Pak. J. Bot., 55(2): 791-797, 2023. DOI: http://dx.doi.org/10.30848/PJB2023-2(3)

ANTI-MICROBIAL ACTIVITY OF JORDANIAN PLANT EXTRACTS AGAINST *HELICOBACTER PYLORI*

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Abstract

Helicobactor pylori (*H. pylori*) is a widespread disease. Medicinal plants provided an important source of many products for the eradication and the treatment of *H. pylori*. The aim of the current study is to assess the efficacy of various fractions from crude extracts of selected plants that grow in Jordan to inhibit the growth of *H. pylori* strains. The ethanol (95%), water, chloroform, methanol, and hexane fractions of the following plant extracts were tested. Plant extracts were from *Artemisia inculata Delile* (Asteraceae), *Inula viscose* (L.) Ait (Asteraceae), *Tecoma stans* (L.) *Kunth ex HBK*, *Atriplex halimus* L., *Marrubuim vulgare* L and *Varthemia iphionoids* Boiss (Asteraceae). The antimicrobial efficacy of crude extracts obtained from the above plants were tested *in vitro* on clinically isolated strains of *H. pylori* using the disk diffusion method. The minimum inhibitory concentration (MIC) was measured by the serial dilution method. We found that for all the six studied plants, ethanol extracts showed high ability to inhibit the growth of the isolated strains of *H. pylori*. From the various studied plants, the most effective fractions against the isolated *H. pylori* strains were the chloroform fraction from *A. inculata*, and *I. viscose* and the ethanol extracts from *M. vulgare*, and *T. stans*. For *V. iphionoids*, and *A. halimu,* the methanol fractions showed highest activity. In conclusion, considering the efficacy of the tested plants in treating several infections due to their antimicrobial properties, Jordanian plants can be used as a raw material for starting the synthesis of new medications against *H. pylori*.

Key words: *H. pylori*, Jordan, Plant extracts, MIC, Fractionation. Gastritis, Triple therapy, resistance

Introduction

One of the most common infections affecting the gastrointestinal system in humans is the Gram negative bacteria *Helicobacter pylori* ((Baker, 2020). Since its discovery in 1938, *H. pylori* remains an important etiology in the pathogenesis and the progression of many diseases including gastric and duodenal ulcer, gastric bleeding, chronic gastritis, and gastric lymphoma ((De Francesco *et al*., 2010; Kawai *et al*., 2010; Touati, 2010; Krueger *et al*., 2011; Baker, 2020). It has been identified as Class 1 carcinogen and has been known as one of the predisposing factors for gastric cancer ((Wroblewski *et al*., 2010). Gastrointestinal infections caused with *H. pylori* are highly prevalent worldwide (Khoder *et al*., 2019). The eradication of *H. pylori*, using effective therapy, is highly crucial in preventing gastric cancer or the development of precancerous lesions ((Lee *et al*., 2013). Although the epidemiological pattern of *H. pylori* has been altered, due to better sanitation techniques and eradication treatment options, the prevalence of *H. pylori* is still very high ((Khoder *et al*., 2019). More importantly, a great variation in its prevalence was seen around the world which could be related to the differences in socioeconomic as well as hygienic conditions ((Hu *et al*., 2017; Khoder *et al*., 2019). Approximately 85% of all the population in developing countries is infected compared to 30-50% in the developed countries ((Hunt *et al*., 2011; Burucoa and Axon, 2017). The highest prevalence is seen in Africa, South America, and Asia when compared to the more developed industrialized countries ((Hooi *et al*., 2017). Successful treatment of *H. pylori* should be directed toward eradicating the bacteria from the gastrointestinal system using effective therapy ((Hu *et al*., 2017). Treatments with either the triple or the quadruple regimens are usually used ((Lee *et al*., 2013). These regimens are a combination of two or three antimicrobial agents with an acid suppressing therapy (usually proton pump inhibitors) ((Poonyam *et al*., 2019). The triple regimen was used for more than 20 years with more than 80% success in eradicating *H. pylori*. However, this regimen is no longer recommended as first line in many countries ((Hu *et al*., 2020). Complete eradication of this bacteria can be problematic with the limited options available ((Graham and Dore, 2016; Wang *et al*., 2017). This can be due to many reasons including inappropriate eradication therapy, poor compliance by the patients, high load of the bacteria in the stomach, biofilm formation, and antimicrobial resistance. The latter is considered by far, the most important and alarming risk factor ((Thung *et al*., 2016). These reasons, in addition to many other reasons such as cost, and possible drug-related adverse effects, make it extremely recommended to search for novel alternative options, with fewer side effects, in managing and eradicating *H. pylori* ((O'Gara *et al*., 2000; Di Mario *et al*., 2006; Alzoubi *et al*., 2007; Masadeh *et al*., 2013; Hu *et al*., 2017).

