REPORT

EFFECT OF NATURAL HONEY ON HUMAN PLATELETS AND BLOOD COAGULATION PROTEINS

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ABSTRACT

Present study was conducted to determine the effects of honey on blood hemostasis, in-vitro effect of honey was observed on platelet aggregation and blood coagulation employing, activated partial prothrombin time (aPTT), prothrombin time (PT), thrombin time (TT) and fibrinogen levels in blood.

Honey samples showed moderate inhibition of platelet aggregation with IC_{50} 5-7.5%. The coagulation assays showed that at higher concentrations (≥15%) honey samples increased whole blood clotting time. When assayed in platelet poor plasma (PPP), honey samples significantly (P≥0.005) prolonged aPTT, PT, and TT. The honey samples (at 3.75% and 7.5% concentrations) cause mean increment of aPTT = 19±10% and 62±10%; PT 6±5% and 40±5%; TT 35±15% and 112±30% respectively. Moreover, PPP isolated from whole blood pre-incubated with honey samples (9.0% for 10 minutes) showed mean prolongation of aPTT, PT and TT of 45±21%, 26±9% and 105±24% respectively. Interestingly, incubation of honey at 6.25% and 11.75% concentrations in PPP considerably (P≥0.005) reduced fibrinogen levels i.e. 13±4% and 86±30% respectively.

The present study outlines the inhibitory effect of natural honey on platelet aggregation and blood coagulation. These observations provide first line data for modulatory role(s) of honey on process of hemostasis.

Keywords: Nutraceuticals; cardiovascular diseases and cerebro-vascular accident; thrombosis; anti-platelet; anticoagulation.

INTRODUCTION

Vascular diseases contribute a major fraction in human diseases. All over the world, major vascular diseases such as cardiovascular diseases (CVD) and cerebro-vascular accident (CVA) accounts for high mortality and morbidity (Kumar and Clark, 2005). Atherosclerotic lesion is the primary pathology found in CVA and CVD (Steinberg, 1992; Wegge *et al.,* 2004).

In the established atherosclerotic disease; disturbed platelet aggregation and blood coagulation complicates the silent atherosclerotic lesion, by the formation of superimposed thrombus (Escandon *et al.,* 1999; Meigs et *al.,* 2000; Carr, 2001; Sobel, 2002). This thrombus formation leads to a variety of acute clinical events such as unstable angina, myocardial infarction or cerebrovascular accidents (Franco et al., 2000; Libby *et al.,* 2002; Levi *et al.,* 2004).

Unhealthy diet is a major cause of high mortality and morbidity of vascular diseases (Pearson, 1999; Kuulasmaa *et al.,* 2000; Kelly, 2003). Therefore, it could be prudent to consume a diet that is supplemented with the food that may interfere physiologically with platelet aggregation and blood coagulation; thus interrupts the thrombotic progress of atherosclerotic disease. As part of normal healthy diet, people around the world use natural honey as a sweetener as well as due to its medicinal benefits (Amy and Carlos, 1996). Honey is a supersaturated sugar solution mainly comprises of D-fructose, D-glucose, sucrose, maltose and higher sugars (~80% of solid mass); while other natural products includes alkaloids, flavonoids / isoflavones, glycosides, phenolics, peptides/proteins are present in minor amounts (White *et al.,* 1962). Health benefits of honey have been reported in a variety of conditions including microbial infections (Cooper *et al.,* 2002), wound healing (Molan, 2001a,b), anti-inflammation and inhibition of reactive oxygen species (Mesaik *et al.,* 2008; Ahmad *et al.,* 2009), glucose tolerance (Ahmad *et al.,* 2008) and analgesia (Azim *et al.,* 2007).

In continuation of our studies, on medicinal attributes of honey, we hypothesized that honey might have effects on blood hemostasis. This effect of honey was assessed *in vitro,* on platelet aggregation and blood coagulation.

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MATERIALS AND METHODS

Honey Samples

Six commercially available unifloral and multifloral honey samples were used in this study. Pakistani honey samples were collected from colonies of *Apis mellifera* bees foraged on *Acacia modesta* **(Sha),** *Plectranthus spp.* **(Swa)** and *Ziziphus spp.* **(Sid).** For comparison, Clover honey **(Clo)**, Eucalyptus honey **(Cap)** and Langnese^{TM} honey **(Lan)** from USA, Australia and Germany respectively were included in the study. Information regarding the floral origin of these commercial honeys was obtained as detailed on the packing labels. The honey samples were diluted with sterile phosphate buffered saline pH 7.4.

