SUPPLEMENTARY INFORMATION

Inhibitory effect of selected Indian honeys in colon cancer cell growth by inducing apoptosis and targeting β-catenin/Wnt pathway

Neeladrisingha Das[1], Neelanjana Ray[1], Abhinandan R Patil[2], Shashank Sagar Saini[3], Bhairavnath Waghmode[3], Chandrachur Ghosh[1], Sunita B Patil[4], Sandeep B Patil[5], Chandrasekhar S Mote[6], Surendra Saini[1], B. L. Saraswat[7], Debabrata Sircar[3] & Partha Roy[1*]

1. Molecular Endocrinology Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Roorkee, Uttarakhand, India, PIN-247667
2. Centre for Interdisciplinary Research, D Y Patil University, Kolhapur, Maharashtra, India, PIN- 416006
3. Plant Molecular Biology Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Roorkee, Uttarakhand, India, PIN-247667
4. Department of Pathology, D Y Patil medical college, Kolhapur, Maharashtra India, PIN- 416006
5. Biocyte Insitute of Research and Development, Sangli, Maharashtra, India, PIN- 416416
6. Department of veterinary pathology, KNP college of veterinary Science, Sirwal, Maharashtra, India, PIN-412801
7. Department of Agriculture, Cooperation & Farmers Welfare (DAC&FW), Ministry of Agriculture and Farmers Welfare, Govt. of India, 150 A, Krishi Bhawan, New Delhi – 110001

Corresponding author: Tel. +91-1332-285686; Fax. +91-1332-273560; Email: partha.roy@bt.iitr.ac.in

1. Supplementary Material Method 1:

Biochemical analyses of honey

1.1. Sample preparation

For this, 5 ml of both mustard (MS) and multi-floral (MF) honey were lyophilized to obtain the powdered form of them. This lyophilized powder was either freshly used or kept at -20 ^{o}C before their biochemical analyses. Then 200 mg of lyophilized powder was dissolved in 1 ml of methanol and filtered through 0.45-micron filter and filtered methanolic extract was used for following biochemical analyses.

1.2. Estimation of total soluble sugar

Total soluble sugar content was estimated by using anthrone reagent. Briefly, 200 µL of the filtered methanolic extract was added into 800 µL of the anthrone solution (0.2% in concentrated H_2SO_4) and then heated for 10 min in a boiling water bath followed by cooling down to room temperature. The absorbance was then measured at 620 nm against reagent blank. The sugar content was estimated from standard curve plotted with glucose as the standard. Total soluble sugar content was expressed as gm glucose percentage (%) (gm glucose equivalent per 100 gm lyophilized honey).

1.3. Estimation of reducing sugar

Reducing sugar content was estimated using Somagiy's reagent and arsenomolybdate reagent. The reaction was started by adding 250 µL of the methanolic honey extract and 250 µL of Somagiy's reagent (4 mL of reagent A and 1 mL of reagent B mixed well just before the reaction), mixed well and then incubated at 100 °C for 10 min in a water bath. After cooling down the contents to room temperature, 1 mL of arsenomolybdate reagent was added to it. The blue green colour was developed which was measured by taking absorbance at 640 nm against reagent blank. The values of reducing sugar were calculated from a calibration curve prepared using standard glucose solution. Reducing sugar content was expressed as gm glucose percentage (%) (gm glucose equivalent per 100 gm lyophilized honey).

1.4. Estimation of non-reducing sugar

Non-reducing sugar content was estimated by subtracting the content of reducing sugar from the total soluble sugar and expressed as gram glucose percentage (%) (gram glucose equivalent per 100 g lyophilized honey).

1.5. Estimation of total phenolic and total flavonoid content

Total phenolic content (TPC) was estimated by using Folin-Ciocalteau reagent. Briefly, 0.1 mL of the methanolic extract was mixed with 0.5 mL of diluted Folin-Ciocalteau reagent (dilution 1:9 with water) and then after 5 min incubation at room temperature, 0.4 mL of sodium carbonate (5% w/v) was added to the mixture. The entire mixture was then kept in dark at room temperature. After 20 min of incubation, absorbance was measured at 765 nm against a reagent blank. TPC was expressed as mg gallic acid equivalents (GAE) per 100 gm lyophilized honey (mg GAE/100 gm). Total flavonoid content (TFC) was estimated by aluminum chloride method. To measure TFC, 0.5 mL of methanolic extract was added to 0.5 mL of 2% $AlCl_3$ solution (prepared in ethanol) and incubated at room temperature for 1 h. After that the absorbance was measured at 420 nm. TFC was expressed as mg quercetin (QE) equivalents per 100 gm lyophilized honey (mg QE/100 gm).

