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Original Research Article (Experimental)

Unifloral *ajwain* honey ameliorates differential inhibition of matrix metalloproteinases 2 and 9 protein, cytotoxicity, and antioxidant potential

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ABSTRACT

Background: Free radicals lead to inflammation, which in turn could intervene several chronic diseases including cancer. The promising scientific finding for anti-cancer properties of honey is an area of great interest.
Objective: The present study was designed to investigate the *in vitro* biological effects (cytotoxic, and anti-inflammatory through differential inhibition of metalloproteinases and antioxidant) of unifloral *Ajwain* honey along with its physicochemical properties (pH, moisture, ash content, electrical conductivity, color, protein).
Materials and methods: Three *Ajwain* honey samples (AJ-1, AJ-2, and AJ-3) were collected from different geographical origins of Western Ghats of India. Melissopalynological analysis was carried out to confirm uniflorality. Physicochemical analysis for ash, moisture content, pH, electrical conductivity, color, and total protein was estimated. Total polyphenol, total flavonoid content, and ferric reducing ability of plasma assay were determined using appropriate methods. The cytotoxic effect was assessed against breast cancer cells (MDA-MB-231) by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the anti-inflammatory activity was evaluated by gelatin zymography of matrix metalloproteinases MMP-2 and MMP-9 proteins.
Results: Melissopalynological analysis confirmed pollens from *Ajwain* plant's and so-called *Ajwain* honey. MTT assay depicted inhibitory trend for all honey samples across the concentrations (6.25–100 mg/ml) as compared to untreated cells. Gelatin zymography of metalloproteinases (MMP-2 and MMP-9) showed inhibitory tendency in all *Ajwain* honey samples. The AJ-3 honey sample had the highest inhibition at 0.625%. A significant correlation between honey color, pH, and protein content was perceived throughout the study.
Conclusion: This study highlights the *in vitro* biological evidence for possible therapeutic application of *Ajwain* honey samples in cytotoxic, anti-inflammatory, and antioxidant management as well as can be considered a potent source of supplements in human nutrition.

1. Introduction

Cancer is a multiphase process involving initiation, proliferation, invasion, and metastasis. Chronic inflammation has always been a

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critical constituent in metastasis of various cancers. The microenvironment surrounding the cancer cells contain several inflammatory cells, inflammatory agents, growth factors, DNA-damage-promoting agents that play crucial roles in the progression and metastasis of tumors [1]. One among these inflammatory agents is matrix metalloproteinases (MMPs), a family of endopeptidases targeting many proteins, including other proteases, protease inhibitors, chemotactic molecules, and cell–matrix adhesion molecules [2]. MMP-2 and MMP-9 have been significantly involved in degrading the collagen type IV, a significant component of extracellular matrix paving the way for tumor cell invasion [3]. Previous studies suggest that natural honey with its various bioactive components helps decrease the inflammation rate, including inhibition of MMP-2 and 9, also impede the cancer progression and metastasis, which makes it a candidate to be explored as a therapeutic agent also as a chemopreventive drug [4].

Honey is a natural food available from nature. There are different types of honey produced worldwide; however, each honey is unique in nature, based on its floral source, the geographical distribution, and composition of sugar content, amino acids, enzymes, proteins, and phytochemicals [5]. The phytochemical composition with higher polyphenols and flavonoid content display it as effective antioxidant agent against oxidative stress [6]. Studies have revealed that honey contains a valuable amount of phytochemical constituents that exhibit many bioactive properties, including antioxidant, anti-inflammatory, and anti-cancer properties [7,8].

Three unifloral *Ajwain (Trachyspermum ammi)* honey samples were collected from Western Ghats - Maharashtra, India and were studied for their possible *in vitro* biological effects. The pollens from *Ajwain* plant comprise more than 45% of this species resulting in it being named as *Ajwain* honey. The *Ajwain* plant is a recognized medicinal plant and the plant parts are previously reported for their several biological properties such as cytotoxic, insecticidal, anti-fungal, antioxidant, anti-microbial, anti-inflammatory, antitussive and also serve as a bronchodilator [9,10]. Hence, the nectar collected by bees from this plant to produce honey gives us the perspective to explore possible biological properties of *Ajwain* honey samples collected from different regions of Western Ghats-Maharashtra, India.

