Inhibitory and bactericidal activity of selected South African honeys and their solvent extracts against clinical isolates of *Helicobacter pylori.*

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Abstract: The growing problem of antibiotic resistance by *Helicobacter pylori* demands the search for novel compounds, especially from natural sources. We evaluated the anti-*H. pylori* activity of six local honeys at different concentrations as well as their solvent extracts by the Hole Plate diffusion method. The minimum inhibitory concentration (MIC_{50}) of the two most active extracts of each honey was determined by the broth microdilution method; and the time kill assay of the most active extract of each honey determined by viability studies. Data were analyzed by one-way ANOVA test at 95% significance level. All the honey varieties as well as their solvent extracts demonstrated varying levels of antibacterial activity based on different mean zone diameters [16.0mm (crude) to 22.2mm (extract)] and percentage susceptibilities [73.3% (crude) to 93.3% (extract)] of the test isolates. The chloroform extracts of Pure Honey (PH) and Champagne Royal Train (CRT) recorded MIC_{50} ranges of 0.01-10% and 0.625-10 % (v/v) respectively; that were not significantly different ($P > 0.05$) from amoxicillin (0.001-1.25mg/mL), the positive control. The most potent bactericidal effect against the test isolates was obtained with 5% v/v (1/2 MIC) concentration of chloroform extract of PH from 42-72hrs. In conclusion, these honeys and their extracts could be leads for further investigation in the discovery of new natural anti-*H. pylori* compounds.

Keywords: Antibacterial activity; time kill; honey; solvent extracts; *Helicobacter pylori*.

INTRODUCTION

Helicobacter pylori (*H.pylori*) is a curved or s-shaped gram-negative, non-capsulated, non-spore forming bacillus (Ghany, 2005) that infects at least 50% of the world's human population (Czinn, 2005).It is incriminated as the main aetiological agent of gastritis (Módena *et al*., 2007) an essential factor in the pathogenesis of peptic ulcer as well as a risk factor in the genesis of gastric carcinoma and mucosa associated lymphoid tissue (MALT) type gastric lymphoma (Roszczenko and Jagusztyn-Krynicka, 2006).

Conventional treatment of *H. pylori* infections is principally based on the use of triple therapies consisting of a combination of two antibiotics (clarithromycin, amoxicillin, and/or metronidazole) with a proton pump inhibitor (Megraud and Lehours, 2007). Unfortunately, *H. pylori* is capable to develop drug-resistance after a period of repeated treatment (Sharara *et al*. 2002). Tanih *et al*. (2010a) reported multidrug resistance of *H. pylori* isolates obtained from patients attending Livingstone hospital, Port Elizabeth, in the Eastern Cape Province of South Africa. Such strains are becoming problematic worldwide (Ochi *et al*., 2005) and may lead to high failure rates of treatment regimens. In addition, non-compliance of patients to the treatment regimen as well as adverse side effects of the drugs may also result in eradication failure. These necessitate the search for new chemotherapeutic agents with excellent activity, which hopefully can eradicate the pathogen.

In a few words, the pathogenic role of *H. pylori* infection in the development of gastro duodenal diseases and the impact of resistance on the clinical outcome (Megraud, 2001) enthused the search for newer treatments and the use of natural agents as alternative therapies (Li *et al*., 2005; Ndip *et al*., 2007; Manyi-Loh *et al*., 2010a, 2010b). Over the years, there has been a lot of interest in the investigation of natural products as sources of new antibacterial agents. In this regard, several natural products have demonstrated *in vitro* antibacterial activity against *H. pylori* (Lesbros-Pantoflickova *et al*., 2007; Ndip *et al*., 2008; Njume *et al*., 2011). Honey, a natural product rich in carbohydrates and produced by bees from floral nectar, with fructose and glucose as major components and other honeybee derived chemical compounds or phytochemicals present in small quantities, has shown promise as an alternative source of treatment against *H. pylori* (Nasir *et al*., 2010). These combinations and a number of other factors, such as low water activity, hydrogen peroxide, low pH, and other partially characterized or uncharacterized compounds, produce a high antimicrobial action in honey (Lusby *et al*., 2005).

Honey has been documented to possess bactericidal activity on many pathogenic micro-organisms including enteropathogens such as *Salmonella*, *Shigella* and *E. coli*

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species and other gram- negative micro-organisms (Jeddar *et al*., 1985). Considering the essential floral origin of honey, its composition in different phytogeographical areas may be expected to vary and, as a consequence, their biological activities (Basson and Grobler, 2008). This is as a result of changeable climatic conditions in different regions or countries. In view of the fact that there may be variation in the chemical composition of different honeys, we decided to investigate six honey varieties obtained from different locations in South Africa. Even though, many authors have demonstrated that honey possesses anti- *H. pylori* potential worldwide (Al Somal *et al*., 1994; Osato *et al*., 1999; Ndip *et al*., 2007); available literature however, indicates that none of the honeys we studied have been tested for anti-*Helicobacter pylori* activity and their possible use as medical grade honeys. In addition, *H. pylori* has been reported to be prevalent as well as multidrug resistant strains present in our study area (Tanih *et al*., 2010b) a situation which merits attention.

