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Abstract

Background: This work aims at describing the traditional uses, to determine the antimicrobial potential of two different extracts hexane, acetone of the leaves of *Citrus unshiu*, *Citrus sinensis*, *Citrus limon*, *Laurus nobilis*, *Citrus paradisi* on clinical strain of *H. pylori* in a bid to identify potential sources of cheap starting materials for the synthesis of new drugs. *H. pylori* strain was a culture collection of Hacettepe University, Turkey.

Methods: The activity was quantitatively assessed on the basis of the inhibition zone, and their activity index was also calculated along with the MIC method.

Results: All the plants demonstrated antimicrobial activity against *H. pylori* with zone of inhibition diameters ranging from 0 - 30 mm and minimum inhibitory concentration (MIC) values ranging from 1:512- 1:4096 dilutions.

Conclusion: The results may serve as scientific validation of the ethnomedicinal uses of the *Citrus unshiu*, *Citrus sinensis*, *Citrus limon*, *Laurus nobilis*, and *Citrus paradisi* in the treatment of *H. pylori*-related infections. However, further investigations would be necessary to determine their toxicological properties, in-vivo potencies and mechanism of action against *H. pylori*.

Key words: *Helicobacter pylori*; antimicrobial activity; plant extracts; acetone; hexane; MIC

Introduction

The use of natural products and search for drugs derived from plants with therapeutic properties is as ancient as human civilization and, for a long time, mineral, plant and animal products were the main sources of drugs (De Pasquale., 1984). In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimicrobial properties (Cowan., 1999). *Helicobacter pylori* is one of the most common chronic bacterial pathogens of humans (Sherif et al., 2004; Tiwari et al., 2005). Colonization is usually life long and may lead to chronic gastritis, duodenal ulceration and gastric cancer in later life (Mackay et al., 2003). Infections have been reported to be higher in the developing than in developed countries. Clarithromycin is recognized as the key antibiotic for *H.pylori* treatment because of its powerful bactericidal effect *in vitro* compared with other available molecules (Megraud, 2004; De Francisco et al., 2007). Considerable attention has been given to the screening of medicinal plants all over the world as a means to identify cheap sources of new drugs against *H. pylori*, a human gastric pathogen with high morbidity rate (Ndip et al., 2004). Medicinal plants may constitute a natural reservoir of therapeutically useful compounds against *H. pylori*. A search for new organic molecules with antibacterial activity, which could be cheap and readily available to the local population, therefore becomes important as it offers the potential of improving primary health care. Due to increasing emergence of drug-resistance in *Helicobacter pylori* isolates, traditional plants are potentially valuable sources of novel anti-*H. pylori* agents.

Materials and Methods

Plant collection and preparation of extracts

Plant names and the areas of collection are shown in Table 1. Herbs used in this study were pressed according to herbarium techniques and air-dried in the dark at room temperature (RT) and then ground to powders (Melaku., 2008). Solvent extractions were carried out according to the method adopted by Freedman et al. (1979) with slight modifications separately within the procedure detailed below. Exactly 50 g of dried powdered plant material was macerated separately in 200 ml of concentrated acetone/ hexane in large labelled glass bottles and put in an orbital shaker for 24h at room temperature. The solvent was then removed under reduced pressure in a rotary evaporator (N-1000S, EYELA, Japan) at 55°C approximately 50-60 min. The procedure was repeated 4 times. Extracts were first filtered using Whatman No. 1 filter papers, filtrates were evaporated to dryness at RT in a steady air current. All dried crude extracts were collected in clean universal bottles and were stored at -20 °C until required for testing. Stock solutions were prepared by dissolving the extracts in dimethyl sulphoxide (DMSO) before use.

Colony selection

Colonies were inoculated on a *H. pylori* agar plate and incubated at 37 °C for 3 days under microaerophilic conditions within GasPak™ Anaerobic System (Oxoid). The isolates were identified based on colony morphology, positive oxidase, urease and catalase tests (Westblom., 1991).

