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ASSESSMENT OF MEMBRANE STABILIZING ACTIVITY FROM HONEY. AN *IN-VITRO* APPROACH

Honnayakanahalli Marichenne Gowda Manukumar, Sharanaiah Umesha✉

Department of Studies in Biotechnology, University of Mysore, Manasagangothri
Mysore-570006, Karnataka, **India**

ABSTRACT

Aim. The present study was conducted to evaluate Manoflora (MF), Polyflora (PF), Polyflora forest (PFf), and Processed (Pro) honey varieties to compare the *in-vitro* anti-inflammatory effects of aqueous honey samples in dose dependent manner. *In-vitro* anti-inflammatory activity was evaluated using membrane stabilization assay of RBCs at different aqueous honey concentrations.

Material and method. The present investigation carried out for selected varieties of honey against erythrocytes exposed to both heat and hypotonic lyses and inhibition of membrane damage was compared to the standard drug acetylsalicylic acid.

Results. Membrane damage was inhibited in both the model hemolysis of erythrocytes *in vitro* in a concentration dependent manner. Hypotonic solution inducing damage was inhibited by aqueous honey sample in ascending order ranged from 8.25% to 97.76% at 10 to 50 mg/ml and standard drug acetylsalicylic acid showing hemolysis protection 96.09% at 100 µg/ml concentration. In heat induced hemolysis model aqueous honey sample exhibited its protecting property during external stress condition in all samples ranged from 0.44% to 21.23% at 10 to 50 mg/ml and acetylsalicylic acid showed 39.38% at 100 µg/ml concentration. Among the variety PFf showed highest protecting nature for hypotonic solution induced lyses (97.76%) and heat induced hemolysis (21.23%) at 50 mg/ml respectively.

Conclusion. With these investigations data conclude that the model exhibits marked anti-inflammatory effect. Future research is to be carried out to identify the molecules responsible in honey and its mechanism involved.

Key words: honey, erythrocytes, anti-inflammatory, hemolysis, hypotonic

INTRODUCTION

Honey is the substance made when nectar and sweet deposits from plants are gathered, modified and stored in the honeycomb by honey bees. The definition of honey stipulates a pure product that does not allow for the addition of any other substance. This includes, but is not limited to, water or other sweeteners (Definition..., 2003). Fructose and glucose are the main carbohydrate constituents of honey. Honey is composed primarily of the sugar glucose and fructose; its third greatest component is water. Honey also contains numerous other types of sugars, as well as acids, vitamins, proteins and minerals (Composition..., 1962; White, 1980). Pure honey contains alkaloids, auterquinone glycosides, cardiac glycosides, flavonoids and reducing compounds (Rakhi et al., 2010). Substituting honey with sugars in processed food can inhibit harmful and genotoxic effects of mycotoxins and improves the gut microflora (Al-Mamary et al., 2002). Honey

✉su@appbot.uni-mysore.ac.in, phone +91 821 241 9884

was the most popular Egyptian drug being mentioned 500 times in 900 remedies (Grossman, 1986).

Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical or microbial agents and body response to inactivate or destroy the invading organisms, to remove the irritants and set the stage for tissue repair. It is triggered by the release of chemical mediators from injured tissue and migrating cells (Umapathy et al., 2010). It is a complex process, which is frequently associated with pain and involves occurrences such as; increase in vascular permeability, increase of protein denaturation and membrane alterations (Umapathy et al., 2010).

The lysosomal enzymes released during inflammation, produce a variety of disorders. The extracellular activity of these enzymes is said to be related to acute or chronic inflammation. Stabilization of lysosomal membrane is important in limiting the inflammatory response by inhibiting the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release or by stabilizing the lysosomal membrane (Vadivu et al., 2008). Human red blood corpuscle (HRBC) or erythrocyte membrane is analogous to the lysosomal membrane (Chou, 1997) and its stabilization implies that the extract well stabilizes lysosomal membranes. HRBC membrane is similar to lysosomal membrane components. Reduced inflammation observed in the clinic following the application of honey is supported by histological evidence of reduced numbers of inflammatory cells present in wound tissue (Molan, 2002).

