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Quercetin -3-*O*-*β*-D-glucouronide butyl ester from *Vitis vinifera* leaves of potent anti-*helicobacter pylori* activity and impact of its combination with clarithromycin

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Abstract

Antibiotic resistance by *Helicobacter pylori* has reduced the eradication rates of multiple therapies. To overcome this crisis, we select safe nutraceutical derived agents of low price for investigation. The hydroalcholic extract of *Vitis vinifera* leaves beside the isolated compounds; quercetin-3-*O*-*β*-D-glucouronide (CPD1) beside its butyl ester (CPD2); which was isolated first time of the plant were investigated against six clarithromycin resistant isolated strains of *H.pylori*. Combination between the antibiotic clarithromycin and extract, CPD1 and CPD2 were also carried out. The hydroalcoholic extract, CPD1 and CPD2 showed distinctive inhibition zones of the growth of *H.pylori* in comparison to clarithromycin. CPD2 only showed synergistic effect on combination with clarithromycin. The mechanism of action was studied virtually against *H. pylori* HyPB and showed docking scores -7.26 and -6.89 Kcal for CPD1 and CPD2 respectively, which mean that both compounds affect colonization *of H. pylori* and in turn prevent formation of new generations of *H.pylori*.

Keywords: *Vitis vinifera;* Quercetin -3-*O*- ***β*** -D-glucouronide butyl ester; *Helicobacter pylori*; Molecular Docking; *Helicobacter pylori* HypB protein

Introduction

Helicobacter pylori is a microaerophilic gastric spiral pathogen which has ability to establish infection in human stomach that can last for years or decades. It is the main pathogenic factor in chronic gastritis and peptic ulcer disease, and is also the initiating factor in gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma. It infects more than 50% of world population. Currently, the most preferred *H. pylori* eradication therapy imply, proton pump inhibitor and more than one antibiotic among them clarithromycin antibiotic. However, using of a multiple therapy regimens have not been very successful in some clinical practice because of the *H. pylori* antibiotic resistance especially clarithromycin [1].

Clarithromycin is one of multiple therapy regimen of *H. pylori* eradication but there is an increasing rate of *H. pylori* resistance towards it. Therefore, the synergism of clarithromycin, with natural products especially nutraceuticals is one of the alternative therapy regimen [2].

Increasing complications in the conventional multiple therapies motivate an urgent need to develop new nonsynthetic or nutraceutical antibacterial agents against *H. pylori* infection that are safe, specific and highly effective. Many natural phenolics especially flavonoids were reported to potentiate antibiotics action towards microbes [3].

Vitis vinifera L. (Vitaceae) is native from Mediterranean region. Since ancient times, *V. vinifera* leaves have been used in medicine due to various biological activities including hepatoprotective, spasmolytic, antidiabetic and vaso-relaxant effects, antimicrobial, antiviral, anti-inflammatory, antinociceptive, and antioxidant activities [4-5]. The leaves juice has been used as an antiseptic for eyewash. In addition, *V. vinifera* leaves are used as food in Egypt, as well as employed to produce dietary ingredients or in the formulation of dietary antioxidant supplements.

Previous chemical investigations have shown the presence of high phenolic contents. It contains organic acids, phenolic acids, flavonols, tannins, procyanidins,

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anthocyanins, lipids, enzymes, vitamins, carotenoids, terpenes, and reducing or non-reducing sugars. [4-5].
Few studies reported the anti-*H. pylori* activity of *V. vinifera* seeds and wine, including an active constituent ; resveratrol which is a stilbene isolated from red wine. Although, *V. vinifera* leaves are rich in phenolics, little is currently known about the anti-*H. pylori* properties of it, making *V. vinifera* leaves candidates for our study [6].
Our aim of this study is to evaluate the anti-*H. pylori* activity of *V. vinifera* leaves and some isolated metabolites. In addition to study the effect of combination between the total extract, isolated metabolites and clarithromycin. Moreover, studying the mechanism of action virtually using molecular docking against *H. pylori* HypB protein**.**

