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Fractionation of acacia honey affects its antioxidant potential *in vitro*

Muhammad Aliyu[1,2,3*], Oyeronke A. Odunola[1], Ahsana D. Farooq[2], Ahmed M. Mesaik[3], Muhammad I. Choudhary[2,3], Ochuko L. Erukainure[2], Shahida Perveen[4], Almas Jabeen[3]

[1]*Department of Biochemistry, University of Ibadan, Ibadan, Oyo State, Nigeria*
[2]*H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan*
[3]*Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan*
[4]*Third World Centre, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan*

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ABSTRACT

Objective: To investigate the effects of fractionation of acacia honey on its antioxidant potential in contrast with the pure honey from whole blood, brain and liver in vitro. **Methods:** Honey was partitioned into three fractions (dichloromethane, ethyl acetate and aqueous). Their immuno-modulatory effect on whole blood was assayed using Luminol-amplified chemiluminescence technique. Their antioxidant activities on rat brain and hepatic tissues which covers for catalase, SOD activities and lipid peroxidation. **Results:** Fractions of the honey enhanced the production of radicals with no significant ($P>0.05$) antioxidant activity on whole blood where as pure honey does. Pure honey significantly ($P<0.05$) stimulates SOD and Catalase activity with no significant ($P>0.05$) effects on lipid peroxidation. **Conclusions:** Fractionation of acacia honey negatively affects its antioxidant potential thereby making it a radical generating agent in contrast with the unfractionated.

1. Introduction

Honey, a natural sweet substance produced by bees via regurgitation has been proven to be beneficial at the level of preventive and curative research. Honey is rich in phenolic compounds, which act as natural antioxidants and are becoming increasingly popular because of their potential role in contributing to human health. It is a natural antioxidant which contains flavinoids, ascorbic acid, tocopherols, catalase, and phenolic compounds all of which work together to provide a synergistic antioxidant effect by scavenging and eliminating free radicals[1]. It is important for the treatment of acute and chronic oxidative stress-mediated diseases and toxicity.

Many studies have shown that regular intake of phenolic compounds is associated with reduced risk of heart diseases. In coronary heart disease, the protective effects of phenolic compounds include mainly antithrombotic, anti-ischemic, anti-oxidant, and vasorelaxant[2]. It has been suggested that flavonoids decrease the risk of coronary heart disease by three major actions: improving coronary vasodilatation, decreasing the ability of platelets in the blood to clot, and preventing low-density lipoproteins (LDLs) from oxidizing[2].

It is a promising antitumor agent with pronounced anti-metastatic and anti-angiogenic effects[3], antibacterial, anti-inflammatory, immune-stimulant, antiulcer and wound/burn healing[4]. Various signaling pathways, including stimulation of tumor necrosis factor-alpha (TNF-α) release, inhibition of cell proliferation, induction of apoptosis and cell cycle arrest, as well as inhibition of lipoprotein oxidation, mediate the beneficial effects exerted by honey and its major components such as chrysin and other flavonoids[5-9]. However, chronic consumption of honey that was mixed with rat pellets has been shown to have a positive impact on the architecture and integrity of hepatocytes in vivo[10]. Its protective role against the kidney dysfunctions induced by sodium nitrite, a known food additives, hepatoprotective, hypoglycemic, reproductive, antihypertensive as well as

*Corresponding author: Muhammad Aliyu, Department of Biochemistry, University of Ibadan, Ibadan, Oyo State, Nigeria.
Tel: +2347038161430.
E-Mail: amachida31@gmail.com
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antioxidant effects has also been reported[11,12]. Honey is produced from many different floral sources and its biochemical and pharmacological activities vary depending on its origin and processing. Honey contains a variety of biologically active compounds such as flavonoids, vitamins, antioxidants as well as hydrogen peroxides[13]. Daily consumption of honey has been shown to have both positive and negative effect on male Wister albino rat[14]. Acacia honey has been reported to reduce proliferation of melanoma cells through alterations in cell cycle progression in vitro[15]. To the best of our knowledge no work has so far been done on antioxidant activity of acacia honey in relation to its fractionation. Therefore, this study was undertaken to investigate the effects of fractionation of acacia honey on its antioxidant potential in contrast with the pure honey from whole blood, brain and liver in vitro.

