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Abstract

Background: It was previously shown that some parts of *Aquilaria agallocha*, which is commonly known as oud or oodh, such as roots have been used as a traditional medical herbal in different countries. In Turkey *A. agallocha* is one of the ingredients while preparing famous Mesir paste, which was invented as a medicinal paste and used from the Ottoman period to now at least for 500 years. The identification of the *in vitro* antimicrobial activity of ethanol extract of *A. agallocha* roots is main purpose of this analysis.

Materials and Methods: By using 17 bacteria and 1 fungi, which include *Bacillus*, *Candida*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Listeria*, *Pseudomonas*, *Salmonella* and *Staphylococcus* genera, the activity of *A. agallocha* root extracts were analysed by the help of the disk diffusion method, that is one of the methods commonly used to determine antimicrobial activities.

Results: As a result of the study it was observed that ethanol extracts of *A. agallocha* roots have a clear antimicrobial activity against nearly all microorganism used in the study, but only two bacteria namely *E. coli* ATCC 25922 and *S. typhimurium* SL 1344.

Conclusion: According to the disk diffusion test results it may be possible to propose that *A. agallocha* roots should have a medicinal uses especially against *E. faecium*, *L. monocytogenes* ATCC 7644, *B. subtilis* DSMZ 1971, *C. albicans* DSMZ 1386, *S. epidermidis* DSMZ 20044 and *S. aureus* ATCC 25923.

Keywords: *Aquilaria agallocha*, Mesir paste, antimicrobial activity, antimicrobial screening, ethanol extract.

Introduction

In several places of the world, there are increasing researches for determination of the unknown activity of medicinal plant (Abbasi et al., 2010). In consequence of the expressly enhancement in infections at developing countries, new explorations for new antimicrobial agents is required. This ingredient is largely significant for medically indigent populations due to extensive bacterial resistance to current antibiotics (Okeke et al., 2005). Natural products have an antimicrobial potential which can be comparable to modern antibiotics and this situation is investigated several decades (Altuner et al., 2010; Clardy and Walsh, 2004).

Humankind without scientific knowledge discovered new treatment methods through trial and error method in the history (Karasu, 2015). Mesir paste is a traditional special mixture founded during Ottoman period about 500 years ago as a medicine, which contains different types of spices and herbs, including *Crocus sativus* (saffron crocus), *Zingiber officinale* (Ginger), *Terminalia citrina* (black chuglam or citrine myrobalan), *Cuminum cyminum* (Cumin) and *Aquilaria agallocha* (oud or oodh). All ingredients of Mesir paste have been used for several diseases healer in Turkish folk medicine for many centuries (Oskay et al., 2010). In addition to this, especially the synergistic antimicrobial effect thought to be the reason of Mesir paste's healing effect.

World Health Organization (WHO) has published a remarkable report in 2007 which not only mentions the microbial evolution but also the possible impact of increasing frequency in resistance against antibiotics for the next century. And these points are described as the critical problem for public health affair (Syed et al., 2010; WHO, 2007). In order to prevent spreading of antibiotic resistant infections, characterization of new antimicrobial substances is significant for intensive researches of scientists (Paudel et al., 2008; Ozkan et al., 2015).

Medical plant is used for thousands of years to different diseases healer related to bacterial and fungal infections and this situation was investigated in huge number of study (Jones, 1996). Current researches represented that medicinal plants have production potential for new antibiotic drug (Cos et al., 2006).

In this study, *A. agallocha* roots, which is one of the ingredients of Mesir paste, ethanol extract is analysed against 17 bacteria and 1 fungi with the disk diffusion method. Although Dash et al. (2008) analysed *A. agallocha* leaf and bark, which is aqueous and methanol extracts, with agar well method against *Bacillus brevis*, *B. subtilis*, *P. aeruginosa* and *S. flexneri*, our analyses are the first report for the activity of *A. agallocha* roots.

