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Assessment of the bio-film inhibition, thrombolytic and cytotoxicity potential of the essential oil from *Zanthuxylum alatum* and *Mentha longifolia*

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Abstract

Zanthoxylum alatum is a popular folk remedy against toothache, colic, and rheumatism whereas *Mentha longifolia* is practiced as an anthelmintic and bactericidal at Rawalakot, Kashmir. Proceeding with the traditional knowledge, the essential oils from *Z. alatum* (leaves and seed) and *M. longifolia* (leaves and roots), were subjected to biofilm inhibition, thrombolytic and cytotoxicity potential. The *Z. alatum* leaves oil (XALO) showed significant potential of *E. coli* film inhibition (69.91±0.5 (%) (Rifampicin: 95.50±0.13); and *M. longifolia* roots oil (MLRO) showed good potential of clot lysis: 32.38±0.66 (streptokinase 89.48±0.55 and distilled water 2.92±0.18 %). Compared to the reference standard: Trition-X 100 (97.21±1.0 %), all the test samples exhibited mild to minimal toxicity (XALO, MLRO, XASO and MLLO: 2.71±0.37, 6.37±1.2 %, 3.32±1.0, 4.74±0.37 % respectively). The selected samples with antibacterial and thrombolytic potential without any potential toxicity will determine the precise quality and safety of the plant to be used by clinicians.

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Introduction

Zanthoxylum alatum, locally known as Timbber, is wildly distributed (altitude: 800-1500 meters) in the foothills of Rawalakot, Kashmir-Pakistan (Majid *et al.,* 2004). The *Zanthoxylum* (Rutaceae) is a big genus with over 200 species distributed worldwide, however, in Asia, most species of the genus are found in Himalayan region (Waterman*, et al.,* 1975). Various species of *Zanthoxylum* is widely used in traditional medicines as tonic, diaphoretic, anthelmintic, antirheumatic, stimulant, and hepatic in China, Pakistan, India and Vietnam.

The seed (powder) of the plant has spice value and are used as a tonic for curing fever, expelling roundworms and treating dyspepsia and cholera etc. (Rout *et al*., 2008). Whereas the branches, thorns and aerial parts are used as stomachic and carminative. The bark is used as circulatory stimulant, diaphoretic, anti-rheumatic, and hepatic (Halliwell & Gutteridge, 1984). In Pakistan, the fruit (dry) of *Z. alatum* is mixed with *Mentha avensis*, *Carumcapticum* and table salt (small quantity) for treating headache and dyspepsia (Majid *et al*., 2004). In Ayurvedic practice in India, the fruit and bark of *Z. alatum* is used as a remedy against bacterial and fungal skin infections, and as carminative, anthelmintic anorexia, and ataxia (Chaudiere& Ferrari, 1999). In Vietnam, the term "Sichuan Peper" is used for spice obtained from a group of closely related plants of Genus Zanthoxylum. America and Africa Zanthoxylum species have not yet been put to culinary use. In many nation bark, carpels (orule bearing leaf of pistil on a flower), carpels of fruit, seeds, essential oil of this species are being used for remedy. The phytochemical analysis of *Z. alatum* fruit constitute 33% monoterpene hydrocarbons out of which 1,8-cineole (15.7%), linalool (18.8%) and undecan-2-one (17.0%) are main constitutes (Weyerstahl *et al*., 1999).

A study carried out at Vietnam for characterizing the chemical composition of *Z. alatum* Roxb leaf yielded 0.52% oil in which 50 compounds were identified. Out of 50 compounds, the major components characterized were 1,8-cineole (41.0%), sabinene (8.4%), terpinen-4-ol (5.2%), linalool (4.5%), α-terpineol (4.1%), β-terpineol (2.1%), 2-tridecanone (1.8%), 2,6-dimethyl-1,3,5,7-octatetraene (1.5%), and β-cymene (1.3%). (Luong *et al*., 2003). The oil obtained from seed and pericarp of fruit of *Z. alatum* consist of linalool a major source. Per Duby and Purohit (1970) analysis of the seed oil, it was revealed that it consists of linalool (34%) as the main components while per Ramidi *et al* (2014) analysis, it consists of .4% of sesquiterpenes and 96.5% of monoterpenes out of which the major components were linalool (72%), (E)- methyl cinnamate (12.2%), limonene (6.2%), *β*-phellandrene (5.3%).