1.6. Estimation of antioxidant activity by oxygen radical antioxidant capacity (ORAC) assay

The ORAC assay was carried out by using the oxygen radical antioxidant capacity kit (ORAC kit, ab233473, Abcam, Cambridge, MA, USA). The analyses were performed at 37°C using a pH 7.4 phosphate buffer. The peroxide radicals were produced by 2,20 -Azobis(2-amidinopropane) dihydrochloride (AAPH), using fluorescein as substrate and Trolox as standard. Each well of the plate reader contained in a final volume of 200 µL assay solutions: fluorescein (16.7 nM), 1–50 mg/mL of honey and AAPH 2.2 mg/mL (final concentration). The fluorescence intensity was measured every 2 min for 1 h. A calibration curve of Trolox in the concentration range 10 to 100 µM was used in each plate read. All determinations were made in triplicates. ORAC values were then calculated as area under the curve (AUC) and expressed as µmol Trolox equivalent (TE)/gm. The blank wells in the experiments contained AAPH, fluorescein and phosphate buffer (pH 7.0).

1.7. Metabolite analyses of honey using HPLC-DAD

Methanolic extract of honey was directly analyzed by high performance liquid chromatography diode array detection (HPLC-DAD) system for detection of secondary metabolites. A Waters (Milford, MA, USA) HPLC system consisting of 1525 binary pump and 2998 photodiode array

detector (PDA) was used for separation. Chromatographic separation was achieved on Waters C18 reversed-phase Xbridge column (150 x 4.6 mm, 5 µm) with mobile phase consisting of 1% aqueous trifluoroacetic acid (solvent A) and acetonitrile (solvent B). The flow rate was 0.7 mL/min and injection volume were 20 µL. A gradient elution was performed by varying the proportion of solvent B to solvent A. The gradient elution was changed from 5% to 10% B for duration of 27 min, from 10 to 40% B in 75 min, 40% B from 75-80 min, 40 to 42% B in 82 min, then 100% B in 84 min and finally 100% B up to 92 min. Data acquisition and an analysis was performed by Empower 3 Software from Waters. All measurements were carried out at room temperature (~ 25 ºC). The peaks were identified by matching with retention time and UV-spectrum with authentic standards.

2. Supplementary Material and Method 2:

2.1. Sample preparation

One mg of lyophilized honey powder was dissolved in 1 mL MeOH and then 2-Phenylphenol (50 µL from 2 mg/mL 2-phenylphenol methanol stock) was added as internal standard to the sample and vortexed vigorously for 5 min followed by overnight incubation at 26 °C at 200 RPM in shaking incubator (Rivotek, India). The mixture obtained was further dried in vacuum concentrator (Eppendorf Concentrator plus™, Eppendorf, USA) at 37 °C until a dried powder was obtained. Finally, the dried material was subjected to double derivatization, first with 35 µL of methoxyamine hydrochloride (20 mg/mL dissolved in pyridine) for 120 min at 37 ºC and then second derivatization with 50 µL of MSTFA (N-Methyl-N-(trimethylsilyl)trifluoroacetamide) for 30 min at 37 ºC. Finally, after the double derivatization, the mixture was centrifuged at 13000 x g for 10 min, supernatant was then taken out in a GC glass vial and analysed by GC-MS. Derivatization reaction prepared with empty tube without sample served as the control.

2.2. GC-MS analyses

GC- MS analysis was performed on Agilent 7890 gas chromatograph coupled with an Agilent 5977C mass detector (Agilent Technologies, CA, USA). For this, 1 µL sample was injected using an automatic sampler (7683 B series, Agilent Technologies, CA, USA) in spitless mode. HP-5 MS column (5% phenyl methyl polysiloxane: 30 m x 0.25 mm i.d. x 0.25 µm, Agilent Technologies, CA, USA) was used for metabolite separation. The temperature program was as follows: initial

temperature of 70 °C for 5 min, followed by temperature increase to 270 °C at the ramp rate of 4°C /min and kept in hold for 1 min. Finally, the temperature was increased to 300 °C at the ramp rate of 10 °C /min and kept on hold at 300 °C for 15 min. Total run time calculated was 74 min. Helium was used as carrier gas at a flow rate of 1 mL/min. The inlet and MS transfer line temperatures were set to 250 °C and 310 °C respectively. The MS unit was tuned to its maximum sensitivity and the mass range for total ion current was m/z 40-600, and the detector voltage was set at 1700 eV. Each sample was replicated three times. The scan was started after solvent delay of 7 min with scan frequency of 3 S^{-1} (2.0 Hz) and with mass range of 30-1000 m/z.