Consequently, this study was designed to screen South African honey varieties and their solvent extracts for anti-*H. pylori* activity, to determine the minimum inhibitory concentration (MIC_{50}) of the two most active extracts of each honey as well as to ascertain the rate of kill of the test strains by the most active extract.

MATERIALS AND METHODS

Between April 2009 and February 2010, the study was conducted at the University of Fort Hare, Alice, South Africa and was ethically approved by the Eastern Cape department of health (E.CDoH-Res0002) and the ethics committee of the University of Fort Hare. A total of thirty clinical isolates of *H. pylori* were employed which were recovered from gastric biopsies obtained from patients presenting with gastro duodenal pathologies at Livingstone hospital, Port Elizabeth, South Africa. This was done after informed consent was obtained as per our previously reported schemes (Ndip *et al*., 2008; Tanih *et al*., 2010a). Confirmed isolates were stored in Brain Heart Infusion broth + 20% glycerol at -80^{o}C for subsequent bioassays. *H. pylori* ATCC 43526 was used as the control strain.

Preparation of bacterial suspensions

An inoculum of each strain used in susceptibility tests was prepared by transferring 3-4 fresh colonies of the strain into tubes containing sterile physiological saline solution and turbidity of the suspension adjusted to 0.5 McFarland standard, which corresponds to approximately 1.8 x 10^{8}cfu/mL of *H. pylori* (Ndip *et al*., 2007).

Source and dilution of honey

In this study, six honey varieties obtained from different localities in South Africa were used (table 1). Each honey was diluted with sterile distilled water to attain the following concentrations: 10, 20, 50 and 75 (%v/v), and sterilized by filtering through a 0.22µm membrane filter (Acrodisc, Pall Corporation, USA) (Al Somal *et al*., 1994), into separate sterile bijou bottles.

Antimicrobial screening of honey varieties

The Hole Plate diffusion method of Dastouri *et al* (2008) was used to screen the honey varieties for antimicrobial activity. The innocula were spread-plated onto Columbia Agar plates (Oxoid, UK) supplemented with 7% laked horse blood (Oxoid, England) and Skirrow's antibiotics (SR 0147E, Oxoid, England). The plates were allowed to dry for 3-5 minutes. Subsequently, five wells were punched in each agar plate with a sterile cork borer of 6mm diameter, and filled with 100µL of the different concentrations of the honey solution. Into the remaining well, clarithromycin (0.05µg/mL) was added as the positive control.

Clarithromycin is recognized as a key antibiotic in conventional triple therapy employed for the eradication of *H. pylori* infections because of its powerful bactericidal effect *in vitro* compared with the other available molecules (De Francisco *et al*., 2007). Our positive controls are antibiotics with well-established antibacterial activity (Inhibitory/bactericidal) against *H. pylori* strains (e.g. clarithromycin & amoxicillin) (Mégraud, 2004). Consequently, they were used to assess the validity of the assays.

The plates were incubated at 37ºC under microaerophilic conditions (CampyGen BR0056A, Oxoid, England) and observed after 2 to 5 days. The tests were performed in triplicate and the antimicrobial activity was expressed in terms of the mean diameter of the inhibition zone around each well. *H. pylori* control strain ATCC 43526 was included in all the experiments.

Solvent extraction of crude honey

Observing that the percentage susceptibilities of the test isolates to honey varieties were greater than 50 based on zone diameter ≥ 14mm, all honeys (except Heritage honey and Citrus blossom) were extracted using different organic solvents. This was done by the method of Manyi-Loh *et al*. (2010b). One hundred grams of crude honey was placed in a 500mL separating funnel, diluted with 150mL of sterile distilled water and extracted with 150mL of the different organic solvents (n-hexane, diethyl ether, chloroform and ethyl acetate). This was performed as three successive extractions using 50mL of solvent each time. The shaking time for each extraction process was 15minutes, after which the mixture was allowed to stand to permit the solvent layer to separate. The three successive layers were collected, mixed and concentrated by evaporation under reduced pressure using a rotary evaporator (Steroglass, Strike 202, Padua, Italy) at 40^{o}C for n-hexane, 30^{o}C for diethyl ether, 50^{o}C for chloroform,

Table 1: Floral sources of honeys and their geographical location

Honey types	Floral source	Physical property (color)	Geographical Location
Pure honey(PH)	Citrus	Dark brown	Eastern cape
Goldcrest (GC)	Citrus	Dark brown	Gauteng
Champagne royal train (CRT)	Vineyards	Dark brown	Western cape
Honeyleine (HL)	Strawberries	Dark brown	KwaZulu Natal
Heritage (HH)	Eucalyptus, fynbos, wildflower	Dark brown	Western Cape.
Citrus blossom (CB)	Berry orchards	Pale brown	Western Cape