Mentha longifolia

Mentha longifolia (mints) with strong aroma, belongs to the family Labiatae (Lamiaceae), are widely distributed in Asia (especially Pakistan), Eurasia, Australia, and Africa (Gulluce *et al*., 2007; Lange and Croteau, 1999). *Mentha longifolia* (L.) Huds., has been commonly used as a kitchen and medicinal plant for centuries. Known as wild mint and horse mint, the plant can reach to 1.5 m high in favorable conditions.

Various other species of *Mentha* have been used as folk remedies for treatment of bronchitis, flatulence, anorexia, ulcerative colitis and liver complaints, due to their anti-inflammatory, carminative, antiemetic, diaphoretic, antispasmodic, analgesic, stimulant, emmenagogue, and anticatharral activities (Sharopov *et al*., 2012). The active virtues of the mints depend on the abundant volatile oils that contain a wide variety of terpenes and terpenoids.

The GC-MSanalysis of *Mentha longifolia* essential oil (84.5-99.0% oil) in Tajikistan resulted in identification of 82 compounds, the major components identified were cis-piperitone epoxide (7.8-77.6%), piperitenone oxide (1.5-49.1%), pulegone (0.3-5.4%), β-thujone (0.2-3.2%), thymol (1.5-4.2%), carvone (0.0-21.5%), menthone (0.016.6%), carvacrol (0.0-2.7%), and (*E*)-caryophyllene (0.92.5%). (Sharopov *et al*., 2012). Based on the traditional

knowledge of usage of *Z. alatum* (leaves and seed) and *M. longifolia* (leaves and roots), essential oil usage against bacteria film inhibition, the four oil samples (XALO, MLRO, XASO and MLLO). Different matrices can be used to incorporate antimicrobial agents in food packaging films like organic acid, enzymes, bacteriocins, polysaccharides and essential oils. Recent foodborne microbial outbreaks are the driving force in the search for innovative ways to inhibit microbial growth in food while maintaining its quality and safety. A new trend in food preservation consists of the incorporation of antimicrobial films on food surfaces. In addition, the topical bacterial infection can be easily treated with essential oil which is more economical and easily obtained from natural sources. Many studies have demonstrated that antimicrobial films and coatings are effective in reducing levels of pathogenic organisms like *E. coli* O157:H7. [Rocha *et al.*, 2013].

The essential oil (natural products) potential as a fibrinolytic drug is very important due to least possible toxicities and here we have found that the four oil samples of essential oil: XALO, MLRO, XASO and MLLO tested for thrombolytic activity on human blood sample has practically showed negligible toxicities. Streptokinase is used as a standard fibrinolytic drug and used a standard in this study. Thrombolytic therapy play an important role in reducing mortality rate and preserving left ventricular function in patients with myocardial infarction. A thrombolytic agents function by activating the enzyme plasminogen that dissolve the cross-linked fibrin mesh. We have found that the essential oil of *Z. alatum* and *M. longifolia* could be incorporated as a thrombolytic agent for the improvement of patients suffering from Atherothrombotic diseases [Prasad *et al.*, 2006].

Material and methods

Plant Collection and Identification

The fresh plant material of the *Zanthuxylum alatum* and *Mentha longifolia* was collected in October 2015 from District Poonch, Tehsil Abbaspur, Kashmir, Pakistan. The plant was identified and authenticated by the plant taxonomist at the campus of sciences faculty, University of Poonch, Rawalakot, Kashmir, Pakistan.

Essential isolation through hydro distillation method

Cedrusdeodara stem and leaves were cut into small pieces. Then these small pieces are air dried in a shady place. These pieces are then immersed in water to carry out hydro distillation (3-5 hours). Clevenger type hydro distillation apparatus was used recommended in British Pharmacopeia (1988). Along with the water vapors essential oils were evaporated and condensed in condenser. From aqueous layer the distillate was separated and over anhydrous Sodium Sulfate it was dried (Irshad *et al.*, 2012).

