

***In vitro* Evaluation of Antibacterial Action of
Thymus schimperi and Garlic (*Allium sativum*)
Against *Staphylococcus aureus*,
Escherichia coli and *Salmonella typhimurium***

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Abstract: Medicinal plants are considered as rich resources of ingredients which can be used in drug development. The gradual rise in resistance of bacterial pathogens for antibiotics highlights the need to find alternative sources from medicinal plants. An *in-vitro* experimental study was conducted from November 2017 to March 2018 in Mekelle on antimicrobial activity of two selected Ethiopian medicinal plants with the objective of screening their antibacterial activity against *E. coli*, *S. typhimurium* and *S. aureus*. The bulb of garlic (*Allium sativum*) and leaves of *Thymus schimperi* were collected; air dried under shade, powdered and soaked in 80% methanol to extract potential anti-microbial substance. Antibacterial activities of both medicinal plants were tested at different concentrations by using disc diffusion method and measuring zone of inhibition. Plant extracts from *A. sativum* showed a significant antibacterial activity against *S. aureus*, *S. typhimurium* and *E. coli*, with maximum mean zone of inhibition value of 16.21 ± 0.52 mm, 15.88 ± 0.25 mm and 15.85 ± 0.7 mm respectively at 200 mg/ml. Growth inhibitory activities were also observed for extracts of *T. schimperi* against *S. aureus* with maximum mean zone of inhibition value of 12.39 ± 0.47 mm at 200 mg/ml but, extracts of *T. schimperi* did not show any antibacterial activity for *E. coli* and *S. typhimurium*. In this finding the highest antibacterial activity 16.21 ± 0.52 mm was observed from *A. sativum* extracts against *S. aureus*, at the concentrations of 200 mg/ml; while, the minimum antibacterial activity was exhibited by the leaf extracts of *T. schimperi* (8.78 ± 0.73 mm) against *S. aureus* at 100 mg/ml. Generally this investigation indicates that *A. sativum* could be a rich source of antibacterial compounds for the tested bacteria. Further studies on the toxicity and identification of active phytochemicals constituents of the test plants are recommended.

Key words: *Allium sativum* • *Escherichia coli* • *Salmonella typhimurium* • *Staphylococcus aureus*
• *Thymus schimperi* • Zone of inhibition

INTRODUCTION

In the world there are about 391, 000 species of vascular plants known to science, of which about 369, 000 species (94%) are flowering plants and About 2, 000 new plant species are discovered or described every year [1]. Ethiopia is reached with a diverse biological resource including about 6, 500 species of higher plants, with approximately 12% are endemic, hence making it one of the six plant biodiversity rich regions. Medicinal plants

comprise one of the important components of the vegetation. There are about 600 species of medicinal plants constituting a little over 10 percent of Ethiopia's vascular flora [2].

Plants have been used for medicinal purposes long before prehistoric period. Medicinal plants are considered as rich resources of ingredients which can be used in drug development. About 85% of world population uses herbal medicines for prevention and treatment of diseases and the demand is increasing in developed and developing

countries. The use of medicinal plants is very wide spread in many parts of the world because it is commonly considered that herbal drugs are cheaper and safer as compared to synthetic drugs and may be used without or with minimum side effects. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases [3]. The active principles of many drugs found in plants are secondary metabolites [4].

Resistance to antimicrobial agents is emerging in a wide variety of pathogens and multiple drugs; resistance is becoming the common problem [5-7]. The appearance of antibiotic resistant pathogens paved the way to the occurrence of infections that are only treated by a limited number of antimicrobial agents. The gradual rise in resistance of bacterial and fungal pathogens for antibiotics and antifungals highlights the need to find alternative sources from medicinal plants [8].

In Ethiopia most of the medicinal plants are confined in southwest region of Ethiopia and have been used as a source of traditional medicine to treat different human and livestock ailments [9, 10]. In Ethiopia different types of medicinal plants are used.

