IZ). Where, *P < 0.05 when compared to cefoxitin treated MRSA. While, **P < 0.01 when compared to modern drug treated K. pneumoniae.

### Table 3: Inhibition zone (mm) of combined essential oils against MDR bacteria at AAU, 2018

| TMP | μl/disc | Gram positive | Gram negative |   |   |
|-----|---------|---------------|---------------|---|---|
|     |         | S.aureus      | MRSA          | E.coli | K. pneumoniae |
|     |         | ATCC          | MDR           | ATCC | MDR |
| T. schimper + B. cuspidata (1:1 ratio) | 5 | 19 ± 1.0 | 18 ± 1.2 | 20 ± 1.0 | 21 ± 0.1 | 27 ± 1.0 | 27 ± 0.8* |
|     | 10 | 29 ± 1.2 | 29 ± 0.5*** | 23 ± 1.2 | 25 ± 0.1* | 29 ± 1.2 | 28 ± 0.3* |
|     | 15 | 33 ± 0.1 | 33 ± 0.6** | 28 ± 0.1 | 26 ± 0.5* | 33 ± 0.7 | 34 ± 0.1** |
|     | 20 | 38 ± 0.3 | 39 ± 0.8*** | 29 ± 0.1 | 29 ± 0.8** | 35 ± 1.4 | 35 ± 0.5** |
| B. cuspidata + B. ogadensis (1:1 ratio) | 5 | 20 ± 0.4 | 20 ± 0.1 | 16 ± 0.4 | 15 ± 0.9 | 19 ± 0.7 | 15 ± 0.4 |
|     | 10 | 25 ± 0.5 | 26 ± 0.3* | 18 ± 0.6 | 23 ± 0.2 | 24 ± 0.4 | 23 ± 0.2 |
|     | 15 | 28 ± 0.3 | 27 ± 0.3** | 24 ± 0.5 | 26 ± 0.4* | 27 ± 1.2 | 26 ± 0.4* |
|     | 20 | 32 ± 0.1 | 32 ± 0.1*** | 26 ± 0.8 | 27 ± 0.1* | 29 ± 0.1 | 29 ± 0.2** |
| B. ogadensis + T. schimper (1:1 ratio) | 5 | 11 ± 0.6 | 11 ± 0.3 | 9 ± 0.1 | 10 ± 0.1 | 12 ± 0.4 | 12 ± 0.4 |
|     | 10 | 14 ± 0.3 | 15 ± 0.7 | 13 ± 0.8 | 13 ± 0.8 | 15 ± 0.6 | 16 ± 0.6 |
|     | 15 | 16 ± 0.4 | 16 ± 0.1 | 15 ± 0.5 | 14 ± 0.4 | 19 ± 0.7 | 20 ± 0.7 |
|     | 20 | 19 ± 0.1 | 19 ± 0.8 | 17 ± 0.7 | 21 ± 0.3 | 21 ± 0.2 | 20 ± 0.7 |
| Modern drug | 24 ± 0.8 | 11 ± 1.3(R) | 22 ± 0.4 | 17 ± 0.4(R) | 23 ± 0.7 | 17 ± 0.2(R) |

The values represent mean ± standard deviation, N NO inhibition zone, R Resistant (≤16 mm IZ), S Susceptible (≥21 mm IZ). Where, *P < 0.05, **P < 0.01, ***P < 0.001 when compared to cefoxitin treated MRSA. While, *P < 0.05, **P < 0.01 when compared to modern drug treated K. pneumoniae and *P < 0.05, **P < 0.01 when compared to modern drug treated E. coli.
**Table 4** Inhibitory concentrations of essential oils (μl/ml) against MDR bacteria in MHB at AAU, 2018