Materials and Methods

Extraction procedure

A. agallocha root samples were purchased from a local company. By using a pestle and a mortar, these samples were ground. Extraction solvent, which is ethanol (Sigma-Aldrich) was selected for receive active substances. Ground samples at room temperature were shaken in the ethanol for 3 days at 90 rpm. Whatman No. 1, which is filter paper, is used in order to extract

filtration into evaporation flasks. By using rotary evaporator (Buchi R3), the filtrate was evaporated at 35°C. The residues were aggregated and it is used to prepare 385 mg.mL⁻¹ of ethanolic extracts after evaporation.

Strains

For the analyses of *A. agallocha* roots, a broad collection of several Gram (-) and Gram (+) strains were chosen. These strains are *Bacillus subtilis* DSMZ 1971, *Candida albicans* DSMZ 1386, *Enterobacter aerogenes* ATCC 13048, *Enterococcus durans*, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium*, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Listeria innocua*, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* DSMZ 50071, *Pseudomonas fluorescence* P1, *Salmonella enteritidis* ATCC 13075, *Salmonella infantis*, *Salmonella kentucky*, *Salmonella typhimurium* SL 1344, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* DSMZ 20044.

Preparation of the strains for the test

All strains were incubated according to their requirements as it was previously mentioned by Canli et al (2015). For the inocula, physiological saline was used and to fix the number of the colonies in the solution it was compared to 0.5 McFarland (Hammer et al., 1999; Altuner et al., 2012).

Disk diffusion test (DDT)

The DDT was applied as it was mentioned in the previous studies (Andrews, 2003). Petri dishes having the same dimensions, which contain equal amount of Mueller Hinton Agar were used in order to standardize the work as described previously by several studies (Ilhan et al., 2006). Thirty and sixty microliters of *A. agallocha* root solutions which contain 11,550 µg and 23,100 µg of extract respectively were loaded on standard sterile cellulose disks (SD) which are regularly used in DDT (Mahasneh and El-Oqlah, 1999; Silici and Koc, 2006). Disks were kept at 40 °C for 24 h in sterile conditions to evaporate ethanol in the extracts as it was stated by Altuner et al (2012) previously. Saline suspension of microorganisms is used for inoculation on the surfaces of the plates. After application of the strains on plates, they were kept in aseptic conditions for couple of minutes before implementing the samples as described in the previous studies (Altuner et al., 2012). Results were identified in mm by the method mentioned by Altuner et al (2012).

Controls

Empty SD and ethanol were utilized as negative controls. Ethanol was loaded on SD first and then removed by evaporating as it was described in previous sections. On the other hand ciprofloxacin 5 µg is used as a positive control to discuss the results.

Statistics

In order to conduct a statistical analysis all tests were done in three parallels. All the results given were the mean values for these parallel studies. To accept the results as significant p was accepted to be lower than 0.05.

Discussion

Antimicrobial activity of *A. agallocha* root ethanol extracts were analysed in our study. SD were used for application of extracts, then they were used for DDT. The results for DDT were given in Table 1. Empty SD and ethanol loaded SD, which were negative controls, had no activity.

Although Dash et al. (2008) studied the antibacterial properties of the leaf and bark of *A. agallocha* by agar well method against *Bacillus brevis*, *B. subtilis*, *P. aeruginosa* and *S. flexneri* previously, our analysis are the very first report of ethanol extracts of *A. agallocha* roots for its antimicrobial activity. Dash et al. (2008) identified that methanol extracts of leaf has an antimicrobial activity only against *B. subtilis* and methanol extracts of bark observed no activity, our study clearly show that ethanol extracts of *A. agallocha* roots are active against most of the strains tested in terms of its antimicrobial activity.

