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Characterization of the Nematicidal Activity of Natural Honey

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Supporting Information

ABSTRACT: Antimicrobial activities of honey against bacteria and fungi are extensively reported in the scientific literature. However, its nematicidal potential has not been characterized so far. This study examined the effect of natural honey on model nematode *Caenorhabditis elegans* and analyzed the honey component(s) responsible for nematicidal activity. Characterization of honey-treated *C. elegans* was done using fluorescence and phase contrast microscopy. Egg-laying and egg-hatching defects of honey-treated *C. elegans* were studied. For identification of nematicidal component(s), bioactivity-directed fractionation of honey samples was carried out using dialysis, ultrafiltration, chromatographic, and spectroscopic techniques. Natural honeys of different floral sources showed nematicidal activity against different developmental stages of *C. elegans*. The nematicidal components of honey induced cell death in intestinal lumen and gonads of *C. elegans* as revealed by microscopy. The nematicidal action of honey was found to be due to reproductive anomaly as manifested by defects in egg-laying and -hatching by *C. elegans*. Honey with concentration as low as 0.03% exerted profound egg-laying defects, whereas 6% honey showed defects in egg hatching. The major sugar components of honey were not involved in observed nematicidal activity. The bioactive components responsible for anti-*C. elegans* activity were found in the 2−10 kDa fraction of honey, which was resolved into ∼25 peaks by reverse phase HPLC. LC-MS followed by further spectroscopic characterization revealed a glycoconjugate with the molecular mass of 5511 as the major nematicidal component of honey.

KEYWORDS: *antinematodal, anthelmintics, glycoconjugates, apitherapy, bee products*

INTRODUCTION

Parasitic nematodes are the common cause of human, livestock, and domestic animal infections. Parasitic nematodes cause low mortality and high morbidity in the human population throughout the tropical and subtropical world. The main species involved are *Trichuris trichiura*, *Ascaris lumbricoides*, and several species of hookworms.[1] It is estimated that 576−740 million people are infected with hookworms, 807−1221 million are infected with *Ascaris*, and 604−795 million are infected with *Trichuris*.[2] Overall >2 billion people are affected by parasitic worms all over the world; of these, 300 million are severely ill (www.who.int/wormcontrol). Parasitic nematodes are also responsible for agricultural losses of U.S. $157 billion annually worldwide.[3] Nematicidal drugs form a significant control measure for parasite nematode infections. Several classes of drugs are being used against nematodes including piperazine, benzimidazole, levamisole, pyrantel, oxantel, morantel, milbemycins, paraherquamide, and avermectins (http://www.wormbook.org/toc_diseasemoddrugdiscov.html). However, continued and heavy reliance on these compounds has led to the development of resistance in many nematode strains.[4−6] Moreover, current anthelmintic drugs also have severe side effects. Therefore, development of safer, environmentally friendly, and nematode-specific drugs is urgently required.

Parasitic nematodes are difficult to maintain in a laboratory setting because of their complex life cycle. *Caenorhabditis elegans* has been used as a model organism to study the molecular mechanism of nematode physiology because of its easy and rapid culturing, low testing cost, simple anatomy, short life cycle, transparent body, and sequenced genome. *C. elegans* is a free living, ∼1 mm long, nematode that lives in temperate soil environment and survives on feeding microbes.[7] *C. elegans* is a good model in drug research for human and veterinary parasites.[8]

Natural honey has long been used for the treatment of various diseases around the world in folk medicine.[9] As a supersaturated sugar solution, honey contains proteins, peptides, glycoconjugates, vitamins, minerals, flavanoids, phenolic acids, and phenolic acid derivatives as minor components.[10,11] Natural honey contains an array of anti-inflammatory and antioxidant compounds[12] that contribute to the prevention of several diseases.[13] It has antibacterial,[14] antifungal,[15] and scolicidal activities[16] and anticancer,[17,18] antinociceptive,[19] and wound healing[20,21] properties.

The antimicrobial activity of honey is an important factor in the bee colony that provides defense against pathogenic microbes including helminthe parasites.[22] Although antibacterial and antifungal activities of honey are well documented, the nematicidal activity has seldom been evaluated. We have previously reported the nematicidal activity of honey[23] using *C. elegans* as a model experimental nematode. Here we present a detailed characterization of the anti-*C. elegans* activity of honey followed by isolation and structural analyses of a nematocidal component of honey.