Searching for better management of *H. pylori* has driven the investigation in the field of medicinal plants ((Zaidi *et al*., 2015). The traditional medicine has always been used for drug discovery. For many years, medicinal plants, due to their bioactive compounds, have provided the starting materials for the synthesis of novel medications

with useful therapeutic effect (Shinwari, 2010; Shinwari *et al*., 2020). Moreover, extracts of several plants were shown to possess an antimicrobial effect against various types of infections ((Martin and Ernst, 2003). In the current research study, we investigated the possible antimicrobial effect of six Jordanian plant extracts to inhibit the growth of *H. pylori*. Three clinically isolated strains of *H. Pylori* were used in the study. Selection of the plants was based on our knowledge of their potential therapeutic effect in the management of various infections, specifically, infections with a bacterial nature. These infections include, but not limited to, gastrointestinal, skin, respiratory, eye and genitourinary systems ((Tabassum and Hamdani, 2014; Semenya and Maroyi, 2018; Karunanidhi *et al*., 2019; Shaheen *et al*., 2019).

Experimental Section: Plants used in this research were obtained from different regions of North Jordan during the summer of 2019 (in the period of May through August). Taxonomic classification was identified for each of the tested plant by Dr. Jammel Lahaam, a professor in the Department of Biological Sciences, Faculty of Science, Yarmouk University (Irbid-Jordan). A representative sample (voucher specimen) from each of the tested plant species was preserved at the Department of Medicinal Chemistry and Pharmacognosy, Faculty of Pharmacy, Jordan University of Science and Technology (Irbid-Jordan). Fractions of the plants were dried in shade, away from sunlight. After drying, they were ground by Wiley grinder (Model 5657 HAAN, Germany) using a mesh of 2 mm diameter in size. For further extraction procedure, percolation of the obtained powder (50 gm for each tested plant) was done, and the powder was submerged in 95% ethanol. To make the extracts dry, vacuum was used to evaporate ethanol and concentrate the samples which were further fractioned according to a two-step fractionation scheme. The first step of fractionation was with 95% ethanol (F001), water (F002) and chloroform (F003). The second step was with for the F003 with either methanol 90% (F005) or hexane (F006) as previously described in (Alkofahi *et al*., 1996). For each plant, a specific part was used for the assessment of the antimicrobial efficacy (leaf, seeds, flower, or fruits). Studying specific parts of each plant was based on several other studies ((Alkofahi and Atta, 1999; Masadeh *et al*., 2014). Following extraction, various concentrations of the same fraction for each studied plant was used ranging from 100000 µg /mL to 3125 µg /mL.

Bacterial culturing, growth media and growth conditions: The *H. pylori* isolates used in this study were obtained from patients attended the teaching hospital King Abdullah University Hospital (KAUH), Irbid/Jordan. Informed consent was obtained from the patients before the procedure took place. Patients presented with gastrointestinal complaints of gastro-duodenal origin, biopsies were taken for histopathologic examination and *H. pylori* testing and culturing.