The commonly used drugs for management of inflammatory conditions are non-steroidal anti-inflammatory drugs, which have several adverse effects especially gastric irritation leading to formation of gastric ulcers (Tripathi, 2008). Natural products have contributed significantly towards the development of modern medicine. Recently traditional medicine worldwide is being re-evaluated by extensive research on different plant species and their active therapeutic principles. It is believed that current drugs available such as opoids and non-steroidal anti-inflammatory drugs (NSAIDS) are useful not in all cases of inflammatory disorders, because of their side effects and potency (Ahmadiani et al., 1998). As a result, a search for other alternatives seems necessary and beneficial. The study of plants that have been used traditionally for curing inflammation is still fruitful and logical research strategy in the source of new anti-inflammatory drugs (Kumarappan et al., 2006) is welcome.

Studies have shown that honey is a significant natural source of antioxidants and has potential therapeutic value in the treatment of cancer, heart disease, cataracts, and several inflammatory diseases (Al-Mamary et al., 2002). However, the actual health benefits derived from honey depend on its quality. Honey anti-inflammatory factor is identified by Professor Peter Malan, University of Waikato published in September 2012 on manuka honey. Manuka honey having Apalbumin-1 protein gets modified by reaction with methylglyoxal, which is present in large quantityonlyin manuka honey, and this modification makes the protein much more potently anti-inflammatory. The erythrocyte membrane resembles lysosomal membrane and thus, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane. Therefore, as membrane stabilizes it interferes in the release and/or action of mediators like histamine, serotonin, prostaglandins, and leukotrienesetc (Shinde et al., 1999).

With this in the background, the aim of our present work was to investigate whether our selected geographically separated varieties of honey, possess *in-vitro* membrane stabilizing activity or not by using an *in-vitro* procedure in terms of RBC membrane stabilization activity.

MATERIAL AND METHOD

Honey samples

Four local honey samples derive directly from beekeepers in Bihar (Monoflora – MF), South Delhi (Poly flora – PF), Sirsi (Poly flora forest – PFf) and Bangalore (Processed – Pro) through Pristine Laboratories based on quality and rich source of phytochemical in nature, Bangalore on April 2013. All of the honey samples were stored at room temperature (22–24°C) in airtight plastic containers until analysis.

Chemicals and reagents

Sodium chloride (NaCl), disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate and acetylsalicylic acid was purchased from Sigma-Aldrich. All chemicals used were of analytical grade.

Effect on hemolysis

Collection of human erythrocyte suspension. The whole blood was collected from a healthy volunteer who had not taken any NSAIDS for 2 weeks prior to the experiment and collected in heparinzed vacutainer. The blood was washed three times with 0.9% saline and centrifuged simultaneously for 10 minutes at 3000 g. The packed cells were washed with 0.9% saline and 40% v/v suspension made using isotonic phosphate buffer which was composed of 154 mM NaCl in 10 mM Sodium Phosphate buffer at pH 7.4 used as stock erythrocyte or RBC suspension.

Hypotonic solution-induced hemolysis. The membrane stabilizing activity of the sample was assessed according to the method described (Shinde et al., 1999) with slight modifications. The test sample consisted of stock erythrocyte (RBC) suspension 0.5 ml mixed with 5 ml of hypotonic solution (50 mM NaCl in 10 mM sodium phosphate buffered saline at pH 7.4) containing honey preparation (HP) ranging from concentration of 10–50 mg/ml. The control sample consisted of 0.5 ml RBC suspension mixed with hypotonic buffered solution alone. The standard drug acetylsalicylic was treated similar to test at 10 µg/ml concentration. The experiment was carried out in triplicate. The mixtures were incubated at 10 minutes at room temperature, centrifuged for 10 minutes at 3000 g and haemoglobin content of the supernatant was measured spectrophotometrically at 540 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated by the following equation.

$$\% \text{ inhibition of haemolysis} = 100 \cdot [A1 - A2 / A1]$$

where: $A1$ – Absorbance of hypotonic buffered solution alone, $A2$ – absorbance of test/standard sample in hypotonic solution.