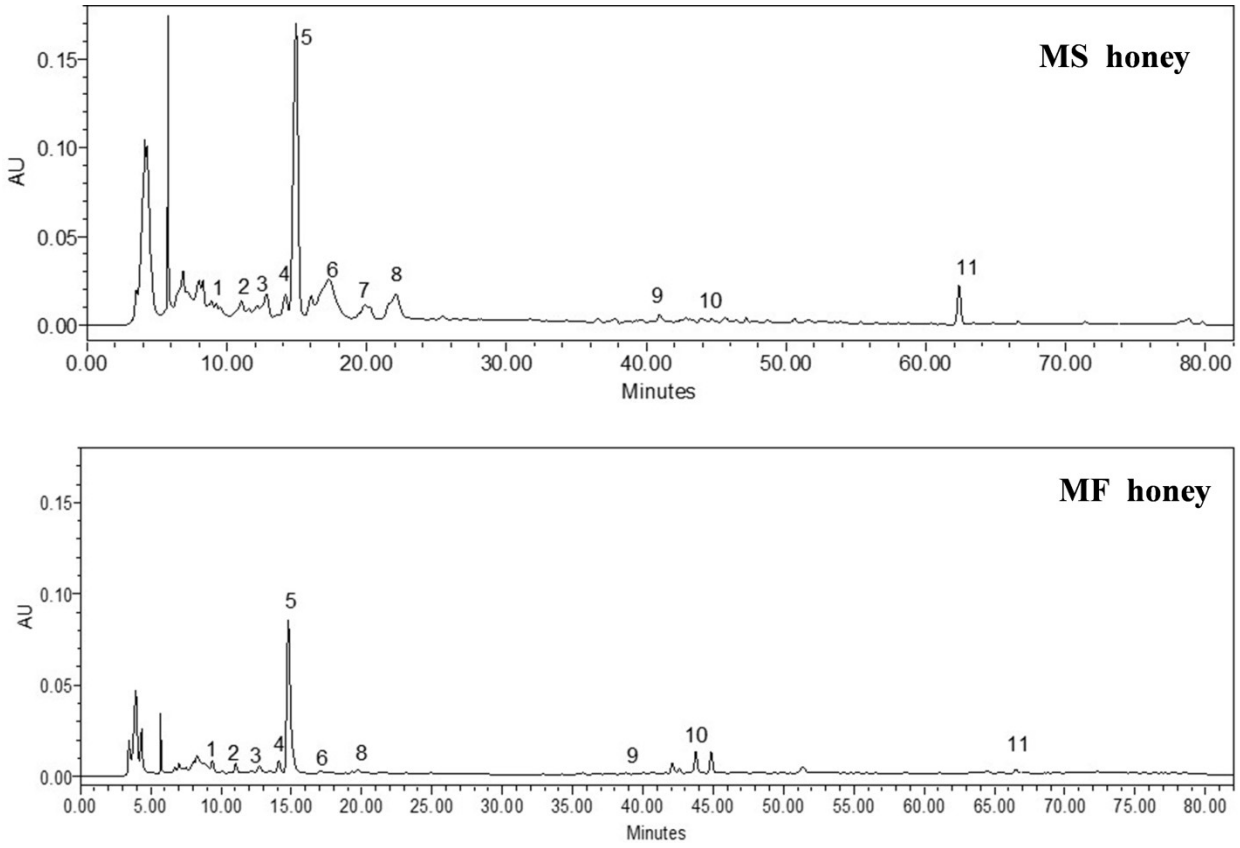
2.3. Metabolite identification

Data acquisition, automatic peak detection, mass spectrum deconvolution, and library search were done by Agilent ChemStation™ software and Wsearch pro (www.wsearch.com.au). The metabolites were identified by matching the mass spectra of target metabolite with the NIST-17 mass spectral library (National Institute of Standards and Technology, Gaithersburg, MD, USA), and our in-house mass spectral database that includes several secondary metabolites, amino acids, organic acids, and sugar standards. Metabolites having matching similarity of 80% or more in library search were only considered. All artifact peaks such as plasticizers, column bleed, derivatizing agent peaks were not considered in final calculation. To obtain accurate peak areas for each metabolite, unique quantification masses were considered and specified. Each mass spectrum was carefully analyzed for co-elution detection.