Antibacterial Activity by Biofilm Inhibition Assay

The biofilm formation was accomplished by method reported by of Shahid *et al.* (2015) and Anjum *et al.* (2014). The wells of a sterile 96-well flat bottomed plastic tissue culture plate were filled with 100 micro litre of nutrient broth (Oxoid, UK), 100 µL testing sample and twenty micro litre of bacterial suspension inoculated. Negative control wells contained nutrient broth only. The plates were covered and incubated aerobically for twenty-four hours at thirty-seven degree centigrade.

The content of each well was washed three times with two twenty Micro letter of sterile phosphate buffer. The plates were vigorously shaken to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 220 µL of 99 percent methanol per well, and after 15 min plates were emptied and left to dry. Then, plates were stained for 5 min with 220 mL of 50 percent crystal violet per well. Excess stain was rinsed off by placing the plate under running tap water. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 220 µL of 33 percent (v/v) glacial acetic acid per well.

The OD of each well was measured at 630 nm using micro plate reader (BioTek, USA). All the tests were carried thrice against both selected bacterial strain and the results were averaged. The bacterial growth

inhibition (INH percent) was calculated as follows:

INH percent = 100 – (OD630 sample*100)/ OD630 control.

Thrombolytic Activity

Five different pre-weighed sterile micro centrifuge tube were used to take 5 mL of venous blood from each volunteer and permitted to incubate at 37℃ for 40 min. From each centrifuge tube, the fluid was completely released after clot formation and the clot weight was determined by subtracting weight of clot containing tube from weight of tube alone. To the centrifuge tubes 100 uL of each sample were added separately, streptokinase and distilled water (100 uL) were taken as positive and negative control respectively. For almost an hour at room temperature these test tubes were incubated and observed for clot lysis. The released fluid was discarded after incubation and tubes were again weighed to assess the differences in weight after clot disruption (Prasad *et al.*, 2007). By following formula, the percentage of clot lysis was determined.

Percent of Clot Lysis = (wt of released clot /clot wt) × 100.

Cytotoxicity by Hemolytic Activity

Hemolytic activity of the compound was studied by the method used by Hussain *et al.*, (2015) and Zuber *et al.* (2014). Three mL freshly obtained heparinized bovine blood was collected from Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan. Blood was centrifuged for five min at thousand xg plasma was discarded and cells were washed three times with 5 mL of chilled (4℃) sterile isotonic Phosphate-buffered saline (PBS) pH 7.4. Erythrocytes were maintained 108 cells per mL for each assay. A 100 µL of each compound was mixed with human (108 cells/mL) separately. Samples were incubated for 35 min at 37℃ and agitated after 10 min. immediately after incubation the samples were placed on ice for 5 min then centrifuged for 5 min at 1000xg. Supernatant 100 µL was taken from each tube and diluted 10 time with chilled (4℃) PBS. Triton X-100 (0.1 percent v/v) was taken as positive control and phosphate buffer saline (PBS) was taken as negative control and pass through the same process.

The absorbance was noted at 576 nm using Quant (Bioteck, USA). The percent RBCs lysis for each sample was calculated using the following formula:

$$\% \, Lysis = \frac{OD_{540} test - OD_{540}(Blank)}{OD_{540}(total \; lysis - OD_{540}(Blank)} \times 100$$

Results

Biofilm Inhibition against E. coli

The result obtained from biofilm inhibition assay against gram negative *E. coli* strains performed with essential oil of *Zanthoxylum alatum* (leaves and seed) and *Mentha longifolia* (leaves and roots) are given Table 1.

Table 1. Biofilm Inhibition of *E. coli* by essential oil from *Z. alatum* and *M. Langifolum*

S #	Sample Name	(%) Biofilm Inhibition of *E.coli*
1	XALO	69.91±0.5
2	XASO	23.87± 0.66
3	MLLO	14.86±0.17
4	MLRO	41.83±0.25
5	Rifampicin	95.50±0.13

Rifampicin as reference standard showed highest inhibition in killing *E.coli* strains (95.50±0.13) which was also microscopically visualized Figure c. Among all test samples, the essential oil of leaves of *Z. alatum* (XALO) showed good potential (69.91±0.5 (%) of biofilm inhibition against *E.coli*, as shown in figure x. On the other hand, essential oil of roots of *M. longifolia* (MLRO) also showed 41.83±0.25 biofilm

Inhibition while *Z. alatum* seed oil (XASO) and *M. longifolia* leaves oil (MLLO) showed mild inhibition 23.87± 0.66 and 14.86±0.17, respectively as compare to the standard: Rifampicin.

