

## Antihelicobacter Activity of a Flavonoid Compound Isolated from *Desmostachya Bipinnata*

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**Abstract:** The study aimed to investigate the potential *in vitro* antihelicobacter activity of selected Egyptian plants, focusing on the determination of the main component responsible for such activity. The main objective is to obtain a natural product have antihelicobacter activity. Antimicrobial screening for wild Egyptian medicinal plant extracts, revealed that five methanolic extracts have good antihelicobacter activity. Determination of MICs, revealed that the wild plant, *Desmostachya bipinnata* (DEM) extract proved to be the most active one, where its MIC was 40 µg/ml. After fractionation of the DEM extract, ethyl acetate fraction exhibited excellent antihelicobacter activity. By further fractionation and purification, using TLC and column chromatography, a flavonoid compound was isolated, with MIC value of 62 µg/ml. The isolated compound was spectroscopically identified as 4'-methoxy quercetin-7-O-glucoside. DEM plant (available as a wild plant in Egypt) containing a flavonoid compound which possesses a good *in vitro* antihelicobacter activity. The isolated compound (Quercetin) might be useful as a chemo-preventive agent for peptic ulcer in *H. pylori*-infected individuals, after its clinical valuation.

**Key words:** Egyptian medicinal plants- *H. pylori*- Microbiological screening- Extraction- Fractionation- Flavonoids-Quercetin- Antihelicobacter activity

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### INTRODUCTION

*Helicobacter pylori* is a truly a matter of worldwide importance, as more than half the world's population is infected. An estimated 10% of those infected with *H. pylori* will develop peptic ulcer disease at some point. These cases comprise 90-95% of all duodenal ulcers and 70-75% of gastric ulcers (Cover and Blaster, 1995). Chronic gastritis induced by *H. pylori* is the strongest known risk factor for adenocarcinoma of the distal stomach (Peek and Crabtree, 2006). Many therapeutic agents are used for eradication of this bacterium, but the widespread use of these agents has increased resistance among the isolated strains of *H. pylori*. Combination of antibiotics, acid suppressors and stomach protectors is reported to be used for eradication of the infection, but this triple therapy is actually cost treatment. It is estimated that less than 10% of patients receive inadequate therapies with eradication rates well under 90%. Higher plants might contain secondary metabolites with good activity against pathogenic organisms. Antimicrobial screening for plant extracts, has yielded some interesting results for the eradication of *H. pylori* (Caceres *et al.* 1993; Paulo *et al.* 1994; Beil *et al.* 1995; Ernst, 1999; Katoaka *et al.* 2001; Fukai *et al.* 2002; Wang and Huang 2005; Ustun *et al.* 2006).

The present study aimed to investigate the potential *in vitro* activity of selected Egyptian plants against *H. pylori*, focusing on the determination of the main component responsible for the antihelicobacter activity, as a natural product, safe and available drug.

### MATERIALS AND METHODS

#### **Bacterial Culture:**

A clinical isolate of *H. pylori* was isolated from endoscopic specimen. Each biopsy specimen was aseptically transferred onto the surface of Columbia agar base (CM331 Oxoid, Basingstoke, UK) supplemented with 5% blood. The inoculated plates were incubated at 37°C under a microaerophilic atmosphere containing 5% oxygen, 10% carbon dioxide and 85% nitrogen (Oxoid generating kit no.1544995). The plates were examined for the characteristic growth of *H. pylori* after 3 to 7 days incubation. The isolate was purified by streaking on the same medium and maintained at -70°C in a liquid medium with glycerol.

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**Plant Extraction:**

Plant materials were collected from various locations in Egyptian deserts. The wild plants (Table 1) were shaded and ground to chaff by a mill, then extracted by maceration with 70% methanol many times until the extracts were clear. The extracts were filtered and the filtrates were concentrated under vacuum followed by drying to produce a solid or paste material.

**Screening for Antihelicobacter Activity:**

Screening for antimihelicobacter activity of plant extracts were carried out by cup diffusion techniques (Bauer *et al.* 1966). Fixed concentrations of the plant extracts were aseptically dropped into cups made in blood agar plates inoculated with a fresh culture of *H. pylori* at a cell density of  $10^7$  to  $10^8$  cfu/ml. The plates were incubated for 3 to 5 days at 37°C under microaerophilic conditions as mentioned before. After incubation, the diameters of inhibition zones were measured.