2. Materials and methods

2.1. Sample collection

Honey was collected from the northwest frontier of Pakistan (N.W.F.P) during spring season of 2012 from acacia flower and maintained at 4 ℃ until analysis. All the chemicals and reagents were of analytical grade.

2.2. Collection of brain and liver from normal rats

The normal male albino rats were collected from the animal house of International Centre of Chemical and Biological Sciences, University of Karachi, Pakistan with the approval of Animal right review committee. They were humanly sacrificed by cervical dislocation. Brain and liver tissues were collected, homogenized in 1:5 of 0.1 M Tris-HCl buffer (pH 7.4). The supernatant was collected after centrifugation at 3 500×g at − 4 ℃ in a refrigerated centrifuge for 5 min and kept at − 80 ℃ until analysis. α-tocopherol at the concentration of 0.5 mg/mL as positive control and 4% (v/v) acacia honey were used for lipid peroxidation, Superoxide Dismutase (SOD) and Catalase activity assay.

2.3. Liquid-liquid fractionation of acacia honey

50 g of honey was weighed and dissolved in 500 mL distilled water, thereafter partitioned by liquid-liquid fractionation using dichloromethane and ethyl acetate separately. The fractions were collected and concentrated using rotary evaporator. The aqueous fraction was later freeze-dried. The % yield of aqueous, dichloromethane and ethyl acetate fractions were 26%, 5% and 3% respectively.

2.4. Luminol-amplified chemiluminescence assay

Luminol-amplified chemiluminescence assay was conducted on Human whole blood from a healthy volunteer as described[16]. Briefly, whole blood (1:20) suspended in modified Hank's solution (MHS) were incubated for 30 min with serial concentrations of honey (0.25%, 0.5%, 1.0%, 1.5, 2.0%, and 2.5% v/v for pure honey. 0.25, 1.25 and 2.5 mg/mL for the fractions. All the dilutions were made using phosphate buffer saline. MHS with cells and no honey was run as control. Then Zymosan (1 mg/mL) (Sigma-Aldrich, Buchs, Switzerland) was added, followed by 25 μL (10^{-5} M) of luminol (G-9382 Sigma-Aldrich). Total chemiluminescence (CL) was recorded with luminometer (Lab system Luminoskan RS, Helsinki, Finland). The luminometer was set to measure the resulting light emission in 96-well plate for a period of 50 min in repeated scan mode with 50 scans, 30 s interval, and one second point measuring time. This method was adopted due to its sensitivity, reproducibility and availability.

2.5. Inhibition of lipid peroxidation

Lipid peroxidation assay was carried out by the modified method of Ohkawa *et al*[17]. Briefly, 100 μL of the sample (tissues) was mixed with a reaction mixture containing 30 μL of 0.1 M Tris-HCl buffer (pH 7.4), sample (100 μL) and 30 μL of the pro-oxidant (7 μM Sodium Nitroprusside). The volume was made up to 300 μL by water before incubation at 37 ℃ for 2 h. The colour reaction was developed by adding 300 μL of 8.1% SDS (Sodium duodecyl sulphate) to the reaction mixture, followed by the addition of 600 μL of acetic acid/HCl (pH 3.4) and 600 μL of 0.8% TBA (Thiobarbituric acid). This mixture was incubated at 100 ℃ for 1 h. The absorbance of TBARS (Thiobarbituric acid reactive species) produced were measured at 532 nm in an ELISA plate reader (Molecular Devices). The percentage inhibition of MDA (Malondialdehyde) produced was calculated using the following formula:

$$\text{Inhibition rate \%} = \left[1-\left(\frac{A_1-A_2}{A_0}\right)\right]\times 100$$

Where A0 = absorbance of control (without the honey); A1 = absorbance of mixture with compound; A2 = absorbance of mixture without sample and honey.

