**JOURNAL OF TROPICAL LIFE SCIENCE**
2024, Vol. 14, No. 1, 77 – 82
http://dx.doi.org/10.11594/jtls.14.01.09

**Research Article**

# Chemical Compounds and Antibacterial Activity of *Thymus Vulgaris* Leaves' Ethanolic Extract Against *Salmonella typhimurium*

Emad Khaleefah Abousouh [1], Agustina Tri Endharti [2], Sanarto Santoso [3], Dewi Santosaningsih [3,4]*

[1] Doctoral Program of Medical Science, Faculty of Medicine, Universitas Brawijaya, Malang 65145, Indonesia
[2] Department of Parasitology, Faculty of Medicine, Universitas Brawijaya, Malang 65145, Indonesia
[3] Department of Clinical Microbiology, Faculty of Medicine, Universitas Brawijaya, Malang 65145, Indonesia
[4] Dr. Saiful Anwar Hospital, Malang 65145, Indonesia

*Article history*:
Submission March 2023
Revised July 2023
Accepted August 2023

**Corresponding author:*
E-mail:
dewi.santosa@ub.ac.id

**ABSTRACT**

Invasive non-typhoidal Salmonella is an important pathogen that causes life-threatening bloodstream infections. Antibiotic resistance in non-typhoidal *Salmonella* has emerged as a result of the increasing use of antibiotics; therefore, an alternative source of therapeutic agents is required. This study aimed to investigate the active compound and antibacterial activity of *Thymus vulgaris* leaves' ethanolic extract against *Salmonella typhimurium*. Thin-layer chromatography was performed to identify the active compound in the ethanolic extract of T. vulgaris leaves. The antibiotic effect of the extract was carried out by agar dilution assay using the following concentrations: 0%, 2.5%, 5.0%, 7.5%, 10.0%, 12.5%, and 15.0% (w/v). Thymol and carvacrol were detected in the thin-layer chromatography. The minimum inhibitory concentration of the extract was 10.0% (w/v). In conclusion, *T. vulgaris* leaves' ethanolic extract demonstrated antimicrobial activity against *S. typhimurium*. Further investigation is required to analyze the role of thymol and carvacrol as active compounds against *S. typhimurium*.

*Keywords: Antibacterial activity, Carvacrol, Minimal inhibitory concentration, Salmonella, Thymol, Thymus vulgaris*

## Introduction

Non-typhoidal *Salmonella* is an important pathogen causing acute mild self-limiting enteritis worldwide [1, 2]. The Salmonella infection presents an acute onset of fever and abdominal symptoms such as nausea, vomiting, abdominal cramping, and diarrhea. Generally, the symptoms subside in 3 – 7 days [1]. However, invasive non-typhoidal Salmonella infections have been frequently found in specific regions, leading to life-threatening bloodstream infections, meningitis, and pneumonia, particularly in children [2–5].

*Salmonellae* are gram-negative bacteria belonging to the family Enterobacteriaceae. Genus *Salmonella* is consisted of two species, including *Salmonella enterica* and *Salmonella bongori*. *Salmonella typhimurium* is a serovar of *S. enterica* that was identified as the most frequent pathogen causing invasive non-typhoidal Salmonella infections since 1966 in African regions [6]. Reddy *et al.* [7] revealed that 65% of invasive non-typhoidal *Salmonella* infections were caused by *S. typhimurium*. The infectious dose of non-typhoidal *Salmonella* is $10^6$ – $10^8$, but a smaller inoculum could produce infection in infants and immunocompromised patients. Therefore, non-typhoidal Salmonella infections are more likely to occur among children, specifically children less than two years old [1]. The ability of *Salmonella* to survive in dendritic cells and macrophages contributes to the pathogen spreading in the bloodstream and migrating to extraintestinal sites [6].

Several risk factors for invasive non-typhoidal

*How to cite:*
Abousouh EK, Endharti AT, Santoso S, Santosaningsih D (2024) Chemical compounds and antibacterial activity of *Thymus vulgaris* leaves' ethanolic extract against *Salmonella typhimurium*. Journal of Tropical Life Science 14 (1): 77 – 82. doi: 10.11594/jtls.14.01.09.

