

Time-kill Study of Ethyl Acetate Extract of Stinging Nettle on *Bacillus subtilis* subsp. *spizizenii* ATCC CRM-6633 Strain NRS 231

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Authors' contributions

This work was carried out in collaboration between all authors. Author AMC performed the study, statistical analysis and wrote the first draft of the manuscript. Author DI designed and managed the study. Author SFS helped us to designed of the methods of extraction and phytochemistry of the study. Author LM wrote some parts of the manuscript and statistical analysis.

Article Information

DOI: 10.9734/ARRB/2015/13571

Editor(s):

(1) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.

Reviewers:

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(4) Anonymous, Nitte University, Mangalore, India.

Complete Peer review History: <http://www.sciencedomain.org/review-history.php?iid=796&id=32&aid=7172>

Original Research Article

Received 23rd August 2014
Accepted 28th October 2014
Published 9th December 2014

ABSTRACT

Aims: This work investigated the antibacterial activity of selected ethyl acetate extract of *Urtica dioica* against *Bacillus subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 (subtilin producer) based on the time-kill approach. Also for further study, the effects of the ethyl acetate extract on *B. subtilis* cells was studied by performing Scanning Electron Microscope (SEM).

Study Design: Prospective

Methodology: According to our previous result, ethyl acetate extract was selected to be more

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potent against *Bacillus subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 based on its inhibition zone diameter. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of ethyl acetate extract against tested bacteria were performed and time-kill study as well as using SEM for further study on structural degeneration of the selected bacterial cells were performed.

Results: The MIC and MBC of the ethyl acetate extract against tested bacteria were found at 8.33 and 16.67 mg/mL from broth micro-dilution assay, respectively. The SEM observations gave ideas on the effects of ethyl acetate extract of *U. dioica* on the growth of *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231. However, the results from SEM suggested that the antibacterial action was due to the internal shrinkage of the cells which collapsed finally. The time-kill approach, determined the kill rate of ethyl acetate extract of *U. dioica* against *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 and significantly inhibited the cell growth and possessed bacteriostatic activity at lower concentration (8.33 mg/mL).

Conclusion: This study showed the potential of *U. dioica* extract as alternative therapy against bacterial infection.

Keywords: Stinging nettles; *Urtica dioica*; ethyl acetate extract; time-kill assay; Scanning Electron Microscopy (SEM).

1. INTRODUCTION

The prevalence of resistant bacteria in the community and hospitals cause severe problems for treatment of patients [1]. The demand for new antimicrobial drugs is higher than last decades because of the appearance of multi-drug resistance in common bacteria [2]. Natural products have been a rich source of antibacterial drugs for many decades, but investments in this area have decreased in the last two decades [3]. Researchers found literally thousands of phytochemical from plants as safe and generally effective alternatives with less adverse impact in recent years [4]. Also *Bacillus subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 produces small quantity of unsuccinylated subtilin. Subtilin (from lanbiotic) is lanthionine-containing peptides which showed antimicrobial like pheromone-like autoinducing activity [5].

Stinging nettle (*Urtica dioica* L.) from Urticaceae family has been used for a long period of time as a medicinal herb in many part of the world especially in Middle East [6,7]. However, this plant is annual and perennial herb which is recognized with its stinging hair, and is used to treat gastritis in Turkish folk medicine [8], and in Iran [9,10]. Besides, it is used to treat rheumatic pain and for common colds and coughs [11].

The present investigation showed the effect of ethyl acetate extract of *U. dioica* against *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 which previously was selected as one of our effective and potent crude extract

based on its high antimicrobial and antioxidant activities [12,13] at ultra-structural level through SEM observation at 8.33 mg/mL concentration with *in vitro* time killing. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values of the extract against *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 were also performed and the time-killed curved as well as structural degeneration of these bacterial cells were studied. The aim of this present study to investigate the antibacterial effect of ethyl acetate extract of *Urtica dioica* followed by effect of subtilin to reach bactericidal effect (synergistic effect) against *Bacillus subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 based on the time-kill approach to determine the kill rate of this extract against selected bacteria as well as structural degeneration of bacterial cells were monitored and studied.

2. MATERIALS AND METHODS

2.1 Preparation of the Extract

The leaves of *U. dioica* were collected from Salmanshahr city in Mazandaran province in Iran in August 2007. The Voucher specimens were deposited by the code of 6725-TEH at the Herbarium of the University of Tehran (Iran) in 2010.