In brief, for bacterial detection, inoculation of the biopsies was made using Columbia agar base plates (Conda Pronadisa, Spain). The used plates were supplemented with the antifungal agent amphotericin B (250 mg), sterile defibrinated sheep blood (7%) and campylobacter supplements which are composed of: vancomycin (10 mg/L), trimethoprim (5 mg/L) and polymyxin B (2500 units/L). The plates were then incubated for three to five days in a microaerophilic environment at 37°C. Since *H. pylori* is a microaerophilic bacteria ((Bury-Moné *et al*., 2006), microaerophilic conditions, which basically depends on providing lower oxygen compared to what it is found in air, were maintained using 5% oxygen and 10% carbon dioxide during the incubation period (Anaerocult Darmstadt, Germany). After incubation, three *H. pylori* subtypes were identified and isolated for antimicrobial sensitivity test.

Testing the antibacterial effect of the plants on the three *H. pylori* isolates: Plants extracts were dissolved in dimethyl sulfoxide (DMSO), then, approximately 60-100 µL of the solution were placed on each 5 mm diameter aseptic filter paper discs. The filter papers were then placed in duplicate into Petri dishes containing Mueller-Hinton agar (MHB) which was inoculated on its surface with 0.2 mL of the tested bacteria (10^8 cells/mL). The Petri plates were then incubated in a temperature of 37°C for 24 hours. Each experiment was carried on in duplicates. Assessing the antimicrobial activity of the plant extracts was based on measuring the zones of inhibition around each filter paper disc which represent the ability of the specific plant to inhibit the growth of the different tested strains of *H. pylori*. Filter paper discs soaked with DMSO were used as negative controls, whereas metronidazole (5 µg/disc), tetracycline (30 µg/disc), clarithromycin (20 µg/disc) and amoxicillin (10 µg/disc) standard antimicrobial agents were used as positive controls. Plant extracts were effective if its zone of inhibition around the disc was a minimum of 15 mm in diameter. Determination of the MIC was carried out based on Clinical and Laboratory Standards Institute Guidelines as previously reported (Masadeh *et al*., 2020). MIC is defined as the lowest possible concentration that inhibits the visible growth of the bacteria ((Andrews, 2001).

Results

In the current research study, the possible antimicrobial activity of six Jordanian plants against three common *H. pylori* strains was examined. Ethanol extracts of various fractions of the tested plants were used to assess the *in vitro* antibacterial effect (Table 1). Table 2 illustrates the zones of inhibition of fractions following the first step of fractionation with 95% ethanol (F001), water (F002) and chloroform (F003) of the several tested plants using different concentrations. As shown from the table, for the plants *Atriplex halimus, Varthemia iphionoides, Artemisia inculata* and *Inula viscosa*, the chloroform fraction was the most active when compared with other fractions of the same plant. This was manifested by the largest zones of inhibition with chloroform fraction in these plants. For *Marrubuim vulgare* L. and *Tecoma stans*, the ethanolic extract was the most active one. In the second phase of fractionation (Table 3), several medicinal plants showed high activity for their methanol fraction against the three isolates of *H. pylori* including *V. iphionoides* and *A. halimus*. Still, the chloroform fraction of *A. inculata* and *I. viscosa*, and the

ethanol fraction of *M. vulgare* L. and *T. stans* recorded the largest diameters for zones of inhibition when compared to the other fractions for the same plant. Regarding the antibiotics used, the three clinical isolates of *H. pylori* responded conventionally to the antibiotics tested except for metronidazole which has almost no activity on the tested strains of *H. pylori*.

Discussion

H. pylori is a common bacteria that impacts the gastrointestinal system ((Aitila *et al*., 2019). Approximately, 70% of people live in developing countries have *H. pylori* in their GI ((Aitila *et al*., 2019; Khoder *et al*., 2019). It is not only a leading cause of peptic and duodenal ulcer, but also a major risk for many other diseases of gastrointestinal origin ((Aitila *et al*., 2019). Developing malignancy either gastric cancer or mucosa-associated lymphoid tissue-lymphoma (MALT) is the most serious complication ((Asano *et al*., 2015). The standard therapy basically depends on two or three antimicrobial agents with an acid reducing therapy ((Mégraud, 2012). For the recent years, many patients treated with the standard regimens of *H. pylori* treatment were not cured ((Mégraud, 2012). This could be related to many reasons with increasing rate of *H. pylori* resistance toward antibiotics is the most important one ((de Boer, 2001). Thus, new options to combat *H. pylori* are needed. In the current study, we were able to show that extracts of some Jordanian plants possess antimicrobial activity against *H. pylori*, with ethanol extract of *T. stans* and chloroform extract of *I. viscosa* were the most active anti-*H. pylori* extracts.