The medicinal plants used in this study were Garlic (*Allium sativum*) and *Thymus schimperi*. *Allium sativum*, commonly known as garlic, is a plant belonging to the family of Liliaceae, which is native to central Asia and nowadays can be found throughout the world. Garlic (*A. sativum*) is a part of the onion family and the 'bulb' of this herb typically consists of 10-20 smaller sections called the 'cloves'. Each small clove is a powerhouse of flavour as well as medicinal properties [11]. Garlic (*A. sativum*) has been used for centuries worldwide by various societies to combat infectious diseases [12]. Over the last centuries, various species of *A. sativum* have been used as spice or condiment for flavoring food. In herbal medicine, *A. sativum* has been prescribed for treating different kinds of diseases [13].

Allicin found in fresh, crushed or chewed garlic (*A. sativum*) has antibacterial and anti-fungal properties and it may help to prevent some forms of cancer. Garlic (*A. sativum*) plant is thought to be regulating the blood sugar and protecting the cardiovascular system. It also bears antibacterial, anticarcinogenic, antioxidant and anti-inflammatory properties [14]. In addition, antibacterial effects of *A. sativum* on various types of bacteria have been reported in some studies [15]. Therapeutic effect of

garlic (*A. sativum*) is possible because of its oil- and water-soluble organ sulfur compounds, which are responsible for the typical odor and flavor of garlic. Thiosulfates play an important role in the antibiotic activity of garlic [16].

The antibacterial properties of crushed garlic (*A. sativum*) have been known for a long time. Various garlic preparations have been shown to exhibit a wide spectrum of antibacterial activity against gram-negative and gram-positive bacteria including species of *E. coli*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Proteus*, *Bacillus* and *Clostridium*. Even acid-fast bacteria such as *Mycobacterium tuberculosis* are sensitive to garlic (*A. sativum*) [17].

The other medicinal plant is *Thymus schimperi* which is found under the genus *Thymus*. *Thymus* is an aromatic plant belonging to the Lamiaceae family, used for medicinal and spice purposes almost everywhere in the world. Thyme is largely distributed in temperate zones and is uncommon in the African tropics. Ethiopia has considerably abundant Lamiaceae family herb growing at different regions and possesses a variety of the wild growing species of this family. Many species belonging to different genera of the family Lamiaceae have been reported to found in different parts of the country. The two species, *T. schimperi* and *T. serrulatus* both locally known as "Tosign", are the endemic species represented in Ethiopia [18].

T. schimperi is rich in medicinally important constituents like thymol and carvacrol. It was found that essential oil obtained from *T. Schimperigrown* in Ethiopia, was rich in carvacrol (66.2%) and thymol (50%) which is responsible constituents of thyme for antibacterial activity against gram positive and gram negative bacteria due to their effect on the bacterial membrane [19].

Even though there have been different studies in different countries on the antibacterial activities of *A. sativum*, but in Ethiopia there is limited studies regarding the antibacterial action of garlic specially the crude extracts. Again few researches have been conducted on the antibacterial action of *T. schimperi* however antibacterial activity of *T. schimperi* essential oil has not been widely investigated. Therefore: the objective of this study was to screen antibacterial action of *Thymus schimperi* and *Allium sativum* against *E. coli*, *Salmonella typhimurium* and *Staphylococcus aureus*.

MATERIALS AND METHODS

Study Area: The study was conducted from November, 2017 to March 2018 in Mekelle University; college of veterinary medicine, microbiology laboratory which is located in Mekelle. Mekelle is the capital city of Tigray Regional state. It is located around 783 kilometers north of the Ethiopian capital city Addis Ababa, at a latitude and longitude of 13°30'N 39°29'E, with an elevation of 2000 meters above sea level [20]. The climate of the study area conforms to that of the highland. Its annual rain fall ranges of 579 to 628.8 mm and average temperature ranges from 17-21°C [21].

Study Design: *In-vitro* experimental study was conducted from November, 2017 to March 2018 to investigate antibacterial activity of garlic (*A.sativum*) and *T.schimperi* against *S. aureus*, *S. typhimurium* and *E. coli* by disc diffusion method.

Medicinal Plants: In this study two medicinal plants were tested namely *T. schimperi* and *A.sativum* for their antibacterial activity against three types of bacteria. *A. sativum*; commonly known as garlic, is a species of the onion family Alliaceae. The bulb grows at the base of a perennial plant with an erect flowering stem that grows 2-3 ft. long. The bulb is made up of several outer thin protective sheaths covering the inner sheath. The inner sheath covers the swollen leaves called as cloves. The mature bulb has around 8 or more cloves in each bulb [22].