2. Materials and Methods

2.1. *General methods*

^{1}H-nuclear magnetic resonance (NMR) spectra were recorded on a Bruker avance III, 400 MHz NMR spectrometer and 100 MHz for ^{13}C NMR. ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C-NMR chemical shifts to dimethyl sulfoxide-d_6 and converted to the TMS scale by adding 39.5, ultraviolet (UV) recordings were made on a Shimadzu UV-Visible-1601 spectrophotometer. Paper chromatographic (PC) analysis and preparative Paper Chromatography(PPC) separation were carried out on Whatman filter paper sheets No. 1 and 3 MM papers, using solvent systems 15% HOAc and BAW (n-BuOH-HOAc-H_2O, 4:1:5, upper layer), [7-8].

2.2. Plant materials

Vitis vinifera leaves were purchased from local farm, Ashmoun city, Menoufia governorate, Egypt.

2.3. Extraction and isolation

Powdered *Vitis vinifera* leaves (500g) were macerated in 5 liter EtOH-H_2O (3:1), for three times. The filtrates were collected and evaporated up to dryness under vacuum to yield a dark brown amorphous powder of the hydroalcoholic extract (77 g). The hydro-alcoholic extract (50g) was suspended in water and defatted by partition with methylene chloride, followed by *n*-butanol (BuOH). The n-butanol extract (10 g) was fractionated on column chromatography on diaion HP-20 column (5 cm × 120 cm, 500 g) using H_2O and EtOH mixtures in order of decreasing polarities as an eluent to yield 4 main fractions (I-IV).Each fraction was examined by two dimension PC using BAW followed by 15% HOAc as eluents [7-8]. Fractions II, III were applied to column Sephadex LH-20 using 50% (H_2O/EtOH), to yield compounds (1-2) as a major products.

3 .Evaluation of anti H.pylori activity.

3.1. *Antimicrobial assay against Helicobacter pylori strains***.**

The strains used in this experiment were *Helicobacter pylori* G27, 7.13, J99, HPAG1, SS1 and 26659 which referred in American Type Culture Collection with the codes J99 (ATCC. 700824), 26695 (ATCC. 700392), G27 (ATCC 43504), SS1 (ATCC. 43579), HPAG1(ATCC. 51652) and 7.13 (ATCC 51111) .
Brucella broth media supplemented with horse serum (10% v/v) and glycerol (10% v/v).The media used was blood agar consisting of Mueller-Hinton Agar and horse blood (10% v/v). Horse blood is mixed aseptically on a warm sterilized agar medium at 56°C. Frozen *H. pylori* stock is inoculated in blood agar media and incubated for 3-5 days at 37°C under micro-aerophilic conditions in 10% CO_2 incubator.
Bacterial suspension solution is made using serum broth (Mueller- Hinton Broth and horse serum (10% v/v) which is mixed aseptically after the sterilization process. Bacterial culture from the blood agar media is suspended in serum broth then incubated for 24 hours in 10% CO_2 incubator at 37°C. The bacterial suspension is diluted with serum broth until the turbidity is equivalent to a standard solution of 0.5 McFarland (108 CFU/ml). In microdilution and turbidimetry testing, the suspension was diluted again 1:20 to produce a colony count of $5x10^6$ CFU / ml [9-10].

3.2. H. pylori Growth Inhibition by Disk Diffusion

We performed several test to check the ability of samples to inhibit the *H. pylori* growth. Firstly, the growth inhibition of *H. pylori* was assayed by the disc diffusion method. A total of 50 µl of bacterial suspension was pipetted into a petri dish then mixed with a serum medium of 15 mL consisting of Mueller-Hinton Agar supplemented with 10% horse serum and homogenized. The media should be left to solidify then prepared 5 sterile paper (d = 6.7 mm).
Disc paper is dipped in each of these solutions then placed on the surface of the media. Petri dishes are stored in a 10% CO_2 incubator at 37°C for 3 days. Then the diameter of the clear zone was observed using a digital calipers [11].