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MATERIALS AND METHODS

C. elegans, Escherichia coli, and Honey Samples. *C. elegans* (strain N2) and *E. coli* (OP50) were received from Caenorhabditis Genetics Center, University of Minnesota, USA. During this study, three natural honey samples obtained from bees foraging on *Plectranthus*, *Ziziphus*, and *Acacia* species (codes Sw-2, Br-2, and Shf-3, respectively) were used. The *Plectranthus* honey (code Sw-2) was obtained from Oriental bees (*Apis cerana*), whereas *Ziziphus* and *Acacia* honeys (codes Br-2 and Shf-3) were from European bees (*Apis mellifera*). These natural untreated honey samples were collected from different locations of northern Pakistan. An artificial honey sample representing the proportions of the four major sugars in the natural honey samples was prepared by mixing D-fructose (45 g), D-glucose (33 g), maltose (7.5 g), sucrose (1.5 g), and 17 mL of deionized water.[14]

Nematicidal Assays. *C. elegans* strain N2 was cultured on NGM plates with *E. coli* (OP-50) as food source. The nematicidal assays were carried out in sterile 96-well microtiter plates (polystyrene, flat bottom wells; Corning, New York, NY, USA). *C. elegans* were exposed to different concentrations of natural honey samples, artificial honey, and fractions of natural honeys.

C. elegans were maintained according to the procedure mentioned in WormBook (http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html). Each sample dissolved in S medium[24] at different concentrations was added directly to the 24/48/96-well plates. Twenty to 50 *C. elegans* worms of different developmental stages (i.e., larvae, adult males, and hermaphrodites) were placed in 200 μL of S medium[24] in well plates containing different concentrations of honey fractions/subfractions/artificial honey/sugars and 10 μL of 5× concentrated overnight culture of *E. coli* maintained in Lauria broth and resuspended in S medium. The plate was incubated at 20 °C. The survival of *C. elegans* was measured after 24 and 48 h of incubation, and the nematode viability was assessed by counting mobile worms using an inverted microscope (Eclipse TS-100, Nikon, Japan).

Effect of Honey on Egg Laying by *C. elegans*. The egg-laying defect was studied by placing worms on honey containing NGM agar plates. The honey samples and artificial honey (see above) were added in NGM separately in the range of 0.01−6% concentration.

To produce age-synchronized worms, a few adult hermaphrodite worms were transferred on fresh NGM plates and allowed to lay eggs for 3 h. After laying eggs, the adult hermaphrodites were removed and the NGM plates were kept at 20 °C for development of eggs into larvae and adult hermaphrodites. Fifteen age-synchronized adult hermaphrodites were transferred to new NGM plates that contained different concentrations (0.01−6%) of natural honeys and artificial honey and incubated at 20 °C. After every 24 h, the number of eggs was counted on each plate, and worms were transferred to fresh NGM plates, containing the same type of honey with same concentration. The counting of eggs and transferring of worms to fresh plates was done for 5 consecutive days. The eggs were counted around the same time every day.

Microscopic Analysis of *C. elegans*. Acridine orange (AO) staining of honey-treated C. *elegans* was carried out according to the method of Hashmi et al.[25] Briefly, adult *C. elegans* grown on NGM plates were transferred on honey-containing NGM plates. The concentration of honey in NGM was in the range of 1−6%. The plates were incubated at 20 °C for 24 h. For staining, 500 μL of AO staining solution (2 μL of 10 mg/mL AO stock solution was mixed per milliliter of M-9 buffer; http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html) was added and evenly distributed on NGM plates. The plates were incubated in the dark for 1 h at room temperature. After incubation, the worms were collected in a tube, washed three times, and then transferred to NGM plates without AO and incubated in the dark for an hour at room temperature. After incubation, worms were mounted on the slides and observed under a fluorescence microscope (Eclipse TE2000-S, Nikon) at 488 nm. The AO staining of honey-affected worms was compared with wild-type *C. elegans* (strain N2) of the same age.

Effects of Natural Honey on Egg Hatching by *C. elegans*. For isolation of eggs, the *C. elegans* (strain N2) was cultured on the NGM plates seeded with an *E. coli* OP50 lawn. Worms were rinsed off the agar plate with M-9 buffer in 50 mL tubes at room temperature and washed twice by centrifugation for 2 min at 1000*g* and then resuspended in M-9 buffer. After the last wash, only 1 mL of M-9 buffer was added in the tube. Then 10 mL of hypochlorite solution (2.4% NaOCl in 0.5 M NaOH) was added to the worms in 1 mL of M-9 buffer, and the tube was shaken for few seconds and then repeated after every 2 min for 20 min. The lysis reaction was stopped by adding 20 mL of M-9 buffer to the reaction mixture followed by centrifugation for 5 min at 10000*g*. The supernatant was removed using a sterile pipet, and the pellets of eggs were resuspended in 20 mL of M-9 buffer. Clumps of eggs were vertexed and centrifuged again at 10000*g* for 5 min. The supernatant was removed, and 2 mL of M-9 buffer was added. These isolated eggs were used to study the effect of honey on the development of *C. elegans*.