Estimation of predominant sugars, water content, pH, ash, viscosity and specific gravity of honey samples

In the present study, determination of amount of simple sugars present in honey samples were done by using high performance liquid chromatography. Simple sugars in honey samples were separated isocratically by reversed phase HPLC on a Prevail Carbohydrate ES, 5µm, 250 x 4.6mm column with acetonitrile/water. Karl Fischer titration method was used to estimate the water content in honey samples (Cedergren, 1974; Hoffmann, 1998), where as estimation of pH in honey samples were carried out by using pH meter. Ash content of honey samples was determined by dry ashing, through Furnace method (Milne *et al.,* 1992). Viscosity was measured using a Brookfield Viscometer (Cheng, 1990) and specific gravity of honey samples was determined by Pycnometer - density bottle method (Wattiaux *et al.,* 1991; Wattiaux *et al.,* 1992; Bailoni and Bittante, 1994).

Determination of anti platelet activity of honey

Human platelets were isolated from blood samples collected from healthy subjects (n=6), aged 35-48, who did not consumed any medication in the last two weeks. Blood was taken by clean vein puncture and immediately mixed with 3.8% (w/v) sodium citrate solution (9:1) and centrifuged at 260×g for 15 minutes at 20°C to obtain platelet rich plasma (PRP).

Platelet count was determined by phase contrast microscopy and all aggregation studies were carried out at 37°C with PRP having counts between 2.5-3.0×10^8/ml of plasma (Shah and Saeed, 1995). All experiments were performed within 2 h of PRP preparation. Platelet aggregation was monitored using Dual-channel lumi-aggregometer (Model 400 Chronolog Corporation, Chicago, USA) using 0.425-0.49 ml aliquots of PRP (Shah and Saeed, 1995, Shah *et al.,* 1996). The final volume was made up to 0.5 ml with the honey samples, dissolved in sterile phosphate buffer saline pH 7.4. Aggregation was induced with 10 µl of adenosine 5'-diphosphate (ADP) solution (4 µM). The time of addition of inducer was defined as 0 time. Various concentrations (2.5, 5, 6 and 7.5%) of honey samples were added following addition of the aggregation agent. Percentage of aggregation inhibition was calculated comparing with the control (no honey).

Determination of effects of honey on blood coagulation

Blood was taken by clean vein puncture from normal human healthy volunteers; who did not consumed any medication in the last two weeks. Platelet poor Plasma (PPP) was obtained by centrifugation at 1500×g for 10 min at 4°C. Effects of honey samples on blood coagulation were carried out by using HemoStat-aPTT-EL, HemoStat Thromboplastin-SI, HemoStat-Thrombin time and HemoStat-Fibrinogen. All reagents were prepared, maintained and performed as directed by the manufacturer (Human Diagonista-Germany GmbH) with some modifications (Posta *et al.,* 2002; Nilsson *et al.,* 1997; Berry *et al.,* 2002; Valeria *et al.,* 2000). Clotting time for all coagulation assays was measured by taking the average of 2-5 measurements using Coagulometer (Human Diagonista-Germany GmbH).

Effect of honey on whole blood coagulation

The extracted blood from the healthy human volunteer (male) was equally distributed (0.3ml) into glass tubes (without anticoagulant) containing diluted honey samples (**Lan** and **Swa**) at the final concentrations of 15, 24 and 30%. Upon addition of blood into tubes, whole blood clotting time was noted by optical method. Whole blood (no honey) was taken as control. For the validity of coagulation test results, sterile phosphate buffer saline was also tested.

Effect of honey on coagulation parameters tested in platelet poor plasma

Blood was collected from 30 healthy human volunteers (20 males, 10 females; aged 32±6). Whole blood was mixed with anticoagulant (sodium citrate) and platelet poor plasma (PPP) was removed by using plastic pipette and kept in covered plastic tube to avoid pH changes. Diluted honey samples were mixed with PPP in pre-warmed tubes and clotting was timed, after addition of reagents for aPTT, PT, TT and Fibrinogen level. For aPTT, PT, TT assays the final concentrations of honey samples were 3.75 and 7.5%; whereas for fibrinogen assays, final concentrations were 6.25 and 11.76%. Platelet free plasma (no honey) was taken as control. For the validity of coagulation test results, sterile phosphate buffer saline and Dextran solution (Clinical grade, M.W. =200,000-300,000) were also tested.

