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Antibacterial and cytotoxic activities of high altitude essential oils from Nepalese Himalaya

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Essential oils of *Rhododendron anthopogon*, *Artemisia vulgaris*, *Zanthoxylum armatum*, *Acrous calamus*, *Cinnamomum glaucescens*, *Nardostachys grandiflora* and *Abies spectabilis* are studied for antibacterial and cytotoxic activities, and are evaluated with standard antibiotics for the synergistic potentiality against human pathogenic bacteria *Staphylococcus aureus, Escherichia coli and Klebsilla pneumonia*. Antibacterial activity was performed by cup-plate method and cytotoxicity was evaluated by brine shrimp lethality test. Essential oils of *C. glaucescens, R. anthopogon* and *Z. armatum* were found to have higher antibacterial activity against *E. coli, S. aureus* and *K. pneumonia*, respectively. The best synergism of *R. anthopogon* oil and erythromycin was found in 20,000 µg/20 µl. Similarly, synergism of essential oil was done in four different concentration (20000/20, 10000/20, 5000/20, 2500/20, 1000/20, 500/20, 250/20 and 125 µg/20 µl). The best result was shown in 125 µg/20 µl against *E. coli*.

Key words: High altitude medicinal plants, essential oils, human pathogen, bacteria, cytotoxicity.

INTRODUCTION

Among natural antimicrobials, plant essential oils (EOs) have been reported to possess a wide spectrum of antibacterial activity (Ormancey et al., 2001; Gianni et al., 2005; Bajpai et al., 2012). EOs and their components are gaining increasing interest because of their relatively safe status and their exploitation for potential multi-purpose functional use. Monoterpenes, sesquiterpenes, alcohols, ethers, aldehydes, esters and ketones are the main constituents (Bakkali et al., 2008). They have also a long history of use for many medical applications. The increased cases of therapy and application have accelerated the resistance of microorganisms against specific antibiotics towards multiple drugs. Out of the various constituents of the plant extract, essential oil due to its lipophilic nature, small molecular structure and different mode of action is thought to be an important contributing factor for therapeutic effect (Cross et al., 2008). Moreover, the screening of such plant products for antimicrobial activity has alway been of great interest to scientists looking for new sources for drugs, for the treatment of various diseases (Wayland, 2004). Therefore, in the last few years, a variety of medicinal plants and their essential oils have been screened for their antimicrobial activity. In developing countries, a high infectious disease burden commonly co-exists with rapid emergence and spread of microbial resistance, while the prevalence of resistance is rising (Okeke et al., 2005). It was found out that infectious disease is one of the leading causes of premature death, and the situation is made more serious due to the emergence of multi drug resistance among pathogens in Nepal (Malla and Dumre, 2008). Synergy assessment has become a key research area in the struggle to overcome the resistance of different pathogens to modern antibiotics. The maximum benefit of combination therapy can be achieved when the synergism of natural product and the antibiotic combination

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matches (Hemaiswarya et al., 2008). We were interested in investigating the essential oil employing this strategy to overcome these resistance mechanisms by essential oils, individually or by use of combination of drugs. Available data on the biological activity of high altitude essential oils is limited, and this is the reason why they are often the first choice of researchers and pharmaceutical companies as precious ingredients.

Due to species climatic and geographical conditions, temperate and alpine plants of the Himalaya offer greater possibilities of having novel molecules and even largest quantities of the active compounds. Herbal drugs of mountain ecosystems, constituting only those traditional medicines which primarily use medicinal plant preparations for therapy, are in vogue in high altitude regions and offer great therapeutic promise. Therefore, the number of people and institutions seeking information on Himalayan medicinal plant is increasing very rapidly. During last decades, some studies have been carried out on antimicrobial properties of Nepalese medicinal plants to assess their properties (Rajbhandari et al., 2001; Parajuli et al., 2001; Bajrachraya et al., 2008; Innocenti et al., 2010). In continuation of our efforts to verify the efficacy of traditional medicine, we have collected several medicinal plants from various geographical locations of Nepal based on the ethnopharmacological information (Gyawali et al., 2008; Gyawali and Kim, 2009; Gyawali et al., 2010). Thus, all these conditions were taken into account in order to conduct this research, aimed to assess the biological properties of some essential oils from medicinal plants; *Zanthoxylum armatum* DC, *Rhododendron anthopogon* D. Don., *Cinnamomum glaucescens* (Mess) meissn, *Acrous calamus* L, *Artemisia vulgaris* L, *Abies spectabilis* (D. Don) Spach, *Nardostachys jatamansi* (D. Don) Candolle which are abundantly used by local people of high altitude region of Nepal for medicinal purpose.

