Original Article

# Chemical Composition, *in Vitro* Antibacterial and Cytotoxicity Effect of *Nectaroscordum tripedale* Extract

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## Abstract

**Background and Aim:** The present study investigated the antimicrobial, and cytotoxic activities of the methanol extract of *Nectaroscordum tripedale* leaves.

**Materials and Methods:** Methanolic extract of *Nectaroscordum tripedale* was investigated for its phytochemical components, antimicrobial activity and cytotoxicity. The antibacterial potentialities of methanol extract of *Nectaroscordum tripedale* leaves were investigated by the disc diffusion and broth dilution method against five bacterial isolates including three food-borne pathogens (*Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli*) and two healthcare-associated pathogens (*Methicillin resistance Staphylococcus and Pseudomonas aeruginosa*) and cytotoxicity activity were evaluated on acute myeloid leukemia cell line (KG-1a) and normal lymphocyte cells and the effect of *Nectaroscordum tripedale* extract and methotrexate in 0.01, 0.1, 1, and 2 mg/ml concentrations on these cells were compared.

**Results:** The extract was found to contain 27 chemical compounds. The maximum zone of inhibition was observed in Methicillin Resistant *Staphylococcus aureus* (MRSA). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Nectaroscordum tripedale* for *E. coli* and MSRA were similar (166 and 322 µg/ml, respectively). While the high level of MIC and MBC is related to *Pseudomonas aeruginosa.* The percentage of lysis for extract and methotrexate on the KG-1a was seen after 24 hours.

**Conclusions:** In comparison with methotrexate, *Nectaroscordum tripedale* had minimal effect on normal cells. The present study revealed that the methanol extract of *Nectaroscordum tripedale* leaves has significant antibacterial activities along with moderate cytotoxicity's on the cancer cells that may lead to new drug development.

**Keywords:** *Nectaroscordum tripedale*, Antibacterial activity, Cytotoxicity.

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## Introduction

*Nectaroscordum tripedale*,belonging to the *Allium* genus, known as "Piaze tabestaneh" and "Aneshk" in Iran. This plant is found in Iran, Iraq, Turkey, North Caucasus and Transcaucasus (1).

Throughout the world, including Iran, the use of herbal medicines is being promoted as safe and sustainable alternative medicines for pharmaceutical care. In the west of Iran (Lorestan province), the traditional healers use the *Nectaroscordum tripedale* for rheumatic and joint pains very commonly (2). Fresh or dried leaves are also used in dietary habits in this region as a spice. Leaves have a very strong and somewhat unpleasant taste and are widely used by the local population as a spicy vegetable (3). The traditional healers also treat the bladder and kidney stones with this plant. Other uses of this plant include as a laxative, expectorant diuretic, parasite repellent, appetizer, stimulant, tonic, muscle, joint pain reliever, and sedative (4).

In recent years, multidrug resistance in human pathogenic microorganisms has been developed due to indiscriminate prescription and malpractice of commercially available antimicrobial drugs, which are mainly used in the treatment of infectious diseases. This situation forced scientist to search for new antimicrobial agents from various sources like medicinal plants that are good sources of novel antimicrobial agents (5).

In many areas, where cancer is common, plants consumption, such as spices and their constituents as potential chemo preventive agents remains as an extensive research topic. Numerous studies have been published in regards to the relation between plants consumption, cancer prevention, antimicrobial effects, and overall protection of human health (6). In Lorestan province, medicinal plant consumption is an integral part of dietary behavior, but relatively little is known about their antimicrobial potential and anti-cancer effects. Although various studies have been conducted to investigate the antibacterial and bioactivity of medicinal herbs, but upon literature review it was found that no research work has been performed on *Nectaroscordum tripedale.* Therefore, in order to provide a scientific basis to their traditional uses, the antibacterial effect was tested on two bacterial pathogens, which was made on some food-borne pathogens. As well as cytotoxic effects of *Nectaroscordum tripedale* on normal cells and leukemic cells (KG-1a) was done and the results compared with the effect of methotrexate as a conventional anti-cancer drug. In addition, a chemical composition of this plant is also described.

## Materials and Methods

**Collection and identifying the plant**

Aerial parts of wild growing *Nectaroscordum tripedale* were collected in May 2012 from Khorramabad mountains (Lorestan Province, Iran) and was air dried in a shadow place. This plant was identified in Herbarium of Agriculture and Natural Resource Research Center, Khorramabad, Iran.