Effect of honey mixed in whole blood and coagulation parameters tested on extracted platelet poor plasma

Blood samples collected from 7 healthy male human volunteers (aged, 25.8±8.2) were taken and placed into two tubes in equal amounts (containing sodium citrate as

anticoagulant). Diluted honey samples (9.0%) were added to one of the tube (T1) and incubated for 10 minutes. After incubation, PPP was removed and aPTT, PT and TT assays were carried out. Platelet free plasma (tube-T2, with no honey) was taken as control. For the validity of coagulation test results, sterile phosphate buffer saline and dextran solution (Clinical grade, M.W. =200,000-300,000) were also tested.

Statistical analysis

Results are mentioned as mean with standard deviation. The mean values were calculated from 2-5 observations. One-way analysis of variance (ANOVA), two-way analysis of variance (ANOVA), test for homogeneity of variance, and post-hoc tests (Tukey HSD and Games-Howell) were all performed wherever applied. All analyses were carried out using Excel (Microsoft Corp., Redmond, Wash., U.S.A.) and SPSS version 12 (Aspire Software Intl., Ashburn, Va., U.S.A.).

RESULTS

Estimation of predominant sugars, water content, pH, ash, viscosity and specific gravity of honey samples

Table 1 shows percentage composition of honey sugars analyzed by HPLC (see methods). During the study, first glucose, fructose, maltose and sucrose separately and then a mixture of four sugars were standardized and considered as control. Later all honey samples were analyzed against the control. Table 2 shows the results of Ash content (%), Viscosity (Poise), pH, Sp. Gravity (20°C) and Water content (% w/w basis) of honey samples.

Effect of honey on blood hemostasis

The results of present study show honey-mediated (a) inhibition of platelet aggregation, (b) prolongation of aPTT, PT, and TT and (c) reduction in Fibrinogen levels.

Platelet aggregation activity of honey

In this study, effect of different honey samples on platelet aggregation assay was carried out. Fig. 1 shows, that addition of honey in the reaction mixture, inhibited ADP-induced platelet aggregation. Table 3 shows IC_{50} dose of honey samples that remained between 5.0-6.5 %. Among the honey samples studied in this experiment, *Plectranthus* (**Swa**) and Euclayptus honeys (**Cap**) showed maximum inhibition of platelet aggregation with IC_{50}~5.0 %. PRP without honey was taken as control. In order to eliminate the false positive effect due to the viscosity of honey, Dextran (9.0 %) was also tested. However, no significant effect was observed supporting the inhibitory activity of honey.

Effect of honey on blood clotting time

Fig. 2 shows, effect of honey on whole blood coagulation. In this study, ***Lan*** and ***Swa*** honey samples were tested in 15, 24 and 30% concentrations. Honey at less than 15% concentration (results not shown) did not show any effect on clotting time. However, after addition of 15% honey in whole blood resulted 2.5 and 5% increase in clotting time. With 24% honey, mean clotting time was increased to 18 and 27% and finally honey at dose of 30% increased the mean clotting time by 38%. Whole blood (no honey) was taken as control.

Table 1: Percentage composition of honey sugars (in %).

Honey Samples	Glucose	Fructose	Sucrose	Maltose	Total sugar %
Reference Ranges					
	28-36%	36-50%	0.8-5.0%	1.7-11.8%	*
Cap	30.09	38.45	5.17	2.1	75.84
Clo	33.33	37.33	3.03	1.83	75.52
Lan	26.78	33.91	6.4	2.59	69.73
Swa	25.09	23.54	4.38	3.67	56.69
Sid	24.3	31.66	9.4	3.79	69.15
Sha	33.63	36.9	2.59	1.87	74.99

Table 2: Composition of honey samples

Honey Samples	Ash (%)	Viscosity (Poise)	pH	Sp. Gravity (20° C)	Water content (% w/w basis)
	Reference Ranges				
	0.04-0.93%	150	3.3-5.6	1.423	15-18
Cap	0.06	130	4.098	1.4335	20.412
Clo	0.07	140	3.859	1.4277	20.364
Lan	0.07	114	4.09	1.4203	21.237
Swa	0.06	140	3.701	1.429	21.814
Sid	0.05	145	5.057	1.422	23.931
Sha	0.06	130	5.216	1.4293	22.798