**Determination of MICs of plant extracts against *H. pylori*:**

The MICs of the tested plant extracts were determined by agar diffusion method according to the method described by Okigbo and Igw (2007). Specific volumes of the plant extracts were dissolved in least amount of the solvent (DMF) to prepare 2-fold serial dilutions of the tested plant extracts. Plates of Columbia blood agar were surface inoculated with *H. pylori* at a cell density of  $10^7$  to  $10^8$  cfu/ml and incubated at 37°C for 3 days under microaerophilic conditions. The cups were filled with respective dilutions of the tested plant extract. Growth control plates of blood agar contained cups filled with DMF without plant extracts were also prepared. The plates were carefully incubated for 3 days at 37°C under microaerophilic conditions. After incubation, the diameters of the inhibition zones were measured. A graph was instructed between the logarithms of the concentrations of each plant extract and the respective diameters of the inhibition zones. MIC was determined from the inhibition zone diameters via linear regression with the corresponding concentrations.

**Fractionation of *Desmostachya bipinnata* extract:**

Separation of the active components of the most active herbal extract with antihelicobacter activity, was carried out by fractionation in a glass separating funnel using solvents with different polarities. A weight of 340 g of the methanolic extract was mixed with water and extracted with petroleum ether, ether, chloroform, ethyl acetate then butanol to obtain the respective fraction (Fig. 1). All the fractions were separately concentrated for susceptibility testing to determine the most active fraction using agar diffusion method. The most active fraction was subjected to silica gel column chromatography to separate the component(s) responsible for the activity (Saieed *et al.* 2006). The main active fraction, separated from the column chromatography, was purified by dissolved in a least volume of the solvent used to be subjected to another column powdered silica gel. Sequential elution of components with appropriate solvents produces many fractions. These fractions were also screened for antihelicobacter activity. The final purification was carried out using many sub columns to produce pure compound. MIC was determined for the pure compound as mentioned before.

**Identification of the Isolated Active Compound:**

Structure elucidation of the pure isolated compounds has been established on the basis of the physicochemical data using the following methods: ultraviolet (UV), nuclear magnetic resonance ( $^1\text{H}$ NMR), and mass spectral measurements (MS).

**Determination of  $LD_{50}$  of *D. bipinnata* extract:**

A preliminary toxicological study was carried out according to the method described by Karber (1931), using adult albino mice of both sexes weighing between 20 to 25gm.

## RESULTS AND DISCUSSION

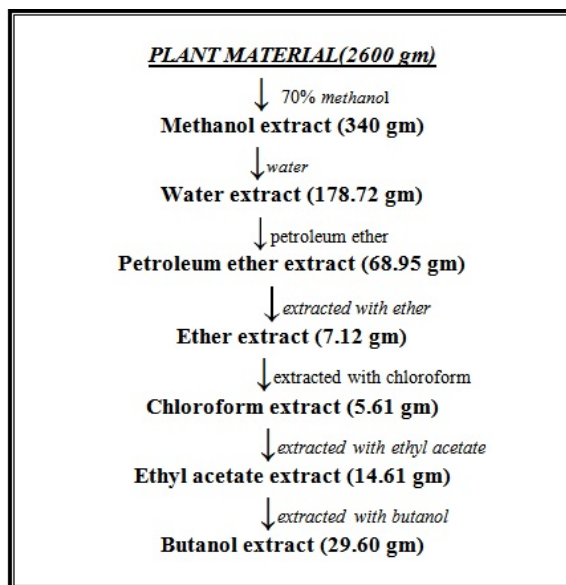
Screening for antihelicobacter activity of 18 plants extracts included in this study, revealed that 5 extracts have good activity against *H. pylori* (Table 2). To assess the antihelicobacter activity of these extracts, MICs were determined by agar diffusion method. The extracts of *Alhagi maurorum*, *Sisymbrium irio*, *Desmostachya bipinnata*, *Marrubium vulgare* and *Cleome africana* extracts exhibited the most activity. Six plant extracts exhibited moderate antihelicobacter activity showing MICs ranging from 1.5 to 10 mg/ml. The most potent antihelicobacter activity was demonstrated by *Desmostachya bipinnata* extract, where its MIC value was very low as much as 40µg/ml.