2. Material and methods

2.1. Reagents and instruments

Triton X 100, amphotericin (5 mg/mL), and gentamycin (4 mg/mL) were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Fetal bovine serum (FBS), Pen Strep (a mixture of penicillin and streptomycin), and Dulbecco's modified Eagle medium (DMEM) were procured from Gibco Life Technologies (Bangalore, India); MTT (methylthiazolyl diphenyl-tetrazolium bromide), DMSO (dimethyl sulfoxide), Gallic acid, Quercetin, $FeSO_4 \cdot 7H_2O$, F–C reagent (Folin Ciocalteu), 2,4,6-Tris (2-pyridyl)-1,3,5-triazine (TPTZ) were purchased from HiMedia Laboratories Pvt. Ltd. The cancer cell line MDA-MB-231 and HT1080 was obtained from National Centre for Cell Science (NCCS), Pune, Maharashtra, India. All other chemicals used were of analytical grade. UV–Visible spectrophotometer (SHIMADZU spectrophotometer UV-1800) was used for detection of absorbance. The anti-inflammatory activity was assessed using electrophoresis assembly (BioBee Tech, Bangalore) and gel documentation (Syngene, G Box, United Kingdom).

2.2. Honey samples: collection and preparation

Honey samples were collected during the month of December 2016 from honey-hunters and apiaries of the Tapola: Satara-Maharashtra area (AJ-1, AJ-2) and Kolad: Raigad-Maharashtra area (AJ-3) of Western Ghats-Maharashtra, India. These collected honey samples underwent a filtration process to remove dirt, wax, and other impurities. Later, the honey samples were kept in storage at room temperature until analysis.

2.3. Melissopalynological analysis

Melissopalynological analysis is the technique of analyzing the botanical origin and geographical distribution of honey and was carried out according to a method of J. Louveaux [11].

2.4. Physicochemical analysis

pH, ash, and moisture content was performed for all the collected honey samples according to Association of Official Analytical Chemists Guidelines [12]. Electrical conductivity (EC) was derived from the equation which is based on a linear relationship between EC and ash content [13,14].

$$EC = 0.14 + 1.74*A$$

where 'A' is the ash content in terms of g/100 g honey and 'EC' is electrical conductivity in mS/cm. Briefly, 2–4 g of honey sample was placed in previously ignited crucible to prevent the honey foaming. Later, the honey sample was incinerated at 500° to 600° in burning muffle for 5 h. After cooling in a desiccator and weighing the crucible, ash value was obtained and applied to the above equation for the calculation of electrical conductivity in mS/cm.

The modified Lowry's method [15] was used to determine honey's total protein content. Bovine serum albumin (0–100 μg/ml) was used as a standard for preparing the calibration curve. The honey color intensity was detected as previously suggested by Beretta et al. [16]. In brief, all honey samples were diluted to 50% (w/v) with warm Milli-Q water (45–50 °C), and the solution was filtered using a 0.45 μm filter to remove large particles. The absorbance was measured at 450 and 720 nm using a spectrophotometer (SHIMADZU spectrophotometer UV-1800), and the difference in absorbance was observed.

2.5. Phytochemical and antioxidant determination

2.5.1. Total polyphenol content

Total polyphenol content was determined by the F–C reagent method in 96 well plates; each well had 150 μL solution containing standard or 10% honey solution, freshly prepared F–C reagent, and 7.5% of sodium carbonate. The plate was incubated in the dark for 30 min, and absorbance was read at 630 nm [17]. Gallic acid (5–160 μg/ml) was taken as standard and results were expressed as mg gallic acid equivalents (GAE) per 100 g of honey.

2.5.2. Total flavonoid content

Total flavonoid content was estimated using the aluminum chloride method [18]. Quercetin (12.5–800 μg/ml) was used as standard, and the results were expressed as mg of Quercetin equivalents (QE) per 100 g of honey.