Salmonella infections include HIV infection, malnutrition, acute severe malaria, and poor sanitation [5, 6]. The HIV – invasive non-typhoidal Salmonella co-infection rate reached 95% among adult patients in Africa [4]. Furthermore, the risk factors for invasive non-typhoidal Salmonella infections among children in Africa were more complicated, including malnutrition and malaria other than HIV infection [4].

Previous studies reported that sub-Saharan Africa was burdened with invasive non-typhoidal Salmonella infections, with 79% of the 535,000 global cases and 85% of the 77,500 mortalities [2, 3, 6]. Ao *et al.* [8] predicted a high incidence of invasive non-typhoidal *Salmonella* infections in European countries, with 102 cases per 100,000 population particularly in Russia, Estonia, and Ukraine. By contrast, large-scale surveillance of invasive non-typhoidal Salmonella infections in Asian countries showed a low number of cases of invasive non-typhoidal Salmonella disease cases [3]. However, an epidemiology study in Thailand-Laos border provinces revealed a high prevalence of *S. typhimurium* among *Salmonella* isolates obtained from animals and humans [9], indicating the potential problem of invasive non-typhoidal Salmonella infections in these countries. Therefore, invasive non-typhoidal Salmonella infections constitute a wide public health threat.

The increasing antimicrobial resistance, particularly in Africa [2, 5], complicates the burden of invasive non-typhoidal Salmonella infections and may be associated with more treatment failures [10]. An epidemic of invasive non-typhoidal Salmonella that was resistant to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole occurred in Malawi. Furthermore, the 3$^{rd}$ generation of cephalosporins and fluoroquinolone were extensively used to treat invasive non-typhoidal Salmonella [11]. Consequently, further resistance may happen. Multidrug-resistant *S. typhimurium* sequence type ST313 has been identified as the predominant sequence type of *S. typhimurium* associated with invasive non-typhoidal Salmonella infections in Africa [4].

In order to control antimicrobial resistance, the development of alternative antibiotic agents against invasive non-typhoidal *Salmonella* is needed. Because of their antimicrobial properties, plant essential oils and extracts have been used as alternative medicine and natural therapies for thousands of years [12]. *Thymus vulgaris*, a member of the Lamiaceae family, originated in Mediterranean countries and North Africa has been traditionally used to treat diarrhea [13]. Several studies performed scientific investigations regarding the antimicrobial activity of the essential oils of *T. vulgaris* extracted by hydro-distillation against *S. typhimurium* [12, 14]. Thymol was detected as the main active compound of the essential oil of *T. vulgaris* obtained from dried aerial *plant*, flowers, and leaves [14–16]. Other active compounds were found in *T. vulgaris* essential oil including p-cymene, γ-terpinene, geraniol, carvacrol, and linalool [12, 14].

Nevertheless, Amhamdi *et al.* [12] reported low concentrations of thymol and no carvacrol in the essential oil of *T. vulgaris*. A previous study found the superiority of the Soxhlet extraction method compared to steam distillation to obtain chemical constituents from plants [17]. However, the superiority of several solvents for the extraction of active compounds in *T. vulgaris* was not described previously. Investigation of active compounds of *T. vulgaris* leaves' ethanolic extract obtained by Soxhlet extraction was scarce. In addition, the antimicrobial activity of the ethanolic leaf extract against *S. typhimurium* is required. Therefore, we aimed to investigate the in vitro antibacterial effect of ethanolic extract of *T. vulgaris* leaves against *S. typhimurium*.

## Material and Methods

### *Plant material and extract preparation*

This study was conducted from March to August 2022. The dry leaves of *T. vulgaris* were obtained from a traditional herbal shop in Libya and verified by a botanist-taxonomist in the Materia Medica Herbal Laboratory, Batu, Indonesia. The extract was prepared as previously described [18]. The leaves were shade-dried for 10-15 days at room temperature and ground into a fine powder. One gram of the leaves powder was macerated in 10 ml of absolute ethanol for 48 hours by the Soxhlet extraction apparatus. The extract solution was evaporated to dry at 40°C using a rotary evaporator, and the crude extract was stored at 4°C for further use.