**Table 1:** Names, families and sources of the tested plants.

Name of plant	Family	Source	Extracted part
<i>Trifolium alexandrinum</i>	Leguminosae	Faiyum	Whole plant
<i>Casimiroa edulis</i>	Rutaceae	Dakahlia	Unripe fruit
<i>Schouwia thebaica</i>	Cruciferae	Sinai	Whole plant
<i>Glossostemon brugueiri</i>	Sterculiaceae	North Sinai	Leaves, roots
<i>Diplotaxis acris</i>	Cruciferae	Sinai	Whole plant
<i>Capparis spinosa</i>	Capparaceae	North costal region	Whole plant
<i>Acacia seyal</i>	Leguminosae	Aswan	Stems, leaves
<i>Centaurea alexandrina</i>	Compositae	North costal region	Whole plant
<i>Thymus capitatus</i>	Labiatae	North costal region	Whole plant
<i>Cleome Africana</i>	Cleomaceae	Gabal elba	Whole plant
<i>Marrubium vulgare</i>	Labiatae	North costal region	Whole plant
<i>Haplophyllum tuberculatum</i>	Rutaceae	Red sea costal region	Whole plant
<i>Hamada elegans</i>	Chenopodiaceae	North costal region	Whole plant
<i>Desmostachya bipinnata</i>	Gramineae	Cairo(from side ways of train roods)	Whole plant
<i>Sisymbrium irio</i>	Cruciferae	Sinai	Whole plant
<i>Bidens bipinnata</i>	Compositae	Gabal elba	Whole plant
<i>Alhagi maurorum</i>	Leguminosae	North costal region	Whole plant
<i>Euphorbia retusa</i>	Euphorbiaceae	Sinai	Whole plant

**Table 2:** Screening of plant extracts against clinical isolate of *H. pylori* and their MICs determination.

Plant Extract	Diameter of zone of inhibition	MIC(mg/ml)
<i>Trifolium alexandrinum</i>	33	25
<i>Casimiroa edulis</i> (unripe fruit)	33	20
<i>Schouwia thebaica</i>	30	25
<i>Glossostemon brugueiri</i> (root)	36	10
<i>Glossostemon brugueiri</i> (leaves)	43	25
<i>Diplotaxis acris</i>	43	10
<i>Capparis spinosa</i>	34	10
<i>Acacia seyal</i> (stem)	35	20
<i>Acacia seyal</i> (leaves)	35	20
<i>Centaurea alexandrina</i> .	34	80
<i>Thymus capitatus</i>	40	12.5
<i>Cleome africana</i>	41	0.158
<i>Marrubium vulgare</i>	35	0.251
<i>Haplophyllum tuberculatum</i>	30	1.58
<i>Hamada elegans</i>	34	10
<i>Desmostachya bipinnata</i>	39	0.040
<i>Sisymbrium irio</i>	31	0.074
<i>Bidens bipinnata</i>	24	25
<i>Alhagi maurorum</i>	38	0.79
<i>Euphorbia retusa</i>	35	2.5



**Fig. 1:** Fractionation of the plant materials using solvents of different polarities.

The result of antimicrobial testing of *D. bipinnata* fractions presented in Table 3. The data showed that the ethyl acetate and butanol fractions were the most active against *H. pylori*. Therefore, purification of their active components was carried out using column chromatography. Ten different fractions were recovered from the column chromatography. However, the results obtained showed that 3 fractions only exhibited antimicrobial activity against *H. pylori*. Purification of the active fraction and separation of the most active compound responsible for the activity were carried out. The isolated compound was identified as a flavonoid with MIC value equal to 62µg/ml.

The structure of the isolated compound was identified using different spectroscopic methods. The UV spectral data (Table 4) were similar to those reported for flavonoid compounds, where addition of methanol the λ max value was an indicator for the presence of flavonol skeleton.

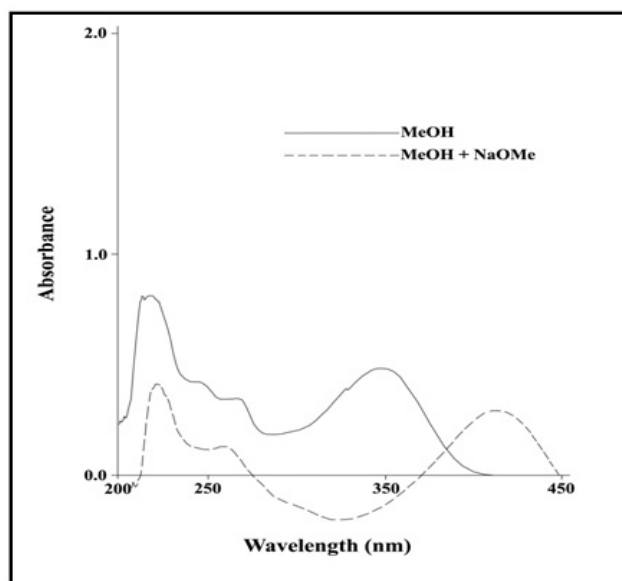
**Table 3:** Susceptibility testing and MICs determination of *Desmostachya bipinnata* fractions against *H. pylori*