Fig. 1: Effect of honey samples on human platelet aggregation

Table 3: Effect of honey on platelet aggregation

No.	Honey Samples	IC_{50} (%)
1.	*Plectranthus* honey (Swa)	5.0±0.1
2.	*Ziziphus* honey (Sid)	5.5±0.15
3.	*Acacia modesta* honey (Sha)	5.5±0.09
4.	Euclayptus honey (Cap)	5.0±0.2
5.	*Clover honey (Clo)*	6.5±0.1
6.	*Langnese*^{TM} honey (Lan)	6.0±0.17

Effect of honey on coagulation parameters tested in platelet poor plasma

In this study, six honey samples were tested in platelet poor plasma using aPTT, PT, TT and Fibrinogen level assays. Results clearly indicate that addition of honey significantly increased measured aPTT, PT, TT and decreased the Fibrinogen levels.

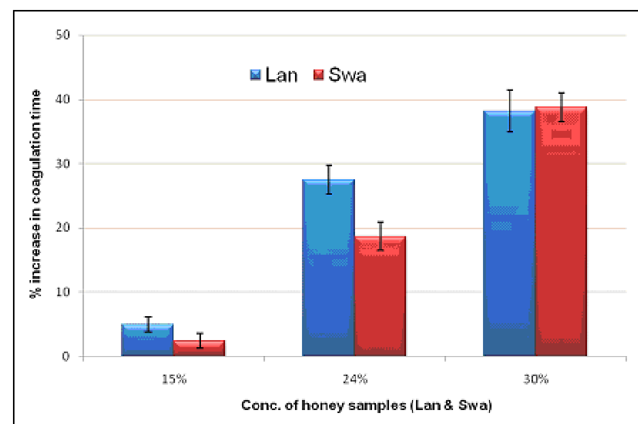

Fig. 2: Effect of honey samples on whole blood clotting time.

Effect of honey on aPTT tested in platelet poor plasma
Fig. 3 shows effect of different honey samples on aPTT. Incubation of natural honey at different concentrations i.e., 3.75 and 7.5% showed progressive prolongation in aPTT. Results shows, the mean increase in aPTT by incubation of 3.75 and 7.5% honey in PPP determined as 19±10% (Swa honey response not included) and 62±10% respectively. PPP (without honey) was taken as control.

Effect of honey on pt tested in platelet poor plasma
Fig. 3 shows, effect of different honey samples on PT. Incubation of natural honey at concentration 3.75 and 7.5% caused progressive prolongation in PT and determined as 6.0±5.0% and 40±5.0% respectively. PPP (no honey) was taken as control.

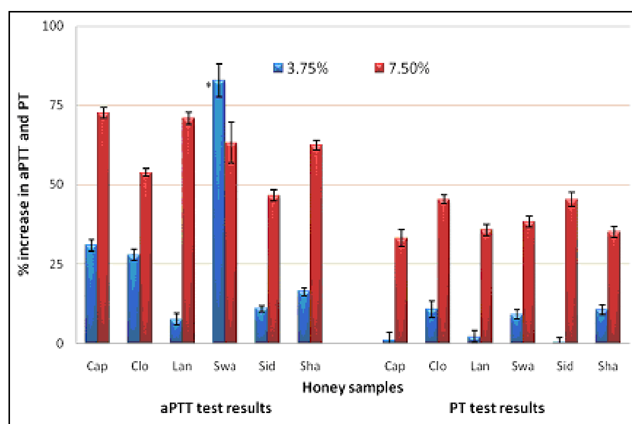

Fig. 3: Effect of honey on activated partial prothrombin time in platelet poor plasma.

Effect of honey on TT tested in platelet poor plasma
Similar to above results, fig. 4, show that incubation of natural honey at concentration 3.75 and 7.5% caused progressive prolongation in TT and determined as 35±15% and 112±30% respectively. PPP (no honey) was taken as control.

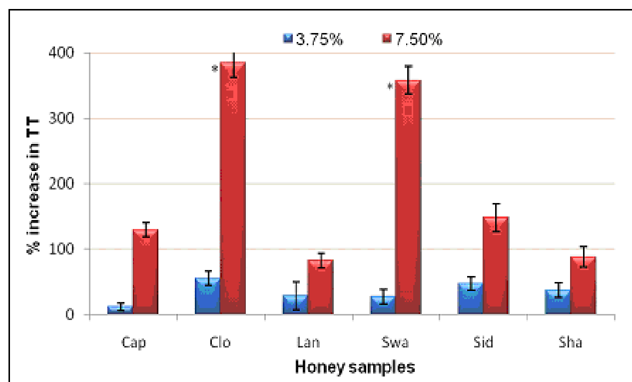

Fig. 4: Effect of honey on prothrombin time in platelet poor plasma.