In agreement with the findings of the current study, the antimicrobial activity, namely anti- *H. Pylori*, of the ethanol extract of *A. inculata* and *V. iphionoides* was previously reported ((Masadeh *et al*., 2013; Masadeh *et al*., 2014). Current results further illustrate that the chloroform fraction of *A. Inculata,* is the most active fraction when compared to ethanol and methanol extracts. In fact, Artemisia extract was shown to mitigate alcoholic gastritis via enhancing heat-shock proteins ((Kim *et al*., 2018). In an animal model where mice infected with *H. Pylori*, chronic administration of Artemisia extract was reported to rejuvenate and prevent chronic gastritis and tumorigenesis ((Jeong *et al*., 2016). The reported anti- *H. Pylori* activity of *A. inculata* could be related to the presence of high amounts of phenolic phytochemicals in addition to artemisinin, a sesquiterpene lactone which was formerly found to have activity against *H. pylori* ((Goswami *et al*., 2012). Their mechanism of action is thought primarily via their antioxidant activity, leading to disruption of the bacterial cell membrane. In addition, their anti-inflammatory effects reduce inflammation and neutrophil infiltration secondary to *H. pylori*, leading to reduction of microbial burden ((Vega *et al*., 2009; Goswami *et al*., 2012; Safavi *et al*., 2015).

For *I. viscosa*, both the antibacterial and the anti- *H. Pylori* activities were previously reported for the ethanolic extract ((Masadeh *et al*., 2013; Abu-Qatouseh *et al*., 2014; Masadeh *et al*., 2014; Lee *et al*., 2016). Results obtained from this study indicate that for this plant, the chloroform extract is the most effective anti-*H. pylori* extract. Such activity could be heavily attributed to the presence of high levels of sesquiterpene compounds which are basically found in leaf extracts ((Cafarchia *et al*., 2002). Several previous studies showed that sesquiterpenes compounds provide a wide range of therapeutic treatment options ((Siedle *et al*., 2004; Crespo-Ortiz and Wei, 2012; Ivanescu *et al*., 2015). One of their studied effects is their antimicrobial efficacy against a wide range of microorganisms ((Siedle *et al*., 2004; Crespo-Ortiz and Wei, 2012). Several types of bacteria were shown to be sensitive on sesquiterpenes, include *Escherichia Coli, Bacillus subtilis, Staphylococcus aureus and bacteroids fragilis* ((Anke and Sterner, 1991; Aljancic *et al*., 1999; Cho *et al*., 2003; Lin *et al*., 2003; Fortuna *et al*., 2011). Moreover, sesquiterpene lactones were found to have a potent antibacterial activity against *H. pylori* ((Konstantinopoulou *et al*., 2003; Vega *et al*., 2009; Goswami *et al*., 2012). In many published studies, extracts obtained from *I. viscosa* were reported to effective for a variety of digestive disorders, this can be due to its antimicrobial activity against several isolates of *H. pylori* and some other microorganisms ((Ali-Shtayeh *et al*., 1998; Stamatis *et al*., 2003).

Current findings also indicate that the most *H. pylori* active fraction of *A. halimus* was the methanolic one, whereas the ethanolic fraction of *M. vulgare*, and *T. stans* was the most active fraction. The ethanolic extracts of the plants were previously shown to possess antibacterial activity against *H. pylori* ((Al-Dabbas *et al*., 2006; Masadeh *et al*., 2013; Masadeh *et al*., 2014). In accordance, eudesmane sesquiterpene isolated from common *V. iphionoids* was previously shown to have antibacterial activity against a wide range of both gram positive as well as gram negative bacteria ((Al-Dabbas *et al*., 2005). Accordingly, results of the current research agree with the previously published studies that found a possible therapeutic effectiveness of the Jordanian plants against the common bacteria *H. pylori.*

Table 1. List of the used plants to test their antimicrobial activity against *H. pylori*.