2.5.3. Ferric reducing antioxidant power (FRAP) assay

The assay was performed with FRAP reagent based on minor modifications [19]. Briefly, the reaction solution contained 200 μL of 10% honey sample and 1800 μL of FRAP reagent. The solution was kept for incubation in dark for 10 min at room temperature, and the absorbance was measured at 593 nm. $FeSO_4.7H_2O$ (100–1000 μM) was used as an assay standard for calibration purpose, and results

were expressed as micromoles of ferrous equivalents (μM Fe (II)) per gram honey solution.

2.6. Anti-cancer activity: MTT cytotoxic assay

The human breast cancer cell line MDA-MB-231 was obtained from NCCS, Pune, India. The honey sample's *in vitro* growth inhibition effect was assessed through spectrophotometric determination of MTT conversion into "Formazan blue" by living cells at a wavelength of 492 nm. Percent cell viability was calculated using the standard formula. Approximately 5×10^3 cells/well were seeded in a 96-well flat-bottom microplate and maintained at 37 °C in 95% humidity and 5% CO_2 overnight. The cells were treated with different concentrations of honey samples dissolved in serum-free DMEM for 24 h. The cells in the well were washed twice with phosphate buffer solution, and 20 μL of the MTT staining solution [5 mg/ml in PBS] was added to each well. After 4 h of incubation at 37 °C, 100 μL of DMSO was added to each well to dissolve the formazan crystals, and the absorbance was recorded at 570 nm using microplate reader [20].

$$\text{Surviving cells } [\%] = \text{Mean OD of test compound} / \text{Mean OD of negative control} \times 100$$

2.7. Gelatin zymography assay

The zymography assay uses gelatin as a substrate for MMP-2 and MMP-9. Fibrosarcoma HT-1080 cells were treated with *Ajwain* honey samples at concentration (0.625%, and 0.3125%) for 24 h. Later the culture media was extracted and loaded for electrophoresis with gelatin substrate gels prepared by incorporating gelatin (2 mg/ml) into 10% polyacrylamide gel. The electrophoresis was done at 120 V for 150 min. The gels were washed in 2.5% Triton X-100 (v/v) for 30 min and then incubated overnight at 37 °C in developing buffer containing 50 mM Tris–HCl, pH 7.6, 200 mM NaCl, 5 mM $CaCl_2$ and 0.2% (v/v) Brij-35. Staining of gels was done using Coomassie Brilliant Blue (CBB) and destained till the clear digestion bands appeared against the dark blue background. Digestion bands were quantified using ImageJ software [21].

2.8. Statistical analysis

All sample analysis was performed in triplicates and data obtained were expressed as the mean ± standard deviations. Correlations were evaluated using Pearson's correlation coefficient (r) ($p < 0.01$). These correlations were analyzed using GraphPad Prism version 7 and SPSS software with statistical significance set at $p < 0.05$.

3. Results

3.1. Melissopalynological and organoleptic characters

Table 1 depicts the percentage of pollen, geographical origin and organoleptic characters such as color, odor, and taste of the three kinds of *Ajwain* honey (AJ-1, AJ-2 and AJ-3). All the three honey samples: [AJ-1 (78.10%), AJ-2 (90.46%), and AJ-3 (99.86%)] had greater number of pollen grains from the flower of the *Ajwain* plant (*T. ammi*); hence, named as *Ajwain* honey.

3.2. Physicochemical characteristics

The results of physicochemical characteristics such as ash, moisture content, pH, and electrical conductivity, color intensity, and protein content are summarized in Table 2. The results of AJ-3 determine maximum physicochemical characteristics than compared with AJ -2 and AJ-3.

3.3. Phytochemical and antioxidant potential

The results of phytochemicals such as total polyphenol and total flavonoid and antioxidant potential by FRAP assay are depicted in Table 3. AJ-3 honey sample expressed highest polyphenol, flavonoid content and FRAP values.