Thrombolytic Activity

As traditionally *M. longifolia* is used for blood thinning application, therefore, a prompt and swift method was used for assessing thrombolytic activity in which streptokinase and water were used as positive and negative control, respectively.

The data of *in vitro* thrombolytic activity of the four essential oil samples of *Zanthoxylum alatum* and *Mentha longifolia* essential oil are given in Table 2.

Table 2. Assessment of Thrombolytic potential of essential oil from *Z. alatum* and *M. Langifolum* (%).

S#	Sample Name	Thrombolytic activity (%)
1	XALO	19.84±0.66
2	XASO	5.89±0.85
3	MLLO	12.27±1.01
4	MLRO	32.38±0.66
5	Streptokinase	89.48±0.55
6	Distilled Water	2.92±0.18

Among the four samples, MLRO of *M. longifolia* and XALO of *Z. alatum* exhibited highest clot lysis potential 41.83±0.25 and 19.84±0.66 respectively. However, the other two samples MLLO and XASO showed insignificant potential of clot lysis 12.27±1.0 and 15.89±0.85, respectively, as compared to standard streptokinase (89.48±0.55), and distilled showed 2.92±0.18 % clot lysis, taken as a negative control.

Cytotoxicity

The data obtained from the cytotoxicity against heparinized bovine blood of the four samples of essential oil of *Z. alatum* and *M. longifolia* are given in Table 3. Trition-X 100 was taken as positive control and phosphate buffer saline (PBS) was taken as negative control. The results showed that all the test samples are in-toxic (XALO, MLRO, XASO and MLLO: 2.71±0.37, 6.37±1.2 %, 3.32±1.0, 4.74±0.37 % respectively), as compare to the reference standard, Trition-X 100 (97.21±1.0).

Table 3. Cytotoxicity (%) by Hemolytic activity.

S#	Sample Name	Cytotoxicity (%) by Hemolytic activity)
1	XALO	2.71±0.37
2	XASO	3.32±1.0
3	MLLO	4.74±0.37
4	MLRO	6.37±1.2
5	Trition-X 100	97.21±1.0
6	PBS	2.5±0.5

Discussion

There is foremost significance to preserve the productiveness of antimicrobial therapy as there is an increase antibiotics resistance due to multidrug resistance bugs development. Most of the antibiotics currently practiced are toxic to some extent to human being and especially when the exposure duration is high. The promising for growing natural product based anti-microbial drug development seems to be worthwhile as it will escort to the blooming of plant

oriented medicines to act against microorganisms; therefore, to attain new horizons, and foundation for new drugs (Evans *et al*., 2002).

Similarly, several researchers have discovered various plants and natural food sources and their supplements having antithrombotic (anticoagulant and antiplatelet) effect and it has been observed that usage of such natural food leads to prevention of coronary events and stroke (Ratnasooriya *et al*., 2008; Joshipura *et al*., 1999; Liu *et al*., 2000; Bazzano *et al*., 2002). Several thrombolytic agents in including obtained through recombinant DNA technology, however, the side effects related (toxicities) to some of these drugs that lead to further difficulties have been reported (Baruah *et al*., 2006; Gallus *et al*., 1998; Wardlaw *et al*,. 2004; Capstick *et al*,. 2005).