| TMP          | Inhibitory concentration | S. aureus ATCC | MRSA | E.coli ATCC | MDR | K. pneumoniae ATCC | MDR |
|--------------|---------------------------|----------------|------|--------------|-----|-------------------|-----|
| T. schimper  | MIC                       | 3.12           | 3.12 | 6.50         | 6.25| 3.12              | 3.12|
|              | MBC                       | 6.25           | 6.25 | 12.5         | 12.5| 3.12              | 3.12|
| B. ogadensis | MIC                       | 3.12           | 3.12 | 6.25         | 6.25| 3.12              | 3.12|
|              | MBC                       | 6.25           | 6.25 | 12.5         | 12.5| 6.25              | 6.25|
| B. cuspidata | MIC                       | 1.56           | 1.56 | 12.5         | 12.5| 3.12              | 3.12|
|              | MBC                       | 3.12           | 3.12 | 25.0         | 25.0| 3.12              | 3.12|
| B. ogadensis + T. schimper | MIC                     | 3.12           | 3.12 | 6.25         | 6.25| 1.56              | 1.56|
|              | MBC                       | 6.25           | 6.25 | 25.0         | 25.0| 1.56              | 1.56|
| T. schimper + B. cuspidata | MIC                     | 0.39           | 0.39 | 1.56         | 1.56| 0.39              | 0.39|
|              | MBC                       | 0.39           | 0.39 | 3.12         | 3.12| 0.78              | 0.78|
| B. cuspidata + B. ogadensis | MIC                     | 1.56           | 1.56 | 6.25         | 6.25| 0.78              | 0.78|
|              | MBC                       | 1.56           | 1.56 | 25.0         | 25.0| 1.56              | 1.56|
| Ciprofloxacin (5 μg) | MIC                     | 0.15           | 10.5 | 0.50         | 8.00| 0.25              | 9.00|
|              | MBC                       | 0.25           | 3.00 | 1.00         | 4.00| N                 | N   |

*N Not done, TMP Traditional medicinal plant, MHB Muller Hinton Broth, MBC Minimal Bactericidal Concentration, MIC Minimal Inhibitory Concentration*
been questionable in the majority of our hospitals where there is no drug susceptibility testing facilities. Unless and otherwise, new drugs or modify currently available antibiotics will be revolutionized, it imposes potentially large health and socioeconomic burden on societies and worries future provision of health care services. This finding substantiates previous studies that clinically isolated bacteria showed resistance to the majority of currently available and affordable drugs. This could be developed by either mutations or exchange of genetic information within and between individuals [3]. Another study showed that evolution of antibacterial resistant in human pathogenic and commensal microorganisms due to interaction between antibiotics exposure and horizontally gene(s) transfer by transformation, transduction and conjugation in very dynamic and unpredictable phenomenon [7, 8]. As a result, they resisted for more than two classes of antibiotics and classified as multi drug resistant bacteria [8, 26, 28]. Another studies showed that multidrug resistant bacteria were bearing different resistant mechanisms such as penicillin-binding proteins, drug modification, mutated drug targets, enhanced efflux pump expression and altered membrane permeability. As a result, it has been created a newly emerging and spreading in the bacterial population that compromised the usefulness of all or a majority of drugs [3, 7–10, 24]. Another report showed that resistant traits are not naturally eliminated or reversed. It may be accumulated over time for variety of antibiotics. This can lead to strains with multiple drug resistance, which is more difficult to kill due to reduced treatment option [8]. Those issues have prompted a search for alternative drug(s) from natural bioactive compounds [10, 14, 28].

The medicinal plants that use by healers in Bale zone are promising antibacterial activities and agrees with many researches presented on antimicrobial activities of medicinal plants on multidrug resistant bacteria. This is due to biodiversity coupled with the chemical diversity found within each species [4, 14, 20, 21]. Other studies shown that medicinal plants synthesize and accumulate some secondary metabolites like alkaloid, sterols, terpenes, flavonoids, saponins, glycoside, cyanogenics, tannins, resins, lactones, quinines and volatile oils; compounds that exhibited a broad spectrum [10, 13, 17, 18, 24, 29]. Many previous studies indicated that a number of essential oils contain aldehydes or phenols that used as antimicrobial properties. In many cases, the effective result from the complex interaction between different classes of compounds such as phenols, aldehydes, ketones, alcohols, esters, ethers or hydrocarbons are found in essential oils [15, 28, 30]. Study done by Virender by different solvents of Euphorbia hirta, Erythrophleum suaveolens and Thevetia peruviana extracts showed antibacterial effect against ESBL producing E.coli, Pseudomonas, Klebsiella, MRSA, Salmonella and Proteus. As a consequence, these bioactive compounds derived from medicinal plants used as starting point for synthetic pharmacophores and as industrial raw materials [1, 2, 11, 18]. In this regard, many researchers argued that there are many mechanisms of antimicrobial interaction that produce synergism. Probably the main reasons for this are sequential inhibition of a common biochemical pathway, inhibition of (enzymes, protein synthesis, nucleic acid synthesis), disintegrated the outer membrane [10, 15, 20]. Other authors proposed that, the synergistic effect could be due to the similarity of their mechanism; or may be due to act on the different targets [2, 4, 17].