### *Active compounds identification*

Thymol and carvacrol, as the active compounds of *T. vulgaris*, were screened by thin-layer chromatography as previously described [19]. The chromatograms of both active

compounds were detected under ultraviolet light 366 nm and 254 nm wavelength after spraying with vanillin-sulphuric acid solution. Moreover, the compounds were interpreted by measuring the retention factors (Rf) values based on the references [20, 21].

### *Bacterial isolate*

*S. typhimurium* was provided by the Department of Clinical Microbiology, Faculty of Medicine, Brawijaya University, Malang, Indonesia and identified using the MicrobactTM Gram-negative system (Oxoid) [18]. Pure culture of *S. typhimurium* obtained within 18-20 hours were used for antibiotic susceptibility tests.

### *Antibacterial susceptibility test of extract*

Agar dilution conventional testing method was carried out to determine the minimum inhibitory concentration of *T. vulgaris* ethanolic leaves extract against *S. typhimurium* [22]. With agar dilution, the antibacterial concentration of *T. vulgaris* leaves' ethanolic extract and *S. typhimurium* was brought together on a Mueller Hinton agar medium. Each concentration of the extract is incorporated into a single agar plate. The final concentration of each agar plate included negative control, 2.5%, 5.0%, 7.5%, 10.0%, 12.5%, and 15.0% (w/v). The volume of the extract poured in a plate varies based on the concentration, as follows: negative control, 0 mL; 2.5%, 0.25mL; 5%, 0.50 mL; 7.5%, 0.75 mL; 10%, 1.00 mL; 12.5%, 1.25 mL; 15%, 1.50 mL. The positive control was not performed in this study; however, it was tested in the in vivo antibacterial susceptibility test that would be published elsewhere. Ten microliters of $10^6$ CFU of *S. Typhimurium were* inoculated on each agar plate and incubated at 37°C for 16-20 hours. Four replications were tested per plate. The minimum inhibitory concentration was determined as the lowest concentration of *T. vulgaris* leaves' ethanolic extract that completely inhibits visible growth of the colonies of *S. typhimurium*.

## Results and Discussion

### *Compounds of Thymus vulgaris leaves' ethanolic extract*

Visually, there are five zones in the chromatogram in our study. However, we characterized two zones with standard material, particularly for thymol and carvacrol. Figure 1 shows that thymol and carvacrol in the ethanolic extract of *T. vulgaris* leaves were detected. The Rf values of thymol and carvacrol were identified at 0.73 and 0.81, respectively. Nevertheless, the compounds were not separated well in the chromatogram.

Herbal plants play an important role in preventing and treating human diseases. People have been using plants as traditional medicine for thousands of years. Plants are considered rich sources of phytochemical ingredients, which enable them to have medicinal value. Medicinal plants are a potential source for the development of new herbal drugs. In the 21st century, the pharmacological effects of medicinal plants have been considered promising future drugs or medicines for the management of health care. In recent years, there has been a resurgence of interest in rediscovering medicinal plants as a source of potential drug candidates [23].

Similar to the previous study, we found both thymol and carvacrol in *T. vulgaris* leaves' ethanolic extract in our study [24]. Thymol and carvacrol are natural monoterpenes that are more soluble in some organic solvents and alcohol [25]. Thymol is detected as the major compound in the essential oil of *T. vulgaris* [14, 26]. Other active compounds contained in the *ethanolic extract of T.*