3.3. *Determination of Minimum Inhibitory Concentration (MIC)*

MIC values were determined through the broth microdilution method using several concentrations of samples (2.5µg-0.078 µg) / ml) in serum broth that contained bacterial suspensions. Then the microdilution plate was incubated at 37°C for 3 days in a 10% CO_2 incubator. The MIC value is shown from the smallest concentration that successfully inhibits bacteria so that there is no precipitation or turbidity is founded in the plate well [12-13].

4. Molecular docking: The crystal structure of HypB from *H. pylori* in complex with nickel (PDB: 4LPS, 2.00 A ° resolution) was chosen as the template for molecular docking study by the MOE ver. (2014.09),

molecular simulation software. The docking protocol was done according to Abdelghfar et al. 2019 and Bankeu et al. 2018 [14-15].

Docking Validation

Validation of the docking protocol was done by the re-docking of the co-crystallized ligand (GDP; nitrogenous base) the pocket of 4LPS. The binding pose of the cocrystallized ligands could be reproduced by the docking program with root mean square deviation (rmsd) values\ 0.77 A°.

5. Data Analysis

The SPSS statistical software package version 25.0 was used for all statistical analyses.

3. Results

Phytochemical investigation of *Vitis vinifera* leaves led to isolation and identification of two flavonoids. All of these structures were identified by 1 D and 2 D NMR including H-H COSY, HSQC and HMBC analyses. The compounds were compared with previous reported data. These flavonoid compounds are identified as quercetin -3- *O-β*-D- glucouronide (**1**) as a major constituent, beside quercetin -3-*O- β*-D-glucouronide butyl ester (**2**) which isolated for the first time from the plant.

3.1. Identification of CPD2:

Compound **2** isolated as yellow amorphous powder, showed dark purple spot under UV lamp, turned to yellow after fuming with ammonia.**^{1}H-NMR** (DMSO-*d*6, 400 MHz): δ ppm 6.21 (1H, br S, H-6), 6.42 (1H, br S, H-8), 7.51 (1H, d, J=2 Hz, H-2'), 6.83(1H, d, J= 8.48 Hz, H-5'), 5.47(1H, d, J= 7.04 Hz, H-1''), 3.98 (t, *J* = 6.4 Hz, 2H, H-1'''), 1.42(2H, m, H-2'''), 1.15(2H, m, H-3'''), 0.73 (3H, t, J= 7.34 Hz, H-4''''); **^{13}C-NMR** (DMSO-*d*6, 100 MHz): δ ppm 156.91 (C-2), 133.56 (C-3), 177.60(C-4), 161.66 (C-5), 99.28 (C-6), 164.81 (C-7), 94.09 (C-8), 156.76 (C-9), 104.33 (C-10), 121.32 (C-1'), 115.62 (C-2'), 145.37 (C-3'), 149.10 (C-4'), 116.52 (C-5'), 122.14 (C-6'), 101.79(C-1''), 74.21 (C-2''), 76.12 (C-3''), 71.85 (C-4''),76.28 (C-5''), 169.41 (COO-), 64.59 (C-1'''), 30.23 (C-2'''), 18.75 (C-3'''), 13.80 (C-4''').

Table 1: **The inhibition zone values (mm) for extract, CPD1 and CPD2, against *H.pylori* strains.**

Strains	Concentration of samples (µg/ml)	Extract	CPD1	CPD2
7.13	2.5	31.00	26.00	38.00
7.13	1.25	29.10	22.10	36.10
7.13	0.625	28.00	19.00	33.00
7.13/	0.312	26.00	15.68	31.68
7.13/	0.165	25.90	28.00	28.90
7.13/	0.0781	24.45	24.45	24.45
26659	2.5	23.00	12.00	38.00
26659	1.25	21.20	12.20	33.20
26659	0.625	20.90	8.90	33.90
26659	0.312	18.00	6.00	30.00
26659	0.165	16.80	20.90	29.80
26659	0.0781	14.00	14.00	24.00
G27	2.5	32.00	28.00	40.00
G27	1.25	28.10	24.10	37.10
G27	0.625	27.20	21.20	35.20
G27	0.312	25.20	17.20	33.20
G27	0.165	29.00	27.20	29.00
G27	0.0781	25.40	25.40	25.40
HPCPD1	2.5	28.00	23.00	37.00
HPCPD1	1.25	27.10	18.10	35.10
HPCPD1	0.625	25.78	14.78	34.78
HPCPD1	0.312	23.80	10.80	32.80
HPCPD1	0.165	21.90	25.78	30.90
HPCPD1	0.0781	19.20	19.20	25.20
J99	2.5	29.00	25.00	40.00
J99	1.25	27.00	20.00	33.00
J99	0.625	25.20	16.20	32.20
J99	0.312	23.90	12.90	30.90