2.4. Multivariate analyses

Raw GC-MS data files were deconvoluted by using Automated Mass Spectral Deconvolution and Identification System (AMDIS) tools available with WsearchPro (www.wsearch.com.au). Resulting peak area data were converted into comma separated values (.csv) format and then uploaded in Metaboanalyst 3.0 (http://www.metaboanalyst.ca) for processing. The data were used with log transformation and pareto scaling. To detect the differences in metabolite profiling between MH and MF honey samples, multivariate method of Partial Least Squares Discriminant Analysis (PLS-DA) were conducted. The PLS-DA is a supervised method which was used to maximize the difference of metabolites between stages to detect those metabolites which were demonstrating key role in discriminating the groups. The output for PLSDA (PC1 Vs PC2) was presented as 2D scores plots. Variable importance in projection (VIP) scores were used to screen

PLS-DA data for important features. Heat maps were created using Metaboanalyst 3.0 by applying Euclidean distance measure, the Ward clustering algorithm, autoscale feature under standardization tool and using original data source.



Supplementary Figure S1. HPLC chromatogram showing separation of major polyphenols from honey samples. Key to peak identity: 1, gallic acid; 2, pinocembrin; 3, protocatechuic acid; 4, caffeic acid; 5,6, unidentified; 7, vanilic acid; 8, vanillin; 9, 4-coumaric acid; 10, syringic acid; 11, chrysin.

Supplementary Table T1. Sequences of primers used in this study

TRAF-2	Forward primer	5'-TCTTCTCCCCAGCCTTCTACAC-3'
	Reverse Primer	5'-GGCCCTTCATCACCACAAAG-3'
CAS-3	Forward primer	5'-GGTTCATCCAGTCGCTTTGTG-3'
	Reverse Primer	5'-GCGTCAAAGGAAAAGGACTCA-3'
CAS-8	Forward primer	5'-CTGCCTACAGGGTCATGCTCTA-3'
	Reverse Primer	5'-AGCAGGTTCATGTCATCATCCA-3'
CAS-9	Forward primer	5'-AGCACTGTCCCTTGCCTCAAT-3'
	Reverse Primer	5'-AGTGGTTGTCAGGCGAGGAA-3'
PARP-1	Forward primer	5'-TACCATCCAGGCTGCTTTGTC-3'
	Reverse Primer	5'-CCTCTGTAGCAAGGAGGCTGAA-3'
β actin	Forward primer	5'-GCATGGGTCAGAAGGATTCCTA-3'
	Reverse Primer	5'-TGTAGAAGGTGTGGTGCCAGAT-3'

Supplementary Table T2. Details of antibodies used in this study

Antibodies	Company	Product code	Dilutions
Bax	Santacruz Biotechnology, Inc	SC-20067	1:500
Bcl-XL	Santacruz Biotechnology, Inc	SC-8392	1:1000
p53	Thermo Fisher scientific	MA5-12453	1:1000
β-catenin	Santacruz Biotechnology, Inc	sc-7963	1:1000
p-β-catenin	Santacruz Biotechnology, Inc	sc-57535	1:500
GSK 3β	Santacruz Biotechnology, Inc	sc-81462	1:500
Axin-1	Thermo Fisher scientific	34-5900	1:1000
Cyclin D1	Thermo Fisher scientific	RM- 9104	1:500
c-Myc	Thermo Fisher scientific	MA1-980	1:500
Survivin	Santacruz Biotechnology, Inc	sc-17779	1:500
GAPDH	Santacruz Biotechnology, Inc	sc-47724	1:1000
β-actin	Santacruz Biotechnology, Inc	sc-47778	1:1000

Supplementary Table T3. Estimation of IC_{50} values of different honey varieties towards HCT15 cell lines after 24 h of incubation

Honey varieties	IC_{50} (in mg/mL)
Litchee	101.4 ± 0.77
Tulsi	105.7 ± 0.26
Mustard	57.55 ± 0.52*
Jujube	111.1 ± 0.25
Eucalyptus	97.47 ± 0.15
Multifloral	73.74 ± 0.29*

Data are the mean ± SEM of three independent experiments. *, indicates significantly different from all other varieties of honey at $p<0.05$.