T. schimperi is found under a crowded inflorescence with pink corollas and have ovate to elliptic leaves with entire margins. They are extensively used by local people as food preservatives; cure for various ailments and for food flavoring [23]. *T. schimperi* was found to have food preservative and antioxidant activity [24].



Garlic (*Allium sativum*) *Thymus schimperi*

Fig. 1: Medicinal plants used for studding antibacterial activity

Plant Collection and Preparation of Crude Extracts: The plants in this study were collected from different sites; Garlic (*Allium sativum*) was purchased from the local market of Mekelle city and the fresh leaves of *T. schimperi* were collected from “*Asagrt Wereda*” North Shoa, Amhara region. After collection the Garlic Bulbs were peeled and washed from the foreign particles under tap water, aseptically and then the bulbs were cut into small pieces with a knife and then kept in the shade for 9 days at room temperature. The semi-dried pieces were then crushed using pestle and mortar and left to dry in the shade at room temperature. The leaves of *T. schimperi* were washed with tap water and kept in shade until fully dried at room temperature. Finally both dried plants were grinded into powder form by grinder machine and sieved by fine mesh.

The extraction was done by maceration method: The obtained powder of each plant was weighed by sensitive balance. About 200 g of powdered plant was taken and soaked in 1,000 (800 ml of 80 % Methanol (1:5) in Erlenmeyer flask and then the flask was plugged with aluminum foil. Then the flasks containing the mixtures were continuously shaken by orbital shaker for 10 minutes in every 4-hours interval for 3 consecutive days. After 3 days the extracts were filtered by using double layer cheese cloth and sieve together. Again the filtrate was filtered by using Whatman #4 filter paper.

The residue of each plant was re-soaked with the same extractors as mentioned above for about 2 days. The residues after second filtration process were discarded while the filtrate was mixed with the previous filtrate. Filtrate was evaporated under reduced temperature by using water bath at 40 to 50°C to reduce the solvent and then the extracts were dried by hot oven at 40°C until the semi-solid material became completely solid and dry. Finally crude extracts of each plant were weighed, labeled and stored at 4°C refrigerator in airtight containers until used for antimicrobial sensitivity test.

Preparation of Antimicrobial Discs from Plant Extracts for *In-vitro* Experiment: Extracts of each plant were dissolved in DMSO to prepare 100 mg/ml, 150mg/ml and 200 mg/ml of stock solution of plant extract. Filter paper discs of 6 mm diameters were prepared from Whatman filter paper no. 1 by using hole puncher. Each filter paper discs were impregnated with equal amounts of crude extracts (20µl) of different concentrations. For each of *A. sativum* and *T. schimperi*; three groups of discs were

prepared; i.e. one group was loaded with 20µl of crude extract containing solution 200mg/ml, second group was loaded with 20µl crude extract containing solution 150mg/ml and the third group was loaded with 20µl crude extract containing solution 100mg/ml.

Finally, the discs were allowed to dry and sterilized under UV for 30 minutes.

Preparation of the Test Bacteria: In this study twogram negative bacteria (*E. coli* and *S. typhimurium*) and one gram positive bacteria (*S. aureus*) were used as test organisms. The test organisms were obtained from Mekelle university, college of veterinary medicine, molecular biology laboratory that were cultured in nutrient broth then, the test microorganisms (*E. coli*, *S. typhimurium* and *S. aureus*) were cultured on nutrient agar and then pure colonies were taken from nutrient agar for each bacterium and suspended into sterile 0.9% saline salt solution. It was standardized according to National Committee for Clinical Laboratory Standards [25] by gradually adding pure bacterial colonies to 0.9% normal saline until the bacterial density or turbidity was comparable with the density of 0.5 McFarland turbidity standard.

Antimicrobial Sensitivity Test Using Filter Paper

Method: In this experiment disc diffusion (Kirby-Bauer disc diffusion susceptibility test) method was used to conduct antibacterial susceptibility test. The test was done according to European Committee on Antimicrobial Susceptibility Testing [26]. For susceptibility testing first Muller Hinton agar was prepared then, adjusted bacterial colonies to 0.5 McFarland turbidity standard were taken

by dipping sterilized swab in to the test organism that was adjusted on 0.9% normal saline solutions. Then pure colonies were spread by the swab on the whole surface of Mueller Hilton agar plate. Then immediately 5 discs per 90 mm diameter plates were placed on Mueller Hilton agar seeded with respective pathogens, with the help of sterile forceps with equal distance.