Intensive care units (ICU) are very serious places for especially nosocomial infections (NI). *S. aureus* is known as one of the common pathogens causing NI especially in ICU (Richards et al., 1999). There are *S. aureus* strains related researchers for investigate antimicrobial activity of some plant extracts. For example, Nair and Chanda (2007) compared 10 medicinal plants antimicrobial effects on *S. aureus* strains, namely *Anethum graveolens*, *Commiphora wightii*, *Emblica officinalis*, *Ficus benghalensis*, *Ficus racemosa*, *Ficus religiosa*, *Ficus tisele*, *Hibiscus cannabinus*, *Mentha arvensis* and *Mimusops elengi*. In this study maximum IZ of ethanol extract was shown by *E. officinalis* with 9 mm. In the present study a 12 mm zone for 11,550 µg of *A. agallocha* roots and 13 mm zone for 23,100 µg of *A. agallocha* roots against *S. aureus* were observed. As the results for disk diffusion tests are compared it is easy to see that *A. agallocha* roots are active against *S. aureus* when compared to some other higher plants. But this result should be supported by MIC values.

Gram (+) microorganisms are known to be more sensitive than Gram (-) microorganism against antibiotics (Faucher and Avril, 2002; Nikaido, 1998). *K. pneumonia* is another important Gram (-) strains which can be found in ICU causing extremely serious infections (Villegas and Quinn, 2004). Ates and Erdogru (2003) analysed 26 mg *Juniperus oxycedrus* ethanol extract and it caused 9 mm of IZ against this strain. However, in this study a 9 mm of IZ was observed with 11,550 µg of *A. agallocha* roots extract. By comparing these studies, ethanol extracts of *A. agallocha* roots have higher antimicrobial activity against *K. pneumonia* than *Juniperus oxycedrus*, because our analysis have lower amount of substance.

Table 1: Disk diffusion test results of 17 bacteria and 1 fungi which are sorted starting from the highest antimicrobial activity to the lowest. (Inhibition zones (IZ) in mm)

	30µL	60µL	Ciprofloxacin
<i>E. faecium</i>	18	20	28
<i>L. monocytogenes</i> ATCC 7644	17	19	20
<i>B. subtilis</i> DSMZ 1971	15	15	36
<i>C. albicans</i> DSMZ 1386	15	15	-
<i>S. epidermidis</i> DSMZ 20044	14	14	34
<i>S. aureus</i> ATCC 25923	12	13	22
<i>E. durans</i>	9	9	24
<i>K. pneumonia</i>	9	9	30
<i>P. aeruginosa</i> DSMZ 50071	9	9	28
<i>E. faecalis</i> ATCC 29212	8	9	19
<i>E. aerogenes</i> ATCC 13048	8	8	30
<i>P. fluorescens</i> P1	8	8	19
<i>S. enteritidis</i> ATCC 13075	8	8	36
<i>L. innocua</i>	7	8	18
<i>S. infantis</i>	7	7	24
<i>S. Kentucky</i>	-	7	34
<i>E. coli</i> ATCC 25922	-	-	-
<i>S. typhimurium</i> SL 1344	-	-	35

“-”: No inhibition

The pathogenicity of *B. subtilis* is normally very important only for immunocompromised patients (De Boer and Diderichsen, 1991; Galièni and Bigazzi, 1998). There are several different studies about anti-infective properties of several compounds from plant origin on these strains. For example, Parekh et al. (2005) used six plants, *Acyranthus aspera*, *Calotropis gigantea*, *Carissa congesta*, *Fagonia cretica*, *Mangifera indica* and *Rauwolfia serpentina* for this purpose. They observed 14 mm and 11mm of IZs in *M. indica* and *C. congesta* respectively, whereas 10 mm of IZs in *A. aspera*, *C. gigantea*, *F. cretica* and *R. serpentina*. In this study a 15 mm IZ was detected both for 11,550 µg and 23,100 µg of *A. agallocha* roots extract. As a result it can be concluded that *A. agallocha* roots may have a potential of using against *B. subtilis* infections.