MATERIALS AND METHODS

Essential oil

The commercially available essential oils *Z. armatum, R. anthopogon, C. glaucescens, A. calamus, A. vulgaris L, A. spectabilis* and *N. jatamansi* were collected from the local trader at Kathmandu, Nepal.

Test organism

Different concentration of essential oil was tested for its antibacterial property against *Staphylococcus aureus, Escherichia coli* and *Klebsilla pneumonia*. These microorganisms were provided by Kathmandu University Teaching Hospital, Dhulikhel, Nepal. The microorganisms were kept under refrigeration (4°C) until use. Experiments were conducted as per the procedure given in literature (Jorgensen et al., 1999; Schwalbe and Steele-Moore, 2007; Maria and Rota, 2008).

Anti-bacterial activity

The cup-plates method was employed for the screening and determination of antimicrobial activity of the essential oil. 0.1 ml of 0.5 McFarland standard of each of the aforementioned species was applied to the Mueller-Hinton agar with a cotton swab. The test organisms were then spread on the surface of the media using a sterile swap stick. Various concentration of essential oil (2000, 1200, 800, 400 µg/ml) were impregnated in 6 mm diameter well each prepared with biopsy punch. Standard antibiotic solution: norfloxacin, 5 µg/ml (against *E. coli*); erythromycin, 30 µg/ml (against *S. aureus*); gentamycin, 30 µg/ml (against *E. pneumonia)* were used as positive controls. Dimethyl sulfoxide (DMSO) and Tween 80 combination was used as negative control. After holding the plates at room temperature for 1 h to allow diffusion of test samples into the agar, they were incubated at 37°C for 24 h. The results were recorded by measuring the zones of growth inhibition around the cup-plates and were presented as the arithmetic average. Overall, cultured microorganisms with halos equal to or greater than 7 mm were considered susceptible to samples tested.

Minimum inhibitory concentration (MIC)

As per the procedure given in literature, agar plates were prepared to contain 500,000 cells/ml microorganism and two fold serial dilution technique was used to prepare different concentration (4000, 2000 and 1000 µg/ml) of essential oil. The negative and positive controls were also performed using 10% DMSO and standard antibiotics. Duplicate wells were run for each concentration of essential oils. The plates were bored with 6 mm diameter borer which was loaded with essential oil. The plates were allowed to set for 2 h at room temperature and then were incubated at 37°C for 24 h. Concentration for which there is no zone of inhibition was noted.

Synergistic test

The synergistic effect is determined by 'L shape' technique as described in literature (http://microblog.me.uk). Agar plates were prepared to contain 2 × 108 CFU microorganisms (as stated by 0.5 McFarland) which were bored in L shape with 6 mm diameter borer. To one side of L antibiotic solution and to the next effective concentration of essential oil was loaded. The test was carried out with two selected essential oils of *R. anthopogon* and *C. glaucescens* with erythromycin estolate (30 µg/ml) against *S. aureus* and *C. glaucescens*, with norfloxacin (5 µg/ml) against *E. coli*. Similarly, synergism of essential oil was done in four different concentration (20000/20, 10000/20, 5000/20, 2500/20, 1000/20, 500/20, 250/20 and 125 µg/20 µl). The best result was shown in 125 µg/20 µl against *E. coli*. Microorganism cultured plates were allowed to set at room temperature for 2 h and then were incubated at 37°C for 24 h. The plates were checked for acute angle and the "L" shape here is important to determine the level of synergy. If the L shape is more like a triangle, then synergy is determined, but if the acute angle is narrow then the antibiotic combination is not synergistic.

Brine shrimp test

As per the procedure stated by Krishnaraju et al. (2005), 0.05 g of dried cyst of *Artemia* was slowly added with stirring in 300 ml of artificial sea water which was then incubated at 37°C. After 12 h of incubation, 10 naupli were concentrated into the pipette and transferred into each test tubes containing 10 ml of different concentration of essential oil (1000, 500, 250, 100 and 10 ppm).