**Extract preparation**

The samples were ground by commercial grinder into fine powder and macerated with ethanol for 24h with occasional shaking and stirring. The extracts were passed through filter paper (Whatman No.3, Sigma, Germany) to remove plant debris and then concentrated in vacuum at 50°C using a rotary evaporator (Heidolph, Germany). The leaves yielded 21.92% extract of dried plant material.

**Chemical analysis**

This part of investigation was carried out by Solid-phase micro extraction (SPME) technique. The samples were first ground into a fine powder. Two grams of the sample was weighed and transferred to a 20ml tube. Then the vial containing the sample was transferred to the ultrasonic device to extract the volatile substances. The temperature of the ultrasonic device was set on 50° C for 15 min. Then, the SPME fiber was placed on the upper space of the sample for 40 min to extract the volatiles. Immediately after the extraction, the SPME fiber was injected into the GC-Mass device for desorbing and identifying the sample. Desorbing was performed in the GC column for 2 min. The SPME fiber holder for manual use and the 100µm Polydimethylsiloxane (PDMS) fibers were obtained from Supelco (Bellefonte, PA, USA) (7).

Isolation and measurement of the sample were done by GC/MS (Shimadzu 17A gas chromatograph and

QP5050A mass spectrometer, USA) and isolation of composition was done in Fused Silica type BP-5 95% polydimethylsiloxane with length of 30m, internal dimensions of 25% mm and film thickness of 25% micrometer. Column temperature increased from 40 to 220°C with speed of 5°C per minute, then the temperature increased to 280°C and kept at 280°C for 2min. Both the injection site and detector (transfer line) temperature was set to 260°C. Helium gas with speed of 0.9 ml/minute with 99/999% purity was used as carrier gas. Spectrometer conditions were exactly in accordance with gas chromatography, just ionization energy of 70 electron volts was used. Also for identification of spectrum with the retention indices, the injection of normal hydrocarbons (C8-C20) under the conditions of sample injection was used.

**Bacterial strains**

The standard bacteria based on the American Tissue Culture Collection (ATCC) were prepared from Microbiology Lab., Reference Laboratories of Iran Research Center and stored in the freezer (-70°C). The species used were *Staphylococcus aureus* (ATCC 25923), Methicillin resistance *Staphylococcus* (MSRA) (ATCC H041940150), *Pseudomonas aeruginosa* (ATCC 27853), *Listeria monocytogenes* (ATCC 35152) and *Escherichia coli* (ATCC 25922).

**Antibacterial screening**

**Disk diffusion assay**

Each bacterial suspension of equivalent to No 0.5 McFarland was prepared in Mueller-Hinton broth, diluted by 1:10, and 0.1 ml of this dilution was streaked on a solid Mueller-Hinton agar medium. A 6 mm diameter of sterilized Wattman filter paper No 1 (Rund filter, Macherey-Nagel, D-5160 Doren Germany, and Werkstrabe 6-8) was soaked in 0.5 ml *Nectaroscordum tripedale* extract, placed on the above-mentioned Mueller-Hinton agar and incubated at 37°C for 48h. The diameter of the zone of inhibition around the disk was measured in mm and recorded. The test was repeated three times and the mean diameter of the zone of inhibition was determined. Filter papers soaked in chlorhexidine and saline were used as positive and negative control, respectively.

**Determination of minimum inhibitory concentration (MIC)**

The MIC of the *Nectaroscordum tripedale* was determined by the broth dilution method. Briefly, each bacterium was grown to stationary phase in tryptic soy broth. Each cell suspension was adjusted spectrophotometrically to approximately $10^4$ CFU/ml. *Nectaroscordum tripedale* extract concentration ranged from 50 to 2 mg/ml, and 25ml of the bacterial cell suspension was added to the serially diluted *Nectaroscordum tripedale* extract. Uninoculated tubes containing growth medium or growth medium and extract were used as controls. All incubations were at 37°C for 24h. The MIC was defined as the lowest concentration of *Nectaroscordum tripedale* extract that completely inhibits the growth of the organisms.

**Determination of minimum bactericidal concentration (MBC)**

Samples from clear wells in MIC test were cultured on nutrient agar (Merck, Germany) to determine the MBC. The lowest concentration of the MIC wells that did not grow on the nutrient agar plate was taken as the MBC.