The assay was conducted in sterile 96-well plates. About 50 eggs of *C. elegans* were placed in 200 μL of M-9 buffer containing 1, 3 and 6% of honey.[24] The plates were incubated at 20 °C for 24 h. After incubation, the numbers of larvae and eggs were counted under an inverted microscope.

Characterization of Nematicidal Component(s) of Honey. The scheme used for the isolation and characterization of nematocidal component(s) of honey is shown in Figure 1.

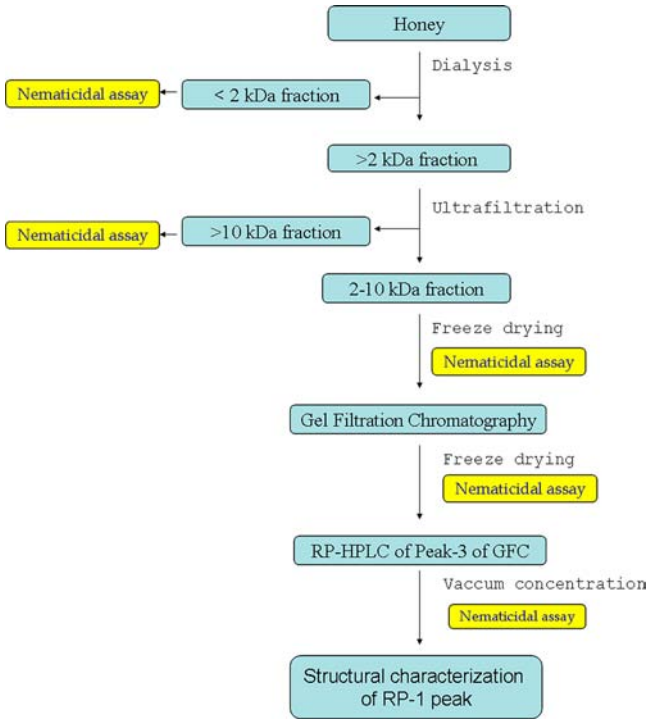

Figure 1. Flowchart of the techniques used for isolation and characterization of nematicidal components of honey.

Fractionation of Honey Components Using Dialysis and Ultrafiltration. The honey samples were fractionated into three fractions, namely, a <2 kDa fraction, a 2−10 kDa fraction, and a >10 kDa fraction, using dialysis and ultrafiltration. For this, honey samples (20 g) were dissolved in 20 mL of 20 mM sodium phosphate buffer, pH 7.0, and centrifuged at 5000 rpm for 5 min to remove pollen grains. The dialysis was carried out using a dialysis membrane (regenerated cellulose membrane) with a cutoff value 2 kDa for 24 h at 4 °C. The dialysate were changed after every 4 h. Dialysis resulted in two fractions, that is, a <2 kDa fraction and a >2 kDa fraction. The <2 kDa fraction was concentrated by rotary evaporation using Rotavapor R-200 (Buchi, Germany), whereas the >2 kDa fraction was subjected

to ultrafiltration using a filtration membrane with a cutoff of 10 kDa. As a result of ultrafiltration two fractions were obtained, that is, 2−10 and >10 kDa. These fractions were lyophilized by freeze-drying. The nematicidal assays were carried out with <2, 2−10, and >10 kDa fractions as mentioned above. The 2−10 kDa fraction showed nematicidal activity against *C.elegans*.

Gel Filtration Chromatography (GFC) of 2−10 kDa Fraction of Honey Samples. GFC of the 2−10 kDa fraction of honey samples was carried out using Superdex-30 (GE Healthcare Life Sciences, Uppsala, Sweden) with sodium phosphate buffer (20 mM sodium phosphate, 100 mM NaCl, pH 7.4) as the mobile phase. Chromatography was carried out at a 0.3 mL/min flow rate, and fractions of 1 mL were collected. Protein estimation of pooled fractions was carried out according to the Lowry method.[31]