2.6. Superoxide dismutase (SOD) activity

The level of SOD activity was determined by the method of Misra and Fridovich[18] with some slight modification. Briefly, 100 μL of tissue sample was diluted in 900 μL of distilled water to make a 1 in 10 dilution. An aliquot of 200 μL of the diluted sample was added to 2.5 mL of 0.05 M carbonate buffer (pH 10.2). 60 μL of the pro-oxidant (7 μM

SNP) was added to the mixture, after which 200 μL of the compound was added and incubated at 37 ℃ for 2 h. 300 μL of freshly prepared 0.3 mM adrenaline was added to the mixture. The blank contained 2.5 mL buffer, 300 μL of substrate (adrenaline) and 200 μL of water. The absorbance was read at 480 nm using ELISA plate reader. SOD activity was measured as the percentage inhibition of adrenaline oxidation using the formula:

$$\text{Inhibition rate \%} = \left[1-\left(\frac{A_1-A_2}{A_0}\right)\right]\times 100$$

Where A0 = absorbance of control (without the honey); A1 = absorbance of mixture with compound; A2 = absorbance of mixture without sample and honey.

2.7. Catalase activity

The Catalase activity of the tissues was determined by adopting the method of Brannan *et al*[19] with some slight modification. Briefly, 200 μL of tissue sample was diluted in 1 800 μL of distilled water. An aliquot of 100 μL of the diluted sample was pipette into a 96-well plate. 30 μL of the pro-oxidant (7 μM SNP) was added to the sample, after which 100 μL of the compound was added and incubated at 37 ℃ for 2 h. 50 μL of 1.2 mM H_2O_2 (in 0.05 M phosphate buffer [pH 7.0]) was added to the reacting mixture and allowed to stand for 25 min. The reaction was stopped by the addition of 40 μL of dichromate/acetic acid to give a red colored compound and absorbance measured at 505 nm using an ELISA plate reader. The enzyme activity was calculated using the molar extinction coefficient of 40.00 M^{-1} cm^{-1} expressed as unit/mg protein

2.8. Statistical analysis

The results were expressed as mean ± Standard deviation. Differences between the groups were analyzed by one-way analysis of variance (ANOVA) with the aid of Statistical Package for Social Sciences (SPSS) software, SPSS Inc., Chicago, Standard version 10.0.1. *P* values<0.05 were considered statistically significant for differences in mean using the least of significance difference (Lsd).

3. Results

For normalizing the Biological variability, experiments were repeated at least three times in triplicates. As seen in Figure 1, dichloromethane and ethyl acetate fractions showed better potency in the suppression of reactive oxygen species production than aqueous fraction. None of the concentration of the fraction was able to cause 50% inhibition. Infact we observed that the aqueous fraction stimulated ROS generation greater than the control probably due to generation of H_2O_2 as result of sugar oxidation.

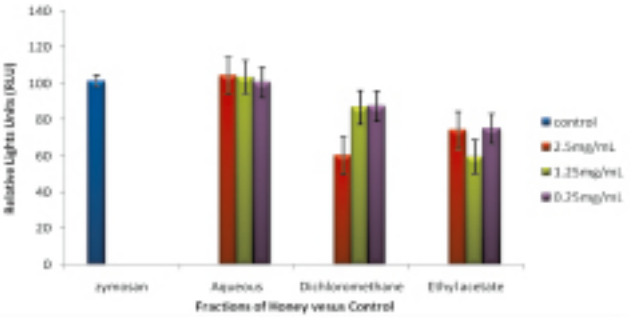

Figure 1. Luminol-amplified chemiluminescence assay results from whole blood using different fraction of acacia honey.