Out of those five discs three discs were impregnated with different concentrations of plant extract. One disc was loaded with DMSO as negative control and sulfamethoxazole-trimethoprim impregnated disc was used as a positive control. Finally, the inoculated plates were incubated at 37°C for 24 hrs. After incubation the diameters of zone of inhibition in mm were measured by using digital caliper.

Data Analysis: All the experimental results were performed in triplicate and the inhibition zones were measured. The data were entered and managed in a Microsoft Excel spread sheet and analyzed using SPSS version 20. The results of zone of inhibition were expressed as mean ± Standard Deviation (SD). Statistical analysis was also undertaken by analysis of variance (one way ANOVA) coupled with Least Significant Difference (LSD) to compare group means between concentrations and test bacteria and $p < 0.05$ was considered as statistically significant.

RESULTS

Yield of Crude Extracts: Out of those two medicinal plants the maximum yield was obtained from *A. sativum* was (15.5%) and the minimum yield from *T. schimperi* was (11.4%) (Table 1).

Table 1: Yield of crude extract of *A. sativum* and *T. schimperi*

Type of plant	Dray powder used in gram	Amount of solvent used in ml	Yield in gram	Yield %	Consistency
<i>A. sativum</i>	200	1000	31	15.5	Solid
<i>T. schimperi</i>	200	1000	22.8	11.4	Solid

Table 2: Mean zone of inhibition of test plants against *S. aureus*, *E. coli* and, *S. typhimurium* at different concentrations

Mean Zone of inhibition mean in mm ±SD								
Test plant	Conc. mg/ml	<i>S. aureus</i>	P-value	<i>E. coli</i>	P-value	<i>S. typhimurium</i>	P-value	Total P -value
<i>A. sativum</i>	100	11.46±2.12	0.002	11.01±1.3	0.001	11.11±1.48	0.00	0.925
	150	13.35±0.65		12.5±1.26		12.63±0.6		0.38
	200	16.21±0.52		15.85±0.7		15.88±0.25		0.597
<i>T. schimperi</i>	100	8.78±0.73	0.002	0.000	-	0.000	-	0.00
	150	10.38±0.96		0.000		0.000		0.00
	200	12.39±0.47		0.000		0.000		0.00
PC(SXT)		19.86±0.68		24±1.08		21.3±0.42		0.0001
NC (DMSO)		0.00		0.00		0.00		

PC=Positive control

NC=Negative control

SXT= Sulfamethoxazole-trimethoprim

Antibacterial Screening: Both medicinal plants (*A. sativum* and *T. schimperi*) showed statically significant action ($p < 0.05$) against *S. aureus* at all the tested concentrations but in a concentration-dependent manner (Table 2). Garlic (*A. sativum*) also showed statistically significant action ($p < 0.05$) against *E. coli* and *S. typhimurium*. However, crude extracts of *T. schimperi* did not show any antibacterial activity against gram negative bacteria (*E. coli* and *S. typhimurium*) (Table 2).

For Both medicinal plants extracted by methanol solvents mean zone of inhibitions were observed with range between 8.78 ± 0.73 mm and 16.21 ± 0.52 mm. The highest antibacterial activity (16.21 ± 0.52 mm) was observed for *A. sativum* at 200mg/ml against *S. aureus* while the minimum antibacterial activity was exhibited by *T. schimperi* at 100 mg/ml against *S. aureus* (8.78 ± 0.73 mm). *A. sativum* exhibited higher antibacterial activity against all tested bacteria (*S. aureus*, *S. typhimurium* and *E. coli*) with mean inhibition zone measured in the range of 11.01 ± 1.3 mm to 16.21 ± 0.52 mm. The extracts of *T. schimperi* also inhibited the growth of the tested gram positive bacteria (*S. aureus*) and its mean zone of inhibition ranged between 8.78 ± 0.73 mm to $12.39 \text{ mm} \pm 0.47$ mm (Table 2).