Heat-induced hemolysis. Aliquots (5 ml) of the isotonic buffer containing 10–50 mg/ml of different honey preparation were put into two duplicate sets of centrifuge tubes (Shinde et al., 1999). The vehicle, in the same amount was added to another tube as control. Erythrocyte suspension (0.5 ml) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54°C for 20 min in a water bath. The other pair was maintained at 0–5°C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition or acceleration of hemolysis in tests and control was calculated according to the equation:

$$\% \text{ inhibition of hemolysis} = 100 \cdot [1 - (OD2 - OD1 / / OD3 - OD1)]$$

where: $OD1$ – test sample unheated, $OD2$ – test sample heated, $OD3$ – control sample heated.

RESULTS

Effect of honey during hypotonic solution-induced hemolysis. Here individual aqueous honey established its potency through protection of RBC hemolysis in hypotonic solution. All the varieties had shown very good dose dependent protection manner. Honey MF, PF, PFf and Pro samples showed maximum protection 80.80%, 86.16%, 97.76% and 92.85% respectively at 50 mg/ml concentration. Also we can match the 'Pro' (92.85%) variety protection to the 'MF' (80.80%) and 'PF' (86.16%);which is justified due to the cocktail of MF and PF. All the results were compared with standard acetylsalicylic acid which showed 99.33% protection against the lyses at 10 µg/ml as shown in Figures 1–4.

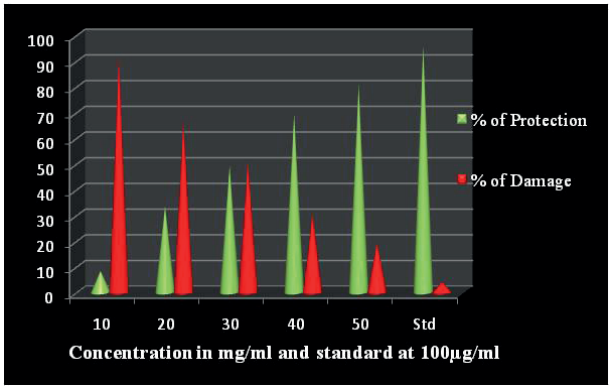

Fig. 1. Effect of MF aqueous honey sample on hypotonic solution hemolysis of erythrocyte membrane

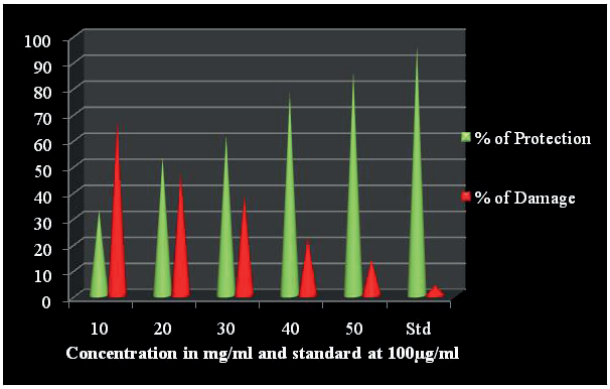

Fig. 2. Effect of PF aqueous honey sample on hypotonic solution hemolysis of erythrocyte membrane