**Antibiotic sensitivity test by agar diffusion technique**

The antibiotic sensitivity profile of the 5 isolates were determined according to the method of Bauer-Kirby (8) using disks of antibiotics placed on the surface of Muller Hinton agar medium seeded with the test organism. Inhibition zones were measured after 24 h of incubation at 37°C. Interpretation of resistance was based on the National Committee for Clinical Laboratory Standards (NCCLS) criteria.

**Determination of cytotoxicity effect**

Four concentrations 2, 1, 0.1, and 0.01mg/ml were prepared in an incomplete culture medium without Fetal Calf serum (FCS).

**Cell line culture**

Acute myeloid leukemia cell line (KG-1a) was prepared from the cell bank of Pasteur Institute in Iran and were used only for this study. The cells were incubated in a 50-ml flask containing 20ml of the RPMI culture medium with 10% FCS (Sigma Corp., Germany) at 37°C with 5% of CO2.

**Human lymphocytes isolation**

The cells were isolated based on the method of separating human mononuclear cells (lymphocytes) as

follows:

10ml of whole blood was taken from a healthy individual using a heparinated syringe in compliance with ethics guidelines. The blood was then diluted in a ratio of 1:2 via the incomplete culture medium. 10ml of the diluted blood was slowly added to a tube containing 5cc of ficoll. The tubes were centrifuged for 30min at 1500 RPM. The cells were removed from the white layer between the two phases using the Pasteur pipette and were washed twice via the incomplete culture medium (9).

**MTT-based cytotoxicity assay**

100 µl of *Nectaroscordum tripedale* extracts in four concentrations of 2, 1, 0.1, and 0.01 mg/ml were used. All the tests were repeated three independent times. A number of $10^4$ of the KG-1a cells and human lymphocytes (100µl) was added to flat-bottom plates. After time courses of 4, 12, 24, and 48 hours, 20µl of the MTT solvent (5mg/ml) was added to the wells and they were placed in the $CO_2$ incubator at 37°C for 4 hours. Then, an Hettich 320R centrifuge in a microwell plate rotor at 500 × g for 10 minutes and the excess MTT solvent was completely emptied.200µl of isopropanol containing 0.05% of chloridric acid was added to all the wells and they were shaken for 30 min. Then, 180µl of all the wells was transferred to another plate. A dual wavelength micro plate ELISA reader (Stat Fax, 2100, USA) was used to measure the optical density, blank against air at 630nm and read at 570nm. The read light absorbance was converted to percentage of cell death by the following formula:

Percentage of cell death = [(absorbance of the test / absorbance of the control)-1] × 100

**Statistical analysis**

Statistical analyses were performed using the SPSS software (version 16) by the One-way ANOVA and Tukey's test as post hoc. In this study, P value of 0.05 was considered as the level of significant difference.

# Results

**Chemical analysis results**

Analysis of *Nectaroscordum tripedale* showed 25 chemical compounds. The result of analysis is show in Table 1.

**Antibacterial results**

Preliminary screening of the antibacterial activity of methanol extract of *Nectaroscordum tripedale* was performed and the results are presented in Table 2.

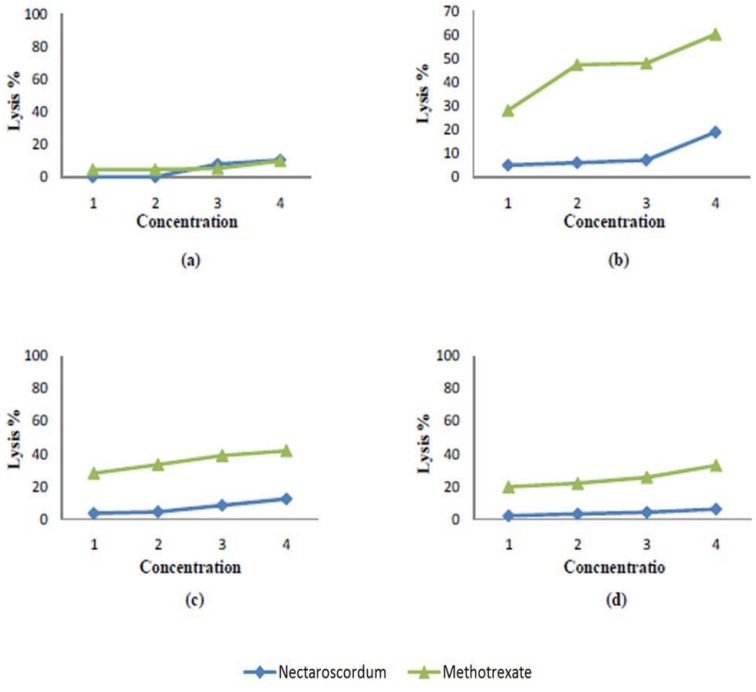


**Fig. 1.** Cytotoxicity effect of Nectaroscordeum tripedale extract and methotrexate in 0.01, 0.1, 1 and 2 mg/mL concentrations on leukemic cell line (KG-1a) after 4hr (a), 12hr (b), 24hr (c) and 48hr (d).