Effect of honey on fibrinogen level tested in platelet poor plasma
Fig. 5 shows results of honey samples with natural honey at concentrations 6.25 and 11.75% that shows decrease in Fibrinogen level and determined as, as 13±4% (Sha honey response not included) and 86±30% respectively. PPP (no honey) was taken as control.

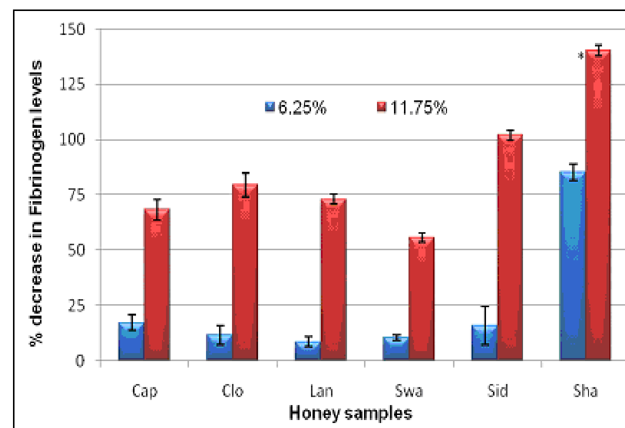

Fig. 5: Effect of honey on thrombin time in platelet poor plasma.

Effect of honey mixed in whole blood and coagulation parameters tested on extracted platelet poor plasma
Fig. 6 shows the results of different honey samples at 9% concentration that were initially incubated in whole blood; later PPP was isolated and assayed for aPTT, TT, PT. This experiment showed that honey treatment of whole blood caused significant prolongation of tested assays. The mean increase in aPTT, PT and TT in extracted PPP determined as 45±21%, 26±9% (Sid honey response not included) and 105±24%, respectively. PPP (no honey) was taken as control.

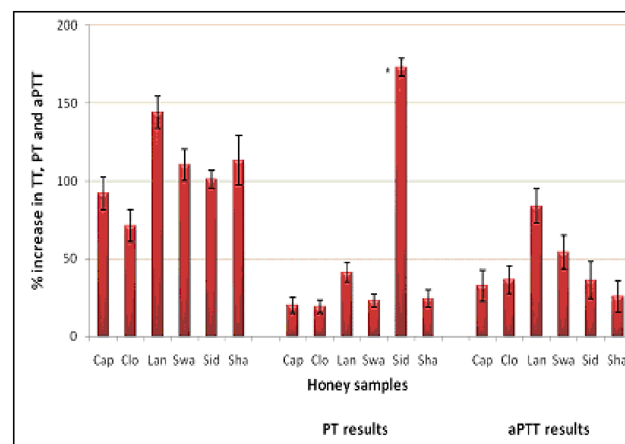

Fig. 6: Effect of honey on fibrinogen level in platelet poor plasma.

DISCUSSION

The present study is the first report about the effect of honey on blood hemostasis. Previously, only anticoagulant properties of honey bee venom were known (Ouyang *et al.,* 1979; Kini *et al.,* 1994; Gowlik *et al.,* 2004; Franco *et al.,* 1994). During the pathogenesis of the vascular disorder, the formation of unwanted thrombus on the pre-existing atherosclerotic plaques can cause partial or complete occlusion of the blood vessel, which leads to sinister clinical events such as acute myocardial infarction or cerebrovascular accidents (Victor *et al.,* 2002; Thomas, 2008). More over independent, abnormal and uncontrolled coagulation termed as hypercoagulability leads to serious clinical events such as deep vein thrombosis (Libby, 2002; Kannel, 2005). The formation of thrombus is initiated by the platelet aggregation (platelet plug); that follows the activation of blood coagulation proteins to further reinforcement platelet plug by forming a mesh work of insoluble fibrin (Kannel, 2005; Rauch *et al.,* 2001; Furie and Furie, 2005).

Platelets play a key role in primary hemostasis, thrombosis and inflammation (Ahmad, 2003). Therefore, the importance of anti-platelet therapy in the prevention of vascular disorder is unquestionable. The results of the present study indicate that addition of natural honey into the reaction mixture; causes the inhibition of platelet aggregation.