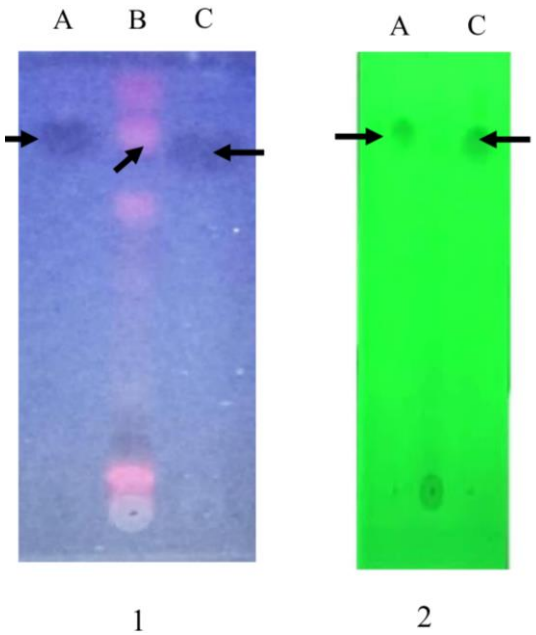


Figure 1. Thin layer chromatography chromatogram of *T. vulgaris* leaves' ethanolic extract and standards carvacrol and thymol. A: standard of carvacrol (Rf= 0.81); B: *T. vulgaris* leaves' ethanolic extract; C: standard of thymol (Rf=0.73).

*vulgaris leaves* obtained by Soxhlet extraction were p-cymene and camphor [24]. In the present study, the composition of thymol and carvacrol was not measured. Therefore, the major active compound in the extract was not identified. Amhamdi *et al.* [12] demonstrated unusual results, showing that camphor is the major compound in the essential oil of T. vulgaris and that carvacrol was not found. Harvesting time and plant location may influence the content and composition of active compounds [12]. Therefore, further investigation should be carried out to identify the composition and interaction of other active compounds in the ethanolic extract of *T. vulgaris* leaves in our study. Furthermore, an active compound related to the antimicrobial effect in the ethanolic extract of *T. vulgaris* leaves could be determined.

***Antibacterial activity of Thymus vulgaris leaves' ethanolic extract***

In this experiment, the agar dilution assay showed the minimum inhibitory concentration value of *T. vulgaris* leaves' ethanolic extract against *S. typhimurium* at 10.0% (w/v) (Figure 2). Dilution method is the best one for determining minimum inhibitory concentration values in antimicrobial susceptibility testing either using broth dilution or agar dilution method. Broth dilution assay is the most frequently used method in antimicrobial susceptibility testing [22]. However, the visual observation to determine the minimum inhibitory concentration in broth dilution assay was constrained by the turbidity factor of the extract. Therefore, an agar dilution assay was performed in this study.