J99	0.165	22.68	25.20	29.68
J99	0.0781	21.60	21.60	24.60
SS1	2.5	26.00	18.00	39.00
SS1	1.25	25, 20	15.20	34.20
SS1	0.625	24.95	11.95	36.95
SS1	0.312	23.20	8.20	29.20
SS1	0.165	22.90	24.95	28.90
SS1	0.0781	20.50	20.50	24.50

Table 2: Minimum inhibitory concentration (MIC) in µg/ml for extract, CPD1, CPD2, the combination of clarithromycin (Clr) with extract, CPD1 and CPD2.

H.pylori Strain	Extract	CPD1	CPD2	Extract + Clr	CPD1+Clr	CPD2 +Clr	Clr
G27	8.20	2.00	2.00	8.20	2.00	2.00	2.81
7.13	8.10	2.21	2.15	8.10	2.21	1.98	2.62
J99	7.80	2.15	1.95	7.80	2.15	1.80	2.57
HPCPD1	8.10	1.90	1.90	8.10	1.90	1.60	2.75
SS1	7.85	2.00	2.20	7.85	2.00	1.40	2.68
26659	7.55	1.98	1.95	7.55	1.98	1.20	2.46

Table 3: Docking of compounds isolated from *Vitis vinifera* against HypB from *H. pylori* in complex with nickel

Compound	Interacting amino acid	Bond Interaction	Bond distance (A)	Bond Energy (Kcal/mol)	Score (Kcal/mol)
Compound 1	MET 213	H-donor	3.74	-1.7	-7.26
	LYS 183	H-acceptor	3.14	-4.8	
	ARG89	H-acceptor	3.17	-3.9	
	GLY 58	H-acceptor	2.92	-5.3	
	LYS 59	H-acceptor	3.10	-2.4	
	LYS 59	H-acceptor	3.28	-7.1	
	MG 301	Metal	2.60	-1.9	
	ARG 89	Ionic	3.17	-3.5	
	MG 301	Ionic	2.60	-7.8	
	LYS 59	ionic	3.28	-2.9	
Compound 2	ASP 185	H-donor	3.30	-1.7	-6.89
	LYS 183	H-acceptor	3.04	-6.4	
	LYS 183	Pi-cation	4.15	4.15	

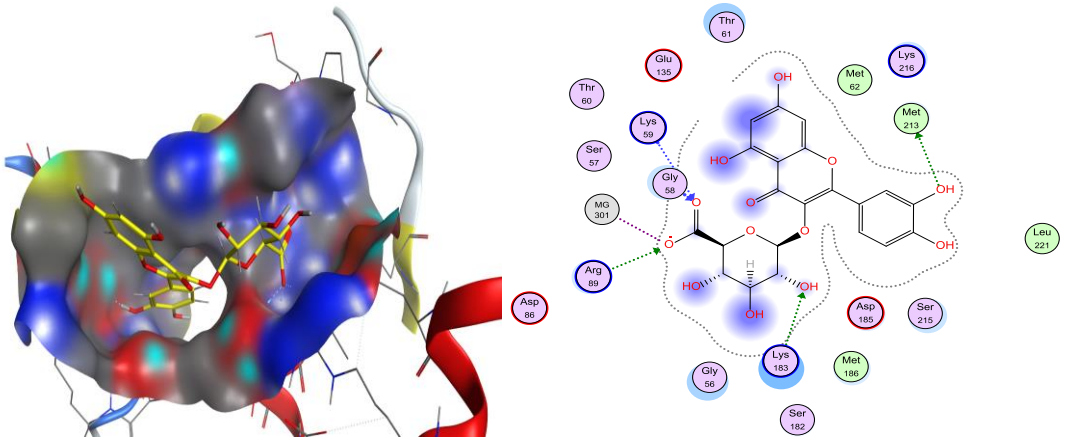

Figure 1: Binding Patterns of quercetin -3-*O*-glucouronide against HypB from *H. pylori* in complex with nickel