Fraction	Diameter of Zone of Inhibition (mm)	MIC (mg/ml)
Petroleum ether	28	1.5
Ether	29	1.0
Chloroform	29	5
Ethyl acetate	35	0.79
Butanol	32	1.3

**Table 4:** Data of U.V absorption spectra of compound (N1)

SHIFT REAGENT	WAVE LENGHT
Me OH	245 sh, 255, 345
NaOMe	260, 325,415
AlCl <sub>3</sub>	275,300sh,370,395
AlCl <sub>3</sub> / HCl	275,300sh,355,385
NaOAc	245,260,350
NaOAc/H <sub>3</sub> BO <sub>3</sub>	245,286,345

On addition of NaOMe there was bathochromic shift with decrease in intensity(Fig. 2a) indicating the 4' occupation at ring B, while the bathochromic in band 1 and 2 on addition of AlCl<sub>3</sub> or AlCl<sub>3</sub> with HCl (Fig. 2b) indicating presence of free OH group at position 3 or 5 on ring A. On the other hand, the absences of bathochromic shift in band 1and band 2 on addition of NaOAc indicating that C7 of ring A and C4' of ring B are occupied. The absences of bathochromic shift in band 1and band 2on addition of NaOAc with H<sub>3</sub>BO<sub>3</sub> indicating the absence of ortho di- hydroxyl groups in ring A and the absence of ortho di- hydroxyl groups at positions 3' and 4' on ring B respectively (Fig. 2c). The U.V spectral data of this compound are similar to those reported for quercetin type compounds with free OH at position 3 and 5, while the positions 4 and 7 were proved to be occupied.



**Fig. 2a:** The U.V spectrum of compound (N1) in Me OH and NaOMe.

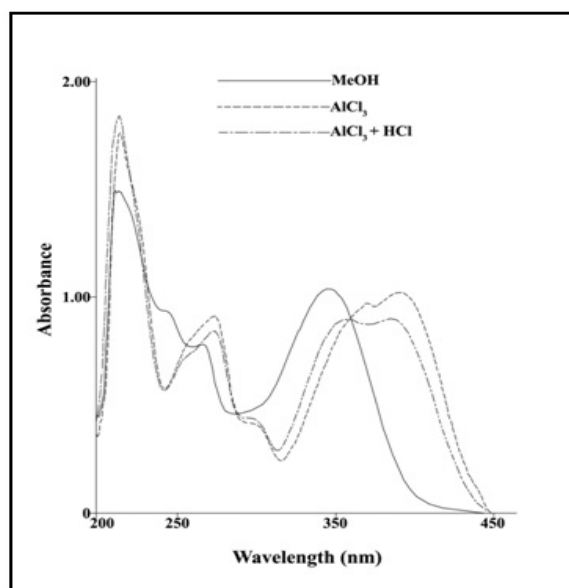


Fig. 2b: The U.V spectrum of compound (N1) in AlCl<sub>3</sub> and HCl.

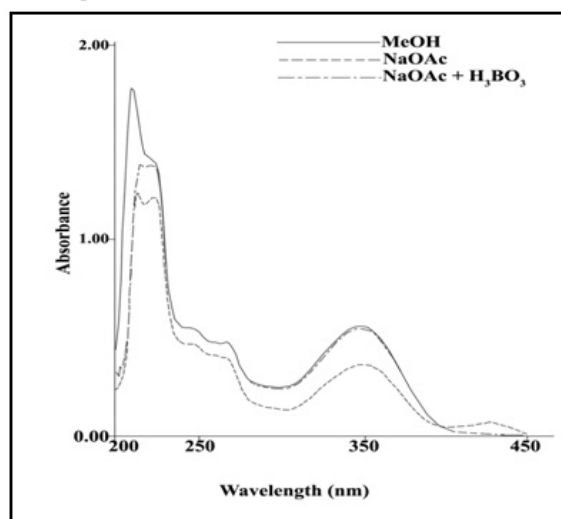


Fig. 2c: The U.V spectrum of compound (N1) in NaOAc and H<sub>3</sub>BO<sub>3</sub>