3.4. Pearson's correlation

Table 4 shows the Pearson r Correlation amongst Total polyphenol content (TPC), Total flavonoid content (TFC), and FRAP values along with pH, and honey color. A positive correlation was observed between polyphenols, flavonoid content ($r = 0.7$) and FRAP and polyphenols content ($r = 0.7$). A highly significant positive correlation observed between FRAP and flavonoid content ($r = 0.9$) indicates high antioxidant activity by phytochemicals in the sample. A significant correlation existed between honey color, pH, and protein content in the present study.

3.5. In vitro cytotoxic effect of Ajwain honeys against MDA-MB-231 cell lines

Fig. 1 (A–C) shows the normalized effect of *Ajwain* honeys against MDA-MB-231 cell lines at 24 h incubation. AJ-1, AJ-2 and AJ-3 sample showed inhibitory effect in dose-dependent manner with IC_{50} concentration at 1.14 mg/ml, 0.75 mg/ml and 0.29 mg/ml respectively.

Table 1
The percentage of pollen, geographical origin and organoleptic characteristics of honey.

T.ammi pollen (%)	Sample identification	Geographical origin	Organoleptic characteristics
78.10%	AJ-1	Tapola (Maharashtra)	Yellowish brown, pleasant, sweet, thick syrupy
90.46%	AJ-2	Tapola (Maharashtra)	Yellowish brown, pleasant, sweet, thick syrupy
99.86%	AJ-3	Kolad (Maharashtra)	Brown, pleasant, sweet, thick syrupy

Table 2
Physicochemical characteristics of *Ajwain* honey.

Samples	pH	Moisture (%)	Ash (%)	EC (mS/cm)	Color Intensity 450 nm	Protein mg/gm of honey
AJ-1	3.28	16.32%	0.07%	0.26	0.316	194.2
AJ-2	3.43	18.53%	0.08%	0.28	0.545	388.1
AJ-3	3.47	19.74%	0.25%	0.58	0.548	388.1

Table 3
Phytochemical and antioxidant potential: Total polyphenol content, Total flavonoid content, and FRAP values.

Sample	Total polyphenol content (mg gallic acid/100 g)	Total flavonoid content (mg of QE/100 g)	FRAP μM Fe(II)/100 g
AJ-1	10.27 ± 0.05	38.52 ± 12.82	760 ± 0.05
AJ-2	12.81 ± 0.03	27.95 ± 4.45	810 ± 0.06
AJ-3	17.15 ± 0.04	208.85 ± 10.98	1050 ± 0.03

Data are expressed as the mean ± SD of three individual determinations. FRAP: ferric reducing ability of plasma.

Table 4
Pearson's correlation amongst TPC, TFC, FRAP values, pH, Honey color.

	TPC	TFC	FRAP	Color	pH	Protein
TPC	1					
TFC	0.777[b]					
FRAP	0.784[b]	0.951[a]				
Colour	0.696[b]	0.509	0.687[b]			
pH	0.764[b]	0.610	0.775[b]	0.981[a]		
Protein	0.740[b]	0.450	0.595	0.973[a]	0.947[a]	1

[a] Correlation is significant at the 0.01 level.
[b] correlation is significant at the 0.05 level.

3.6. Effect of Ajwain honeys on MMP 2 and 9 protein

Fig. 2 (A and B) depicts the effect of all the three *Ajwain* honey samples on fibrosarcoma HT-1080 MMP-2 and -9 secretions by gelatin zymography. The zymography results reveal that at a lower concentration of 0.3125%, AJ-3 and AJ-2 honey samples had approximately 30% and 20% inhibition of MMP-2 and MMP-9, respectively (Fig. 2A). At a concentration of 0.625%, though, all *Ajwain* honey samples displayed significant inhibitory potential, and AJ-3 honey showed the highest inhibition (nearly 50%), making it the most potent inhibitor (Fig. 2B).