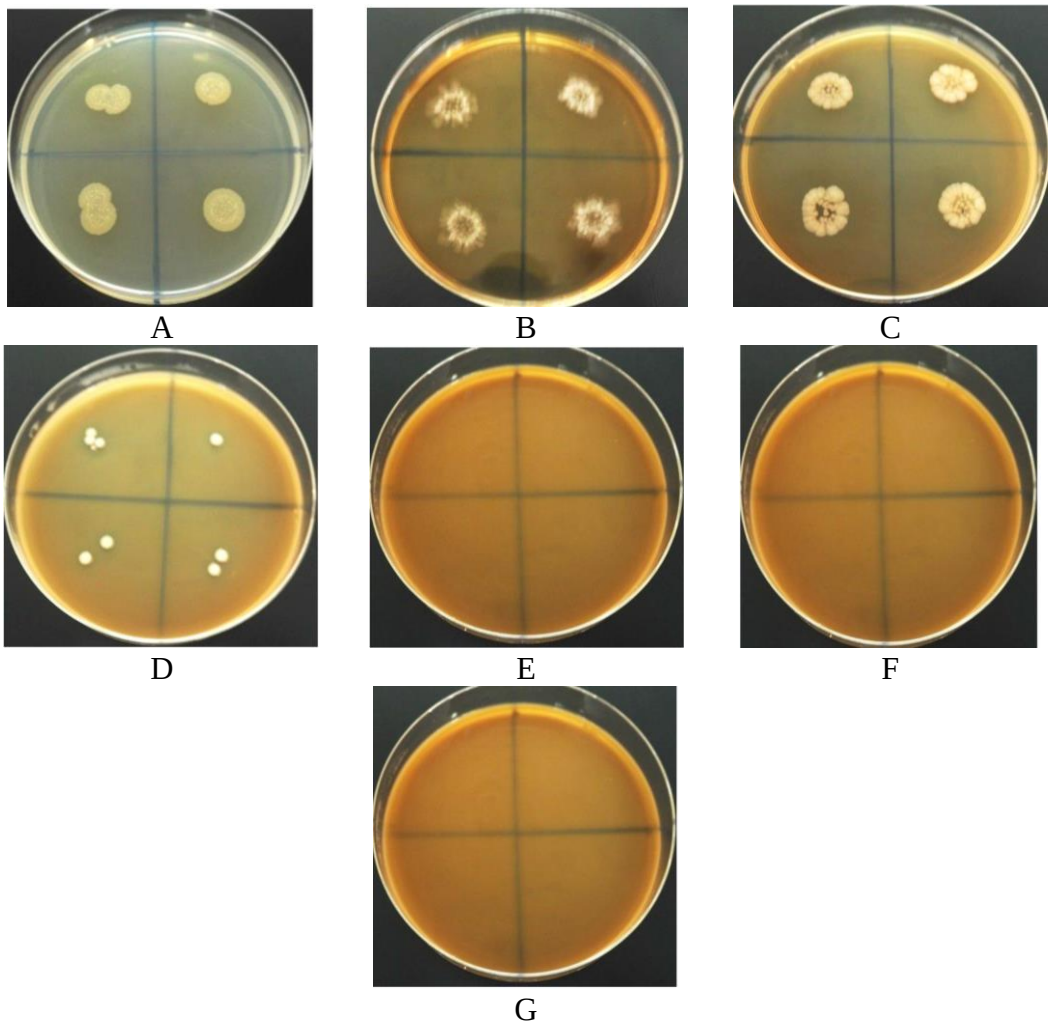


Figure 2. Agar dilution assay of *Thymus vulgaris* leaves' ethanolic extract against *Salmonella typhimurium*. A: negative control; B:2.5%; C:5.0%; D:7.5%; E:10.0%; F:12.5%; G:15.0%

To the best of our knowledge, this study is the first agar dilution assay of *T. vulgaris* leaves' ethanolic extract against *S. typhimurium*. Previous studies reported the antimicrobial activity of *T. vulgaris* essential oil against *S. typhimurium* using broth dilution assay and agar diffusion assay [14, 25, 26]. In this study, we used the agar dilution method to determine the MIC because of the turbid extract, therefore. it was not possible to determine the MIC using the broth dilution method.

The antimicrobial effect of *T. vulgaris* leaves' ethanolic extract might be associated with the thymol and carvacrol in the extract. Multiple studies have found that thymol, alone or in combination with other substances such as carvacrol, has antibacterial properties [25, 27]. Some researchers hypothesized that thymol's antibacterial mechanism was caused, at least in part, by a change in the lipid fraction of the bacterial plasma membrane, resulting in changes in membrane permeability and the escape of intracellular content [25]. Recently, it has been demonstrated that stress induced by thymol influences the *S. typhimurium* proteome, downregulating genes involved in chemotaxis, motility, and virulence. In addition, it is reported that these substances can affect quorum sensing of bacteria which, in turn, controls virulence factor production [28]. Carvacrol revealed antimicrobial activity against multidrug-resistant Gram-negative bacteria. The antimicrobial effect of carvacrol could be related to the interaction between carvacrol and lipid bilayer leading to the bacterial lysis due to cytoplasmic membrane expansion and destabilization [29].

The synergy effect of thymol and carvacrol in combination as antibacterial against S. Typhimurium was investigated by the previous study. The efficacy of ampicillin against *S. typhimurium* was increased by the combination with thymol and carvacrol [25]. The synergy of antibacterial activity against Gram-negative bacteria is not only between thymol and carvacrol but also between thymol and gamma-terpinene [14]. However, we did not identify active compounds other than thymol and carvacrol in this study. It is suggested that *T. vulgaris* leaves' ethanolic extract may have the potential as antibacterial therapy for *S. typhimurium* infection.

The present study has some limitations. First, we screened thymol and carvacrol in *T. vulgaris* leaves' ethanolic extract but no other active compounds. Therefore, the chemical composition of the extract was not identified in the chromatogram. Second, the chromatogram of thymol and carvacrol was not separated well due to technical restrictions. Further thin layer chromatography should be performed to analyze the active compounds in *T. vulgaris* leaves' ethanolic extract in detail. In addition, statistical analysis should be carried out to measure the effect of T. vulgaris leaves' ethanolic extract concentration on the growth of *S. typhimurium* colonies. It is concluded that *T. vulgaris* leaves' ethanolic extract has the potential as an antibiotic against *S. typhimurium*. Further study is required to investigate the antibacterial effect of *T. vulgaris* leaves' ethanolic extract against multidrug-resistant *S. typhimurium* to develop an alternative therapy for non-typhoidal Salmonella infections, particularly caused by resistant strains of *S. typhimurium*.