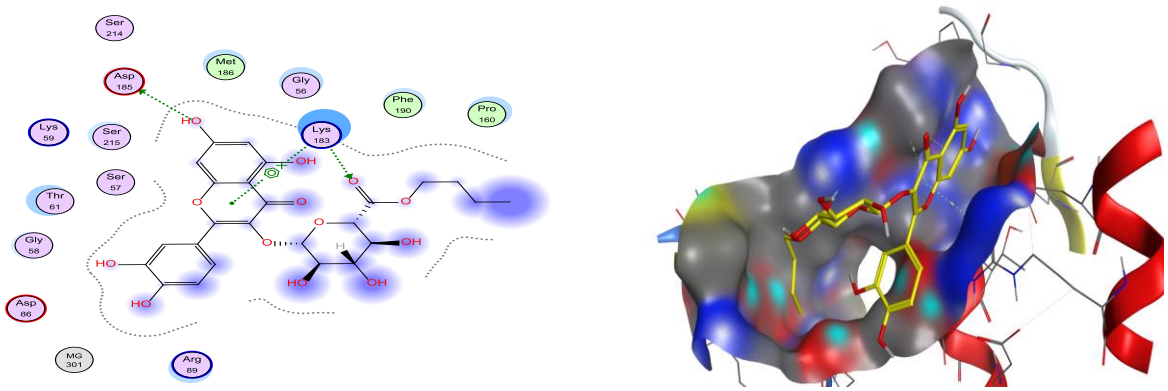

Figure 2: Binding Patterns of quercetin -3-*O*-glucouronide butyl ester against HypB from *H. pylori* in complex with nickel

4. Discussion

4.1. Identification of isolated compounds:

Vitis vinifera cultivars reported to include the 3-*O*-glucosides and 3-*O*-glucuronides of quercetin as a major constituents [16]. CPD1 was isolated before from the plant. CPD2 isolated for the first time from the plant and identified by 1D and 2D NMR according to the previously reported data [17].

4.2. *Antimicrobial activity against H.pylori*

The results of growth inhibition for *H. pylori* showed that the hydroalcoholic extract, CPD1 and CPD2 showed positive results with different levels of effect for all concentrations in all dilutions of the tested samples towards all *H. pylori* strains that were used in the experiment (Table 2).

However, it is noticeable that the inhibitory effect of the growth of the *H. pylori* bacterium differs from one concentration to another for the three tested samples hydroalcoholic extract, CPD1 and CPD2. The inhibitory zone for CPD2 is larger than the inhibitory zone of the hydroalcoholic extract and CPD1. It was observed that the CPD2 concentration of 2.5 µg/ml gave the highest rates of inhibition of microbial growth for different strains, and the levels of inhibition varied from 40 mm with G27 and 38 mm with 7.13, 40 mm for J99, 37 mm with strain HPAG1, 39 mm with strain SS1, and 38 mm with strain 26659.

While, the inhibitory zone of the CPD2 concentration: 1.25 µg/ml was the second highest zone towards the investigated strains of *H. pylori*. The level of the inhibitory effect of the concentrations used in the dilutions down to the lowest level of the inhibitory effect is graded at a concentration of 0.078 µg/ml. It is noted that there is no inhibitory effect of the DMSO solvent, which was used as a negative control in the experiments.

This indicates the extent of the potency of CPD2 as a *H. pylori* growth inhibitor, which makes the use of this compound a promising agent in the eradication of *H.pylori*.