Reverse Phase HPLC. Reverse phase HPLC was carried out for purification of peptides and glycopeptides present in peak P-3 from GFC of 2−10 kDa fractions from acacia honey (code Shf-3). The HPLC system (LC-20AT, Shimadzo, Japan) equipped with a diode array detector (SPD-M20A, Shimadzu, Japan) and a 20 μL loop was used. Samples were dissolved in 0.1% TFA solution and filtered by a 0.45 μm syringe filter (Millipore, Bedford, MA, USA). The reverse phase C-18 column (Hibar 150-4.6 mm, Purospher STAR RP-18e, Merck, Darmstadt, Germany) was equilibrated with 0.1% TFA. The 20 μL sample was injected, and elution was done by using the acetonitrile gradient at a flow rate of 1 mL/min. All peaks were collected and concentrated by using a centrifugal vacuum concentrator (model 5301, Eppendorf, Germany). Protein estimation was carried out according to the Lowry method.[31]

Mass Spectrometry, NMR Spectroscopy, and Fluorescence Spectrometry. The mass spectrometric measurements of peak RP-1 from RPLC (which showed strong nematicidal activity) were carried out using a LC-ESI-MS system (QSTAR-XL, Applied Biosystem, USA). The molecular mass determination was carried out using an electrospray ionization technique in the positive ion mode. Online separation was performed on a Perkin-Elmer series 200 micropump liquid chromatographic system (Perkin-Elmer, USA). The samples were eluted at a flow rate of 50 μL/min using an acetonitrile/H_2O mixture (1:1 v/v) containing 0.1% formic acid. The data were acquired using Analyst 1.4.1 software over a mass range of 400−2000 amu. The molecular mass of proteins/peptides was generated from several multiply charged peaks using Analyst 1.4.1 software.

The NMR spectrum of peak RP-1 was recorded for structural characterization of molecule(s) present in this peak. The ^{1}H NMR (see Supplementary Figure 1 in the Supporting Information) and ^{13}C NMR (see Supplementary Figure 2 in the Supporting Information) spectra were recorded in D_2O on the 300 MHz NMR spectrometer (Bruker, France).

Fluorescence emission properties of peak RP-1 were analyzed by using an LS55 spectrofluorometer (Perkin-Elmer, USA). Fluorescence emission spectra were obtained at 254, 272, and 280 nm excitation wavelengths. For comparison, fluorescence spectra of glucose, fructose, maltose, and sucrose were obtained.

RESULTS AND DISCUSSION

Previously, we have shown that natural honey has strong paralyzing (nematicidal) effects on all developmental stages of *C. elegans* with LD_{90} values ranging between 0.75 and 2.9% and a mean value of 1.5%.[23] During the present study, the effects of honey on the morphology and reproduction of *C. elegans*, that is, egg-laying and egg-hatching behaviors, were analyzed. Later, fractionation of honey components was carried out to determine the bioactive compound(s) responsible for the nematicidal effect. Structural characterization of the chromatographic peak showing strong nematicidal activity was done.

Microscopic Analysis of Honey-Treated *C. elegans*. To characterize the nematicidal effect of honey, fluorescence microscopy of AO-stained *C. elegans* was carried out. Fluorescence microscopy provided intriguing information regarding the effect of honey on *C. elegans* anatomy. Fluorescence microscopy of AO-stained honey-treated *C. elegans* showed that worms grown in NGM plates containing 1 and 3% honey have yellowish green fluorescence in the intestine, indicating dead cells in the intestinal lumen. However, the pharynx appeared normal (Figure 2a). Apparently, honey

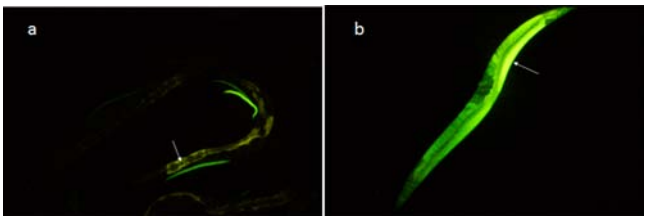

Figure 2. *C. elegans* showing the yellowish green fluorescence in intestinal and gonadal cells after incubation for 24 h on honey. Worms were incubated on NGM Petri plates containing (a) 3% and (b) 6% acacia honey (code Shf-3) for 24 h and stained with Acridine orange. The same effect was observed with *Ziziphus* and *Plectranthus* honeys (codes Br-2 and Sw-2, respectively).

entered the body of *C. elegans* by ingestion via the pharynx followed by entry into the intestinal lumen and absorption into the intestinal cells. It seems that the nematicidal component of honey initiated an apoptotic/necrotic response in intestinal cells, leading to cell death. In some cases intense yellowish green fluorescence in gonadal cells was observed in worms grown in plates containing 4−6% honey, indicating gonadal cell damage (Figure 2b). The intense fluorescence was seen in worms placed in NGM plates containing 6% honey, indicating massive cellular damage. In some stained worms the eggs were black in color and nonfluorescent, but in other worms they were fluorescent green.