## Conclusion

It is concluded that *T. vulgaris* leaves' ethanolic extract has potential as an antibiotic against *S. typhimurium*. Further study is required to investigate the antibacterial effect of *T. vulgaris* leaves' ethanolic extract against multidrug-resistant *S. typhimurium* to develop an alternative therapy for non-typhoidal *Salmonella* infections particularly caused by resistant strains of *S. typhimurium*.

## Acknowledgment

We thank the Rector of Brawijaya University and the Dean of the Faculty of Medicine, Brawijaya University, who supported our study. The laboratory assistant of Slamet Riyanto is appreciated.

## References

1. Chen HM, Wang Y, Su LH et al. (2013) Nontyphoid Salmonella infection: Microbiology, clinical features, and antimicrobial therapy. Pediatrics and Neonatology 54: 147–52. doi: 10.1016/j.pedneo.2013.01.010.
2. Tack B, Vanaenrode J, Verbakel JY et al. (2020) Invasive non-typhoidal Salmonella infections in sub-Saharan Africa: A systematic review on antimicrobial resistance and treatment 18 (212): 1-22. doi: 10.1186/s12916-020-01652-4.
3. Balasubramanian R, Im J, Lee JS et al. (2019) The global burden and epidemiology of invasive non-typhoidal Salmonella infections. Human Vaccines and Immunotherapeutics 15 (6): 1421–6. doi: 10.1080%2F21645515.2018.1504717.
4. Gilchrist JJ, MacLennan CA (2019) Invasive Nontyphoidal Salmonella Disease in Africa. EcoSal Plus 8(2): 10-1128. doi: 10.1128/ecosalplus.esp-0007-2018.
5. Feasey NA, Dougan G, Kingsley RA et al. (2012) Invasive non-typhoidal salmonella disease: an emerging and

