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Anti Ulcerogenic and Cytoprotective Effects of Saharian (Sidr) Honey from Algeria

Noureddine Djebli[a], Mohammad Rais Mustafa[b] , Merve Keskin[c,*] and Sevgi Kolayli[d]

[a]*Pharmacognosy & Api-Phytotherapy Laboratory, Mostaganem University –Algeria;* [b]*Faculty of Medicine University of Malaysia, 50603 Kuala Lumpur, Malaysia;* [c]*Vocational School of Health Service, Bilecik Seyh Edebali University, 11230, Bilecik, Turkey;* [d]*Department of Chemistry, Faculty of Sciences, Karadeniz Technical University, 61080 Trabzon, Turkey*

Abstract: ***Aim and Objective*:** This study aimed at investigating the gastro-protective effects of Algerian Sahara (Sidr) honey from *Apis mellifera intermissa* against HCl/Ethanol-induced gastric ulcers in rats.

***Material and Methods*:** Total phenolic content, antioxidant activity and phenolic compounds were determined. Than, Three groups of rats (control, HCl/ Ethanol induced ulcer, and orally administered honey) were used for the determination of gastro protective effect of Sidr honey.

***Results*:**Total phenolic content, total flavonoid content, and DPPH activity of honey sample was determined as 47.35±3.35 mg GAE/ 100 g, 2.13±0.17 mg QE/ 100 g, and 229.24±0.02 mg/mL, respectively. Oral pretreatment of rats with honey (1.2 g/Kg body weight orally at interval of 2 days) protected gastric mucosa against HCl/Ethanol induced damage by decreasing ulcer score, the volume and acidity of gastric juice and increasing pH.

***Conclusion*:** These results were confirmed by the histological assessment which demonstrated a significant gastro protective activity of Saharian (Sidr) honey against HCl/Ethanol-induced stomach ulcer. Plasma tumor necrosis factor-α, IL-6 and PGE_2were also measured. Sahara honeys significantly decreased the plasma TNF-α, PGE2, and IL-6 concentrations.

Keywords: Gastric ulcer, Sahara honey, HCl/Ethanol, ulcer score, TNF, IL-6, PGE2

1. INTRODUCTION

Honey is an important bee product collected by honey bees and stored in honeycomb [1]. Honey is an important foodstuff and sweetener. It has been used in Apitherapy applications since ancient times. The composition of the honey varies according to the botanical origin; the period and the method of production, but the main ingredient in its structure are certain carbohydrates. In general, honey contains 80% sugars, 17% water, and the remaining 3% are valuable components such as minerals, vitamins,

*Address correspondence to this author at the Vocational School of Health Service, Bilecik Seyh Edebali University, 11230, Bilecik, Turkey; E-mail: merveozdemirkeskin@gmail.com

organic acids, amino acids and phenolic components. Various benefits of honey have been reported in recent years such as anti-inflammatory, anti-microbial, anti-mutagenic, antioxidant, and anti-tumoral effects [2, 3]. The most important factor finding out the antioxidant and antimicrobial effects of honey is the source from which honey is obtained [3].

It is clear that honey's therapeutics effects depend on its composition, and its composition depends on the geographical origin, the species of bee and the plants the bees feed on [4].

Antimicrobial, anti-inflammatory and antioxidant properties of honey were found to contribute to wound healing, especially in ulcers and burns [5].

Gastric ulcer is a lesion that affects the entire gastrointestinal mucosa and muscularis mucosa [6]. Approximately 25 percent of patients with this disease have a serious complication, such as bleeding, perforation, or gastric outlet obstruction. [5]. Nowadays, there are difficulties in the treatment of stomach ulcers due to the limited effects of drugs on the market and side effects. [7]. Therefore, more effective and safe antiulcer agents with fewer side effects should be identified than synthetic drugs from natural products.

In this study, it was investigated the gastro protective effects of Sidr honey sample from Algeria against Ethanol-induced gastric ulcers in rats. In addition, the mechanism is elucidated in terms of pro-inflammatory cytokines production response (TNF-α, IL-6 and PGE_2).