With other respects, synergistic combination of essential oils of oregano/basil, basil/ bergamot, oregano / bergamot and oregano / perilla against S. aureus, E. coli, B. subtilis and S.cerevisiae respectively shown that significantly disrupted the integrity of cell membranes [23, 24]. Another study showed that alkaloid in combination with conventional antibiotics (methicillin, ampicillin) exhibited antimicrobial effects against microorganisms [15, 17, 22, 29]. The antimicrobial effect of mixture of the LGEO and amoxicillin indicated synergistic effects against MRSA [23]. However, the commercial turmeric essential oil alone did not show bactericidal effect against the microorganism (L.monocytogenes & S. typhimurium) but when combined with ascorbic acid, it showed significant antibacterial effect [10, 15, 18, 24].

Inversely, the combined essential oil from B.ogadensis and T. schimper had antagonistic relations with a FIC index greater than 2 for almost all tested bacteria and resulted less effective in their combination [19, 24]. This result argues with many studies, this might be due to the

### Table 5

| Combined                  | Gram positive MRSA | multdrug resistant gram negative bacteria |
|----------------------------|--------------------|------------------------------------------|
|                            |                    | E.coli (R) | K. pneumoniae (R) |
| B. cuspidata + B.ogadensis | 0.38^a             | 0.75^a    | 0.25b            |
| B. cuspidata + B.ogadensis | 1.50^d             | 3.00^d    | 0.50^d           |
| B. ogadensis + T.schimper   | 3.00^d             | 3.00^d    | 1.00^4           |

The values represent mean fractional inhibitory concentration index (n = 6), where, ^aX^ represents partial synergy while ^bX^ synergy interaction. ^cX^ represents indifference interaction while ^dX^ represents antagonistic interaction of essential oil.
combination of the essential oils of bactericidal and/or bacteriostatic agents act on the same target of the microorganism and/or chemical interaction between compounds [18]. A lot has to be done to investigate the undiscovered medicine plants and their valuable chemicals that can potentially curb multidrug resistant bacteria. Essential oils obtained from these medicinal plants have significant potential against MDR bacteria. Their cumulative synergistic effects were inhibited the growth of reference and multidrug resistant bacteria. This interaction may be resulted due to the new structure or reaction or different mechanism of action which lead to easy lethal action of all tested bacteria. The plant may possess therapeutic properties or exert beneficial pharmacological effects on mentioned human pathogen. Yet, their phytochemical composition hasn’t been studied. This is reminding us for searching antibacterial compound and/or secondary metabolites from plants to overcome the problem from multidrug resistant bacteria.

Conclusions
Based on the present study combined essential oils were found to have more antibacterial effect than single EO. Even, it is promising anti-bacteria for multi-drug resistant bacteria and the ways to overcome difficulty caused by them. Hence, essential oil contains different bioactive that may be applied to a pharmaceutical composition as modulator or adjuvant or precursor for synthesis new antibiotics in future activities.

Abbreviations
ATCC: American Type Culture Collection used as reference strain for respective bacteria; CLSI: Clinical Laboratory Standard Institute; DMSO: Dimethyl Sulfoxide; EOS: Essential Oils; ESBL: Extended Spectrum Beta Lactamase; FICI: Fractional Inhibitory Concentration Index; I2: Inhibition Zone; MBC: Minimal Bactericidal Concentration; MBC: Minimum Bactericidal Concentration; MDR: Multidrug Resistant; MHA: Muller Hinton Agar; MHb: Muller Hinton Broth; MIC: Minimal Inhibitory Concentration; MRSA: Methicillin Resistant Staphylococcus aureus; TMP: Traditional Medicine Plant

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Availabilty of data and materials
All data and materials of this work are available from the corresponding author on request.

Authors’ contributions
EG designed the study, performed the MIC and MBC, susceptibility testing and analyze the data. GW and KD were guiding and consulting on laboratory work. GT and SH interviewed local healers and farmers. KL, and AT participated in the extraction, susceptibility testing and analysis of data. All authors read and approved the final manuscript.

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Not applicable.

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