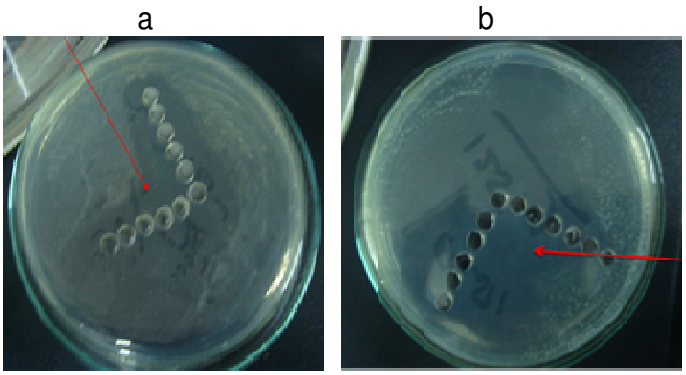

Figure 1. The synergistic effect of (a) *R. anthopogon* oil and erythomycin on *S. aureus* and (b) the synergistic effect of *C. glaucescens* oil and norfloxacin on *E. coli*

Total 10 naupli were transferred into 10 ml of artificial sea water as positive control .The tubes were then incubated at 37°C for 24 h. Percentage of death of naupli was calculated. Test was repeated for three times.

$$\text{Mortality (\%)} = \frac{\text{Number of dead naupli}}{\text{Initial number of naupli}} \times 100\%$$

RESULTS

The antibacterial activities of *Z. armatum*, *R. anthopogon*, *C. glaucescens, A. calamus, A. vulgaris L, A. spectabilis* and *N. jatamansi* essential oils were assayed *in vitro* by a cup plate method against three pathogenic bacteria. Microbial inhibition by essential oil from different plant species at different concentration and result are also evident as shown in Figure 1. According to the result, it was found out that *R. anthopogon, C. glaucescens and Z. armatum* are the most effective against *S. aureus, E. coli and K. pneumoniae*, respectively with MIC value of 125/20, 3.9/20 and 800 µg/20 µl, respectively. In addition, the lower concentration of all oils was found effective against the *E. coli and S. aureus. K. pneumoniae* was found to be strongly resistant even at very higher concentration of treated oil. *Z. armatum* oil was relatively found to be strongly efficient against all treated bacteria.

The brine shrimp lethality assay has been used to assess the toxicity towards brine shrimp, which provides an indication of possible cytotoxic properties of the test materials. According to Figure **3,** the brine shrimp lethality test showed that *R. anthopogon* and *C. glaucescens* has lethal effect to naupli with LC_{50} value of 87.69 and 124.47 ppm, respectively, whereas *Z. armatum* was non toxic with LC_{50} value of 430.6 ppm.

The synergistic test was found less effective, though slight concavity was seen. As demonstrated in Figure 1a, at concentration of 20000 µg/20 µl, best synergistic effect was seen for *R. anthopogon* with erythromycin treated against *S. aureus*, and as shown in Figure 1b, 125 µg/20 µl of *C. glaucescens* showed best effect with norfloxacin (5 µg/20 µl) against *E. coli*.

DISCUSSION

In the present study, most of the essential oil at certain concentration was able to inhibit the growth of bacterial pathogens. This can be due to difference in the chemical constituent of the essential oil, qualitatively and quantitatively. In classifying the antibacterial activity as Gram positive or Gram negative, it would generally be expected that a much greater number would be active against Gram positive than Gram negative bacteria (McCutcheon et al., 1992). Also, as seen, most of the essential oil has greater zone of inhibition to *S. aureus* species (Gram positive) as compared to *E. coli* and *K. pneumonia* (Gram negative). It is in agreement with previous report that Gram positive bacteria are more susceptible to essential oils than Gram negative bacteria (Al-Bayati, 2008). The tolerance of Gram negative bacteria to essential oils has been described to the presence of a hydrophilic outer membrane that blocks the penetration of hydrophobic essential oils into target cell membrane (Al-Bayati, 2008). *R. anthopogon, C. glaucescens* and *Z. armatum* has the effective antimicrobial property against *S. aureus*, *E. coli* and *K. pneumonia*, respectively. *E. coli* was intermediate susceptible to the essential oil of *C. glaucescens* and the maximum zone of inhibition of 17 mm (Figure 2). the Present finding is also in agreement with previous report on *R. anthopogon* oil (Innocenti et al., 2010). These tested plants can be used as the potential source, as a new antimicrobial agent. The mode of action of antimicrobial agents depended on the type of microorganism, and evidence indicates that in the case of essential oils, it may be associated with cell membrane damage. Their chemical constituents are characteristically hydrophobic and will accumulate in the lipid-rich environments of cell membrane structures and cause structural and functional damage (Cross et al., 2008; Lambert et al., 2001).