2. MATERIALS AND METHODS

2.1. Chemicals

Ethanol and HCl was purchased from, (Millipore, Davmstadt, Germany), Ketamine/Xylazine was from (Troy Laboratories Pty Limited., NSW, Australia), and formalin was from (LabchemSdn. Bhd, Petaling Jaya, Malaysia), paraffin wax, hematoxylin, NaOH, phenolphthalein, eosin and sodium pentobarbitone was all purchased (Sigma-Aldrich, St. Louis, USA). All other chemicals were of analytical grade.

2.2. Honey Samples

Sidr honey was obtained directly from beekeepers practicing transhumance to Algerian Sahara. It was not heated treatments or pasteurized or any other operation able to alter natural composition. Honey sample was stored at 4°C in the dark until further analysis. In order to determine total phenolic, flavonoid, condense tannin and phenolic compounds Sidr honey sample was extracted. For this purpose, 3 grams of honey sample was mixed with 30 mL methanol and shaked for 24 h on a shaker at room temperature. Than, the exract was filtered to remove particles and the filtrate concentrated in a rotary evaporator (IKA-Werke Staufen Germany) under pressure at 40 ^{0}C. The residue divided into two parts for antioxidant and HPLC analyses.

2.3. Determination of Total Phenolic Contents

Total phenolic content (TPC) of the methanol extract of honey sample was determined by using Gallic acid as standard in the Folin–Ciocalteu method [8, 9]. TPC was expressed as mg of Gallic acid equivalents per 100 g samples (mg GAE/ 100 g sample).

2.4. Determination of Total Flavonoid Contents

The total flavonoid content was measured according to a spectrometric assay [10]. Quercetin was used a standard and the results were expressed as mg of quercetin equivalents per 100 g of sample (mg QE/ 100g sample).

2.5. Determination of Condense Tannin

The amount of condensed tannins of honey sample was determined according to a spectrometric method [11]. (+)-Catechin was used as standard. Results were expressed as mg of catechin equivalents per g of sample (mg CE/ 100 g sample).

2.6. Determination of Antioxidant Capacity

Antioxidant activity of honey sample was measured by using ferric reducing antioxidant power (FRAP) [12]. Trolox was used as a positive control in order to construct a reference curve. Results were expressed as $\mu molFeSO_4.7H_2O$ equivalents per g of sample ($\mu molFeSO_4.7H_2O/g$).

2.7. 1,1-diphenyl-2-picrylhydrazyl (DPPH)-free radical scavenging assay

DPPH assay was performed according to [13]. For the calculation of DPPH radical scavenging activity, equal volume of honey extracts at different concentration and fresh DPPH solution was mixed separately. The absorbance of the mixtures was measured at 517 nm after 50 min incubation. The DPPH scavenging activity was calculated as SC_{50} (mg of sample per mL).

2.8. Phenolic Profile of Sidr Honey

Honey extract was re-extraction for High Performance Liquid Chromatography (HPLC) analysis. The residue was weighed and dissolved in methanol for HPLC analysis for identification of phenolic compounds. Concenrated methanolic residue was dis-

solved in 10 mL distilled water at pH 2 and stirred for 1h. The mixture was extracted with 3 mL of diethyl ether at least three times, stirred for 15 min and then shaken. The upper phase was placed into a 50 mL flask and extracted three times with 3 mL of ethyl acetate for 15 min. [14]. The upper phase, consisting of organic solvent, was evaporated in a rotary evaporator under reduced pressure at 40°C. Seventeen polyphenolic standard compounds were determined using HPLC at 280 nm (Elite LaChrome; Hitachi, Tokyo, Japan) fitted out with a reverse phase C18 column (150 mm* 4.6 mm, 5 μm; fortis). Acetonitrile, water and acetic acid were used as mobile phase. The mobile phase consisted of (A) 2% acetic acid in water and (B) acetonitrile: water (70:30). 20 μL of samples was injected individually at room temperature and flow rate was set as 0.75 mL/min [14]. Calibration curve for each compounds were found between 0.998 and 1.000.