The leaves were washed, rinsed and dried under sunlight before ground to powder form. The dried samples were grinded into powder form. Then the residue of the sample was allowed to dry off

before extraction. The Soxhlet apparatus was selected for the method of extraction by following solvents from non-polar to polar [14]. These solvents were namely; hexane, chloroform, ethyl acetate and methanol extract. The dried ethyl acetate extract was weighed and then kept in 4°C. The ethyl acetate was selected due to its high antioxidant and antimicrobial activity.

2.2 MIC and MBC Determination

The broth micro-dilution assay was conducted for this study to detect minimum inhibitory concentration (MIC). The bacterial extract at two-fold dilution was prepared with sterile medium. Then 100 µL of ethyl acetate extract was distributed into each well (96 microtiter plate). Later 100 µL of bacteria inoculums (approximately 1.0×10^5 CFU/mL bacterial cells) was dispensed to each well and volumes of each well become 200 µL as a final. For positive control, Amoxicillin was selected and a negative control (with 5% DMSO) and bacterial inoculums were used for the study. About 24h of incubation in rotary shaker at 37°C, 40 µL of 0.2 mg/mL p-iodonitro tetrazolium violet salt (INT) (Sigma) which dissolved in 99.5% ethanol was added to each well. The MIC was evaluated as the lowest concentration of the ethyl acetate extract where the colour of INT shifted from yellow to purple and at this point inhibited the growth of selected bacteria. For detecting minimum bactericidal concentration (MBC), after recording of MIC value at 24 h, the viable cells of bacteria were seeded on SDA plates by a standard plates count. Then inoculated plates were placed at incubator at 37°C for 24 h. The MBC also was recorded as the lowest concentration of ethyl acetate extract against *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 which occurred in 99.9% growth reduction [15].

2.3 Time-kill Assay

A time-kill study was used in order to observe the dynamic pattern of bactericidal activity of ethyl acetate extract of *U. dioica*. A series of concentrations were performed for this experiment, varied from range of half of the MIC ($\frac{1}{2}$ MIC), MIC and double time of MIC (2MIC) with NB broth against *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231.

After 16 h initial incubation period, bacterial cells grown on NA plates were distributed in 10 mL of sterile distilled water and were adjusted the

turbidity was compared with 0.5 McFarland standard solutions at 1.0×10^5 cell/mL. 1.0 of inocula plus 1.0 of ethyl acetate extract was mixed to aliquots of 10 mL Nutrient Broth (NB) in a flask that obtained the final concentration at retained ratio for *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 were 4.16 mg/mL ($\frac{1}{2}$ MIC), 8.33 (MIC) and 16.67 (2MIC). All flasks were shaken at 150 rpm at 37°C for 48 h. Also the test was performed in triplicate [16,17]. Viable cells were calculated every 4 h for 48 h to give exact cfu/mL, meanwhile kill curves were plotted with time against the logarithm of the viable cell count. In order to accurately, each experiment was carried out twice on separate occasions. Generation time is following by formula by Todar (2012) [18]; (G) is expressed as the time, (t) per generation. Hence, $G = t/n$ is equation for calculations of generation time; while (G) is showed generation time, (t) is expressed as the time and (n) as a number of generations.

n (number of generations) =

$$\frac{(\log \text{ cells at the end of incubation}) - (\log \text{ cells at the beginning of incubation})}{0.301}$$

2.4 Preparation of bacteria cells for Scanning Electron Microscope (SEM)

For the preparation of bacterial cells as a sample for SEM analysis, 1 mL aliquots of bacterial cell suspension (1×10^5 cell/mL) were seeded on Nutrient Agar plates from the culture bottles under sterile conditions. The bacteria cell suspensions were taken at the late exponential growth phase and were incubated at 37°C for 24 h. For samples, two mL of the extract at concentration of MIC value, 8.33 mg/mL was pipette onto the NA plate and was incubated for 12, 24 and 36 h at 37°C. The plate without any treatment was performed as control cells. After period of times, a small block around 1.0 cm³ of NA (containing bacterial cells) was cut at 0, 12, 24 and 36 hours and was fixed for further observation under SEM (Fesem Leo Supra 50 VP, Carl Zeiss, Germany).