The worms incubated in 3−6% honey containing NGM plates for longer periods, that is, 48 h, showed abnormality in egg-laying behavior manifested by hatching inside the body (Figure 3). Several larvae and large numbers of eggs were seen inside the bodies of treated worms compared to controls, whereas very few larvae and eggs were seen outside the worms on honey-containing NGM plates. Weakening of worms and rupturing of vulva were also seen in some honey-affected worms. Hence, it was concluded that honey exerted a nematicidal effect on *C. elegans* by causing defects in

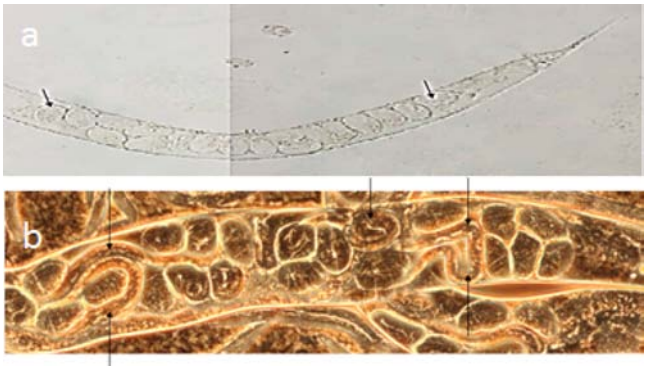

Figure 3. Phase contrast microscopy of *C. elegans*: worms were incubated on (a) 3% and (b) 6% acacia honey (code Shf-3) containing NGM Petri plates for 48 h. Large numbers of eggs in the body indicate an egg-laying defect. The arrow shows the hatched larvae in the body of *C. elegans*.

reproduction (egg laying and hatching). The egg-laying defect exerted by honey was characterized. Moreover, the effect of honey on the development of egg to larva was also assessed.

Effect of Honey on Reproduction of *C. elegans*. *Effect of Honey on Egg-Laying Behavior of C. elegans.* The hermaphrodite *C. elegans* are self-fertile and produce sperm during the fourth larval (L4) stage and store them in spematheca.[26] When the worms go through their final molt to develop into adults, they switch to producing oocytes. The mature oocytes are ovulated and pass through the spermathecae, where they come in contact with sperm and are fertilized. The fertilized eggs are stored in the uterus of adult hermaphrodites. Generally, a young adult hermaphrodite stores 10−15 eggs in its uterus (http://www.wormbook.org/toc_neurobiobehavior.html).

We studied the effect of natural honeys and artificial honey on the egg-laying behavior of *C. elegans*. The adult age-synchronized worms were incubated on honey-containing NGM plates and allowed to lay eggs. The egg-laying behavior of *C. elegans* during 5 days on NGM plates containing different concentrations of natural honey is shown in Figure 4. In the

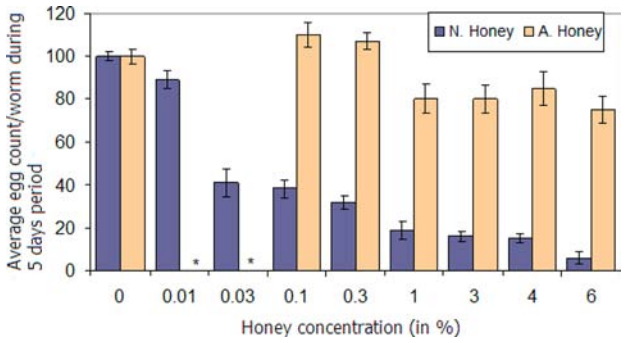

Figure 4. Average egg count laid by age-synchronized *C. elegans* during 5 days at different concentrations of acacia honey (code Shf-3) and artificial honey (∗, not tested; N, honey, natural honey; A, honey, artificial honey).

presence of honey, the egg-laying behavior was disrupted after 24 h of incubation with a concentration-dependent manner. The honey with a concentration as low as 0.03% exerted a profound effect on egg laying by *C. elegans* (60% decrease in egg laying at 0.03% honey in NGM medium). Egg laying was abolished at 6% honey. It was observed that the number of eggs laid by *C. elegans* on honey-containing NGM plates decreased as the incubation time increased.

The effect of artificial honey (representing the proportions of the four major sugars in natural honey; see Materials and Methods) was also tested. However, no considerable effect of artificial honey on egg laying was observed (Figure 4).