Screening of crude extracts for anti-*H. pylori* activity

Antimicrobial activities of different extracts were evaluated by the agar well diffusion method, and minimum inhibitory concentration (MIC). For the agar well diffusion method antimicrobial susceptibility was tested on solid media in petri dishes as previously reported (Boyanova et al., 2005). Briefly, *H. pylori* inocula prepared at McFarland's turbidity standard 2 was plated onto Mueller- Hinton agar (CM0337, Oxoid, England). The inocula was evenly spread on the plate from subcultures of bacteria by sterile cotton swab and allowed to dry for 5-8 min. Wells (6 mm in diameter) were punched into the agar using a sterile stainless steel borer and filled with 50 µL of the extract at 100 mg/mL. DMSO (10%) was used as a negative control and 0.05 µg/mL clarithromycin was added as positive control included in all experiments respectively. The plates

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were incubated under microaerophilic conditions GasPak™ Anaerobic System (Oxoid) at 37 °C for 72 hours after which the diameters of zones of inhibition were measured in millimetres. The experiment was repeated once and mean zones recorded. The results of the agar well diffusion method were used to detect active extracts for MIC determination using the microdilution method. This was necessary to cut down on wastage of the microtitre plates.

Determination of Minimum inhibitory concentration (MIC)

Dilution susceptibility testing method was used for MIC determination in reference to the cited literatures (Bonacorsi et al., 2009; CLSI, 2003) wherein, 160 µl of sterile Brucella broth media was decanted into first well and the other wells were filled with 100 µl of sterile Brucella broth media. 40 µl of an 18-h old broth culture of *H. pylori* (McFarland's turbidity standard 2) suspension was added to the first well. After mixing of the above, 100 µl of the same was transferred to the second well and in this way, the dilution procedure was continued for the subsequent wells to attain a series of dilutions of 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024, 1/2048, 1/4096 respectively. Inoculum solution at 4 µl was added to every well. Being incubated for 72 h at 37°C under microaerophilic conditions, the plates were monitored for turbidity growth and non-turbidity as no growth. The MIC values were interpreted as the highest dilution (lowest concentration) of the sample, which showed clear fluid with no development of turbidity. Solvent blanks and positive controls were also included. All the tests were tested in duplicate.

Results and Discussion

The use of medicinal plants as traditional medicines in the treatment of numerous human diseases for thousands of years is a common phenomenon in many parts of the world especially in areas where medical health facilities are not readily accessible or affordable. In the developing world, especially in rural areas, herbal remedies continue to be a primary source of medicine (Afolayan and Lewu., 2009). Scientifically, medicinal plants have proven to be a source of many potent powerful drugs and abundant source of biologically active compounds, many of which have already been formulated into useful therapeutic substances or have provided a basis for the development of new lead molecules for pharmaceuticals (Srivastava et al., 1996).

Table 1: Native Turkish plants chosen for the study, collection areas and plant parts collected.

<i>Taxa</i>	Province/Collection area	Parts collected
<i>Citrus limon</i>	Hatay/Erzin	Leaf
<i>Citrus sinensis</i>	Hatay/Erzin	Leaf
<i>Citrus unshiu</i>	Hatay/Erzin	Leaf
<i>Laurus nobilis</i>	Hatay/Erzin	Leaf
<i>Citrus paradisi</i>	Hatay/Arsuz	Leaf

Table 2: Inhibition zones diameter of acetone extracts against *Helicobacter pylori*

extracts	concentrations (mg/mL) (diameter: mm)			
	50	100	150	200
<i>L. nobilis</i>	*	*	*	24±0.3
<i>C. paradisi</i>	*	*	*	29±0.3
<i>C. sinensis</i>	27±0.2	*	*	*
<i>C. unshiu</i>	26±0.3	*	*	*
<i>C. limon</i>	*	30	*	*