DISCUSSION

Due to gradual rise in resistance of bacterial pathogens for antibiotics, much attention has been focused toward plant extracts and biologically active compounds isolated from popular plant species [27]. In this study, the antibacterial effect of crude extracts from, *T. schimperi* and *A. sativum* were tested by disc diffusion method.

The results indicated that the extracts isolated from *T. schimperi* and *A. sativum* showed antibacterial activity against one or three of the tested bacteria which was indicated by clear zone of inhibition. These are in line with the previous researches that *A. sativum* has antimicrobial activity [28] and *T. schimperi* also has antimicrobial action [29]. The results also showed the antibacterial activities of *T. schimperi* and *A. sativum* extracts showed concentration dependent inhibition zone. For both medicinal plants zone of inhibition in all tested bacteria increased with increment of the extract concentration, this might be due to the reason that, as the concentrations of plant extracts is increased active ingredients also increased.

In the current study, among the two tested plant extracts, *A. sativum* extracts showed high antibacterial

activity against all the test organisms while *T. schimperi* extracts showed antibacterial activity against only *S. aureus*. The high antibacterial activities exhibited by *A. sativum* compared to *T. schimperi* may be due to the presence of sulphur-based compounds such as allicin which possess strong antibacterial activities [30].

The present study clearly indicated that concentrations of 100 mg/ml, 150 mg/ml and 200 mg/ml of *A. sativum* extracts were active against all tested bacteria (*S. aureus*, *E. coli* and *S. typhimurium*). But the degrees of zone of inhibition were different for the tested bacteria. This may be due to difference in bacterial strain and concentrations. The antibacterial activity observed by *A. sativum* could be attributed by biologically active compound (Allicin) which is responsible for the anti-microbial properties of this plant [31].

The present study showed that *A. sativum* extracts were able to inhibit both gram negative and gram positive bacteria (*S. aureus*). This implies that the test plant could be a potential source of antibacterial agents. Similar findings have been reported by Bakri and Douglas [17] on antibacterial, antifungal and antiviral activity *A. sativum*, Bokaeian and Bameri [32] on antibacterial properties of aqueous garlic extract against multidrug-resistant *enterococcus*, Abiy and Berhe [33] on antibacterial actions of garlic against clinical isolates of *staphylococcus aureus* and *E. coli* and Kheira *et al.* [34] on antimicrobial effect of *A. sativum* against *E. coli* and *S. aureus* isolated from mastitis cow milk.

In this study *A. sativum* extracts caused higher mean zone of inhibition with the range of 11.01 ± 1.3 mm to 16.21 ± 0.52 mm against all tested bacteria at different concentrations. The maximum mean zone of inhibition of *A. sativum* extracts was recorded for *S. aureus* (16.21 ± 0.52 mm) at 200mg/ml, followed by *S. typhimurium* (15.88 ± 0.7 mm) and *E. coli* (15.85 ± 0.25 mm). While the minimum mean zone of inhibition was recorded for *E. coli* (11.01 ± 1.3 mm) at 100 mg/ml.

The above results suggest that *A. sativum* extracts showed slightly higher mean zone of inhibition against (*S. aureus*). While, slightly lower inhibition zone against *E. coli* and *S. typhimurium*. These results agree with the finding of previous studies reported by Kheira *et al.* [34] in which *A. sativum* showed slightly higher zone of inhibition for gram positive bacteria than gram negative bacteria. The observed difference in antibacterial activities between gram negative and gram positive bacteria was attributed due to the difference in structure of the bacterial outer membrane and cell wall [35].

In this finding the extracts of *A. sativum* have broad - spectrum antibacterial activities. However, the antibacterial activities of *A. sativum* were lower than standard antibacterial drug sulfamethoxazole-trimethoprim which was used as a positive control. This is probably due to the fact that these extracts were crude preparations which may not contain enough of the active chemicals. DMSO loaded discs did not show inhibition against the test organisms which implies that the observed inhibition zone was exclusively by the crude extracts.