Scientific Name	Family	Part Used	Voucher specimen number	Yield (g/kg)
Atriplex halimus L.	Chenopodiaceae	Leaf	72	131.0
Marrubuim vulgare L.	Lamiaceae	Leaf	63	59.4
Varthemia iphionoides Boiss	Asteraceae	Aerial parts	97	93.0
Tecoma stans (L.) Kunth	Bignoniaceae	Aerial parts	122	146.0
Artemisia inculata	Asteraceae	Aerial parts	90	131.0
Inula viscosa (L.) Ait	Asteraceae	Leaf	112	216.2

Table 2. Antimicrobial activity of the three fractions F001, F002, and F003 of the six studied plants using various concentrations of the extracts against the three isolates of *H. pylori*.

Plant/Concentration in µg /ml	Diameter of zone of inhibition (mm)																	
	F001						F002						F003					
	Stock	C1	C2	C3	C4	C5	Stock	C1	C2	C3	C4	C5	Stock	C1	C2	C3	C4	C5
***Atriplex halimus* L.**																		
H. pylori Isolate 1	17	15	13	0	0	0	16	0	0	0	0	0	20	17	15	14	13	12
H. pylori Isolate 2	12	16	14	11	0	0	20	17	0	0	0	0	26	20	19	16	14	0
H. pylori Isolate 3	23	20	18	15	13	11	18	15	11	0	0	0	26	25	21	18	16	14
***Marrubuim vulgare* L.**																		
H. pylori Isolate 1	47	45	41	34	29	25	19	0	0	0	0	0	44	40	39	33	28	22
H. pylori Isolate 2	49	43	39	37	35	30	39	31	24	0	0	0	41	39	37	35	33	27
H. pylori Isolate 3	35	31	29	26	24	21	17	0	0	0	0	0	32	31	30	29	28	25
Varthemia iphionoides																		
H. pylori Isolate 1	34	30	27	24	22	20	25	23	20	18	15	12	41	36	35	29	23	14
H. pylori Isolate 2	40	38	36	34	30	27	22	20	19	18	0	0	43	39	33	31	29	27
H. pylori Isolate 3	33	31	29	27	24	19	26	21	18	17	16	0	49	46	37	25	20	18
***Tecoma stans* (L.) Kunth**																		
H. pylori Isolate 1	47	35	31	25	23	15	42	31	27	18	0	0	39	34	30	23	19	15
H. pylori Isolate 2	49	47	39	31	29	19	45	41	37	31	27	21	38	32	29	25	24	18
H. pylori Isolate 3	61	55	49	47	40	33	51	49	47	45	42	30	45	38	31	28	24	19
Artemisia inculata																		
H. pylori Isolate 1	19	17	15	14	12	11	26	22	18	13	11	10	34	33	32	31	30	27
H. pylori Isolate 2	32	29	23	14	12	11	46	40	35	27	23	20	51	47	39	32	30	26
H. pylori Isolate 3	33	25	19	12	11	0	41	39	33	29	21	17	36	32	29	23	22	18
Inula viscosa																		
H. pylori Isolate 1	47	43	41	34	28	24	27	21	13	0	0	0	50	43	40	39	32	29
H. pylori Isolate 2	50	49	44	41	35	33	38	33	30	28	24	15	55	52	43	40	35	30
H. pylori Isolate 3	53	49	45	36	32	26	49	45	41	36	30	25	59	56	55	49	45	41

Table 3. Antibacterial activity of fraction F005, F006, versus the most potent of F001 or F003 of the six tested plants extracts with different concentrations against *H. pylori* Isolates.