*: not determined

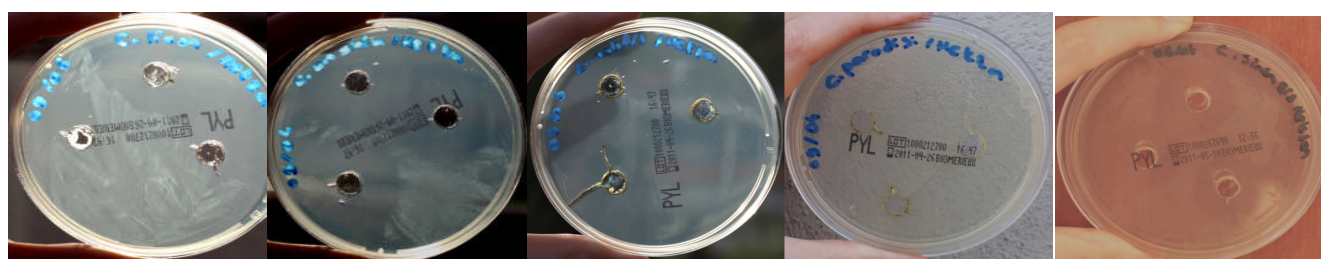
Table 3: Inhibition zones diameter of hexane extracts against *Helicobacter pylori*

extracts	concentrations (mg/mL) (diameter: mm)			
	50	100	150	200
<i>L. nobilis</i>	*	*	*	26±0.1
<i>C. paradisi</i>	*	*	*	24±0.5
<i>C. sinensis</i>	27±0.2	*	*	*
<i>C. unshiu</i>	25	*	*	*
<i>C. limon</i>	*	*	*	*

*: not determined

Table 4. Minimum inhibitory concentrations of acetone and hexane results

Plant extracts	Acetone	Hexane
<i>C. sinensis</i>	1:512	1:512
<i>C. unshiu</i>	1:1024	1:512
<i>C. limon</i>	1:2048	1:1024
<i>C. paradisi</i>	1:1024	1:4096
<i>L. nobilis</i>	1:4096	1:2048

**Figure 1:** Antimicrobial activity of acetone extracts against, *Helicobacter pylori*a) *C. sinensis*, b) *L. nobilis*, c) *C. unshiu*, d) *C. limon*, e) *C. paradisi***Figure 2:** Antimicrobial activity of hexane extracts against, *Helicobacter pylori*a) *C. limon*, b) *C. unshiu*, c) *L. nobilis*, d) *C. paradisi*, e) *C. sinensis*

Measurement of Antimicrobial Activity using Agar Well-Diffusion Method

In the present investigation, the inhibitory effect of different extracts (hexane, acetone) of the *in vivo* leaves from *L. nobilis*, *C. paradisi*, *C. sinensis*, *C. unshiu*, *C. limon* were evaluated against *Helicobacter pylori*. The antimicrobial activity was determined using the agar well-diffusion method. Agar dilution is a reliable technique, which is usually carried out as the reference method for evaluating the accuracy of other testing methods. Although this method proves well suited in large studies on stored strains, it is laborious, time-consuming, and thus not very practicable in routine laboratories. Recently, the National Committee for Clinical Laboratory Standards (NCCLS) now known as the Clinical Laboratory Standard Institute (CLSI) approved the agar dilution method as the test of choice for susceptibility testing of *H. pylori* to clarithromycin (Megraud and Lehours, 2007). The zone of inhibition was calculated by measuring the diameter of the inhibition zone around the well (in mm) including the well diameter. The readings were taken in three different fixed directions in all three replicates and the average values were tabulated. The antimicrobial potential of both the experimental plants was evaluated according to their zone of inhibition against various pathogens, and the results (zone of inhibition) were compared with the activity of the standards, namely, clarithromycin. The results revealed that all the extracts were potent antimicrobials against *Helicobacter pylori*. In the presence of the acetone extract, the maximum inhibition zone diameter at 50 mg/mL was obtained, that is, 27 ± 0.2 mm in *C. sinensis* and 26 ± 0.3 mm in *C. unshiu*. *C. limon* showed an inhibition zone with 30 mm diameter at 100 mg/mL. *C. paradisi* showed an inhibition zone with 29 ± 0.3 mm and *L. nobilis* with 24 ± 0.3 mm diameter at 200 mg/mL (Figure 1; Table 2). In the hexane extract, the antibacterial activity was obtained in *C. sinensis*, with the inhibition zone diameter of 27 ± 0.2 mm and *C. unshiu* with 25 mm diameter at 50 mg/mL, at 200 mg/mL *L. nobilis* showed an inhibition zone with 26 ± 0.1 mm diameter, and *C. paradisi* showed an inhibition zone with 24 ± 0.5 mm diameter. *C. limon* showed no domain at any concentrations of hexane extracts (Figure 2; Table 3).