This finding on calamus oil is in contrast with the literature report on calamus oil with same pathogens (Bajrachraya et al., 2008; such discrepancy in the results may be due to the type of sample taken and geographical origin. From the brine shrimp lethality test, *R anthopogon and C. glaucescens* have LC_{50} value less than 250 ppm which indicates the possibility of these oils to show positive result towards the cell line assay for cytotoxicity (Bhattari et al., 2010). Limonene present in *R. anthopogon* could exhibit inhibitory effect in the present study, since it is known for its strong effect against tumor cell line (Innocenti et al., 2010).

L-shaped synergistic test showed the effect of *C. glaucescens* on *E. coli* at 125 µg/20 µl and *R. anthopogon* on *S. aureus* at 20,000 µg/20 µl, but not being too remarkable. Regardless of the United States National Cancer Institute (NCI) recommendation that a plant extract should be considered as active if it inhibits less

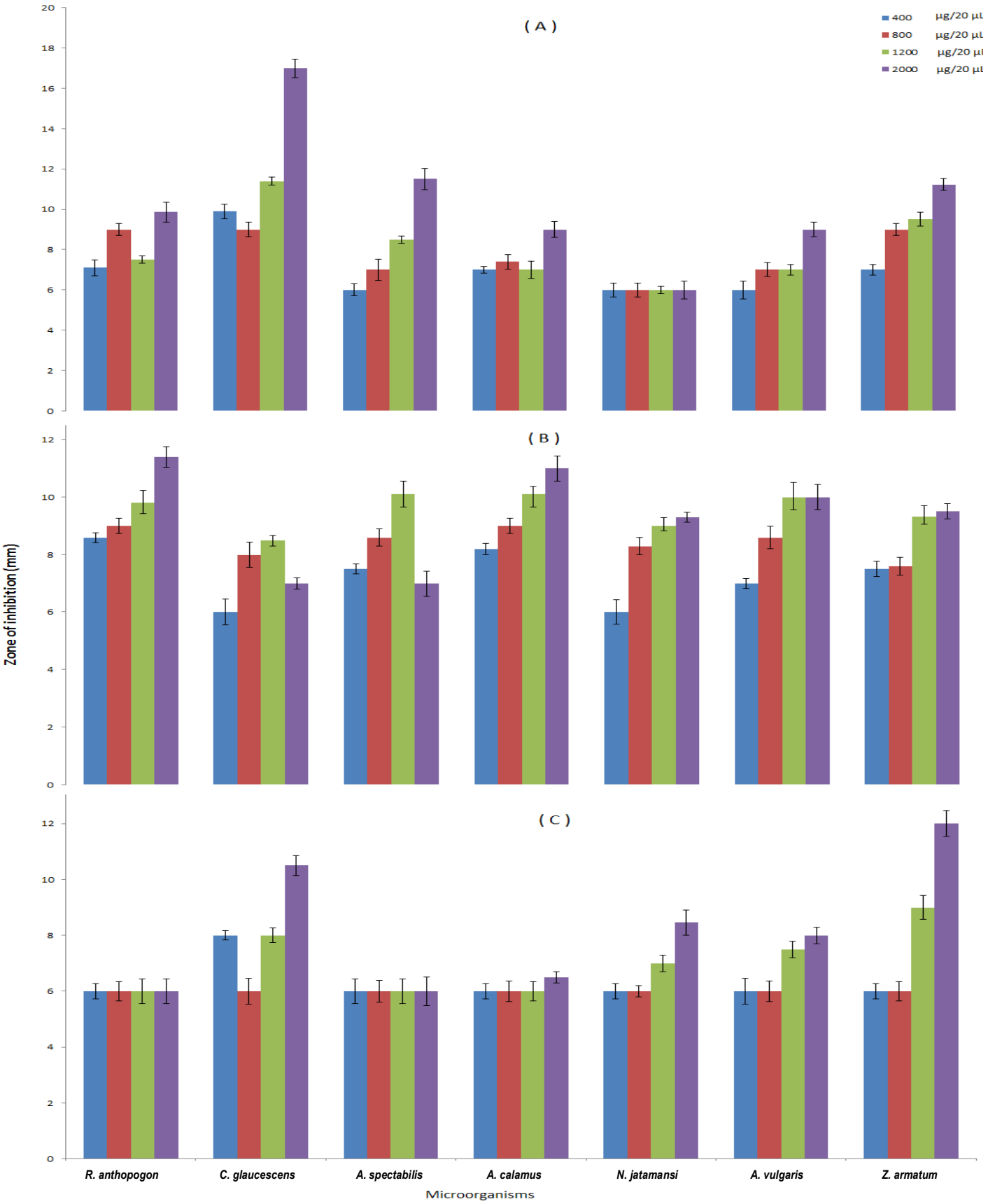

Figure 2. Zone of inhibition (mm) exhibited by essential oils from Nepalese Himalaya against human pathogenic bacteria.

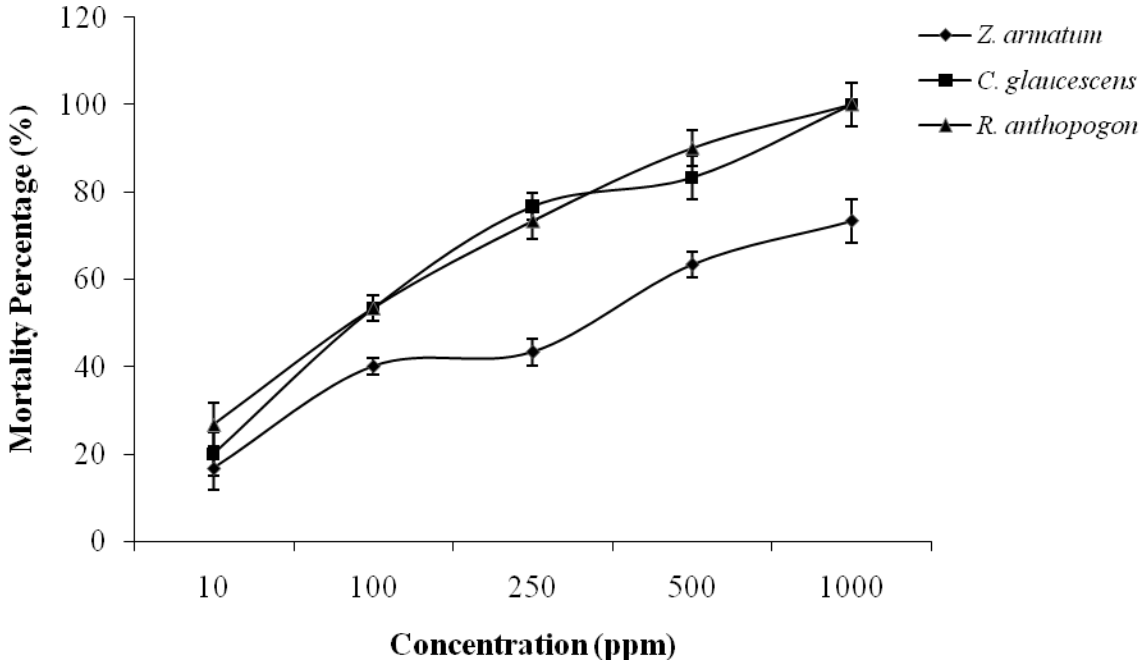

Figure 3. Cytotoxic effect of essential oils from Nepalese Himalaya.

inhibits less than 50% at 50 µg/ml concentration. In this study, the value of 52.53% was considered as "borderline" activity, and LC_{50} was determined for the ethanol extract o*f Laurus nobilis* (Boik, 2001). The concavity of the curve was not so remarkable and the synergism may not be actually effective. Hence, it is necessary that either the test be conducted for other groups of antibiotic on same essential oil or different essential oil for same microorganism. These synergy effects, understood not only as additive effects but also as a true synergism, may not only cause a better effect with lower dosage of the single component, but also lead to a reduction of adverse reactions. It could be therefore expected that at correctly chosen combination of natural products with antibiotics, the potential of side effects of the synthetic antibiotic can be reduced simultaneously (Ulrich-Merzenich et al., 2010). The present finding support the traditional knowledge of local users and it is a preliminary scientific validation for the use of these essential oils for antibacterial activity against human pathogenic bacteria. To promote proper use of such resources, awareness of local communities should be enhanced incorporating the traditional knowledge with scientific findings.

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