2.9. Experimental Animals

Four week old male Sprague Dawley rats weighing 100g-150g were obtained from Monash University, Kuala Lumpur, Malaysia. The rats were fed with standard rat chow pellet and water *ad libitum*. The animals were kept in a standard animal house at a controlled ambient temperature of 25 ± 2 ° C and 12 hours in a dark / light cycle. The study was carried out after obtaining the necessary permission from the Corporate Animal Ethics Committee. Three groups of rats were used: Control (normal saline), HCl/Ethanol induced gastric lesion, HCl/Ethanol induced gastric lesion and orally administered honey. HCl/Ethanol mixture: 0.6M HCl/ 80% Ethanol solution.

2.10. Experimental Design

The rats were divided into three groups comprising of six rats each.

- Group I: Normal control (Normal saline).
- Group II: Gastric ulcer (1mL HCl – Ethanol mixture on 2nd day Single dose).
- Group III: Animals treated with Sidr Honey (1.2 g/Kg body weight orally) for 2 days and 1mL HCl – Ethanol mixture (on 2nd day (Single dose) (1mL (HCl / Ethanol) 0.6M HCl / 80% Ethanol solution. Honey 1 mL (1.2 g/Kg body weight orally) low *honey* dose)

The determination of ulcer index in gastric tissues was followed by the method described by Jothi et al. [15]**.** After treatment of animals, they were subjected to surgery under sodium pentobarbitone anesthesia. The 4-hour gastric juice collection was poured into a graduated test tube and centrifuged at 224 g for 10 minutes. Supernatant volume and pH were recorded. The total acid content of gastric juice was also determined by titration to pH 7.0.

0.05 N NaOH, using phenolphthalein as indicator. At the end of the experimental period, the animals were sacrificed by Ketamine/Xylazine overdose (80/8mg/kg body weight). Blood was collected and used for cytokines measurements.

The stomach was removed, opened along the greater curvature and washed it slowly under the running tap water. Placed it on the glass slide and observed under microscope (20x) for ulcers.

Mean ulcer score for each animal is expressed as ulcer index. Mucosal lesions were scored independently (in mm) by two commentators. The severity of gastric damage was determined by measuring the length of each lesion over its largest length.

In the case of petechial hemorrhage, five such lesions were taken equivalent to 1 mm [16]. Index of ulceration was calculated as the total lesion lengths divided by the number in each group [17]

2.11. Histopathological Examination of Gastric Mucosa

Each freshly excised clean stomach was used for the histopathological examination; tissues were fixed in a 10% buffered formalin solution. Formalin-fixed stomach sections were embedded in paraffin wax and serially sectioned (3–5 μm) and stained with hematoxylin , eosin and observed for pathological changes using ordinary light microscopy [18].

2.12. Quantification of Serum Tumor Necrosis Factor alpha (TNF-α), Interleukin-6 (IL-6) and Prostaglandin E2 (PGE_2)

Blood samples were collected from post vena cava and transferred into heparinised tubes immediately. A portion of the whole blood collected without anticoagulant centrifuged after coagulation and serum was separated. The serum TNF-α, IL-6 and PGE_2 were quantified by sandwich ELISA method (R&D Biosystems, Minneapolis, MN, USA). The serum were diluted and incubated with pre-quoted anti TNF-α, IL-6 and PGE_2 in 96 well plates for two hours in room temperature. The wells were washed with washing buffer. Then the respective antibodies were added 100 µL diluted in dilution buffer and the wells were washed with washing buffer. The secondary antibody was incubated and the TMB was added. The color developed was measures by ELISA reader (Hidex Chameleon, Cornaredo (MI),Italy).

2.13. Statistical Analysis

Data are expressed as an average of 6 standard deviations (SD). The data were processed using statistical analysis software SPSS version 16.0 (SPSS, Chicago, IL). All p values of less than 0.05 were considered significant, treated by honey against no treated and control.