According to the results, the MIC and MBC of *Nectaroscordum tripedale* for *E. coli* and MSRA are similar and were 166 µg/ml and 322µg/ml, respectively. While the high level of MIC and MBC is related to *Pseudomonas aeruginosa*. The diameter of the zone of inhibition around the *Nectaroscordum tripedale* disk varied from 6.4 to 10 mm, indicating that *Nectaroscordum tripedale* has good inhibitory activity on growth of the bacteria tested. Overall, gram-positive bacteria were more sensitive to the extract than the gram-negative bacteria. However, gram-negative *E. coli* was the most susceptible bacterium ($P<0.05$) to the action of these plant.

**Cytotoxicity of Nectaroscordum tripedale extract**

Cell cytotoxicity assay for four different concentrations (0.01, 0.1, 1, and 2 mg/ml) of *Nectaroscordum tripedale* extract showed that the percent lysis of KG-1a cancer cell line was 20%, 70%, 85% and 98%, at 0.01, 0.1, 1 and 2 mg/ml, respectively. As can be seen in Figure 1, the percentage of lysis for *Nectaroscordum tripedale* extract and *methotrexate* is dependent to the time and the highest effect of cytotoxic on leukemic cell line (KG-1a) was seen after 24 hours. Figure 2 showed the effects of cytotoxic of *Nectaroscordum tripedale* extract and methotrexate (in four same concentrations) on normal cells after different times. In comparison with methotrexate, *Nectaroscordum tripedale* has minimal effect on normal cells (Figure 2).

## Discussion

The discovery of effective antibiotics, vaccines and other products or methods has decreased the devastating impact of infectious diseases and improved quality of life. However, the efficacy of many drugs and antibiotics is being threatened by the emergence of microbial resistance to existing chemotherapeutic agents because of their indiscriminate and inappropriate use (10). The use of some antibiotics is associated with side effects, including allergy, immune suppression, and hypersensitivity (11). Many populations who live in developing countries are deprived of the advantages of modern medicine because of its high cost; hence, poor people are more vulnerable to infectious diseases. Besides these, co-infection with multiple diseases is an obstacle to infection prevention and treatment. For all

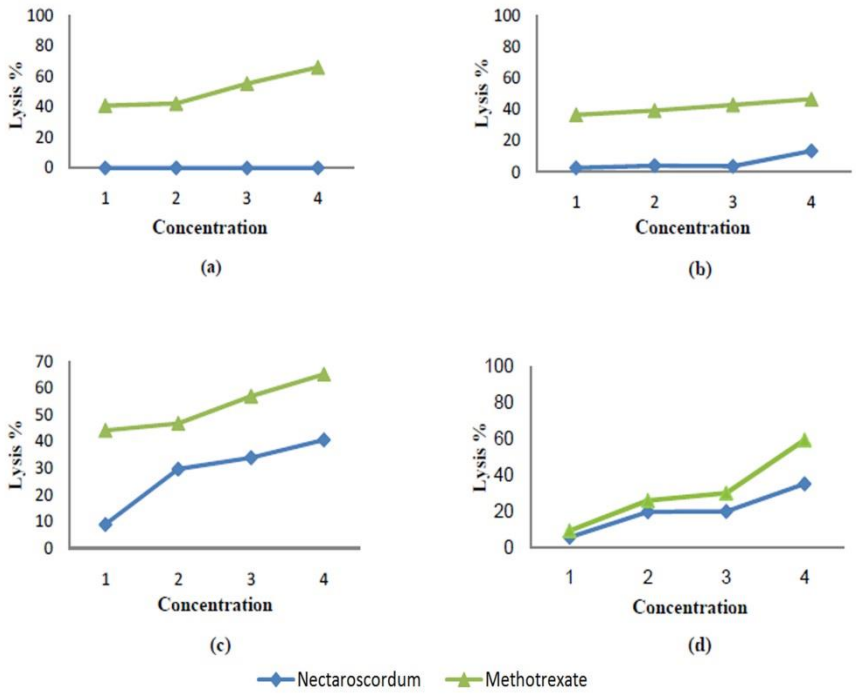


**Fig. 2.** Cytotoxicity effect of Nectaroscordeum tripedale extract and methotrexate in 0.01, 0.1, 1 and 2 mg/mL concentrations on normal lymphocyte cells after 4hr (a), 12hr (b), 24hr (c) and 48hr (d).