Effect of Honey on Egg Hatching of C. elegans. After fertilization, the eggs of *C. elegans* start to develop in the body. They remains in the uterus for approximately 150 min after fertilization. First, cleavage takes place at 40 min postfertilization. The eggs are expelled to the environment after reaching the ∼30-cell stage, that is, gastrula stage. Cell proliferation and organogenesis occur, and the eggs develop into larva approximately 9 h after expulsion to the environment (www.wormbook.org).

We analyzed the effect of natural honeys on the hatching of eggs of *C. elegans*. The eggs were exposed to 1, 3, and 6% honey for 24 h. The "harmful" effect was observed at 3 and 6% of honey. At 3% honey concentration, 76% of eggs were hatched, but in 6% honey concentration only 21% of eggs were hatched compared to control (IC_{50} value was calculated as 4.5% honey). Interestingly, at 6% honey solution all of the larvae died and some larvae were seen with abnormal morphology.

Identification and Characterization of Nematicidal Component(s) of Honey. The natural honey mainly contains four simple sugars, that is, D-fructose, D-glucose, maltose, and sucrose. It was therefore important to study the effect of individual sugars as well as the combination of these sugars (artificial honey; see Materials and Methods[14]) on *C. elegans*. For this purpose, an artificial honey sample was prepared representing the proportions of the four major sugars in the natural honey samples.[17]

The results showed that major sugar constituents of honey have a much lesser paralyzing effect on *C. elegans* compered to natural honey itself (Figure 5). At 3% concentration, artificial

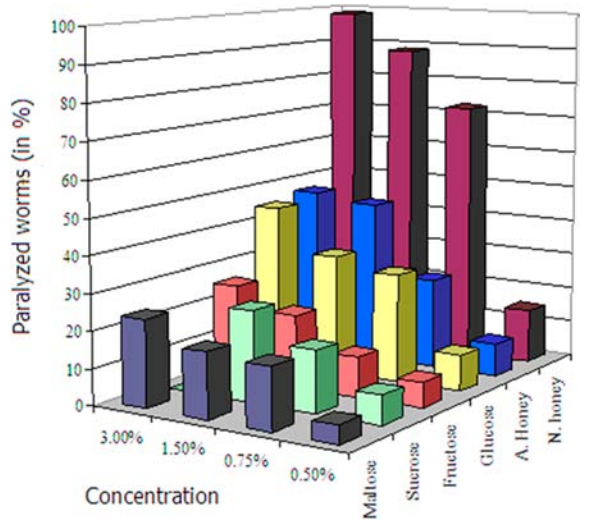

Figure 5. Nematicidal activity of artificial honey (A. honey) and major sugar constituents of honey in comparison with acacia honey (N. honey; code Shf-3).

honey, D-glucose, D-fructose, sucrose, and maltose exerted paralyzing effects of 47, 45, 26, 25, and 24% on *C. elegans*, respectively. These findings pointed out that among the major sugar constituents of honey, D-glucose has a comparably greater effect on *C. elegans* physiology. This observation can be explained by the adverse effect of a high amount of glucose on energy metabolism. The assays further indicated that tested sugars and artificial honey did not exert irreversible cellular damage as 80−100% of the paralyzed worms recovered after washing with buffer. Figure 6 clearly points out that ∼80% of the paralyzed worms incubated in wells containing 3 and 6% artificial honey/sugars regained activity after washing with M-9 buffer, whereas in the case of natural honey only 15% worms placed in the wells containing 6.25% honey regained activity.

Therefore, the major sugar components of honey were not found to be involved in nematicidal activity. Hence, we speculated that minor constituents of honey, such as plant phenolics, flavonoids, oligosaccharides, peptides, and proteins, might be responsible for this activity. Antioxidant potentials of phenolic constituents of honey have been demonstrated earlier.[27] Several nematicidal peptides from natural sources have been reported.[24,28,29] A 5.8 kDa bioactive component

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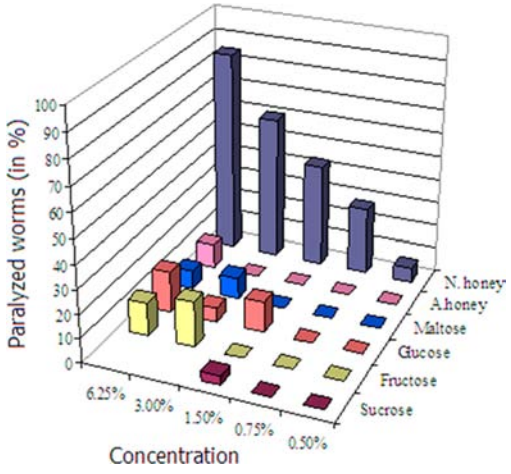

Figure 6. Percentage mortality of *C. elegans* after incubation in NGM Petri plates containing acacia honey (N. honey; code Shf-3), artificial honey (A. honey), and sugars for 24 h followed by washing with M9 buffer and incubation at 20 °C for 2 h.

from manuka honey has been reported.[30] We therefore moved forward in the identification of minor constituent(s) responsible for nematicidal activity.