3. RESULTS AND DISCUSSION

Based on various different studies, some medicinal plants might indeed be capable sources of new antibacterial agents even against some antibiotic-resistant strains [19,20]. The antimicrobial activity of *U. dioica* may due to the

presence the activity of 5-hydroxytryptamine (5-HT) [21,22]. Lass-Flörl et al. [23] and Perkhofer et al. [24] found antifungal activity of 5-HT (serotonin) against *Candida* and *Aspergillus* species, respectively. Hence, serotonin may affect growth inhibition by disrupting cell membrane synthesis.

Ethyl acetate extract of *U. dioica* showed bacteriostatic effect against *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231. Based on the graph (Fig. 1), it is expected that the increase in concentration up to 4 times MIC, 8 times MIC and above would cause bigger log cycle reduction and therefore drop the extract near the bactericidal effect. A quantity of biologically active compounds can saturate more target sites and cause rapid bactericidal action against the bacteria [25]. The MBC value was found at 16.67 mg/mL.

The time-kill study was performed over a period of 48 h with bacteria being exposed to ½MIC, MIC and 2MIC values. In this research, a time-kill assay was carried out against *B. subtilis* subsp. *spizizenii* ATCC 6633 with ethyl acetate extract of *U. dioica* to determine whether this extract had antibactericidal activity. Fig. 1 shows the viable cells of *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 were reduced by 5.57 log₁₀ CFU/mL within 16 h after exposure to MIC and 2 times the MIC of ethyl acetate extract of *U. dioica*. Although, control (without ethyl acetate

extract) showed a 0.82 log₁₀ increase in CFU/mL by 12 hours. Bacterial cultures were monitored for up to 48 h, and no regrowth was observed. At control and ½MIC, ethyl acetate extract did not reduce bacterial CFU during first 24 hour after incubation. At MIC and 2MIC, ethyl acetate extract showed a 1 log₁₀ CFU/mL decrease in colony counts after 12 h but at MIC (8.33 mg/mL) with a subsequent minor regrowth of 0.14 log₁₀ CFU/mL from 16 h to 24 h. However, the bactericidal effect was achieved at MIC and 2MIC after 16h by this extract. The results showed that the addition of the extract definitely affected the growth of the tested bacteria. The higher concentration of the extract added, the severe inhibition to the cells occurred.

The results for confirmation of the MBC value by spread plating is shown in Table 1 which shows mean counts and reductions achieved after exposing the *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 to different concentration of ethyl acetate extract for 24 and 48h. In contrast, when investigating the MBC of *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231, the result showed a bit higher than our confirmation obtained from Estimated Reduction (ER). The MBC of this bacteria was 16.66 mg/mL but compared to ER, achieved at 8.33 mg/mL after 48 h (for 24 h, the value of ER showed 33.33 mg/mL at 2MIC, but still less than 4 log₁₀ CFU/mL).

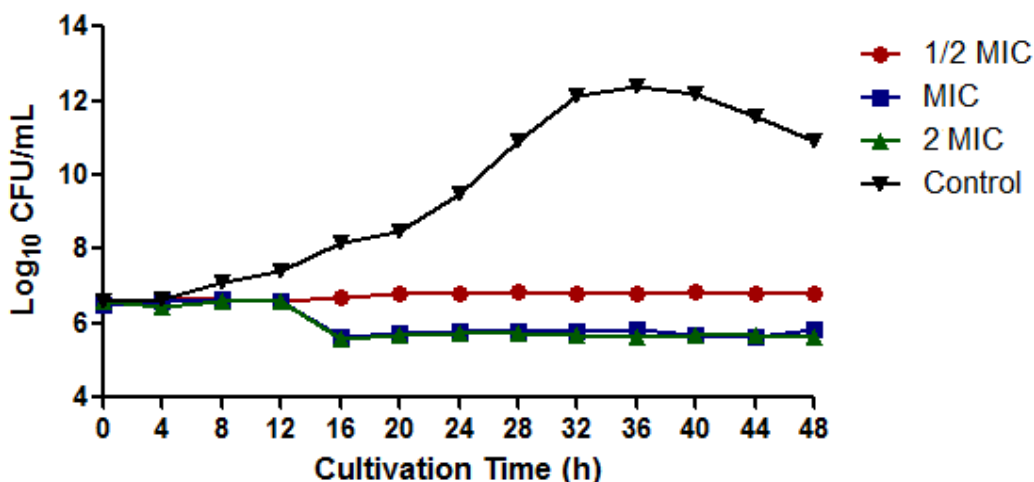


Fig. 1. Representative time-kill curve plot of ethyl acetate extract of *U. dioica* against *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 at the following concentrations: control (without extract); 4.16 mg/mL (½MIC); 8.33 mg/mL (MIC) and 16.67 mg/mL (2MIC)