4. Discussion

Honey is a natural food with the various phytochemicals and bioactive compounds exhibiting different biological properties [22–24]. Each honey is unique in nature with the difference in geographical and floral origin. The present study aimed to explore *Ajwain* honey for its *in vitro* biological effects (cytotoxic, anti-inflammatory by differential inhibition of metalloproteinases and antioxidant) along with its physicochemical properties (pH, moisture, ash, electrical conductivity, color, protein). The melissopalynological analysis determines the uniflorality. As per recommendations of the International Commission for Bee – Botany, honey samples, which had pollen count of similar plant species with more than 45% was considered as unifloral honey [11]. Among the three *Ajwain* honey samples, AJ-3 has been found to have the maximum percentage of pollen with a darker color. The physicochemical parameters like ash, moisture content, pH, and electrical conductivity, color intensity, and total protein were assessed. The ash content of the *Ajwain* honey samples is according to the reference range of < 0.6%, and also moisture content was within limits recommended by Codex Alimentarius Commission [25]. Moisture content is a critical quality parameter and determines the shelf-life of honey. The pH was found to be in the acidic range and AJ-3 showed the highest value which is comparable with other kinds of honey from different geographical origins [26]. Electrical conductivity was observed maximum in AJ-3 honey sample which is consistent with other published studies [5]. The electrical conductivity is one of the parameter assessed in routine honey quality control. The electric conductivity of honey is useful for discriminating honeys of different floral origins as it is influenced by mineral content, organic acid, proteins, and storage time [27]. Floral origin honeys have electrical conductivity less than 0.8 mS/cm in comparison to the adulterated or artificial honey hence, affirming floral origin of *Ajwain* honeys [28]. Honey contains proteins in the form of enzymes and royal jelly. The protein content of *Ajwain* honey was within the limits established by the official standard methods [29]. The main enzymes present in honey are diastase (amylase) and invertase (saccharase or α-glucosidase) in addition to glucose oxidase and catalase added by the honey bee, which regulate the production of hydrogen peroxide; which serves as predominant anti-bacterial factor in honey [30]. The phytochemicals estimated are total polyphenols and total flavonoid