**Table 1:** Phytochemical constituents of aqueous methanolic extract of *Nectaroscordum tripedale*.

| No. | Name | KIx | KIs | Similarity | Area% |
|---|---|---|---|---|---|
| 1 | Tetramethylpyrazyn | 1080 | 1086 | 92 | 1.50 |
| 2 | n-Nonanal | 1100 | 1101 | 94 | 4.38 |
| 3 | n-Decanal | 1195 | 1202 | 93 | 1.42 |
| 4 | Pipertitone Oxide | 1237 | 1231 | 85 | 7.14 |
| 5 | 2-Decenal | 1252 | 1261 | 95 | 3.73 |
| 6 | 2,4-Dcadienal | 1284 | 1291 | 93 | 3.81 |
| 7 | Disulfide, Dibutyl | 1291 | - | 88 | 3.04 |
| 8 | 2,4-Decadienal,(E,E) | 1307 | 1314 | 94 | 11.11 |
| 9 | 1-Undecene,8-methyl | 1333 | - | 83 | 1.38 |
| 10 | Trans-2-Undecenal | 1354 | - | 93 | 6.15 |
| 11 | 4-Heptenal | 1370 | - | 86 | 1.86 |
| 12 | Neryl Acetone | 1441 | 1436 | 91 | 2.30 |
| 13 | Pentadecane | 1487 | 1487 | 96 | 2.85 |
| 14 | Gamma, Cadinene | 1498 | 1499 | 89 | 2.80 |
| 15 | 2(4H)-Benzofurane,5,6,7,7 | 1513 | - | 81 | 1.67 |
| 16 | Caryophyllene Oxide | 1575 | 1575 | 90 | 6.02 |
| 17 | Hexadecane | 1600 | 1600 | 96 | 4.71 |
| 18 | Delta,Cadinol | 1635 | 1636 | 89 | 3.44 |
| 19 | Heptadecane | 1689 | 1700 | 96 | 9.55 |
| 20 | Octadecane | 1788 | 1800 | 95 | 2.43 |
| 21 | Octadecane | 1797 | 1800 | 89 | 1.69 |
| 22 | Isopropyl Myristate | 1816 | - | 94 | 1.73 |
| 23 | 2-Undecanone,6,10-dimethyl | 1834 | - | 90 | 2.85 |
| 24 | 1,2-Benzenedicarboxylic Acid | 1953 | - | 87 | 1.88 |
| 25 | Hexadecanoic Acid | 1963 | 1946 | 93 | 10.29 |

these reasons, there is a pressing need to identify new, safe, and cost-effective antimicrobial agents which would help to alleviate the problems of infectious diseases and cancer. Plant-derived natural products represent an attractive source of antimicrobial agents because they are natural and affordable, especially for rural societies (12). Acceptance of medicines from such plant origins as an alternative form of healthcare is increasing because they are serving as promising sources of novel antibiotic and anticancer prototypes. Moreover, these compounds may have different mechanisms of action than conventional drugs and could be of clinical importance to improve health care (13).

Continuous efforts to identify new and novel bioactive materials have encouraged us to evaluate the activities of *Nectaroscordum tripedale* against an array of microorganisms and cytotoxicity.

Some of the phytochemical compounds, e.g., glycoside, saponin, tannin, flavonoids, terpenoid, and alkaloids respectively, have been reported to have antimicrobial activity (14). In the present study we identified a total number of 27 compounds in *Nectaroscordum tripedale* extract by SPME technique. The highest values were 2, 4-Decadienal (11.11%), Hexadecanoic acid (10.29%), Piperitone oxide (7.14%), Caryophyllene oxide (6.02%), disulfide compound (3.04%). Some studies indicate an association of 2, 4-decadienal with genotoxic effects (15), development of atherosclerosis (16), cytotoxic effect (17). Researchers also found that this aldehyde induces DNA damage in A-549 cells, which was related to the reactive oxygen species formation (18).