Conter et al. (2009) proposed that *L. monocytogenes* is a microorganism which are sensitive to the antibiotics which is generally used in human listeriosis treatment, but *L. monocytogenes* is slowly becoming antibiotic resistant. There are researches about effective treatment of human listeriosis which is related to antimicrobial resistance of this pathogen. Therefore antibacterial activity against *L. monocytogenes* is significant. Ates and Erdogru (2003) identified that ethanol extract of *Juniperus oxycedrus* caused 7 mm of inhibition zone against *L. monocytogenes* whereas *Cinnamomum cassia*, *Glycyrrhiza glabra*, *Coriandrum sativum* and *Pimpinella anisum* observed no activity. In this work a 17 mm of IZ was found for 11,550 µg of *A. agallocha* roots and 19 mm IZ for 23,100 µg of *A. agallocha* roots. Comparing these results clearly presents that *A. agallocha* roots are highly active against *L. monocytogenes*.

Conclusion

Consequently, *A. agallocha* roots have obviously antimicrobial activity against most of tested strains. Our study clearly presents that *A. agallocha* roots should have a possible medicinal uses especially against *E. faecium*, *L. monocytogenes* ATCC 7644, *B. subtilis* DSMZ 1971, *C. albicans* DSMZ 1386, *S. epidermidis* DSMZ 20044 and *S. aureus* ATCC 25923.

However, further researches are needed in order to analyse the active substances and their activity mechanisms in details and also the MIC values.

On the other hand while comparing the results with the previous studies, it should always kept in the mind that the results may differ due to geographical differences of the places where the plant samples were collected from.