Table 1. Estimated reduction and mean populations of *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 against ethyl acetate extract of *U. dioica*

	Ethyl acetate extract of <i>U. dioica</i> against <i>B. spizizenii</i> ATCC 6633			
	Mean ± SEM		ER	
	24 h	48 h	24 h	48 h
Control	9.494±0.02	10.893±0.01	NA	NA
2 MIC	5.710±0.20	5.657±0.07	3.8	5.2
MIC	5.792±0.02	5.822±0.12	3.7	5.1
½ MIC	6.821±0.03	6.808±0.03	2.7	4.1*

NA = Not applicable, Control = Initial inoculum, ER = Estimated reduction = (Log₁₀ CFU/mL of control at 24/48 h) – (Log₁₀ CFU/mL after 24/48 h of exposure to the treatment, * MBC since > 4 log₁₀ CFU/mL reduction was obtained

Fig. 2, shows the results from SEM study. The untreated cells (A) show a normal and typical *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 with rod shaped and regular surface appearance that remains unaltered. The individual cells were normal and some were

undergoing dividing process which finally from new rod-shaped cells that were seen to be uniformly next to each other.

The concentration used was 100 mg/mL. Control bacteria (10000×) (A); *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 treated for 12 hours (20000×) (B); *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 treated for 24 hours (10000×). The arrows indicated the formation of cavities on bacteria cell (C) and *B. subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 treated for 36 hours (20000×). The arrows indicated the formation of cavities on the bacterial cell (D).

Fig. 2 B shows the 12 hour of ethyl acetate extract treated cells which had undergone some changes in their cell morphology. The cell surface demonstrated irregularities and the rod-shaped cells began to shrink. These conditions if contrasted with the control cells, was indicating that the concentration of the extract affect on the cells.