Table 2: Analysis of the anti-*Helicobacter pylori* activity of honey varieties by Hole Plate assay

Honey types	Zones of inhibition (mm) [a] and Percentage susceptibility (%)			
	Concentrations of honey(% v/v)			
	10	20	50	75
Pure honey(PH)	12.0 ± 8.9 16/30(53.3)	13.3 ± 7.8 19/30 (63.3)	12.7 ± 9.9 16/30(53.3)	16.0 ± 7.5 22/30 (73.3)
Citrus blossom (CB)	13.3 ± 7.5 17/30 (56.7)	12.6 ± 8.0 17/30 (56.7)	15.1 ± 8.7 20/30 (66.7)	15.5 ± 8.5 21/30 (70.0)
Goldcrest(GC)	13.3 ± 7.8 18/30(60.0)	12.9 ± 7.9 16/30(53.3)	14.4 ± 9.0 18/30(60.0)	13.7 ± 10.0 20/30(66.7)
Champagne royal train (CRT)	11.0 ± 8.6 16/30 (53.3)	12.5 ± 9.1 15/30 (50.0)	12.7 ± 8.6 17/30 (56.7)	16.0 ± 7.4 16/30 (53.3)
Honeyleine(HL)	11.0 ± 8.7 14/30 (46.7)	11.7 ± 8.5 17/30 (56.7)	13.0 ± 9.2 19/30 (63.3)	14.8 ± 8.6 18/30 (60.0)
Heritage(HH)	11.6 ± 9.2 16/30 (53.3)	12.4 ± 8.4 17/30 (56.7)	13.1 ± 9.2 18/30 (60.0)	15.1 ± 6.1 16/30 (53.3)
Clarithromycin (CLR); 0.05µg/mL	18.0 ±7.3 23/30(76.7)			

Table 3: Analysis of the anti-*Helicobacter pylori* activity of solvent extracts by Hole Plate assay

Honey types	Zones of inhibition (mm) [a] and Percentage susceptibility (%)			
	Solvent extracts of honey varieties			
	n-Hexane	Diethyl ether	Chloroform	Ethyl acetate
Pure honey(PH)	15.8 ± 7.9 16/30 (53.3)	18.8 ± 8.3 21/30 (70)	16.9 ± 6.6 19/30 (63.3)	16.3 ± 7.6 18/30 (60.0)
Goldcrest (GC)	17.9 ± 8.7 19/30 (63.3)	19.9 ± 10.1 22/30 (73.3)	15.2 ± 8.7 17/30 (56.7)	16.7 ± 9.3 18/30 (60.0)
Champagne royal train (CRT)	18.5 ± 6.6 25/30 (83.3)	22.2 ± 6.1 28/30 (93.3)	21.2 ± 6.4 27/30 (90.0)	16.2 ± 8.6 23/30 (76.7)
Honeyleine (HL)	14.5 ± 9.3 20/30 (66.7)	17.9 ± 11.0 20/30 (66.7)	15.9 ± 9.1 19/30 (63.3)	17.5 ± 8.9 22/30 (73.3)
Clarithromycin (CRT);0.05µg/mL	18.0 ±7.3 23/30(76.7)			

[a] mean of triplicate assay ± standard deviation; Zone of sensitive isolate ≥ 14mm; n, total number of *H. pylori* isolates equals 30.

and 60°C for ethyl acetate. Water contaminating extracts was removed by filtration over anhydrous sodium sulphate. The complete extraction plan is as shown on the flow diagram (fig. 1).

Antimicrobial susceptibility testing of honey solvent extracts

The different solvent extracts of each honey at its most active concentration (50% v/v for CRT, HL; 75% v/v for PH, GC) were tested against the isolates using the method described for crude honey above. The respective pure solvent used for the extraction was tested side by side with its extract. Diameters of zones of inhibition of extracts were measured; averaged and resultant values recorded in millimeters.

Determination of the minimum inhibitory concentration (MIC)

The two most active extracts of each honey were employed in broth microdilution assay to determine their

Table 4: MICs of the two most active extracts of each honey and reference antimicrobials at 50% bacterial growth inhibition