The mechanism by which honey inhibits the platelet aggregation can be explained by many factors such as, honey contains hydrogen peroxide (Molan, 2001a, b); studies shows that exogenous exposure to hydrogen peroxide result in platelet inhibition (Ferroni *et al.,* 2004a), therefore it can be assumed that the presence of hydrogen peroxide might be the underlying basis of honey induced inhibition of platelet aggregation. Moreover, it can be suggested that honey is competitively antagonizing ADP receptor mediated aggregation. Natural honey is known to have suppressive effects on reactive oxygen species (Ahmad *et al.,* 2009). Here it would be meaningful to mention, that activated platelets during and after the platelet aggregation release different cytokines which in turn activates phagocytes. Thus platelet activated phagocytes results in increase production of free oxygen radicals (Pervushina *et al.,* 2004; Kazemi *et al.,* 2008). Since honey inhibits the aggregation of platelets therefore it can be assumed that it indirectly inhibits the production of free oxygen radicals. Interestingly free oxygen radicals indirectly act on platelet function via oxidative modification of low-density lipoproteins or oxidation of lipids and their derivatives (Ferroni *et al.,* 2004a). Hence it can be assumed that honey can influence the platelet function by inhibition of LDL oxidation (Hegazi and El-Hady, 2007) that indirectly affects platelets function. Natural honey causes physiological euglycemia (Ahmad *et al.,* 2008) and it has been reported that hyperglycemia causes redox activation of platelets (Ferroni *et al.,* 2004b). The honey induced physiological euglycemia might be additional factor, affecting the functions of platelets.

After the process of primary hemostasis induced by platelets; secondary hemostasis is achieved by the activation of blood coagulation that resulted in the formation of fibrin mesh work. Therefore, the importance of anti-coagulant therapy in the prevention of vascular disorder is also without a shred of doubt.

Results of present study demonstrate that honey inhibited the coagulation proteins of all three coagulation pathways i.e., intrinsic pathway (assayed by aPTT); extrinsic pathway (assayed by PT) and final common pathway (assayed by TT). Moreover, during this study honey-induced decrease in fibrinogen levels that is in agreement with the prolongation of aPTT, PT and TT observed.

There could be several reasons for natural honey to have anticoagulant attributes such as, honey contains variety of flavonoids that may affect the activity of coagulation factors like fibrinogen and factor VII (Cazenave, 1988; Middleton and Kandaswami, 1992; Beretz and Cazenave 1991). Similarly different types of sugars affect the process of blood coagulation. Honey contains glucose (28-36%) and it has been suggested that high level of glucose interferes with coagulation through different mechanisms such as, non-enzymatic glycation, the development of increased oxidative stress, and a decrease in the levels of subendothelial heparin sulphate (Carr, 1996; Bakaltcheva and Reid, 2003). Moreover, honey contains maltose (1.7-11.8%) that also reported to interfere with blood coagulation (Carr and Carr 1995; Carr *et al.,* 1996; Bakaltcheva and Reid, 2003).

The results of present study, provides clear evidence for multi-dimensional anti-haemostatic properties of honey; that encompasses interference with the intrinsic, extrinsic, common coagulation cascades as well as platelet aggregation. It can be assumed that observation of median levels of efficacy in honeys originated from different floral sources and geographical regions indicated that anti-platelets / anticoagulant activity might be a general property of honey. These observations provide first line evidence for modulatory role(s) of honey on process of hemostasis.

Medicinal properties of honey, encompass various mechanisms that might play a role in the prevention of atherosclerotic vascular disorders e.g., cardiovascular and cerebrovascular disorders. Honey has reported to inhibit thrombin (main enzyme of blood coagulation) induce formation of reactive oxygen species from phagocytes; as

free oxygen radicals particularly superoxide and hypochlorous acid provides the nidus for the development of atherosclerotic plaque, thus honey might interrupts the nidus formation of atherosclerotic plaque (Ahmad *et al.,* 2009). Honey independently inhibits LDL oxidation that also prevents the development of primary atherosclerotic lesion (Hegazi and El-Hady 2004). It is reported that fasting blood sugar is an independent predictor of platelet dependent thrombosis in patients with coronary artery (Shechter *et al.,* 2000). Honey has reported to have physiological euglycemia in fasted human subjects (Ahmad *et al.,* 2008). This effect also possibly interferes with the process of thrombosis.

On the basis of present and previous results, it can assumed that honey might interferes at several steps in the formation of atherosclerotic disease this effect finally translates into the prevention of vascular disorders such as cardiovascular and cerebrovascular disorders.

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