The recorded zone of inhibition in present finding by *A. sativum* for the three tested bacteria is lower than the previous finding in Nigeria in which zone of inhibition for *S. aureus*, *S. typhi* and *E. coli* were 24.66 ± 5.9 mm, 21.86 ± 6.6 mm and 20.86 ± 6.1 mm respectively [14]. The observed difference may arise from that in their studies they used aqueous extract of *A. sativum* to test its antibacterial activity but in this study methanol extract was employed. As Research has found aqueous extract of *A. sativum* to be more potent than organic extracts [27, 36]. This could be as a result of the fact that when plant materials are grinded in water, a number of phenolases and hydrolases are released and these enzymes might serve to modulate the activity of the active compounds in the extract [37]. The other factor that makes the difference in the antimicrobial activities between the same plants might be difference in the environment where the *A. sativum* was collected and genetic variations of plant.

The other plant used in this study is crude extracts of leaves of *T. schimperi* which showed that some antibacterial effect against gram positive bacteria (*S. aureus*) at all concentrations. The maximum mean zone of inhibition exhibited by *T. schimperi* extract obtained for *S. aureus* was (12.39 ± 0.47) mm at 200mg/ml and the minimum mean zone of inhibition was $8.78 \text{ mm} \pm 0.73$ against *S. aureus* at 100mg/ml. However methanol extracts of *T. schimperi* did not show any zone of inhibition for gram negative bacteria (*S. typhimurium* and *E. coli*). This result agrees with the previous reports in which both methanol and ethanol extracts of *T. schimperi* could not inhibit the growth of gram negative bacteria. However, chloroform extract inhibits the growth of both gram negative and gram positive bacteria [38].

The observed difference in zone of inhibition between gram positive and gram negative bacteria may be attributed to the difference in the outer membrane of gram negative and gram positive bacteria. Gram-positive bacteria were more susceptible to the extract than the

gram-negative bacteria [39]. The present findings also agree with the previous reports in that the resistance is due to the differences in their cell wall composition. In Gram-negative bacteria, the outer membrane acts as a great barrier to many environmental substances including antibiotics [40]. Furthermore, presence of the thick murine layer in the cell wall inhibits the entry of inhibitors [27].

The other difference might arise from methanol and ethanol is not a good solvent for extraction of *T. schimperi*. Harmala *et al.* [41] reported that 20 different solvents were evaluated and chloroform is the best solvent for extraction of non-polar biological active compounds that were lethal to many bacteria.

But the present study disagrees with the previous studies which were reported by Awol *et al.* [42] and Chalachew *et al.* [43]. In those studies *T. schimperi* extracts exhibited antibacterial activity against all the tested gram positive and gram negative bacteria. The observed difference may be due to: first the extraction methods used, on both previous studies hydro distillation method was used but, in this study maceration method was used. Second, as Awol *et al.* [42] reported in their studies agar well diffusion method was used and undiluted essential oil of plant extract was dispensed into respective wells however, in this study disc diffusion method was employed and crude extracts were diluted to lower its concentrations. The other factors might be geographical and ecological difference on distribution where *T. schimperi* was collected that varied the concentration of the active ingredients.

CONCLUSION AND RECOMENDATIONS

The present study evaluates *in-vitro* antibacterial activities of *A. sativum* and *T. schimperi*, against *E. coli*, *S. aureus* and *S. typhimurium*. Both *T. schimperi* and *A. sativum* showed antibacterial activity which was evidenced by clear zone of inhibition and the pattern of inhibition varied with bacterial strain and concentration of crude extracts. *A. sativum* has showed antibacterial activities against *E. coli*, *S. aureus* and *S. typhimurium*. Extracts isolated from *T. schimperi* showed antibacterial activity against *S. aureus* only. From the findings of this study it is possible to conclude that *A. sativum* could be a rich source of antibacterial compounds for the tested bacteria. Among the two plants for antimicrobial properties, *T. schimperi* was found with few reports on its chemical constituents.

Therefore from the findings of this study the following recommendations are forwarded.

- Further studies especially on *T. schimper* should be made in order to identify the phytochemical constituents that are responsible for their antibacterial activity
- In addition toxicity tests of the active parts and *in-vivo* experiments of these plants are required to assure safety and effectiveness.

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List of Abbreviations

<i>A. sativum</i>	<i>Allium sativum</i>
DMSO	Dimethylsulphoxide
<i>E. coli</i>	<i>Escherichia coli</i>
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. typhimurium</i>	<i>Salmonella typhimurium</i>
<i>T. schimper</i>	<i>Thymus schimper</i>

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