H. pylori isolates	MIC_{50} values in different concentrations									
	Concentrations of solvent extracts (%v/v)								Antibiotics (mg/mL)	
	Pure Honey (PH)		Champagne (CRT)		Honeyleine (HL)		Goldcrest (GC)		Amoxicillin	Metronidazole
	CHLO	DEE	CHLO	DEE	EA	DEE	n-HEX	DEE	AMOX	MET
PE11A	-	-	-	-	-	-	-	-	0.039	5
PE11C	-	-	-	-	-	-	5	5	0.078	5
PE26A	-	-	1.25	-	-	-	-	-	0.02	5
PE76A	1.25	10	10	-	-	-	-	-	0.039	5
PE84C	-	-	-	10	1.25	-	10	-	0.01	2.5
PE93A	1.25	1.25	0.625	0.625	-	0.625	5	5	0.625	5
PE102C	10	-	-	-	10	-	-	10	0.039	5
PE115A	10	-	-	-	10	-	10	-	0.02	5
PE162C	-	-	-	-	5	-	5	5	0.001	5
PE219C	10	1.25	0.625	0.625	2.5	1.25	-	-	0.625	5
PE252C	1.25	-	0	10	2.5	-	2.5	1.25	0.039	5
PE258C	1.25	2.5	2.5	2.5	-	1.25	-	-	0.313	10
PE308C	1.25	10	-	10	2.5	-	2.5	1.25	0.078	5
PE369A	1.25	1.25	1.25	1.25	-	1.25	-	-	0.039	5
PE369C	-	-	-	10	0.625	-	10	0.625	0.02	5
PE397C	1.25	-	-	-	1.25	10	5	5	0.625	5
PE406C	2.5	10	-	10	10	-	5	1.25	0.156	5
PE407C	-	10	-	10	-	-	-	-	0.313	5
PE411C	-	1.25	1.25	1.25	-	1.25	-	-	0.039	5
PE430A	10	-	-	10	-	-	2.5	-	0.01	5
PE430C	-	10	-	10	0.156	-	-	0.078	0.039	5
PE435A	2.5	1.25	1.25	1.25	-	1.25	-	-	0.039	5
PE436A	-	0.625	0.625	0.625	2.5	1.25	1.25	1.25	0.625	5
PE462A	-	-	-	-	-	-	0.039	-	0.625	5
PE462C	-	-	0.625	-	-	10	-	-	1.25	2.5
PE464A	-	10	-	10	10	-	-	-	0.078	5
PE464C	1.25	-	-	-	2.5	10	1.25	1.25	0.002	2.5
PE466A	10	-	-	-	10	-	10	10	0.01	5
PE466C	0.01	-	-	-	-	-	-	-	0.625	5
PE473A	2.5	-	-	10	2.5	-	2.5	2.5	0.156	5

MIC_{50} after triplicate assay; -, value not within susceptible range; CHLO, chloroform; DEE, diethyl ether; HEX, hexane; EA, ethyl acetate; AMOX, amoxicillin; MET, metronidazole.

MICs against the test isolates, according to the method of Njume *et al*. (2011). Two-fold dilutions were prepared in 96-well-round-bottom microtitre plates (Greiner Bio-One, Frickenhausen) in BHI broth (Oxoid, England); the final extract concentration was 0.01-10%v/v. Similarly, amoxicillin (0.0012-1.25 mg/mL) and metronidazole (0.01-10 mg/mL) were two-fold diluted and tested on the same plate with the honey solvent extracts as reference antimicrobials. Control wells were also prepared with culture medium only, culture medium with honey extract and culture medium with bacterial isolate only. The inoculum of each strain was diluted tenfold in sterile normal saline. Twenty µL of the bacterial suspension (10^8 CFU/mL) was aliquoted into each well. The final volume in each well of BHI broth, honey extract, and inoculum was 120µL. The absorbencies were read using an ELISA microtitre plate reader (Model 680, S/N 19138, Biorad, Japan) adjusted at 620nm. The micro plates were sealed and incubated at 37°C under microaerophilic conditions for 2-3days, agitated and the absorbencies were again read at the same wavelength. The absorbencies were compared to the values obtained before incubation to detect any increase or decrease in bacterial growth. The lowest concentration of the extract resulting in inhibition of bacterial growth by 50% was taken as the MIC_{50}.

Time-kill assay of extract

Assay for the rate of kill of *H. pylori* isolates by the most active extract of each honey was determined in accordance with the method of Akinpelu *et al.* (2008) with modifications. Each isolate was subcultured on CBA (Oxoid, England) plates and incubated at 37°C under microaerophilic conditions for 2-3 days. Growth of each isolate was transferred into BHI broth (Oxoid, England) and incubated overnight under the same growth conditions. The turbidity of an 18 h old broth culture of the test isolate was standardized to contain approximately 1.8×10^8 cfu/mL. A 0.5mL volume of the standardized suspension was added to 4.5 mL of different concentrations of the extracts (1/2MIC, MIC, 2xMIC and 4xMIC).

Table 5(A-D): Bactericidal activity of honey extracts at different concentrations against *H. pylori* isolate (PE 252C)

A.