4.2.1. Combination with clarithromycin.

The combination of the antibiotic clarithromycin (1volume:1volume) with the hydroalcoholic extract and CPD1 separately, showed no effect on their MICs. While the combination between CPD2 and clarithromycin increase inhibition of *H.pylori* growth zones values and MICs values (Table 3), and this reflects the catalytic and effective role in a one-to-one ratio between the CPD2 and the antibiotic.

This reflects the supportive effect of CPD2 to the antibiotic clarithromycin.

This study represents the antimicrobial effect investigation against resistant strain of *H.pylori*.

MIC value of CPD2 used microdilution method were in the range of 2.30 to 1.56 µg/mL (Table 2). According to those reported before CPD2 has potential anti-*H.pylori* activity. This conclusion derived from that: if the natural products possess MICs less than100 µg / mL; it will be significantly active. While the natural products of MICs between 100 and 512 µg/mL; it will be quite active. On the other hand if the natural products possess MICs more than 512 µg/mL, it will have a weak antibacterial effect [13].

According to this criteria the all samples which were tested against the six *H. pylori* strains showed a quite active antibacterial activity (Table 2).

The mixture of samples with the antibiotic used in a ratio of one volume to one volume, for which MIC was decreased only for CPD2, and this corresponds to the rates of increase in the inhibition zone values as in Table 1.

4.3. Virtual screening and the mode of action

Urease and [NiFe]-hydrogenase are *Helicobacter pylori* nickel-containing enzymes; which are, used for successful colonization of the *H. pylori* in the human gastric mucosa [18-20].

Urease is responsible for preventing the cytosolic pH by catalyzing the hydrolysis of urea to produce ammonia and bicarbonate. [NiFe]-hydrogenase provides *H. pylori* with an energy source by catalyzing the oxidation of molecular hydrogen. Both enzymes require multiple dedicated accessory proteins for

biosynthesis of their intricate metallocenters. The *H. pylori* genome contains genes encoding the UreEFGH and HypABCDEF accessory proteins responsible for the maturation of urease and [NiFe]-hydrogenase, respectively. *Helicobacter pylori* HypB is a metal-regulated GTPase needed for the biosynthesis of [NiFe]- hydrogenase and urease [21-22].

For understanding the potent activity of CPD2 and CPD1 for eradication of *H. pylori.*

Inhibition of both compounds to HypB from *H. pylori* in complex with nickel was studied. Both compounds showed inhibitory activity towards HypB as shown in table 3 and Fig. (1-2).

Bankeu et al. 2019 [15] reported that the Key H-bond interactions between the nitrogenous base; GDP and amino acid residue in the binding site could be reproduced, including those with Ser215, Thr61, Asp185, Gly56, Lys183, Arg89 and Glu135 via the Mg^{2+} cation (Fig.1 and Table 3). For CPD1 there is an interactions with LYS 183, ARG89, GLY 58 and LYS 59 while CPD2 showed only interactions with ASP 185 and LYS 183 only (Fig.2) and Table 3) .

Moreover, it should be noted that quercetin derivatives are reported to have metal chelation activity which in turn prevent the maturation of both urease and hydrogenase by chelation of iron and nickel [23].Although, CPD1 showing docking score of -7.26 more than CPD 2 of docking score -6.89 but the *in vitro* studies showed that CPD2 is more active. These findings may be explained as result of the hydrophobicity of butyl group in CPD2, which in turn increase the anti-adhesion activity and hence the inhibition of *H. pylori* of CPD2 increased [24].

These finding are already matched with previously reported that flavonoids inhibit HypB protein [25].

5. Conclusions

Vitis vinifera is a nutraceutical plant can provide us with potential agents for eradication of *H.pylori*. Both of hydro alcoholic extract of the *Vitis vinifera* leaves and quercetin-3-*O*- *β*-*D*-glucouronide derivatives are potent *H.pylori* inhibitors. *H.pylori* resistance towards clarithromycin can be overcome by combination with quercetin-3-*O*-*β*-*D*-glucouronide butyl ester. Quercetin-3-*O*- *β*-*D*-glucouronide alkyl esters derivatives are candidate to be lead compounds for new safe and cheap drugs for eradication of *Helicobacter pylori* infections.

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Conflicts of Interest: "The authors declare no conflict of interest."

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