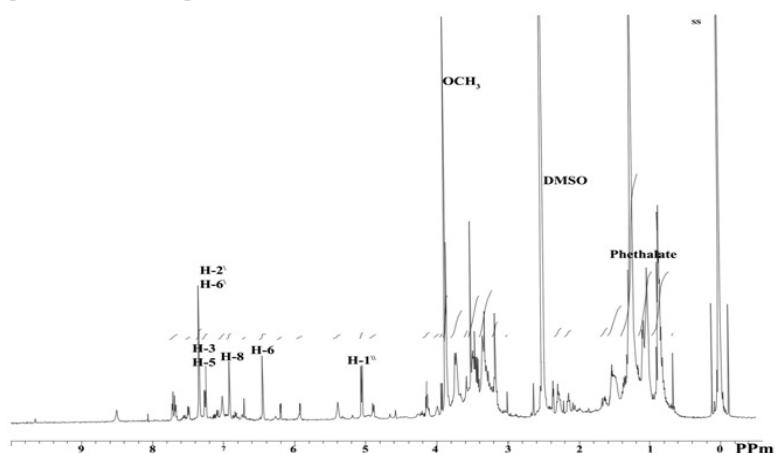


Fig. 3: The <sup>1</sup>H NMR spectrum of compound (N1).

<sup>1</sup>HNMR spectral data (Fig. 3) showed that one meta-coupled doublet of H-8 and H-6 at 6.9 and 6.4 respectively indicating substitution at position 5 at ring A. A doublet H-5 at 7.2 ppm indicating the ortho coupling with H-6'. At 3.8, the protons of the methoxy group appeared indicating that there is a substitution at ring B with methoxy group. One anomeric proton appeared at 5.1 ppm (1H, d, J= 7.3 Hz, H-1'') showing that there is only one sugar moiety identified as glucose.

The results of mass spectroscopy of compound (N1) were determined as follows: [M]<sup>+</sup> molecule ion peak was observed at 478 with 17.5% relative intensity (Fig. 4). The [M-1H] molecule ion peak was observed at 477 with 12.2 % relative intensity. These fragments ascribed for methoxy quercetin + glucose (316 + 162 = 478). Comparing the obtained pervious data with the published data (Mabry and Markham, 1970), the compound (N1) could be identified as: 4'-methoxy quercetin-7-O-glucoside (Fig.5).

The results of the toxicity test revealed that the *D. bipinnata* extract is non toxic and can be used safely after its therapeutic evaluation, where the compound possessing LD50 bigger than 50mg/kg.b.wt is considered non-toxic.

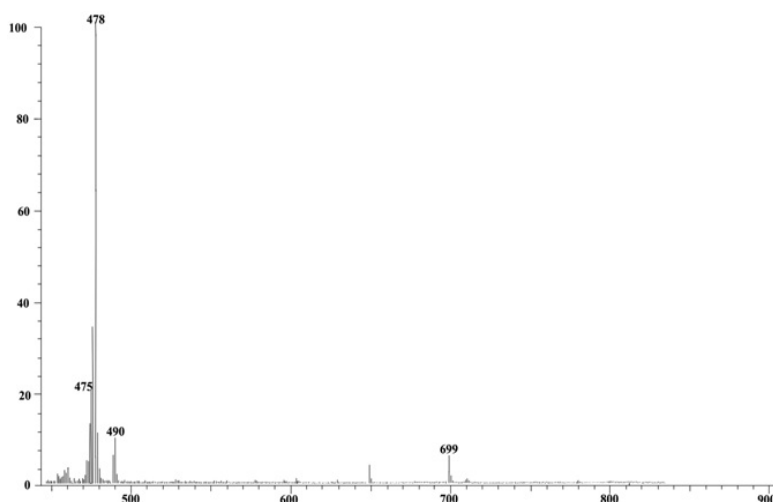


Fig. 4: The mass spectrum of compound (N1).

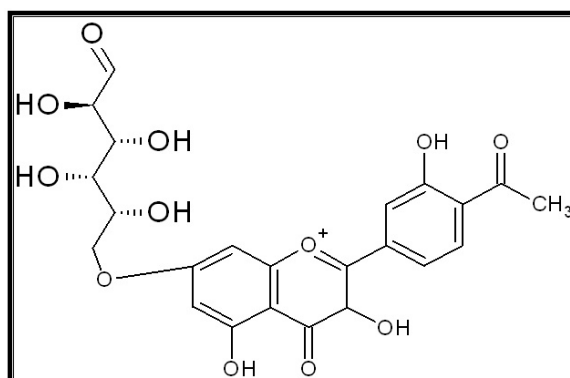


Fig. 5: The chemical structure of the compound (N1).