To see whether the decreased antioxidant activity was as result of fractionation, we used the pure acacia honey as depicted in Figure 2. There was a significant (P<0.05) inhibition in a dose-dependent manner with an IC_{50} of <0.25% (v/v). By implication this suggests that the fractionation had negatively affected the antioxidant activity, thus suggesting a synergetic activity of the whole honey.

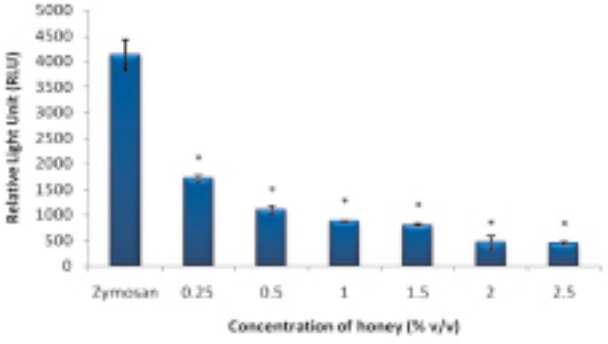

Figure 2. Luminol-amplified chemiluminescence assay results from whole blood using different concentration of acacia honey.

To further confirm our observation, we attempted using 4% (v/v) of acacia honey to see its effect on the antioxidant activities of brain and hepatic tissue after inducing the oxidative stress with sodium nitroprusside. Our result (Figure 3) shows that honey was able to induced SOD activity by about 43% and 42% for hepatic and brain tissues respectively as compared with α-tocopherol. Similarly, in Figure 4, significant (P<0.05) catalase activity was observed in both tissues. However, we could not detect appreciable inhibition of lipid peroxidation in both brain and hepatic tissues as depicted in Figure 5. This is not surprising due to earlier induction of SOD and catalase activity. This can be attributed to the fact that catalase reduces H_2O_2 to H_2O and

O_2, thereby making H_2O_2 unavailable for Fenton and Haber Weiss reactions which could generate hydroxyl radical leading to lipid peroxidation.

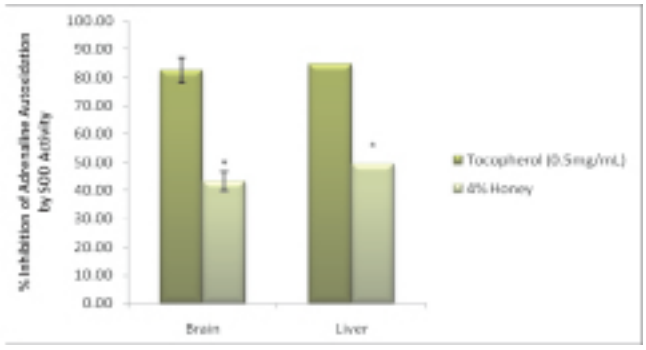

Figure 3. % inhibition of adrenaline autoxidation by SOD in the presence of acacia honey and α-tocopherol in brain and liver homogenate.

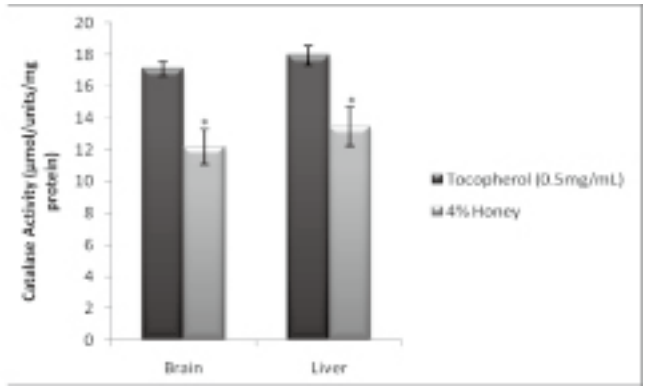

Figure 4. Catalase activity in brain and liver homogenate due to acacia honey and α-tocopherol.