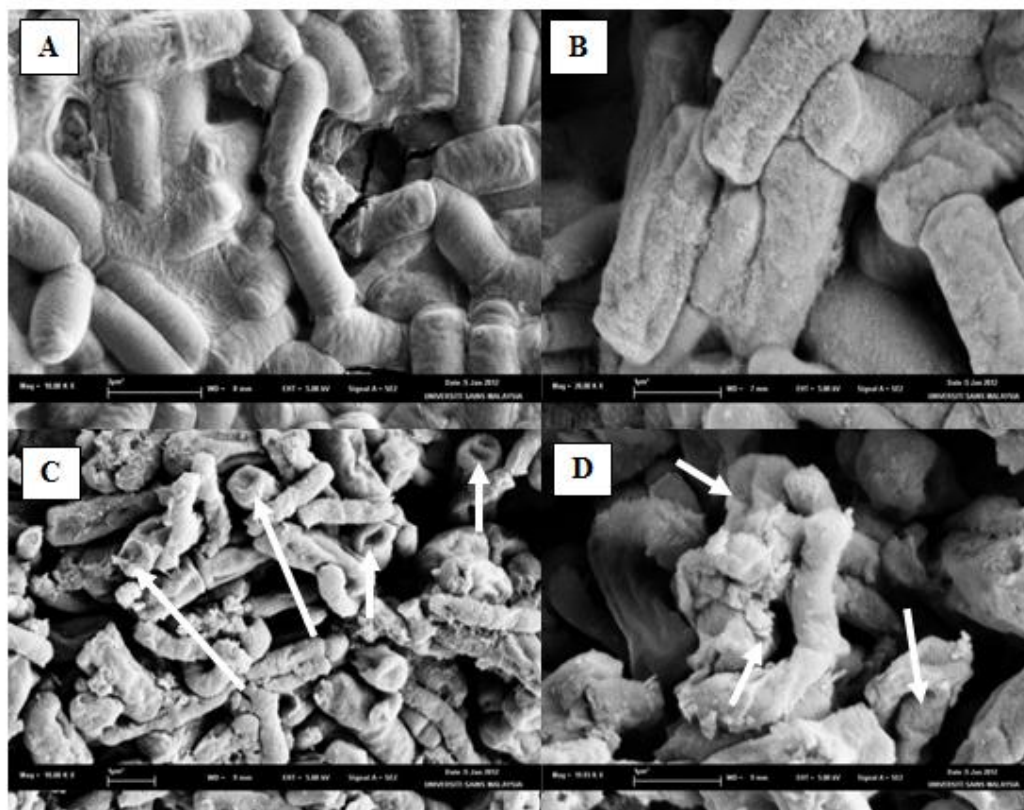


Fig. 2. SEM micrographs on the effect of ethyl acetate extract of *U. dioica* on *Bacillus subtilis* subsp. *spizizenii* ATCC® CRM-6633™ Strain NRS 231 cells at different exposure time

At 24 hours of exposure to the extract (Fig. 2 C), the cells became swollen and altered in its shaped. The shape of the cells became irregular and values in shape and sizes. Some of the cells were shrunken and collapsed. In fact, some of the cells were necrotic. AT 36 hours of exposure (Fig. 2 D) the cells became shrunken and seem to lose their structure and their ability to function normally and eventually unable to resume their growth. The cells became lysed and crumpled revealing that the severity of damages occurred to the cell. The cells showed significant morphological changes with the formation of collapsed cells. At least, only dead cells were observed and this condition was beyond repair.

From our previous study, we found out that this extract showed moderate total phenolic and flavonoid contents at 31.75 ± 0.59 GAE/g extract and 36.19 ± 2.37 QE/g extract, respectively [12]. However, the antimicrobial mode of action is related to the phenolic compounds. The relationship between phenolics compounds and their antimicrobial activity has been reported. Also flavonoids as potent and major therapeutics has been well distinguished [26] and some researchers such as Cushnie and Lamb [27] found that flavonoids can related to damage of bacterial membrane. In this case, flavonoids would help an increase in the permeability of the inner bacterial membrane including loss of membrane potential. There is no general consensus on the actual mechanisms underlying flavonoids antibacterial activities [28].

Some studies also mentioned that oxidized phenols inhibit metabolic enzymes causing an inactivation of the reproductive activity of the cell [29]. Phenolic compounds interact with membrane function through electron transport, protein and nucleic acid synthesis, enzyme activity and nutrient uptake. It seems that chelation of transition reactive metals ions such as iron and copper, by them reduces bioavailability for bacterial growth [30]. Some studies revealed that the Gram positive bacteria are more sensitive than Gram negative bacteria.

Cáceres et al. [31] found that *Urera baccifera* from same family exhibited no antimicrobial activity against some pathogenic microorganisms. Their study on *U. dioica* extracts showed same results on tested microorganisms. Dulger and Gonuz [32] were tested 80% ethanol of *U. dioica* extract on some tested bacteria but extract did not show any inhibition zone. In other study by Steenkamp et

al. [33] on *U. urens* (Urticaceae family), they found this plant no possess antimicrobial activity which their results supports other studies [34,32]. However, Janssen and Scheffer [35] found that *Urtica dioica* had noticeable antibacterial activity against *Staphylococcus aureus*. Our previous results demonstrated that the ethyl acetate and hexane extract exhibited highest inhibition against some pathogenic bacteria such as *B. subtilis*, *B. cereus*, MRSA and *Vibrio parahaemolyticus* [13]. The mode of action of the ethyl acetate extract against *B. subtilis* was not studied, but it could be assumed that the effects of the ethyl acetate extract of *U. dioica* was exerted on the outer membrane of the cell wall which then changed the membrane structure and permeability of the cell. According to Zao et al. [36], this alteration of the membrane structure may be referred to the breakage of the hydrogen bonds which functions in maintaining the hardness of the membrane. Further study is needed for the isolation and identification of bioactive compounds including the *in vivo* evaluation of antimicrobial activity, along with toxicity experiments, present in this extract before it is used for marketing in the making of pharmaceutical drugs.