Conc. of chloroform extract of PH (%v/v)	Percentage of bacterial cells killed												
	Time interval (hrs)												
	0	6	12	18	24	30	36	42	48	54	60	66	72
5	NG	NG	0	70	30	20	0	100	100	100	100	100	100
10	NG	NG	0	30	60	30	0	0	20	0	20	80	0
20	NG	NG	50	0	30	0	0	0	30	18	0	18	0
40	NG	NG	100	100	30	100	0	100	60	60	100	100	0
Neg. control	G	NG	0	0	0	0	0	0	0	0	0	0	0

NG, No growth; G, growth; 5%v/v, 1/2xMIC; 10%v/v, MIC; 20%v/v, 2xMIC; 40%v/v, 4xMIC

Table 5 B:

Conc. of ethyl acet. extract of HL (%v/v)	Percentage of bacterial cells killed												
	Time interval (hrs)												
	0	6	12	18	24	30	36	42	48	54	60	66	72
5	NG	NG	0	100	10	0	0	0	0	100	30	0	100
10	NG	NG	0	0	0	0	0	0	0	30	0	0	100
20	NG	NG	100	0	0	0	0	0	0	30	70	0	30
40	NG	NG	100	100	100	100	80	90	90	100	100	100	100
Neg. control	G	NG	0	0	0	0	0	0	0	0	0	0	0

NG, No growth; G, growth; 5%v/v, 1/2xMIC; 10%v/v, MIC; 20%v/v, 2xMIC; 40%v/v, 4xMIC.

Table 5 C:

Conc. of chloroform extract of CRT (%v/v)	Percentage of bacterial cells killed												
	Time interval (hrs)												
	0	6	12	18	24	30	36	42	48	54	60	66	72
5	NG	NG	0	0	0	0	0	0	0	0	20	0	0
10	NG	NG	0	0	0	0	0	0	10	0	20	0	0
20	NG	NG	0	0	0	0	0	0	0	0	20	0	0
40	NG	NG	0	100	100	100	100	100	100	90	80	100	100
Neg. control	G	NG	0	0	0	0	0	0	0	0	0	0	0

NG, No growth; G, growth; 5%v/v, 1/2xMIC; 10%v/v, MIC; 20%v/v, 2xMIC; 40%v/v, 4xMIC.

Table 5 D:

Conc. of n-hexane extract of GC(%v/v)	Percentage of bacterial cells killed												
	Time intervals (hrs)												
	0	6	12	18	24	30	36	42	48	54	60	66	72
5	NG	NG	20	15	0	0	40	100	30	40	0	100	0
10	NG	NG	10	0	40	0	0	0	30	0	0	0	0
20	NG	NG	80	40	30	0	30	70	0	0	20	20	30
40	NG	NG	100	80	50	100	50	100	100	100	100	100	100
Neg. control	G	NG	0	0	0	0	0	0	0	0	0	0	0

NG, No growth; G, growth; 5%v/v, 1/2xMIC; 10%v/v, MIC; 20%v/v, 2xMIC; 40%v/v, 4xMIC.

These were incubated at 37^{o}C under microaerophilic condition in an orbital shaker at 120rpm and the killing rate was determined over a period of 72hrs. Exactly 0.5mL volume of each suspension was withdrawn at 6h intervals and transferred to 4.5mL of BHI broth recovery medium containing 3% "Tween 80" to neutralize the effects of the antimicrobial compound carry-overs from the test isolates. The suspension was serially diluted and 100µL plated out for viable counts. The plates were later incubated at 37°C for 72hrs. The control plates contained the bacterial cells without the extract. The emergent bacterial colonies were counted and compared to the counts of the culture control. Time-kill assays were carried out in duplicate.