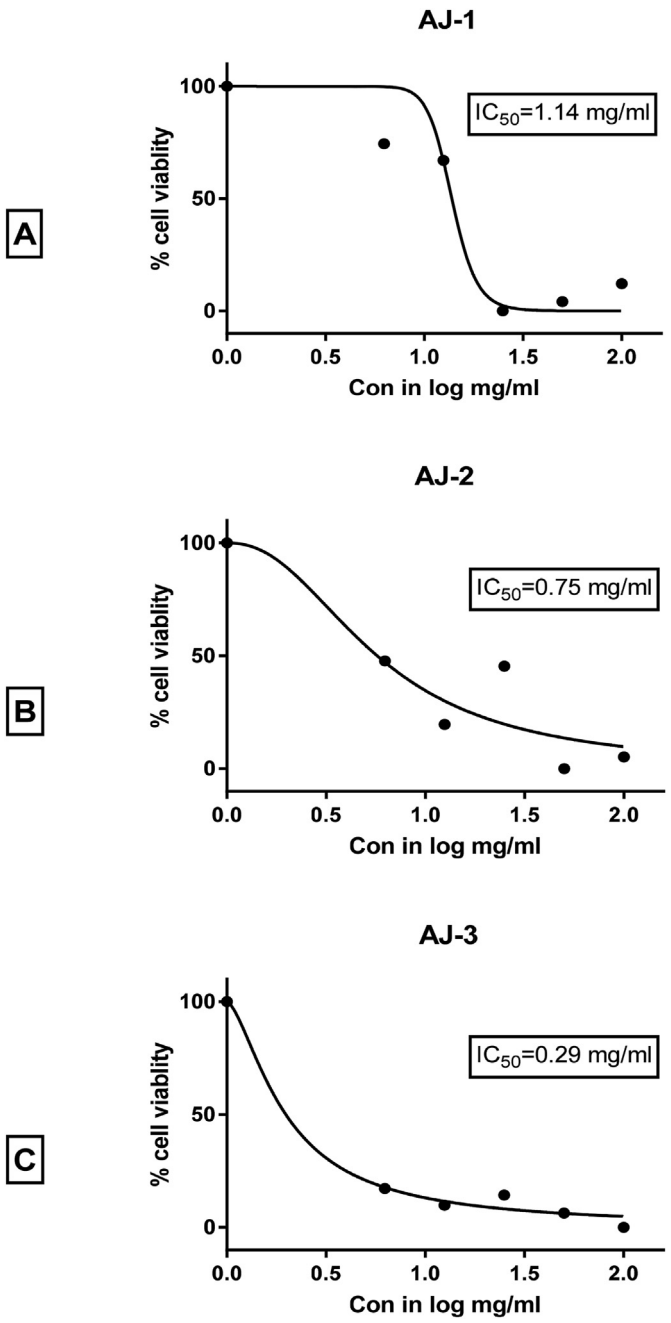

Fig. 1. Normalized results of *in vitro* cytotoxic activity of (A) AJ-1, (B) AJ-2 and (C) AJ-3 against MDA-MB-231 cell lines derived from breast cancer cells. [*In vitro* cytotoxic activity was evaluated by % cell viability considering viability of control as 100%].

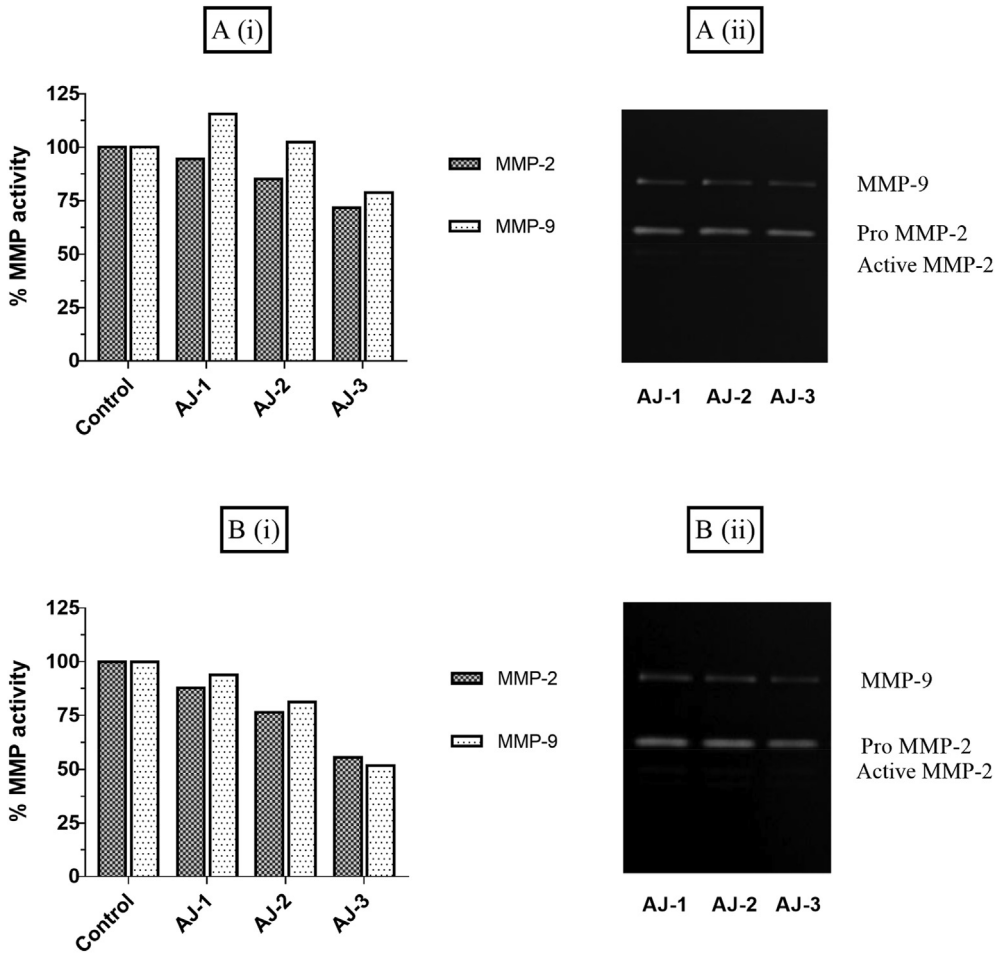

Fig. 2. Effect of *Ajwain* honey (AJ-1, AJ-2 and AJ-3) on fibrosarcoma HT-1080 MMP-2 and -9 secretions. A (i) Densitometry analysis and A (ii) gelatinase zymogram at 0.3125%. B (i) Densitometry analysis and B (ii) gelatinase zymogram at 0.625%. Control: without sample/FBS.