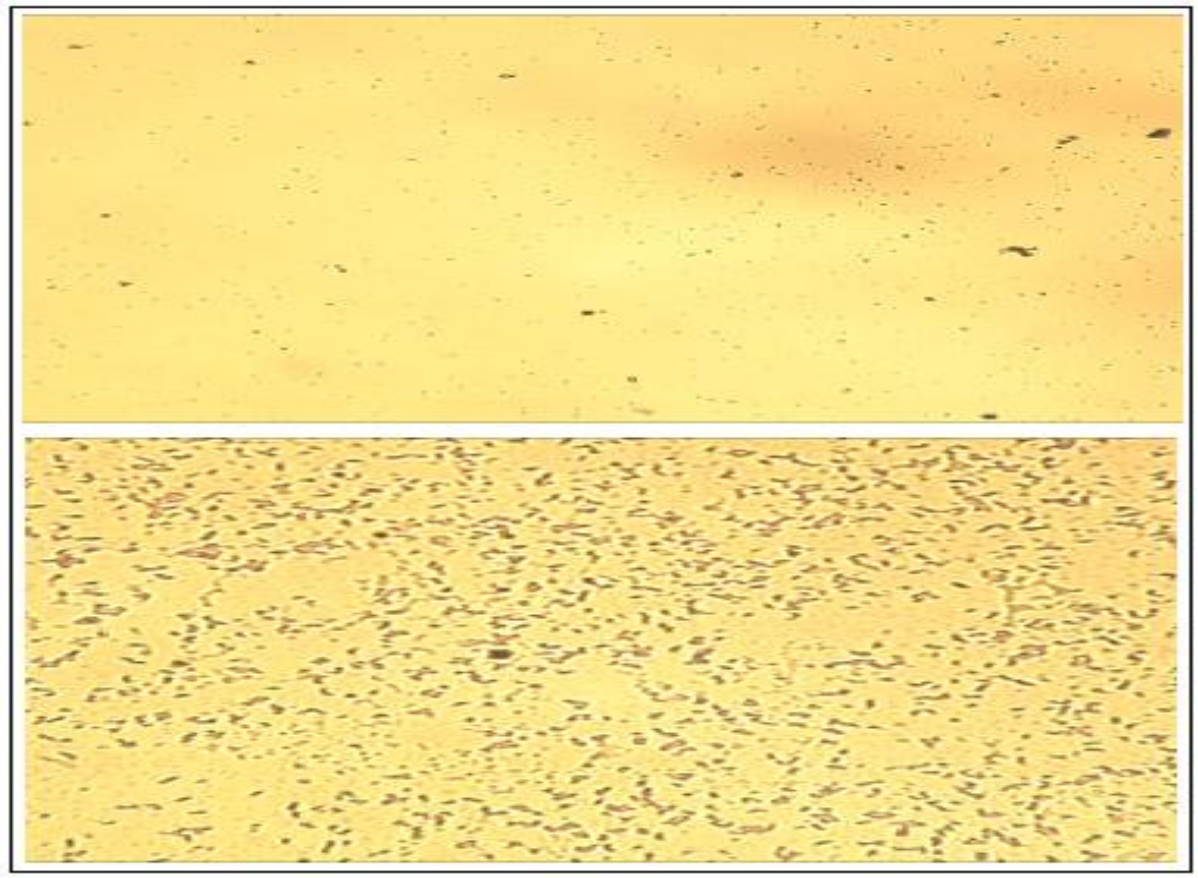

Fig. 1. Negative Control Nutrient broth only.

Platelets regulates the development process of atherothrombosis as well as counteract the peripheral areas of endothelial (produced by reactive oxygen species). This stimulation causes platelets to platelets binding, as well as to leucocytes carrying them into an intricate process of plaque development and progression (Prentice *et al*., 1999). Plasmin, a natural fibrinolytic agent, lyses clot by breaking down the fibrinogen and fibrin contained in a clot. Streptokinase forms a 1:1 stoichiometric complex with plasminogen that can convert additional plasminogen to plasmin (Banerjee *et al*., 2004). Moreover, phlorotannin, isolated from marine brown algae, have a unique property in promotion of dissolution of intravascular blood clot via antiplasmin inhibition (Prasad *et al*., 2007). Several studies reveal that *A. bilimbi,C. viscosum* and *D. quercifolia* possesses tannin, alkaloid saponin (Hasanuzzaman *et al*., 2013; Runa *et al*., 2013; De *et al*., 2013) which could be participated for its clot lysis activity (Ali *et al*., 2013). Keeping in view the traditional knowledge of usage of the essential oils of *Z. alatum* (leaves and seed) and *M. longifolia* (leaves and roots), these were subjected to biofilm inhibition, thrombolytic and cytotoxicity

potential. The *Z. alatum* leaves oil (XALO) showed significant potential of *E. coli* film inhibition (69.91±0.5 (%) (Rifampicin: 95.50±0.13) while the other three samples: XASO (23.87± 0.66), MLLO (14.86±0.17), MLRO (41.83±0.25) did not show good activity. The *M. longifolia* roots oil (MLRO:32.38±0.66) showed good potential of clot lysis,(streptokinase 89.48±0.55 and distilled water 2.92±0.18 %) while the rest of the three samples: XALO (19.84±0.66), XASO (5.89±0.85), MLLO (12.27±1.01), showed insignificant potential of thrombolysis. Compared to the reference standard: Trition-X 100 (97.21±1.0 %), all the test samples exhibited mild to minimal toxicity (XALO, MLRO, XASO and MLLO: 2.71±0.37, 6.37±1.2 %, 3.32±1.0, 4.74±0.37 % respectively).