#### Discussion:

*Helicobacter pylori* is an important etiological agent of peptic ulcer and is a risk factor for gastric cancer (Forman *et al.* 1991; Parsonnet *et al.* 1991; Mendall and Goggin 1992; Mitchel 1993). Combination of antibiotics, acid suppressors and stomach protectors (triple therapy) is commonly used for eradication of *H. pylori* (Malfertheiner *et al.* 2002). The widespread use of these agents has increased resistance among the isolated strains of *H. pylori* (Jenks 2002). Two main problems of using triple therapy, is the antibiotic side effects as well as cost treatment (Meurer *et al.* 2002).

For general eradication of *H. pylori*, there is a need for new drugs which can be easily and cheaply produced locally in order to treat symptomatic and asymptomatic carriers, especially in developing countries and to fight the resistant strains of *H. pylori* to different types of antibiotics. This underlies the importance of using natural products in the treatment, since they are more effective, safe as well as fewer side effects than antibiotics

It is well known that most of higher plants contain secondary metabolites with good activity against microorganisms (Fabry *et al.* 1996). Thus, Thyme extract is proved to be potent as *in vitro* inhibitor of growth and urease production of *H. pylori* (Tabak *et al.* 1996). Therefore the objective of this study was to determine the antihelicobacter activity of some wild Egyptian medicinal plant extracts against *H. pylori*.

The *In vitro* screening of 18 wild plant extracts against *H. pylori*, revealed that five of these extracts have good antihelicobacter activity. The data obtained showed that these extracts have good *in vitro* antihelicobacter activity where their MICs values, were less than 1.0 mg/ml. Many studies were reported the antihelicobacter activity of plant extracts. Thus, Fabry *et al.* (1996) found that *Terminalia spinosa*, *Harrisonia abyssinica* and *Ximania caffra* exhibited potent antihelicobacter activity with MIC values less than 2.5 mg/ml. Licorice extract was reported to have good antihelicobacter activity (Fukai *et al.* 2002). Furthermore, Taiwanese folk medicinal extracts named *Paederia scandens*, *Plumbago zeylanica*, *Anisomeles indica*, *Bombax malabaricum*, *Alpinia speciosa* and *Bombax malabaricum* demonstrated strong antihelicobacter activities with MIC values ranged from 0.64 to 10.24 mg/ml (Wang and Huang 2005).

Although 5 plant extracts have good antihelicobacter activity, only *Desmostachya bipinnata* proved to be the highly active against the tested strain where its MIC was very low as much as 40µg/ml. This plant seems to contain highly active secondary metabolites of good antihelicobacter activity. To determine the active component responsible for the antihelicobacter activity of *DEM*, fractionation and purification of the crude extract were performed. The fractionation process revealed that the ethyl acetate and butanol fractions, which include most the flavonoids of the plant, had excellent antihelicobacter activity where their MICs were 0.79 mg/ml and 1.3 mg/ml respectively. Similar findings were reported by Wiart *et al.* (2004) whom proved that the ethyl acetate fraction has the highest antibacterial activity in comparing with other solvent extracts.

Further fractionation and purification using column chromatography, a pure flavonoid compound was recovered. Determination of MIC of the pure compound revealed that this compound has the maximum antihelicobacter activity, where its MIC was 62µg/ml. The pure compound was characterized using spectroscopical methods to determine the main nucleus responsible for the observed antihelicobacter activity. Comparing the obtained previous data with the published data (Mabry and Markham, 1970), the compound (N1) could be identified as: 4'-methoxy quercetin-7-O-glucoside. The quercetin is belonging to flavonoid groups. It is well known that this flavonoid, has the capacity to inhibit the growth of *H. pylori* (Bae *et al.* 1999, 2001), as well as ease the pain of ulcers with their anti-inflammatory (Beil *et al.* 1995 and Konstantinopoulou *et al.* 2003). Although no clinical data have been reported on flavonoids for eradication of *H. pylori*, several *in vitro* studies point to their potential benefit. *In vitro* inhibitory effect against *H. pylori* have been reported for flavonoids (Bae *et al.* 1999 and Kataoka *et al.* 2001). To our knowledge, this is the first report about *Desmostachya bipinnata* as antihelicobacter agent. This compound might be useful as a chemopreventive agent for peptic ulcer or gastric cancer in *H. pylori*-infected individuals, after its clinical valuation.

#### ACKNOWLEDGEMENT

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