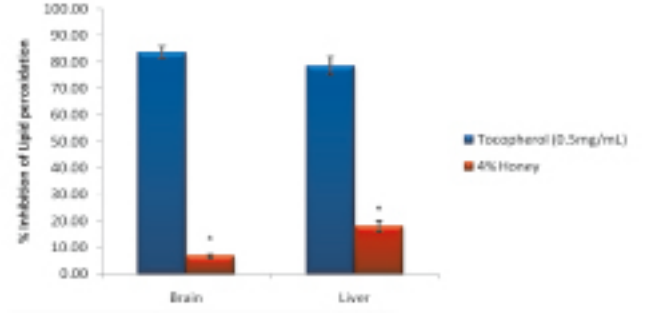

Figure 5. % inhibition of lipid peroxidation by α-tocopherol and acacia honey in brain and liver homogenate.

4. Discussion

Functional foods are characterized by the fact that they have health promoting or disease preventing properties apart from being nutritional and of such type is honey. Blood phagocytes respond to particulate or soluble stimuli by the oxidative burst associated with increased production of reactive oxygen species (ROS). NADPH oxidase located in the plasma membrane and in the membranes of specific granules produces superoxide anions from which other free radicals arise. ROS are released into the surrounding medium or into a membrane-enclosed subcellular organelle[20]. Dismutation of $O_2^{\bullet -}$, either spontaneously or catalyzed by superoxide dismutase, results in the formation hydrogen peroxide (H_2O_2), which acts as a substrate for the myeloperoxidase system (MPO) and this H_2O_2 converts to hypochlorous acid (HOCl) [21]. Emission of photons which takes place during ROS production can be measured easily as chemiluminescence. This effect can be amplified by the use of chemiluminescent probes, i.e. luminol[22]. An important and a sensitive method of measuring the generation of these metabolites is chemiluminescence assays as described by Allen *et al*[23], luminol-amplified chemiluminescence. Luminol is specific for the detection of hypochlorous acid, hydroxyl free radical *etc.*, which are primarily produced at the later phase of oxidative burst by phagocytic myeloperoxidase (MPO)[24-29].

Our findings clearly indicate that none of the fractions of acacia honey was able to confer a significant antioxidant activity. This supports the findings from other types that the antioxidants activity of honey is due to synergistic effects of wide range of compounds present in it[5]. These wide ranges of compounds include phenolics, peptides, organic acids, enzymes, Millard reaction products, and possibly other minor components and that the phenolics compounds contribute significantly to the antioxidant capacity of honey but are not solely responsible for it[30].

However, the pure acacia honey without being fractionated shows good antioxidant activity by decreasing the level of ROS generated from whole blood due to Zymosan stimulation in a dose-dependent manner. The results of this present study support previous data which reported that honey caused scavenging and quenching of ROS[5,31,32]. Moreover, honey has been shown to decrease the zymosan-stimulated human monocyte cell line-based ROS production[33]. By implication our study has clearly indicate that the interactions between different components found in acacia honey is important towards its antioxidant activity.

Reactive oxygen species spontaneously reacts with nitric oxide (NO) generated by sodium nitroprusside producing cytotoxic reactive nitrogen species capable of nitrating proteins and damaging other molecules like lipids and nucleic acids. We demonstrated that acacia honey stimulated the activity of SOD and Catalase as well as inhibition of lipid peroxidation against sodium nitroprusside-induced oxidative stress in brain and liver homogenates. In the liver of young and middle-aged rats, honey supplementation was reported to restore activities of catalase[34], SOD[35] and inhibition of lipid peroxidation[36], which invariably have supported our findings.

We conclude that the fractionation of acacia honey negatively affects its antioxidant potential thereby making it a radical generating agent instead in contrast with the unfractionated one *in vitro*.

Conflict of interest statement

We declare that we have no conflict of interest.

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