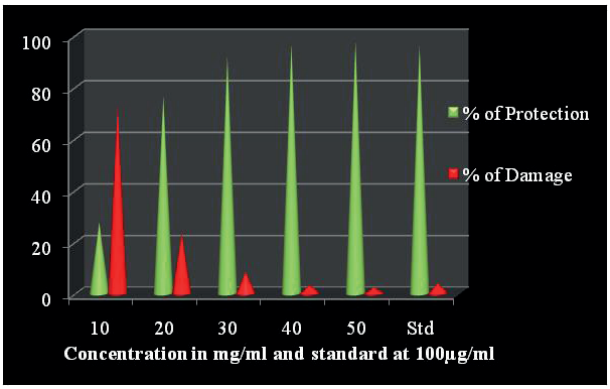

Fig. 3. Effect of PFf aqueous honey sample on hypotonic solution hemolysis of erythrocyte membrane

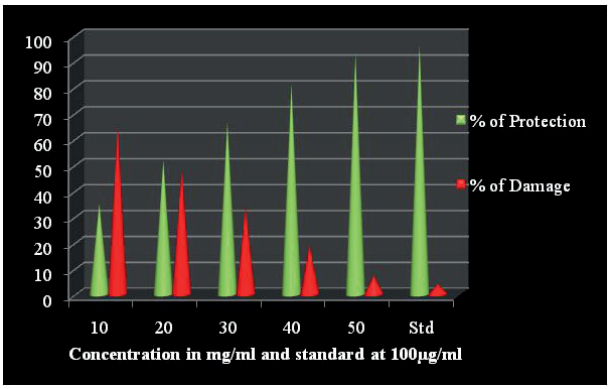

Fig. 4. Effect of Pro aqueous honey sample on hypotonic solution hemolysis of erythrocyte membrane

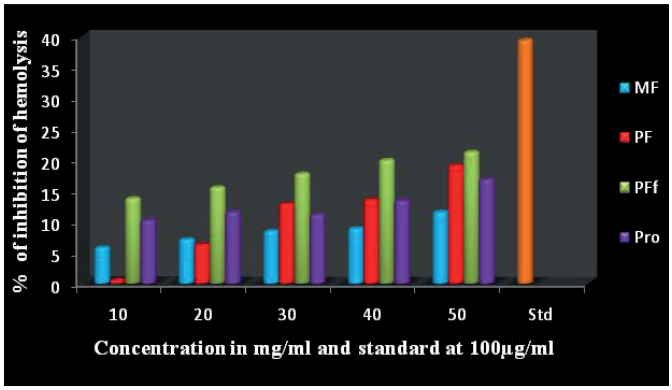

Fig. 5. *In-vitro* anti-inflammatory potency of aqueous honey sample evaluated by heat induced – hemolysis methods: MF – Monoflora (Bihar); Pf – Poly flora (South Delhi); PFf – Poly flora forest (Sirsi) and Pro – Processed (Bangalore

Effect of honey during heat-induced hemolysis. The honey was also effective in inhibiting the heat/stress induced hemolysis at different concentrations. Test sample (10–50 mg/ml) inhibited the heat induced hemolysis of RBCs to varying degree as shown in Figure 5. All varieties of honey samples showed inhibiting potency in increasing order. Hemolysis inhibition of honey varieties ranged from 0.44% to 21.23% at 10 to 50 mg/ml concentrations. Among the variety PFf the maximum inhibition of 21.23% was shown at 50 mg/ml. Acetylsalicylic acid, standard drug showed the maximum inhibition of 39.38% at 100 µg/ml.

DISCUSSION

There are certain problems associated with use of animals in experimental pharmacological research such as ethical issues and the lack of rationale for their use when other suitable methods are available, or could be investigated. Hence, in the present study the hemolysis assay was selected for in vitro assessment of anti-inflammatory property of honey MF, PF, PFf and Pro varieties. Denaturation of tissue proteins is one of the well-documented causes of inflammatory and arthritic diseases. Production of auto-antigens in certain arthritic diseases may be due to denaturation of tissue proteins in vivo (Opie, 1962; Perez et al., 1995). Agents that can prevent protein denaturation therefore, would be worthwhile for anti-inflammatory drug

development. During inflammation, lysosomal hydrolytic enzymes are released which causes damages of the surrounding organelles and tissues with a variety of disorders present (Sadique et al., 1989). Various methods were employed to screen and study drugs, chemicals, herbal preparations and natural products which exhibit anti-inflammatory properties or potentials. In the present study, stabilization of erythrocyte membranes exposed to both heat and hypotonic induced lyses was employed.