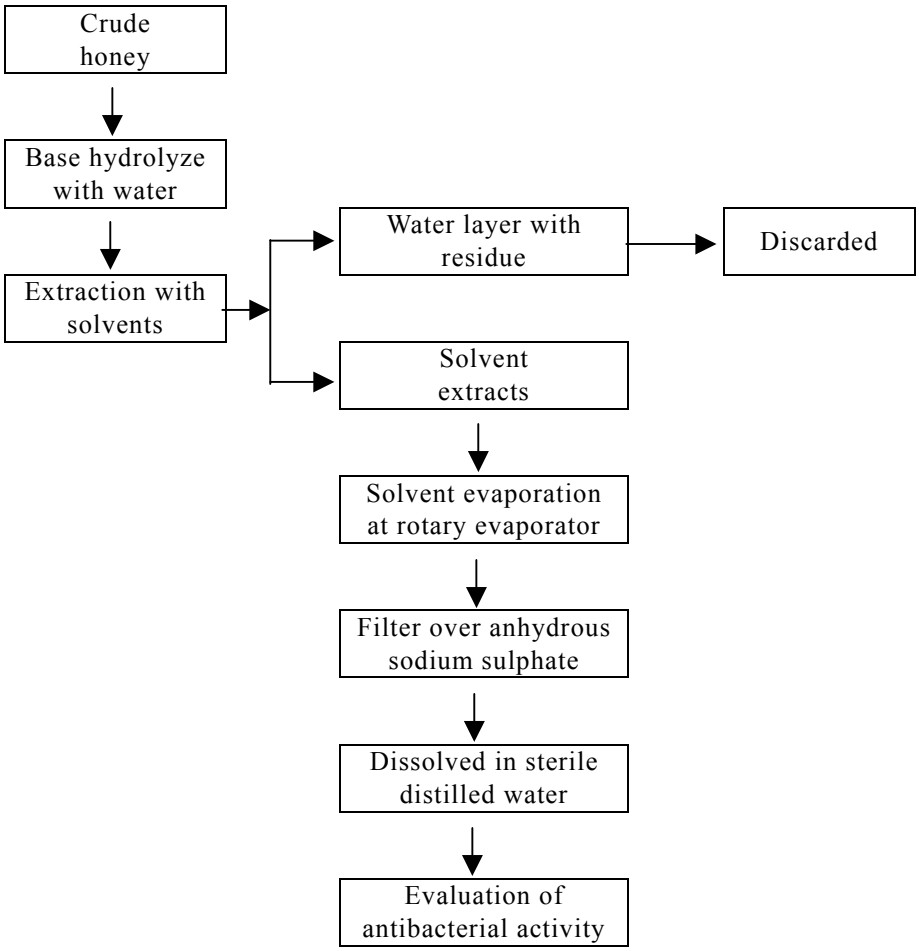

Fig.1: Flow diagram for the extraction of honey

STATISTICAL ANALYSIS

Diameters of zones of inhibition (excluding the diameter of the well) were expressed as mean±Standard deviation. One-way ANOVA test was used while employing SPSS (version 18.0, Illinois, USA) to determine any statistically significant difference by comparing zone diameters of the different honeys at various concentrations; zone diameters of clarithromycin to different solvent extracts, zone diameters of different extracts as well as the MIC values of these extracts to amoxicillin and metronidazole at 95% significance level.

RESULTS

Antimicrobial screening of honey varieties

All the honey samples at the various concentrations (10, 20, 50, 75%v/v) exhibited varying levels of antibacterial activity against the test isolates as indicated by mean ± S.D of zone of inhibition and percentage susceptibilities (table 2). PH, CB and GC honeys were most active at 75% v/v concentration whilst CRT, HL and HH were most active at 50% v/v concentration. Consequently, there was no statistically significant difference ($P > 0.05$) recorded when the mean zone diameters of these honeys obtained at their most active concentrations were compared to that of clarithromycin (positive control).

Antimicrobial susceptibility testing of honey solvent extracts

All the solvent extracts of the honeys demonstrated anti-*H. pylori* activity with mean zone diameter of inhibition and percentage susceptibility of isolates in the ranges 14.5-22.2 mm and 53.3-93.35% respectively (table 3). Based on the percentage susceptibility and mean zone diameter of inhibition, diethyl ether extract of CRT honey was the most active (22.2±6.1mm (n=30); 28/30, 93.3%) while the least antibacterial activity was noted for n-hexane extract of PH honey (15.8±7.9mm (n=30); 16/30, 53.3%). However, no statistically significant difference ($P>0.05$) was reached comparing the mean zone diameters of solvent extracts to clarithromycin.

Determination of the minimum inhibitory concentration

The MIC50 values of all the extracts ranged from 0.01-10% v/v concentration (table 4). The two most active

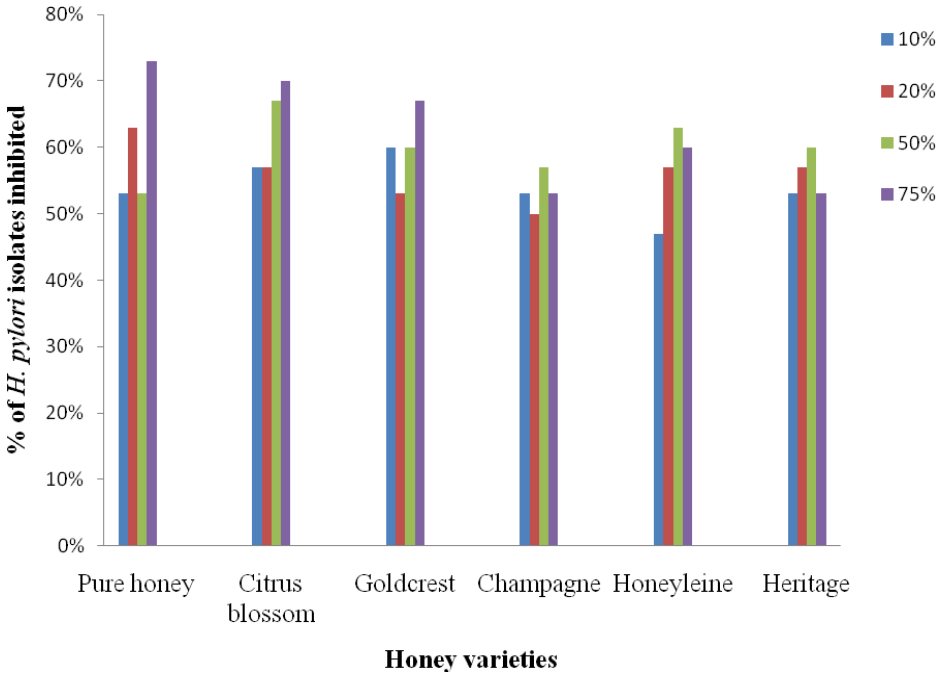

Fig. 2: Antimicrobial activity of honey varieties at 10% v/v, 20% v/v, 50% v/v, 75% v/v against *H. pylori* isolates. Zone diameters of sensitive isolates were ≥ 14.

extracts were the chloroform extract of PH and CRT honeys with MIC50 ranges 0.01-10% v/v and 0.625-10% v/v respectively; since there was no statistically significant difference (P>0.05) recorded when their MIC50 values were compared to amoxicillin (0.001-1.25mg/mL).