Fractionation of Honey Components. The fractionation of natural honey was carried out to identify the nematicidal component(s). Three honey samples [*Plectranthus* honey (code Sw-2), *Ziziphus* honey (code Br-2), and acacia honey (code Shf-3)] were selected for fractionation. The fractions obtained after dialysis, ultrafiltration, and chromatographic procedure were tested for nematicidal activity using *C. elegans*.

The dialysis and ultrafiltration of honey samples resulted in three fractions, namely, <2, 2−10, and >10 kDa fractions. The <2 kDa fraction contained small molecules including major sugar constituents and other natural products. The >10 kDa fraction comprised macromolecules including proteins, glycoproteins, and proteoglycans,[10] whereas the 2−10 kDa fraction was composed of glycopeptides, peptides, glycoconjugates, etc.

Nematicidal assays of these fractions from three honeys were carried out at 100 μg/mL concentration. Survival of the worms was checked after 12, 24, and 48 h of incubation. The nematicidal assays of three fractions from honey samples showed predominant activity in the 2−10 kDa fraction (Figure 7). More than 90% worms were found paralyzed in wells containing the 2−10 kDa fraction within 12 h of incubation, whereas only ∼10% worms were paralyzed in wells containing the <2 and >10 kDa fractions during the same period. Hence, the results showed that nematicidal activity was predominately due to the bioactive molecules present in the 2−10 kDa fraction of honeys.

Differential interference contrast (DIC) microscopy was used to analyze the morphological defects in *C. elegans* caused by the 2−10 kDa fraction of honey. The DIC image of an adult hermaphrodite *C. elegans* after exposure to the 2−10 kDa fraction of honey showed defects in gonads and muscles, whereas the pharynx appeared to be normal (Figure 8).

GFC of the 2−10 kDa fraction of honey was done for further fractionation. The gel filtration chromatogram showed a major peak (P-3) eluted in the third void volume. The nematicidal activity was confined to peak P-3. Figure 9 shows a plot of the percentage of paralyzed worms as a function of peak P-3 concentration. The LD_{90} was calculated using nonlinear regression analysis. The LD_{90} values of P-3 after 12 and 48 h

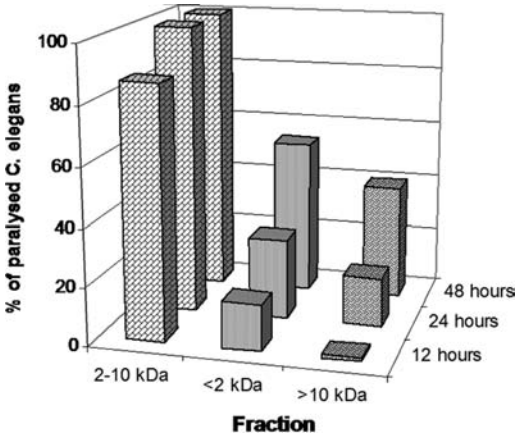

Figure 7. Nematicidal activity of the <2, 2−10, and >10 kDa fractions of acacia honey (code Shf-3) at 100 μg/mL concentration. The same effect was observed with *Ziziphus* and *Plectranthus* honeys (codes Br-2 and Sw-2, respectively).

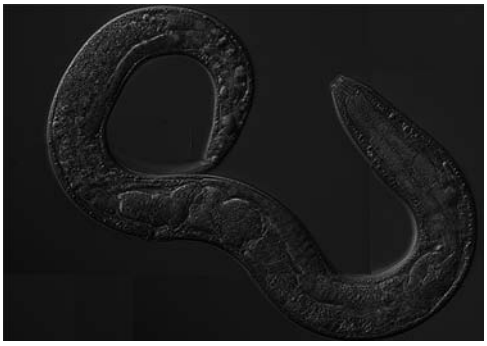

Figure 8. Differential interference contrast of *C. elegans* affected by the 2−10 kDa fraction of acacia honey (code Shf-3).