The selected samples with antibacterial (XALO: 69.91±0.5) and thrombolytic (MLRO: 32.38±0.66) potential without any potential toxicity will explain the specific quality and safety of the plant to be used by clinicians against certain ailments.

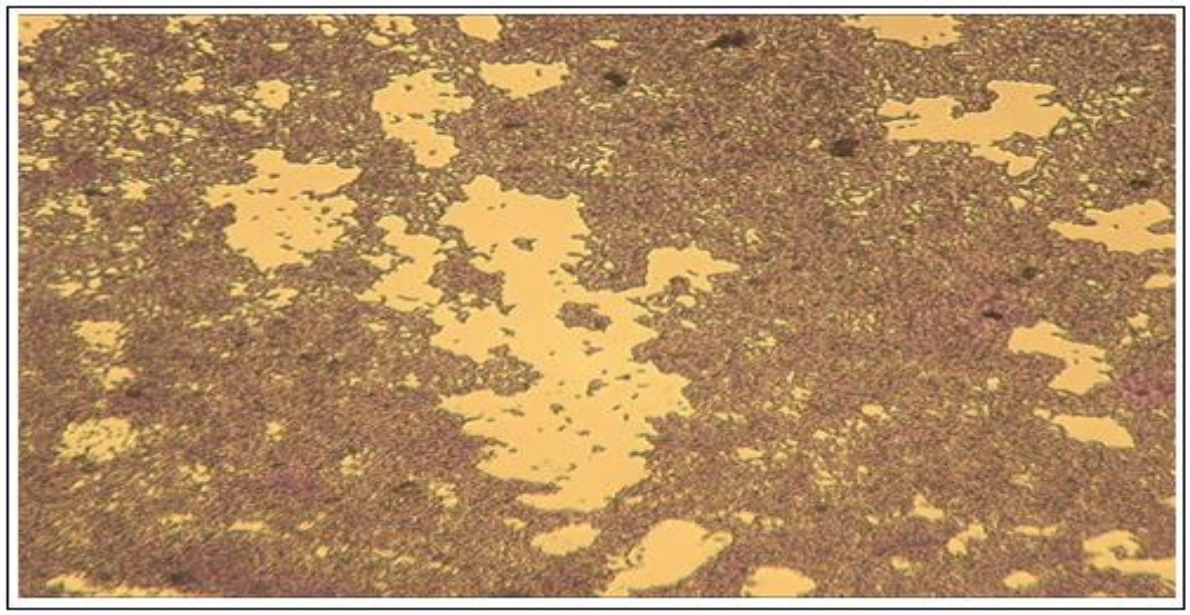

Fig. 2. Positive Control Rifampicin in biofilm inhibition assay.

Streptokinase (SK), is used as a standard thrombolytic drug and here it is used as a positive control (Prasad *et al.,* 2007) while water was used as a negative control.

The comparison of positive control with negative control clearly confirmed that clot dissolution does not occur when water was added to the clot. By comparing with this positive & negative control, a significant thrombolytic activity was observed after treating the clots with *M. longifolia* roots oil (MLRO:32.38±0.66).

Conclusion

In conclusion from our recorded data, it can be demonstrated that our findings will help the status of cardiovascular health. In addition, this finding will probably indicate the possibility of developing novel thrombolytic compounds from the *M. longifolia* roots oil (MLRO:32.38±0.66). Further studies will be focused to isolate and characterize the compounds responsible for thrombolytic activity. Similarly, the *Z. alatum* leaves oil (XALO) showed significant potential of *E. coli* film inhibition (XALO: 69.91±0.5 (%) (Rifampicin: 95.50±0.13) which will further be explored for active constituent.

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Conflict of interest

The authors declare no conflict of interest with this study.

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