3. RESULTS

3.1. Biochemical characterization of Sidr Honey

The total amount of phenolic content of Sidr honey was determined 47.35±3.35 mg GAE / 100 g sample and the total amount of flavonoid content was 2.13±0.17 mg QE / 100 g sample ant condense tannin was determined as 0.09±0.003 mg CE/ 100 g sample. Antioxidant capacity of honey was found as 1.504± 0.070 µmol $FeSO_4.7H_2O$ / g, whereas the effect of scavenging free radicals (DPPH) was determined as 229.24±0.02 mg/ mL (Table **1**). Sidr honey was determined to be rich in p-OH benzoic acid, mrysetin, chyrisin and pinocembrin (Table **2**).

Table 1. Biochemical characterization of Sidr honey

Honey	TP mgGAE/ 100 g sample	TF mgQE/ 100 g sample	CT mg KE/ 100 g sample	FRAP (µmolFeSO4.7H2O/g)	DPPH SC_{50} mg/mL
Sidr	47.35±3.35	2.13±0.17	0.09±0.003	1.504±0.070	229.24±0.02

Table 2. Phenolic compounds of Sidr honey

Phenolic Compounds	Amount of Compounds (µg/100 g)
Gallic acid	45.40±0.11
Protocatechuic acid	90.10±0.23
p-OH Benzoic acid	229.90±0.19
Catechin	39.50±0.08
Caffeic acid	59.60±0.10
Syringic acid	50.00±0.12
Epicatechin	32.90±0.07
p-coumaric acid	51.50±0.02
Ferulic acid	54.50±0.01
Mrysetin	214.90±0.13
Daidzein	26.80±0.02
Luteolin	107.60±0.03
t-cinnamic acid	42.40±0.01
Hesperetin	12.50±0.01
Chrysin	520.60±0.24
Pinocembrin	168.20±0.16
Caffeic acid phenethyl ester (CAPE)	583.55±0.21

3.2. Anti-ulcerogenic and Cytoprotective Effect of Algerian Honeys

In this study oral administration of HCl and Ethanol (0.6M HCl / 80% Ethanol solution), precipitated a significant enhance in the number of lesions in the gastric mucosa, increases in volume of gastric juice and acidity Vs control. The animal groups pre-treated with Sahara honeys (1.2 g/Kg body weight orally) showed increase in the pH and a reduction in the volume of the gastric juice, ulcer score and total acidity (Table **3**). These results were confirmed by the histological assessment which established an important gastroprotective activity of Sahara honeys against HCl/Ethanol-induced stomach ulcer (Figure **1**).

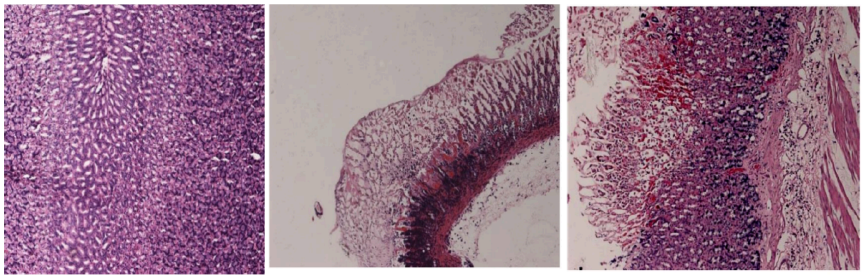
Ethanol is the best damaging agent to the gastric mucosa, used in animals and clinical studies [19]. The model of an ethanol-induced gastric ulcer is used for the evaluation of the gastro protective activity of many new therapeutics and natural products [20]. The effects of Algerian honey on gastric lesion induced in Sprague dawley rats are shown in Table 3. Oral observation of the HCl / Ethanol solution to the control group produced a characteristic mucosal lesion, as evidenced by the number of lesions

in the stomach. Algerian Sidr honey (group III) pretreatment caused a significant (P <0.05) protective effect against stomach lesion from HCl / Ethanol.