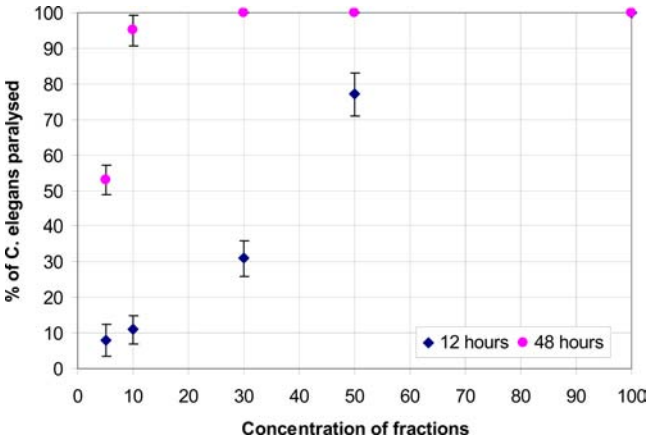

Figure 9. Nematicidal activity of peak P3 obtained from gel filtration chromatography of the 2−10 kDa fraction of acacia honey: percentage of *C. elegans* paralyzed as a function of different concentrations of P3 after 12 and 48 h of incubation.

of incubation were 70 and 8 μg/mL, respectively. Further fractionation of molecules present in peak P-3 was carried out using C-18 reverse phase HPLC (Figure 10a). The total LC time program was set up for 40 min, and 23 distinct peaks were collected. The peaks were collected, dried under vacuum, and dissolved in buffer. The RP-LC peaks were then used for nematicidal assay at 10 μg/mL concentration. Peaks RP-1, RP-

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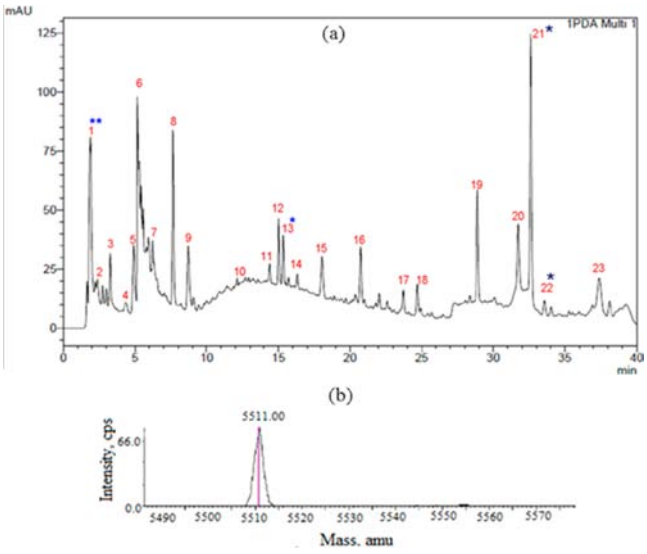

Figure 10. (a) RP-HPLC chromatogram of peak P3 (∗ indicates peaks showed nematicidal activity); (b) mass spectrum of peak RP-1 obtained from LC-ESI-MS system.

13, RP-21, and RP-22 exhibited considereable nematicidal activities after 12 h of incubation (RP-1, 100%; RP-13, 5%; RP-21, 32%; and RP-22, 26% of worms paralyzed). However, RP-1 showed more potent nematicidal activity after 48 h of incubation (all worms paralyzed) compared to other peaks. Hence, RP-1 was considered to be an important contributor of nematicidal activity displayed by honey.

Detailed spectroscopic analysis of peak RP-1 was carried out. The behavior of this peak during RP-LC and the UV spectrum obtained from the diode array detector pointed out that peak RP-1 contain polar, nonpeptidic molecule(s) with λ_{max} = 210. Mass spectrometery showed a molecule of mass 5511 amu as the major component of peak RP-1 (Figure 10b). The ^{1}H NMR and ^{13}C NMR spectra revealed the presence of polyhydroxy compounds. In proton and carbon NMR spectra the signals appeared in the ranges of δ 3.41−4.20 and 65.3−78.2, respectively (see the Supporting Information). Fluorescence spectroscopic analysis of this peak revealed fluorescence emission in the range of 340−480 nm with λ_{max} at 430 nm when excited at wavelengths 280, 270, and 254 nm. The sugars (glucose, fructose, maltose, and sucrose) gave no fluorescence emission in this range. These observations indicated the presence of nonglycosidic groups attached to polyhydroxy compound(s) in peak RP-1. Hence, it was concluded that the peak RP-1 contains glycoconjugate(s).

Conclusion. A series of experiments carried out during this study clearly demonstrated the nematicidal activity of natural honey using *C. elegans* as the model system. Microscopic analysis showed apoptosis/necrosis in the intestine and gonads caused by honey constituents. Furthermore, the nematicidal activity of honey was found to be due to defects in reproduction. The major sugar components of honey did not show nematicidal activity. Bioactivity-directed fractionation of honey and structural characterization identified a glycoconjugate responsible for nematicidal activity.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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