MICs of the plant extracts

Both acetone and hexane extracts of *C. sinensis* showed growth inhibitions with 1:512 dilutions on *H. pylori* which shows that these extracts dilutions can play a major role as an inhibitor agents on this pathogen. *C. unshiu* acetonic extract showed MIC values at the dilution of 1:1024 and hexanic extract with the dilution of 1:512. Effectiveness of *C. paradisi* acetone extract with 1:1024 dilution on *H. pylori* is important especially in comparison with hexane extract such as 1:4096 dilution. *C. limon* hexane extract with 1:1024 dilution and acetone extract with 1:2048 were effective against *H. pylori* as well. *L. nobilis* hexane extract with 1:2048 turbidity and acetone extract with 1:4096 was effective on *H. pylori*. According to the MIC the tested herb extracts showed effective results on *H. pylori* as opportunistic agents. Inhibitory activity of the acetonic and hexanic extracts of plants are shown at the table 4. Results obtained in this study indicate that *Citrus unshiu*, *Citrus sinensis*, *Citrus limon*, *Laurus nobilis*, *Citrus paradisi* extracts were effective at inhibiting *H. pylori in vitro* with acetone and hexane extracts. Water or 50% DMSO as the negative controls did not show any inhibition zones of the test strain. Of the herbal extracts tested, however, were found to have antibacterial activity against *H. pylori* tested; inhibition zones ranged from 24 to 30 mm. The inhibition zones produced by these extracts indicated that both showed effective antimicrobial activities, although the acetonic extracts showed slightly higher activity, based on inhibition zone sizes.

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According to MIC determinations with the 1:512 dilutions *C. sinensis* hexane/acetone extracts showed that these extracts dilutions can play a major role as an inhibitor agents on *H. pylori*. Although anti-*H. pylori* activities by individual compounds were reported (Vattem et al., 2005), it is believed that a synergistic mode of action is more likely responsible for the extract's antimicrobial activity. Due to increasing antibiotic resistance in clinical isolates of *H. pylori*, the search for new and safe anti-*H. pylori* compounds are of at most importance and native medicinal plants seem to be a logical source for seeking new agents.

The findings of our study demonstrated the *in vitro* activities of the crude extracts of these plants and provide evidence to justify the use of such plants in traditional medicine. For a different perspective we used the leaves of *Citrus unshiu*, *Citrus sinensis*, *Citrus limon*, *Laurus nobilis*, *Citrus paradisi* and demonstrated the extracts have significant antimicrobial properties against *Helicobacter pylori*, and it could be a promising native herb treatment for patients with gastric ulcer caused by *Helicobacter pylori*. The plants may provide novel or lead compounds, which could become starting materials for the synthesis of new drugs. Although evidence on efficacy of the tested plants have been obtained, further investigations are necessary prior to their recommendation for use as safe and effective agent.

Conclusion

Plants of the genus *Citrus* are prized throughout their distribution for their fruits, their essential oils, their medicinal properties and are known to have long history of use in Turkey. *Citrus unshiu*, *Citrus sinensis*, *Citrus limon*, and, *Citrus paradisi* are well known plants with the widespread potential for growth in this region. Lauraceae family member *Laurus nobilis* is also well known plant with the diuretic properties it is also used to treat earaches, lowers high blood pressure, aromatherapy and ulcers. Of these plants, only about 6% have been screened for biologic activity, and a reported 15% have been evaluated phytochemically. With high throughput screening methods becoming more advanced and available, these numbers will change, but the primary discriminator in evaluating one plant species versus another is the matter of approach to finding leads. There are some broad starting points to selecting and obtaining plant material of potential therapeutic interest. However, the goals of such an endeavor are straightforward. These plants have an advantage in this area based on their long-term use by humans. This study reports some preliminary results of herbal extracts of these plants on the inhibition of *H. pylori* and may thus serve as preliminary scientific validation of their folkloric uses in the treatment of infections due to *H. pylori*. Tested plants may contain compounds that could be used as lead molecules for the synthesis of novel drugs against this carcinogenic organism. For further studies the effective ingredients from these plants will be studied to explore whether they have potential toxic effects or not.

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