Table 3. Effect of Algerian Honey (Sidr Honey) on Ulcer score, Gastric Juice volume, pH and Total acidity

Groups	Ulcer score	Gastric juice volume	pH	Total Acidity
Control	1.0±0.28	2.6±0.25	5.86±0.32	2.5±7.5
GU	9.33±2.4 a***	4.43±0.5 a***	3.4±0.55 a***	149±5.9 a***
GU+H	11.71±2.60 b***	3.43±0.59 b***	4.16±0.62 b***	135.66±6.88 b***

Values are mean ±S.D (n = 6). '*' indicates statistically significant (P<0.05).



A) Control Group B) GU C) GUH

Figure 1. Histological assessment of stomach in control and experimental rats. Control picture showing normal architecture. HCl/Ethanol induced rats showed severe damage in the stomach and mucous layer was damaged due to the severity of the HCl/Ethanol induction. Algerian honey treated group showed much protection and the damage was reduced. Hematoxylin & eosine (H&E) staining (G X 400).

3.3. Histological Assessment

Photomicrographs revealed that the mucosa of ulcer control animals have hemorrhagic erosion, discontinuity in the lining of epithelium cells and significant damage in sub mucosa in HCl/Ethanol-induced rats. Animals that have received honey reduced gastric lesion formation and sub mucosal edema. This indicates honey is actively involved in protection of the stomach from HCl/Ethanol exposure.

3.4. Algerian Honeys Reduced the Serum Levels of TNF-α, IL-6 and PGE2

Ethanol essentially results in a rush in neutrophil infiltration into an injury site acute inflammatory reaction Ethanol also causes changes in the cytokine balance responsible for inflammation in the gastric mucosa [21]. The proinflammatory cytokine tumor necrosis factor-α (TNF-α) was found to play an important role in Ethanol induced apoptosis during gastric ulcer formation [22]. Inflammation is one of main event in the ulcer of stomach. In that scenario increased levels of pro-inflammatory cytokines such as TNF-α and IL-6 were reported earlier. Cyclooxygenase (COX)-2 is a key enzyme the play major role in regulating the inflammation. COX-2 is involved in the conversion of arachidonic acid to prostaglandins. The increased level of PGE2 is responsible for the inflammation. In our observation the levels of TNF-α, IL-6 and PGE2 were increased in HCL/Ethanol-induced rats when compared to control rats. In case of Sidr honey treated to HCl/Etanol-induced rats the levels of TNF-α, IL-6 and PGE2 were decreased significantly (P<0.05).

4. DISCUSSION

In addition to effective drugs, many bioactive components such as phenolic compounds prevent stomach ulcers. Although phenolic compounds show different activities, they have essential structural properties with aromatic ring, bearing one or more hydroxyl groups and they are generally classified into two groups that call phenolic acids and flavonoids. Each member they were justified by the existing findings that they carried out different biochemical activities [3]. Total phenolic content is directly related to the floral richness of honey and is responsible for the antioxidant capacity of honey. In a study, it is stated that the total amount of phenolic content in honey samples varies between 33 mg GAE / 100 g and 81 mg GAE / 100 g [23]. It is reported that the total phenolic content of honey samples collected from different parts of Turkey varies between 34.37 to 470.70 mg / 100 g sample [4]. It is reported that the total amount of phenolic content was 540.720±3.035 mg Vanillin / kg sample [24]. In a study, total phenolic content changed between 63.93 and 95.36 mg / 100 g honey as gallic acid equivalent

[25]. They determined that the DPPH radical scavenging assay was found for an average 30.14% ± 9.28 [25]. It is stated that DPPH activity of Sidr honey is 91.64± 1.245% in their study [26]. It is reported that the honey samples they analyzed were rich in terms of chlorogenic acid, vanillic acid, p-coumaric acid, vanillin and rutin [27]. It is stated that the Turkish honeys that they analyzed were rich in gallic acid, p-OH benzoic acid, caffeic acid, ferulic acid and benzoic acid [3].