Time-kill assay of extract

The most active extract of each honey (i.e. chloroform extract of PH and CRT honeys, n-hexane extract of GC honey as well as ethyl acetate extract of HL honey) was employed in this assay to ascertain the rate of kill of the test isolates. The results of the bactericidal activity of these solvent extracts over the period of 72hrs are presented on table 5(A-D). As shown in all the tables, the lag phase of *H. pylori* strain PE 252C was between 6 and 12hrs as there was no growth on plates inoculated with extract constituted broth as well as on the control plate. With the exception of chloroform extract of PH, the most potent bactericidal effect was established at 4xMIC (40%v/v) against the test isolates by all the honey solvent extracts at different time interval. Interestingly, at 1/2 MIC (5%v/v) the chloroform extract of PH honey demonstrated a profound bactericidal activity over a time interval of 42-72hrs.

DISCUSSION

The pathogenic role of *H. pylori* infection in the development of gastro duodenal diseases and the impact of resistance on the clinical outcome (Megraud, 2001) enthused the search for newer treatments and the use of natural agents as alternative therapies (Li *et al*., 2005; Ndip *et al*., 2007; Manyi-Loh *et al*., 2010a, 2010b). Honey has been documented to possess bactericidal activity on many pathogenic micro-organisms including enteropathogens such as *Salmonella*, *Shigella* and *E. coli* species and other gram- negative micro-organisms (Jeddar *et al*., 1985).

In the preliminary screening of PH, CB, GC, CRT, HL and HH honeys at various concentrations (10, 20, 50 and 75% v/v), the data showed that these honeys possess anti-*H. pylori* activity exhibited by zones of inhibition that ranged from 11.0-16.0 mm. This inhibition gives credence to the fact that these honeys could be used as antibacterial agents in the treatment of ailments caused by *H. pylori*.

Furthermore, this result is congruent with the work of Saraf and colleagues (2009) that evaluated the antibacterial activity of local Fijian honeys on both gram-positive and gram-negative bacteria at varying concentrations. With the exception of the other three honeys, the anti-*H. pylori* activity of PH, CB and GC honeys was concentration dependent since they were most active at 75% v/v concentration (fig. 2). In addition, there was no statistically significant difference (P>0.05) recorded when the mean zone diameters of these local honeys at their most active concentrations were compared to that of clarithromycin. This may advocate that these honeys at their specified concentrations could elicit antibacterial potential seeming that of clarithromycin, a

key antibiotic in *H. pylori* treatment regimen (Megraud and Lehours, 2007).

In spite the fact that all the honeys demonstrated antibacterial activity against our test isolates, there was variation in the percentage susceptibilities of the isolates to the different honey types (fig.2). This variation has purportedly been attributed to the floral sources and plant species foraged by the bees to produce honey (Yao *et al*., 2003). Moreover, the floral and plant distribution is affected by the climatic conditions prevailing in a certain locality (Ndip *et al*., 2007; Manyi-Loh *et al*., 2010b). Apparently, the environmental conditions would influence the chemical composition of the honeys and as a consequence their biological properties/functions would differ. This may hold true for our study since the honeys were from different localities in South Africa.

In the past, several studies have reported the antimicrobial activity of only the aqueous solution of honey (Taormina *et al*., 2001; French *et al.*, 2005; Ndip *et al*., 2007). It is obvious that honey possesses antimicrobial potential but it is not clear whether it is the mass of the honey or some fraction of it. In view of the fact that there might be some specific components (phytochemicals or non-peroxide factors) such as flavonoids and phenolic acids which may be contributing to the antimicrobial activity, we decided to use four organic solvents viz n-hexane, diethyl ether, chloroform and ethyl acetate that have been previously employed by Zagloul *et al*. (2001) to possibly extract the afore mentioned phytoconstituents.

Accordingly, PH, GC, CRT and HL honeys were employed in the extraction process except HH and CB honeys that were obtained from the same locality as CRT honey. The physical properties (particularly color) credited the continued usage of CRT honey in the subsequent bioassays. The color of honey is closely related to its chemical composition, primarily to the presence of carotenoids, flavonoids and derivatives of tannins and polyphenols (Kaškonienė *et al*., 2009). The solvent extracts of the honeys were evaluated for anti-*H. pylori* activity. The resulting data showed that the most potent antibacterial activity against the test isolates was demonstrated by the diethyl ether extract of CRT honey (22.2±6.1mm (n=30); 28/30, 93.3%) unlike the n-hexane extract of PH honey (15.8±7.9 mm (n=30); 16/30, 53.3%) that presented the least antibacterial activity. This was based on mean±S.D. of zone diameter of inhibition observed with each solvent extract as well as the percentage susceptibilities of the test isolates to each extract (table 3).