4. CONCLUSION

From the above results, it can be concluded that the ethyl acetate extract derived from *U. dioica* leaves and young stems could be considered as potential antimicrobial agent with its possible applications in pharmaceutical industries.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Maya J, Chan CH, King A, Williams L, French GL. Time-kill studies of tea tree oils on clinical isolates. J. Antimicrob. Chemother. 2000;45(5):639-643. PMID: 10797086.
2. Spellberg B, Powers JH, Brass EP, Miller LG, Edwards JE Jr. Trends in antimicrobial drug development: Implications for the future. Clin. Infect. Dis. 2004;38(9):1279-1286. PMID: 15127341.
3. Brown DG, Lister T, May-Dracka TL. New natural products as new leads for antibacterial drug discovery. Bioorg. Med.

- Chem. Lett. 2014;24(2):413-418. PMID: 24388805.
4. Sasidharan S, Chen Y, Saravanan D, Sundram KM, Latha LY. Extraction, isolation and characterization of bioactive compounds from plants` extracts. Afr. K. Tradit. Compliment. Altern. Med. 2011;8(1):1-10. PMID: PMC3218439.
 5. Fuchs SW, Jaskolla TW, Bochmann S, Kötter P, Wichelhaus T, Karas M, Stein T, Entian KD. Entianin, a novel subtilin-like lantibiotic from *Bacillus subtilis* subsp. spizizenii DSM 15029^T with high antimicrobial activity. Appl. Environ. Microbiol. 2011;77(5):1698-1707. DOI: 10.1128/AEM.01962-10.
 6. Chaurasia N, Wichtl M. Flavonol glycosides aus *Urtica dioica*. Planta Med. 1987;53:432-434.
 7. Ibrahim D, Modarresi Chahardehi A, Sulaiman SF. Effects of butanol extract of *Urtica dioica* on MRSA: Structural degeneration study. Journal of Applied Biological Sciences. 2013;7(3):39-44.
 8. Gülçin İ, Küfrevioğlu Öİ, Oktay M, Büyükkuroğlu MF, Büyükkuroğlu MF. Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica* L.). J. Pharmacol. 2004;90:205-215. PMID: 15013182.
 9. Pourmorad F, Hosseinimehr SJ, Shahabimajid N. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. Afr. J. Biotechnol. 2006;5:1142-1145.
 10. Monfared M, Kamkar A, Ghaffari-Khaligh S, Jebelli-Javan A, Asadi F, Akhundzadeh-Basti A. Antioxidative effects of Iranian *Urtica dioica* L. extracts on the oxidation of sunflower oil. J. Med. Plants Res. 2011;5:4438-4445.
 11. Sezik E, Yeşida F, Tabata M, Honda G, Takaishi Y, Fujita T, Tanaka T, Takeda Y. Traditional medicine in Turkey VIII. Folk medicine in East Anatolia Erzurum Ağrı, kars, Iğdır provinces. Econ. Bot. 1997;51:195-211.
 12. Modarresi Chahardehi A, Ibrahim D, Sulaiman SF. Antioxidant activity and total phenolic content of some medicinal plants in Urticaceae family. Journal of Applied Biological Sciences. 2009;2(3):01-05.
 13. Modarresi-Chahardehi A, Ibrahim D, Fariza-Sulaiman S, Mousavi L. Screening antimicrobial activity of various extracts of *Urtica dioica*. Rev. Biol. Trop. (In. J. Trop. Biol). 2012;60(4):1567-1576.
 14. Das K, Tiwari RKS, Shrivastava DK. Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. J. Med. Plants Res. 2010;4(2):104-111.
 15. Jorgensen JH, Turnidge JD. Susceptibility test methods: Dilution and disk diffusion methods, In Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA. (ed.), Manual of clinical microbiology, 9th ed. ASM Press, Washington, D.C. 2007;1152-1172.
 16. Sasidharan S, Ibrahim D, Jain K. Screening of antimicrobial activity of various extracts of *Gracilaria changii*. Pharm. Biol. 2009;47:72-76. DOI: 10.1080/13880200802434161.
 17. Darah I, Lim CL, Nurul Aili Z, Nor Afifah S, Shaida Fariza S. Effects of methanolic extract of a soft sponge. *Haliclona* sp. on bacterial cells: structural degeneration study. Pharmacie Globale (IJCP). 2011;7(03):1-6.
 18. Todar K. Todar`s online textbook of bacteriology; 2012. Available:http://textbookofbacteriology.net/growth_3.html
 19. Kone WM, Atindehou KK, Terreaux C, Hostettmann K, Traore D, Dosso M. Traditional medicine in North Cote-d'Ivoire: Screening of 50 medicinal plants for antibacterial activity. J. Ethnopharmacol. 2004;93:43-49. PMID: 15182903.
 20. Thakare M. Pharmacological screening of some medicinal plants as feed additives. Master of science thesis, Virginia Polytechnic Institute and State University. 2004;21-24.
 21. Collier HOJ, Chesher GB. Identification of 5-hydroxytryptamine in the nettle sting. Br. J. Pharmacol. 1956;11:186-189. PMID: 13329337.
 22. Kulze A, Greaves M. Contact urticaria caused by stinging nettles. Br. J. Dermatol. 1988;119:269-270. PMID: 3166946.
 23. Lass-Flörl C, Fuchs D, Ledochowski M, Speth C, Dierich MP, Würzner R. Antifungal properties of 5-hydroxytryptamine (serotonin) against *Candida* species in vitro. Journal of Medical Microbiology. 2003;52(2):169-171. DOI:10.1099/jmm.0.04987-0.
 24. Perkhofer S, Niederegger H, Blum G, Burgstaller W, Ledochowski M, Dierich MP, Lass-Flörl C. Interaction of 5-hydroxytryptamine (serotonin) against