Plant/ Concentration in µg /ml	Diameter of zone of inhibition (mm)																	
	F001or F003						F005						F006					
	Stock	C1	C2	C3	C4	C5	Stock	C1	C2	C3	C4	C5	Stock	C1	C2	C3	C4	C5
***Atriplex halimus* L. (F003)**																		
H. pylori Isolate 1	20	17	15	14	13	12	22	20	19	18	17	16	18	17	16	15	14	13
H. pylori Isolate 2	26	20	19	16	14	0	25	21	19	12	11	10	17	15	13	12	11	10
H. pylori Isolate 3	26	25	21	18	16	14	32	30	25	21	19	17	17	16	14	13	12	11
***Marrubuim vulgare* L. (F001)**																		
H. pylori Isolate 1	47	45	41	34	29	25	36	35	34	32	31	27	19	18	17	14	12	11
H. pylori Isolate 2	49	43	39	37	35	30	40	36	33	30	25	23	17	16	15	15	14	10
H. pylori Isolate 3	35	31	29	26	24	21	40	37	34	25	23	22	21	20	19	17	13	11
***Varthemia iphionoides* (F003)**																		
H. pylori Isolate 1	41	36	35	29	23	14	45	43	36	27	24	19	18	16	15	14	13	12
H. pylori Isolate 2	43	39	33	31	29	27	35	33	26	25	22	19	16	15	14	13	12	11
H. pylori Isolate 3	49	46	37	25	20	18	37	33	31	27	23	21	22	19	17	16	15	14
***Tecoma stans* (L.) Kunth (F001)**																		
H. pylori Isolate 1	47	35	31	25	23	15	42	39	37	30	26	20	18	17	16	15	12	11
H. pylori Isolate 2	49	47	39	31	29	19	40	39	33	31	27	23	15	14	13	12	11	0
H. pylori Isolate 3	61	59	47	44	40	33	44	38	37	30	27	24	37	22	19	15	14	13
***Artemisia incula ta* (F003)**																		
H. pylori Isolate 1	34	33	32	31	30	27	21	20	19	18	15	13	21	19	17	16	15	14
H. pylori Isolate 2	51	47	39	32	30	26	23	22	20	17	15	12	20	16	15	13	11	0
H. pylori Isolate 3	36	32	29	23	22	18	24	23	21	14	12	11	20	18	16	13	12	11
***Inula viscose* (F003)**																		
H. pylori Isolate 1	50	43	40	39	32	29	55	46	40	35	30	27	28	20	14	13	12	11
H. pylori Isolate 2	55	52	43	40	35	30	37	30	28	25	20	20	40	31	26	25	24	21
H. pylori Isolate 3	59	56	55	49	45	41	45	42	40	39	31	27	46	40	27	24	20	17
Amoxicillin	*H. pylori* Isolate 1: 60						*H. pylori* Isolate 2: 55						*H. pylori* Isolate 3: 65					
Clarithromycin	*H. pylori* Isolate 1: 58						*H. pylori* Isolate 2: 52						*H. pylori* Isolate 3: 52					
Metronidazole	*H. pylori* Isolate 1: 0						*H. pylori* Isolate 2: 0						*H. pylori* Isolate 3: 0					
Tetracycline	*H. pylori* Isolate 1: 48						*H. pylori* Isolate 2: 40						*H. pylori* Isolate 3: 30					

Stock = 100000 µg /ml, C1= 50000 µg /ml, C2= 25000 µg /ml, C3= 12500 µg /ml, C4= 6250 µg /ml, C5= 3125 µg /ml, Amoxicillin = 10 µg/disc, Metronidazole = 5 µg/disc, Clarithromycin = 20 µg/disc, and Tetracycline = 30 µg/disc.

Conclusions

As a conclusion, different extracts of Jordanian plants that are commonly used in folk medicine, namely *T. stans* and *I. visoca* can be a starting point toward making new antimicrobial agents with an effect against *H. pylori*.

Acknowledgement

The authors would like to thank and acknowledge the Higher Council for Science & Technology (Amman; Jordan) for their financial support of this research (Grant No 20040010).

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(Received for publication 2 September 2021)