Due to lifestyle changes and eating habits, an increase in the frequency of the stomach that is affected by ulceration is observed [28]. It is known that gastric acid is an important marker in normal upper gastrointestinal functions for many years, including protein digestion, calcium and iron absorption, and provides some protection against bacterial infections. However, unfavorable gastric acid levels are the underlying factors of wide spread pathological conditions [29]. The fact that acid gastric juice plays an important role in peptic ulcer formation is supported by clinical, pathological and laboratory-based evidence [30]. Mucosal cell death results from increase in H^+ concentration in its immediate environment (decrease pH) [31]. It is also well known that gastric acid secretion take place an important function in gastric ulcer and anti-ulcerogenic drugs reduce acid secretion and accelerate recovery [32].

A number of chemically synthesized antiulcer drugs are used for reduction volume and neutralization of gastric acid, but clinical evaluation of these drugs has shown side effects and drugs interaction [28]. The results obtained from the use of natural honey are consistent with others, which have esthabilished the gastroprotective activity of several types of honey, either unifloral or multifloral, and from different botanical origin [18,33-36] TNF-α is a pro-inflammatory cytokine and several polymorphisms of gene TNF-α are known. The TNF-α cytokine influences gastric production and lowers acid output by gastric parietal cells [32]. This cytokine was found to play an important role in Ethanol induced apoptosis during gastric ulcer formation [22]. TNF-α also shares an important inflammatory property with IL-6, i.e. the induction of acute phase reactant protein production by the liver [37]. COX-2 is the key enzyme that catalyzes the conversion of arachidonic acid to PGE2 in the prostaglandin (PGE2) biosynthesis pathway [38].

PGE2 is an important mediator of all types of inflammation [39]. Changes in acute phase proteins reflect the presence and intensity of inflammation and have long been used as a clinical guide for diagnosis and treatment [40].