neglected tropical disease in Africa. Lancet 379 (9835): 2489–99. doi: 10.1016%2FS0140-6736(11)61752-2.
6. Piccini G, Montomoli E (2020). Pathogenic signature of invasive non-typhoidal Salmonella in Africa: implications for vaccine development. Human Vaccines and Immunotherapeutics 16 (9): 2056–71. doi: 10.1080%2F21645515.2020.1785791.
7. Reddy EA, Shaw AV, Crump JA (2010) Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis. Lancet Infectious Disease 10(6): 417–32. doi: 10.1016/s1473-3099(10)70072-4.
8. Ao TT, Feasey NA, Gordon MA et al. (2015) Global burden of invasive nontyphoidal salmonella disease, 2010. Emerging Infectious Disease 21 (6): 941–9. doi: 10.3201/eid2106.140999.
9. Sinwat N, Angkittitrakul S, Coulson KF et al. (2016) High prevalence and molecular characteristics of multidrug-resistant Salmonella in pigs,pork and humans in Thailand and Laos provinces. Journal of Medical Microbiol. 65 (10): 1182–93. doi: 10.1099/jmm.0.000339.
10. Tadesse G, Tessema TS, Beyene G et al. (2018) Molecular epidemiology of fluoroquinolone resistant Salmonella in Africa: A systematic review and meta-analysis. PLoS One 13 (2): e0192575. doi: 10.1371/journal.pone.0192575.
11. Gordon MA, Graham SM, Walsh AL et al. (2008) Epidemics of invasive *Salmonella enterica* serovar enteritidis and *S. enterica* serovar typhimurium infection associated with multidrug resistance among adults and children in Malawi. Clinical Infectious Diseases 46 (7): 963–9. doi: 10.1086/529146.
12. Amhamdi H, Wathelet JP, Ankit M et al. (2009) Chemical composition of the essential oil of thyme (*Thymus vulgaris*) from Eastern Morocco. International Journal of Agriculture and Biology 11:205–8.
13. Fani M, Kohanteb J (2017) In Vitro Antimicrobial Activity of *Thymus vulgaris* Essential Oil Against Major Oral Pathogens. Journal Evidence-Based Complementary and Alternative Medicine. 22 (4): 660–6. doi: 10.1177/215658721770077.
14. Jianu C (2014) *Thymus vulgaris* essential oil: chemical composition and antimicrobial activity. Journal of Medicine and Life 7 (3):56-60.
15. Sokovicǵ M, Glamočlija J, Marin PD (2010) Antibacterial effects of the essential oils of commonly consumed medicinal herbs using an in vitro model. Molecules 15 (11): 7532–46. doi: 10.3390/molecules15117532.
16. Micucci M, Protti M, Aldini R et al. (2020) *Thymus vulgaris* l. Essential oil solid formulation: Chemical profile and spasmolytic and antimicrobial effects. Biomolecules 10 (6): 860. doi: 10.3390%2Fbiom10060860.
17. Okonkwo CO, Ohaeri OC (2019) Comparative Study of Steam Distillation and Soxhlet for the Extraction of Botanical Oils. Asian Journal of Biological Sciences 13 (1): 62–9.doi: 10.3923/ajbs.2020.62.69.
18. Ulhaq ZS, Hendyatama TH, Hameed F et al. (2021) Antibacterial activity of *Citrus hystrix* toward *Salmonella* spp. infection. Enferm Infecc Microbiol Clin 39 (6): 283–6. doi: 10.1016/j.eimc.2020.05.0.
19. Valle DL, Puzon JJM, Cabrera EC (2016) Thin Layer Chromatography-Bioautography and Gas Chromatography-Mass Spectrometry of Antimicrobial Leaf Extracts from Philippine *Piper betle L.* against Multidrug-Resistant Bacteria. Evidence-Based Complementary and Alternative Medicine 2016: 1–7. doi: 10.1155/2016/4976791.
20. Wahyuni D, Susanti DA, Elmaghfuroh DR (2019) The Physical Properties and Phytochemical Exploration of Bioinsecticide Granules Mixed Betel Leaf Extract (*Piper betle*) And Srikaya Seed Extract (Annonasquamosa). International Journal of Scientific & Technology Research 8 (6): 242-247.
21. Wagner H, Blandt S (2001) Plant Drug Analysis: A Thin Layer Chromatography Atlas. Springer.
22. Forbes BA, Sahm DF, Weissfeld AS (2007) Bailey and Scott's Diagnostic Microbiology. 12th ed. Missouri: Mosby Elsevier. 87–213.
23. Singh A, Bra C, Jeet D et al. (2019) Antibiotic Resistance in *E. coli* Isolated from Poultry. International Journal of Current Microbiology and Applied Sciences 8 (10): 89–94. doi: 2F10.20546%2Fijcmas.2019.810.010.
24. Samein NM, Kamel FH (2019) Extraction of Compounds from Thyme Leaves and Thier Antimicrobial Activity. Diyala Agricultural Sciences Journal (DASJ) 11(2): 18-24. doi: https://doi.org/10.52951/dasj.19110203.
25. Memar MY, Raei P, Alizadeh N (2017) Carvacrol and thymol: strong antimicrobial agents against resistant isolates. Reviews in Medical Microbiology 28 (2): 63–8. doi: 10.1097/MRM.0000000000000100.
27. Palaniappan K, Holley RA (2010) Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. International Journal of Food Microbiology 2010 140(2–3): 164–8. doi: 10.1016/j.ijfoodmicro.2010.04.001.
28. Qi Y, Zhao W, Wang T, et al. (2020) Proteomic analysis of the antimicrobial effects of sublethal concentrations of thymol on *Salmonella enterica* serovar Typhimurium. Applied Microbiology and Biotechnology 104 (8): 3493–505. doi: 10.1007/s00253-020-10390-9.
29. de Almeida de Souza GH, dos Santos et al. (2021) In vitro and in vivo antibacterial activity assays of carvacrol: A candidate for development of innovative treatments against KPC-producing Klebsiella pneumoniae. PLoS One 16 (2): e0246003. doi: 10.1371/journal.pone.0246003.