Notwithstanding, all the solvent extracts presented good anti-*H. pylori* activity implying that the antimicrobial components were extractable into the various solvent extracts. This corroborates the finding of Chauhan *et al*. (2010) that equally reported significant antibacterial activity of solvent extracts of honey against gram negative micro-organisms. In addition, there was no statistically significant difference ($P>0.05$) recorded when the mean± S.D of diameters of zones of inhibition were compared to the positive control (clarithromycin); suggesting that the specific solvent extracts may contain putative antimicrobial compounds whose therapeutic potential are highly comparable to clathromycin.

Furthermore, we realized an increase in the antimicrobial activity of the solvent extracts of these honeys. Based on mean zone diameter and percentage susceptibilities of test isolates, there was an increase from 16.0mm (crude) to 22.2mm (extracts) and 73.3% (crude) to 93.3% (extract) respectively. This may suggest that the putative antimicrobial components would have been concentrated in the honeys after extraction (Manyi-Loh *et al*., 2010b).

Guided by the work of Tanih *et al*. (2010a) who reported amoxicillin and metronidazole as the most sensitive and resistant antibiotics, respectively, against *H. pylori* in our environment, we included both antibiotics as reference antimicrobials in the assay to determine the MICs of the two most active extracts of each honey. The lowest MIC_{50} range (best antibacterial activity) of 0.01-10% was obtained for the chloroform extract of PH honey. Both the ethyl acetate extract of HL and n-hexane extract of GC honeys recorded MIC_{50} values of 0.156-10% v/v and 0.039-10%v/v respectively. In addition, no statistically significant difference ($P> 0.05$) was recorded when MIC_{50} values of the chloroform extracts of both PH and CRT honeys were compared to amoxicillin (0.001-1.25mg/mL). This may suggest that of the four solvents used for extraction in this study, chloroform extracts demonstrated the highest relative antibacterial activity; insinuating that chloroform solvent might have had higher solubility for antimicrobial phytoconstituents because it has been reported that different solvents have the capacity to extracts different phytoconstituents based on their solubility or polarity (Doughari, 2006). This may further indicate that these chloroform extracts would contain most active substances (Yeşilada *et al*., 1999).

The bactericidal activity of the chloroform extracts of PH and CRT honeys, n-hexane extract of GC honey as well as ethyl acetate extract of HL honey was determined using viability studies.

At different time intervals during cultivation, broth constituted with different concentrations of the solvent extracts were sampled, diluted and the total colony counts on the plates were enumerated in order to determine the relationship between the concentration of the solvent extracts, treatment time and bactericidal activity (Wang and Huang, 2005).

The periods 6 -12 hrs could be considered as the lag phase of the isolate since there was no growth on the negative control plate as well as on plates inoculated with sample from extract constituted broth. At the time interval of 42-72hrs, the test isolate was killed at a concentration as low as 5% v/v (1/2xMIC) of the chloroform extract of PH honey, thus it was the most effective bactericidal extract (table 5A). At the highest concentration of 40% v/v (4x MIC), ethyl acetate extract of HL, chloroform extract of CRT and n-hexane extract of GC presented potent bactericidal activity over the time interval of 12-72hrs,18-72hrs and 30-72hrs respectively (table 5 B-D). This may suggest that with a further increase in extract concentration better results would be obtained. In addition, there was growth of bacterial cells at 10%v/v (MIC), showing they were inhibited as expected.

The ability of honey to inhibit (bacteriostatic) and or kill (bactericidal) a micro-organism has been accredited to its high osmotic effect, high acidic nature (pH being 3.2-4.5), hydrogen peroxide concentration and its phytochemical nature i.e. flavonoids, phenolic acids and many unidentified compounds (Bogdanov, 1984; Osman *et al*., 2003).The contributory effects of the other components of the extracts cannot be ignored in view of the presence of bioactive substances that have antimicrobial effects (Subrahmanyam *et al*., 2001).

CONCLUSION

The need for an alternative antibacterial substance derived from natural products on a sustainable manner as an alternative/complementary source of treatment for *H. pylori* infections has become a subject of interest for scientists' world over because of multidrug resistance presented by this micro-organism of medical importance (Megraud and Lehours, 2007). The results of this study unequivocally shows that PH, GC, CRT and HL honeys (bulk components) as well as the necessary antibacterial components extracted in n-hexane, diethyl ether, chloroform and ethyl acetate solvents from these honeys could become potential candidates to explore be exploited for further investigation in the discovery of novel natural anti-*H. pylori* compounds, which would be useful on a sustainable basis.

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