This study reports the anti-inflammatory activity of aqueous honey sample by *in vitro*. Inhibition of heat induced erythrocyte membrane lyses by honey is considered to be an index of anti-inflammatory activity (Perez et al., 1995). Drugs act either by inhibiting or degrading these lysosomal constituents or by stabilizing the lysosomal membrane (Gandhisan et al., 1991).

Aqueous honey at a concentration of 10–50 mg/ml was studied for MF, PF, PFf and Pro varieties and well established in the Figures 1–4. The extracts exhibited membrane stabilization effect by inhibiting hypotonicity induced lyses of erythrocyte membrane of maximum 97.76% at 50 mg/ml for PFf honey variety. The erythrocyte membrane is analogous to the lysosomal membrane (Chou, 1997) and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophils (Murugasan et al., 1981). Heat induced hemolysis stabilization provide evidence for membrane stabilization as an additional mechanism of their anti-inflammatory effect. The present finding demonstrated that the aqueous honey has the capacity to stabilize red blood cell membrane against stress, which indicates the ability of the honey to prevent hemolysis or rupture of RBCs. The honey may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutrophil lysosomal constituents include bactericidal enzymes and protease, which upon extracellular release cause further tissue inflammation and damage (Chou, 1997).

Although the precise mechanism of this membrane stabilization is yet to be elucidated, it is possible that the honey produced this effect surface area/volume ratio of the cells, which could be brought about by an expansion of membrane or the shrinkage of the cells and an interaction with membrane proteins (Shinde et al., 1999). The significant membrane stabilizing activity of the aqueous honey may be due to the presence of polyphenolic content of the extract. Several reports have shown that herbal drugs are capable to facilitate the stabilization of red blood cell membrane and possess anti-inflammatory activity (Sadique et al., 1989). These varieties were compared to the result of hemolytic study indicating that maybe it is due to the presence of polyphenolic compounds, especially phenol. But as the amount of flavonoids (compared with the colour of the honey) decreases, it probably indicates an increase in the membrane rupture as shown in the results in Figures 1–5. Stabilization of the RBCs membrane was studied to further establish the mechanism of anti-inflammatory action of honey.

The present findings concluded the potency of honey for anti-inflammatory property by confirming the membrane stabilizing activity of MF, PF, PFf and Pro honey were observed in the above mentioned models and experiments were performed to assess erythrocyte membrane variation. A possible explanation for the stabilizing activity of the honey due to an increase in the surface area/volume ratio of the cells could be brought about by an expansion of membrane or shrinkage of the cell and an interaction with membrane proteins. The present investigation suggests that the membrane stabilizing activity of our collected Indian honey samples plays a significant role in its anti-inflammatory activity and possesses very good membrane stabilizing property which is one of the preliminary steps involved in the screening of anti-inflammatory property. Protective effect on heat and hypotonic saline-induced erythrocyte lyses is known to be a very good index of anti-inflammatory activity of any agent. Since the membrane of RBC is structurally similar to the lysosomal membrane, the effect of any substance on stabilization of RBC membrane may be extrapolated to the stabilization of lysosomal membrane. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release. With this background we conclude that, honey dosage in high

concentration is good during inflammatory period because it has a capacity to stabilize the RBCs membrane and inhibit elevation of inflammation. Dosage concentration effect is shown in the study and documented clearly. Further study is needed to identify the compound responsible for this activity in honey and its mechanism of action.

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