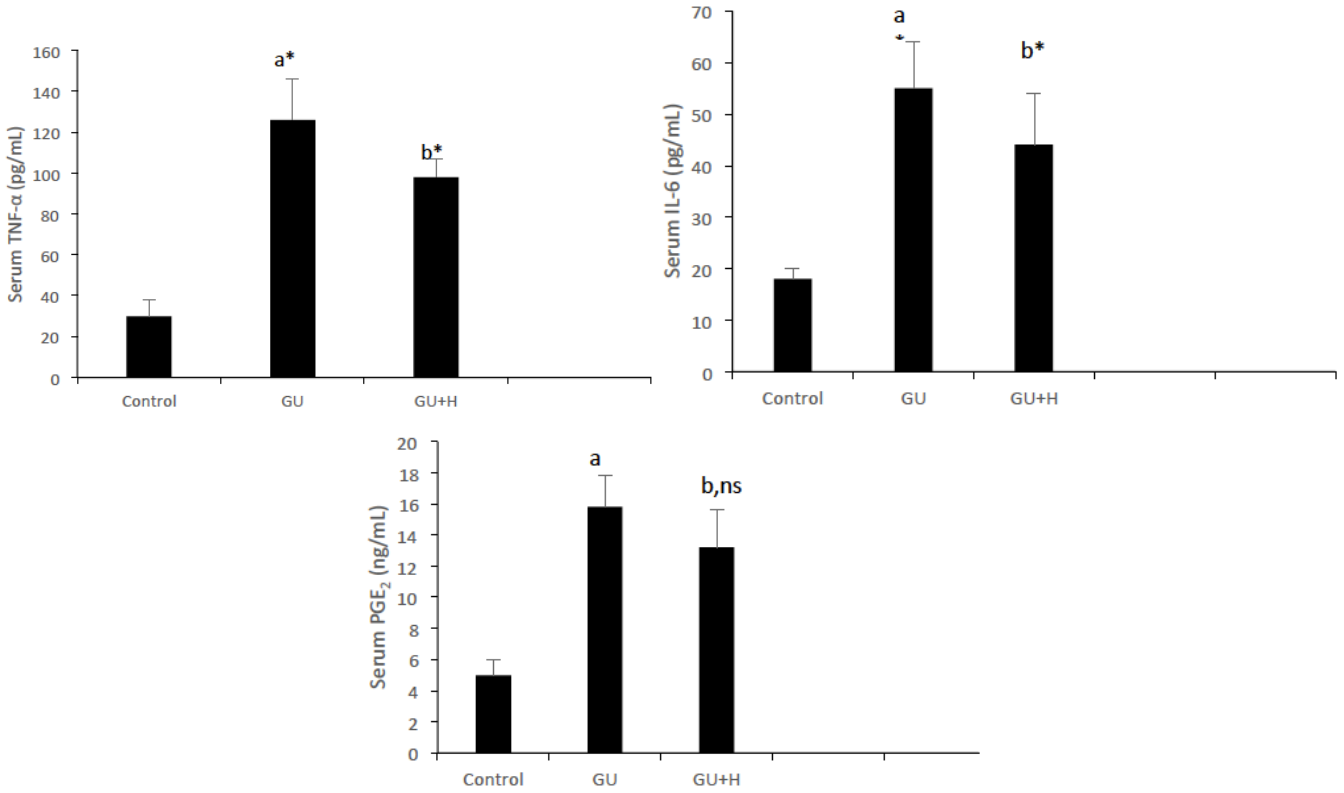


Figure 2: The effect of Sidr honey on the serum levels of TNF-α, IL-6 and PGE2 Rats were pretreated orally with Sidr honey (1.2g/kg of body weight) for 2 days before HCl/Ethanol induced gastric ulcer induced. Serum levels of TNF-α, IL-6 and PGE2 were decreased significantly ($P<0.05$).

It has been reported that Australian jelly bush honey, New Zealand manuka and pasture honeys (1%) stimulated grown producing of the cytokines TNF-α, interleukin-1 (IL-1β) and IL-6 by MM6 cells and human blood monocytes [41], and that

Thyme honey (2.5%) induced an increase of TNF-α and PGE2 productions in macrophages (Raw 264.7 murine macrophages) [42]. Acacia honey (1%w/v) earned from Slovakia stimulates TNF-α, transforming growth factor beta (TGF-β), IL-1β and matrix metalloproteinase [43]. It is stated that New Zealand Manuka and clover honeys (4% w/v) stimulated TNF-α release from monocytic cell lines THP-1 [44]. At first sight, these findings seem contradictory with our study (Figure **2**) but it is worth noting that these studies were carried out in vitro with honeys at very low concentrations. It is reported to be raw Manuka honey with a high level of methyl glioxin (MGO) neutrophils had inconsistent effects on TNF-α production. Both MGO250 ™ and MGO400 ™ Manuka honey samples got inhibition of TNF-α production by neutrophils when determined at 400 μg / mL honey concentrations, but 100 μg / mL MGO250 ™ honey concentrations stimulated TNF-α production by neutrophils [45]. In *in vivo* studies it has been shown that nnGelam honey reduced the plasma levels of inflammatory mediators including IL-6, TNF-α, PGE2 and NO in carrageenan-Induced Acute Paw Edema in Rats [46]**,** Manuka honey decreased plasma TNF-α, IL-1β, and IL-6) in Ethanol-induced gastric ulcers in rats [18]. Ingestion of diluted natural honey can lower the concentrations of PGE2 in plasma of normal individuals [47] and shows potent inhibitory activities against TNF-α and PGE2 in air pouch model of inflammation [48].

CONCLUSION

Our results showed that Sidr honey possess a potential *in* vivo antiulcerogenic consequence in acute inflammation model of gastric ulcer. The gastroprotective effect may be due in large part or in part to inhibition of TNF-α, PGE2, and IL-6. Hence, Sidr honey can be considered a powerful potential therapeutic agent against gastric ulcer.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

There is no need Ethics Approval and Consent to Participate

HUMAN AND ANIMAL RIGHTS

The study was carried out after obtaining the necessary permission from the Corporate Animal Ethics Committee.

CONSENT FOR PUBLICATION

There is no individuals' data.

AVAILABILITY OF DATA AND MATERIALS

"The data supporting the findings of the article is available in the [repository name] at [URL], reference number [reference number]".

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

There is no Supplementary Material

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