Hexadecanoic acid or Palmitic acid (C16H32O2) is a common saturated fatty acid found in fats and waxes including olive oil, palm oil, and body lipids. In a study showed the injection of this fatty acid, is effective for treating systemic *staphylococcus aureus* infection in a mouse model (19).

Antibacterial and antiparasitic properties of some plants have been attributed to Piperitone oxide (20, 21). Another study showed Piperitone completely inhibited *Aspergillus flavus* at low concentrations (22). Extract of *Nectaroscordum tripedale* showed good

**Table 2:** Antimicrobial activities of the methanolic extract of *Nectaroscordum tripedale* and known antibiotics against five bacteria isolates.

| Bacteria isolate | Maximum zone of inhibition by antibiotic | | Maximum zone of inhibition by NT (mm) | MIC (µg/ml) | MBC (µg/ml) |
|---|---|---|---|---|---|
| | Antibiotics | Diameter of zone (mm) | | | |
| *Pseudomonas aeruginosa* | Gentamicin | 24 | 6.4 | 664 | 664 |
| *Listeria monocytogenes* | Ciprofloxacin | 24 | 9 | 332 | 664 |
| *Staphylococcus aureus* | Penicillin | 19 | 6.4 | 332 | 664 |
| MRSA | Vancomycin | 18 | 10 | 166 | 332 |
| *E. coli* | Ciprofloxacin | 29 | 8 | 166 | 332 |

NT: *Nectaroscordum tripedale*
MRSA: Methicillin resistant *Staphylococcus aureus*

activity against all bacteria isolates but poor activity was found against *Pseudomonas aeruginosa* according to MIC and MBC tests. Antibacterial effects of this plant extracts against *E. coli*, *Pseudomonas aeruginosa*, *listeria monocytogenes*, and *staphylococcus aureus* suggest that they may possess remarkable therapeutic action in the treatment of gastrointestinal infection and diarrhea in human and skin diseases.

The high potency of *Nectaroscordum tripedale* against these bacteria gives scientific basis for it uses in folk medicine in the treatment of abscesses, bilious conditions, cancer, cough, dyuria, scurvy and stangury and cancer (23).

In antibiotic sensitivity test by disk diffusion method, the maximum zone of inhibition is related to Methicillin-resistant *Staphylococcus aureus* (MRSA) isolate. The increasing antibiotic resistance of pathogens that associated with infectious disease suggested the use of *Nectaroscordum tripedale* as a safe and cheap alternative antibiotic. However, further researches are required to evaluate the practical values of therapeutic application.

There is no paper concerning the therapeutic activity of *Nectaroscordum tripedale* in human or animal. Therefore, we could not compare our results to them. The cytotoxicity test results indicate that the plant extract, perhaps, possess the potential to kill cancer cells. These activities may lead to conclude the presence of secondary metabolites responsible for showing such biological effects. Some of this compound such as tannins and flavonoids might have the involvement in showing antioxidant, cytotoxic and antimicrobial activities of *Nectaroscordum tripedale* extract. However, studies are still needed to evaluate the biological activities and toxicological evaluation of the active constitution of the plant. Based on our results we can conclude that *Nectaroscordum tripedale* extract appears to satisfy criteria for antibacterial and anticancer agents, being cheap and safe. Since the introduction of antibiotics, there has been tremendous increase in the resistance of many bacterial pathogens. Scientists advance in their search for new bacterial targets to attack, bacteria evolve and as a result a large number of bacterial species have become resistant to antibacterial drugs. Hence, search for new antimicrobials is very important in recent times. Maybe *Nectaroscordum tripedale* act synergically with antibiotics, and resistance was not seen for *Nectaroscordum tripedale*, more dose-response preclinical studies and eventually clinical studies should be done to assess the use of an antibiotic/*Nectaroscordum tripedale* combination for bacteria that are difficult to eradicate. In view of the strong antibiotic properties and the complete absence of development to resistance future investigation upon

the principle of the antimicrobial activity of juices from Allium species merits consideration.

## Conclusion

Pharmacological evaluation of *Nectaroscordum tripedale* extract reveals some interesting activities like cytotoxic and antibacterial activities of this plant. Since, methanol extract of *Nectaroscordum tripedale* showed antibacterial and cytotoxic effect, we assume that different active secondary metabolites are present in its extracts and perhaps some of these compounds may function in a synergistic manner. However, further studies are necessary to elucidate the mechanism lying with this effect. This report may serve as a footstep regarding the biological and pharmacological activities of *Nectaroscordum tripedale*.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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