References

1. Abbasi, A. M., Khan, M.A., Ahmad, M., Jahan, S. & Sultana, S. (2010). Ethnopharmacological application of medicinal plants to cure skin diseases and in folk cosmetics among the tribal communities of North-West Frontier Province. Pakistan. J Ethnopharmacol, 128: 322–335.
2. Altuner, E. M., Akata, I. & Canli, K. (2012). *In vitro* antimicrobial screening of *Cerena unicolor* (Bull.) Murrill (Polyporaceae Fr. Ex Corda). Fresenius Environmental Bulletin, 21(1B): 3704-3710.
3. Altuner, E. M., Cetin, B. & Cokmus, C. (2010). Antimicrobial Screening of Some Mosses Collected From Anatolia. Pharmacognosy Magazine, 6 (22): 56.
4. Andrews, J. M. (2003). BSAC standardized disc susceptibility testing method (version 6). Journal of Antimicrobial Chemotherapy, 60, 20-41.
5. Ates, D. A. & Erdogru, O. T. (2003). Antimicrobial activities of various medicinal and commercial plant extracts. Turk J Biol, 27: 157-162.
6. Canli, K., Altuner, E. M. & Akata, I. (2015). Antimicrobial screening of *Mnium stellare*. Bangladesh Journal of Pharmacology, 10(2):321-325.
7. Clardy, J. & Walsh, C. (2004). Lessons from natural molecules. Nature, 432, 829-837.
8. Conter, M., Paludi, D., Zanardi, E., Ghidini, S., Vergara, A., & Ianieri, A. (2009). Characterization of antimicrobial resistance of foodborne *Listeria monocytogenes*. International journal of food microbiology, 128(3): 497-500.
9. Cos, P., Vlietinck, A. J., Vanden Berghe, D. & Maes, L. (2006). Anti-infective potential of natural products: How to develop a stronger *in vitro* 'proof-of-concept'. Journal of Ethnopharmacology, 106, 290-302.
10. Dash, M., Kumar, P.J., Panda, P. (2008). Phytochemical and antimicrobial screening of extracts of *Aquilaria agallocha* Roxb. Afr. J. Biotechnol, 7: 3531-3534.
11. De Boer, A. S. & Diderichsen, B. (1991). On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: A review. Appl Microbiol Biotechnol, 36, 1-4.
12. Faucher, J. L. & Avril, J. L. (2002). Bactériologie générale et médicale. Tome 1, (Ellipses (Ed.), Paris), 214p
13. Galieni, P. & Bigazzi, C. (1998). Recurrent septicemia in an immuno-compromised patient due to probiotic strains of *Bacillus subtilis*. J Clin Microbiol, 36, 325- 326.
14. Hammer, K. A., Carson, C. F. & Riley, T. V. (1999). Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology, 86, 985 - 990.
15. Ilhan, S., Savaroglu, F., Çolak F., Iscen C. F. & Erdemgil F. Z. (2006). Antimicrobial activity of *Palustriella commutata* (Hedw.) Ochyra extracts (Bryophyta). Turk J Biol, 30, 149-152.
16. Jones, F. A. (1996). Herbs - useful plants. Their role in history and today. European Journal of Gastroenterology and Hepatology, 8: 1227-1231.
17. Karasu, A. (2015). Bulgaristan Tırgovışte'den Osmanlı Dönemine Ait Bir Tıp Metni, Turkish Studies, 10(8), 1597-1612.
18. Mahasneh, A. M. & El-Oqlah, A. A. (1999). Antimicrobial activity of extracts of herbal plants used in the traditional medicine of Jordan. Journal of Ethnopharmacology, 64, 271-276.
19. Nair, R., Chanda, S. V. (2007). Antibacterial activities of some medicinal plants of the Western Region of India. Turkish Journal of Biology, 31: 231–236.
20. Nikaido, H. (1998). Antibiotic resistance caused by Gram-negative multidrug efflux pumps. Clin Infect Dis, 27: 32-41.
21. Okeke, I. N., Laxmanarayan, R., Bhutta, Z. A., Duse, A. G., Jenkins, P., O'Brien, T. F., Pablos-Mendez, A. & Klugman, K. P. (2005). Antimicrobial resistance in developing countries. Part 1: recent trends and current status. Lancet Infectious Diseases, 5, 481- 493.
22. Oskay, M., Karayıldırım, T., Ay, E., & Ay, K. (2010). Determination of some chemical parameters and antimicrobial activity of traditional food: Mesir paste. Journal of Medicinal Food, 13 (5): 1195–1202.
23. Ozkan, O. E., Zengin, G., Akca, M., Baloglu, M. C., Olgun, C., Altuner, E. M., Ates, S., Aktumsek, A. & Vurdu, H. (2015). DNA protection, antioxidant, antibacterial and enzyme inhibition activities of heartwood and sapwood extracts from juniper and olive woods. RSC Advances, 5: 72950-72958.
24. Paudel, B., Bhattarai, H. D., Lee, J. S., Hong, S. G., Shin, H. W. & Yim, J. H. (2008). Antibacterial Potential of Antarctic Lichens against Human Pathogenic Gram-positive Bacteria. Phytotherapy Research, 22, 1269-1271.
25. Parekh, J., Nair, R. & Chanda, S. (2005). Preliminary screening of some folkloric plants from Western India for potential antimicrobial activity. Indian J Pharmacol, 37: 408-409.
26. Richards, M. J., Edwards, J. R., Culver, D. H. & Gaynes R. P. (1999). Nosocomial infections in medical intensive care units in the United States: National Nosocomial Infections Surveillance System, Crit Care Med, 27: 887–892.
27. Silici, S. & Koc, A. N. (2006). Comparative study of *in vitro* methods to analyse the antifungal activity of propolis against yeasts isolated from patients with superficial mycoses. Letters in Applied Microbiology, 43, 318-324.
28. Syed, G. W., Syed, A. S. & Oh, L. A. (2010). Risk Evaluation Under Various Speculations of Antibiotic Usage; A Cohort Survey Among Outpatients of Pinang, Malaysia. Eur J Gen Med, 7, 303-309.
29. Villegas, M. V. & Quinn, J. P. (2004). An update on antibiotic-resistant gram-negative bacteria. Infections in Medicine, 21, 595-599.
30. WHO. (2007) The world health report 2007: A safer future: global public health security in the 21st century.