- Aspergillus* spp. in vitro. Int. J. Antimicrob. Agents. 2007;29(4):424-429. DOI: 10.1016/j.ijantimicag.2006.12.003.
25. Satta G, Cornaglia G, Mazzariol A, Golini G, Valisena S, Fontana R. Target for bacteriostatic and bactericidal activities of β -lactam antibiotics against *Escherichia coli* resides in different penicillin-binding proteins. Antimicrob. Agents Chemother. 1995;39:812-818. PMID: 7785976.
 26. Ratnam DV, Ankola DD, Bhardwaj V, Sahana DK, Ravi Kumar MNV. Role of antioxidants in prophylaxis and therapy: A pharmaceutical perspective. J. Control Release. 2006;113:189-207. PMID: 16790290.
 27. Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. Int. J. Antimicrob. Agents. 2005;26:343-356. PMID: 16323269.
 28. Draughon FA. Use of botanicals as biopreservatives in foods. Food Technol. 2004;58(2):20-28.
 29. Cuervo-Pliego MP. Effect of natural antimicrobials against *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*. Thesis of Master of Science, Texas A&M University. USA. 2007;8:26.
 30. Sato M, Ramarathnam N, Suzuki Y, Ohkubo T, Takeuchi M, Ochi H. Varietal differenced is the phenolic content and superoxide radical scavenging potential of wines from different sources. J. Agric. Food Chem. 1996;44:37-41.
 31. Cáceres A, Giron LM, Alvarodo SR, Torres MF. Screening of antimicrobial activity of plants popularly used in Guatemala for the treatment of dermatomucosal disease. J. Ethnopharmacol. 1987;20:223-237. PMID: 3682847.
 32. Dulger B, Gonuz A. Antimicrobial activity of certain plants used in Turkish traditional medicine. Asian J. Plant Sci. 2004;3(1):104-107.
 33. Steenkamp V, Mathivha MC, Gouws MC, van Rensburg CE. Studies on antibacterial, antioxidant and fibroblast growth stimulation of wound healing remedies from South Africa. J. Ethnopharmacol. 2004;95(2-3):353-357. PMID: 15507359.
 34. Rabe T, van Staden J. Antibacterial activity of South African plants used for medicinal purposes. J. Ethnopharmacol. 1997;56:81-87. PMID: 9147258.
 35. Janssen AM, Scheffer J. Acetoxychavicol acetate, an antifungal component of *Alpinia galangal*. Planta Med. 1985;51:507-511. PMID: 17345272.
 36. Zao HX, Rinaldi AC, Rufo A, Bozzi A, Kinnunen PKJ, Di-Giulio A. Structure and charge requirements for antimicrobial peptide insertion into biological and model membranes. In: Memestrina G, Dalla-Serra M, Lazarovici P. (eds.) Pore Forming Peptides and Protein Toxins. New York, Harwood Academic Publishers. 2003;151-177.

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