content in all the three *Ajwain* honey samples. AJ-3 showed the highest contribution in polyphenols and flavonoids when compared with other two *Ajwain* honey samples. The antioxidant potential was analyzed by FRAP assay which clearly demonstrated that the AJ-3 honey sample showed the highest content of FRAP value compared with the other honey samples. The darker color of AJ-3 honey indicates the predominance of flavonoid and polyphenol. The color of honey is attributed due to the presence of Millard reaction products (MRPs) and polyphenols. Presence of MRPs corresponds to the strong absorbance seen at 560–720 nm and presence of polyphenols at 420–450 nm (Supplementary Fig. 1). The color intensity is the net absorbance obtained as difference between the readings at 450 nm and 720 nm [16]. The color signifies the antioxidant capacity; darker the color higher the antioxidant capacity [31]. According to previous literature, flavonoids and phenolic compounds have been identified in various honeys which include kaempferol, quercetin, chrysin, pinobanksin, vanillic acid, and caffeic acid [32]. These act as predominant bioactive compounds exhibiting various activities including anticancer, anti-inflammatory, and antioxidant agents [33,34]. This bioactive capacity of honey with various modes of action contributes to the possible prevention of several acute and chronic diseases. AJ-3 honey sample had the highest antioxidant capacity which was comparable with other honey such as Malaysian Tualang [35], Indian Jamun [22], and Artisanal honey [36]. A significant correlation between flavonoids, polyphenols, and FRAP values further affirms *Ajwain* honey to be considered as potential antioxidant supplements in human nutrition.

Honey has been extensively studied for their wound-healing and anti-microbial properties [37,38], but recently their role in prevention or as a therapeutic agent in various cancers are being explored [29–31]. In the current study, we evaluated *Ajwain* honey as a potent anti-cancer therapeutic by assessing its inhibitory activity on inflammatory MMPs along with the cytotoxic potential exhibited against MDA-MB-231 cell line derived from breast cancer cells. Honey around the world has demonstrated promising inhibitory effect on various cancer cell lines. Previous studies have elaborated the possible mechanism of cytotoxicity exhibited against various cancer cells which include inhibition of cell proliferation, induction of apoptosis pathway, cell cycle arrest, as well as inhibition of lipoprotein oxidation and disruption of mitochondrial membrane potential. Studies have also expressed the ability of honey contents to target specific signaling pathways or target the suppression of particular type of cancer [39–41].

Honey samples from India have been previously studied for present chemical constituents and anti-cancer potential along with the possible mechanism of action. Jaganathan et al. [42] studied caffeic acid, one of the phenolic constituents of honey-induced anti-proliferative effect on the HCT-15 colon cancer cells

using MTT assay. This finding was further confirmed by cell cycle analysis using flowcytometry. The study concluded that caffeic-acid-treated cells confirmed apoptosis with increasing reactive oxygen species generation and reduction in the mitochondrial membrane potential in dose- and time-dependent manner. In another study by Amruta et al. [43], the anti-cancer ability and phytochemical characterization of few Indian honey samples such as *Jambhul*, rubber, litchi and drumstick was documented. In the study, HeLa cell lines were treated with these honey samples to explore the effect on the expression of proteins Cyclin B1 and p53 which play a major role in the event of cell cycle and apoptosis. In conclusion, these honey samples exhibited encouraging anti-cancer potential along with the ability of altering the expression of Cyclin B1 and p53 proteins. Antiproliferative effect is attributed to the total polyphenols and flavonoid content of honey. Hence, honey samples of different geographical areas are being explored against varying cancer cell lines whose promising results warrants present study [4,44].

MMPs are elevated in many pathological and physiological conditions including chronic inflammation and cancer metastasis [2,45]. Among the MMPs, gelatinases i.e., MMP-2 and MMP-9 perform a predominant role in degrading the major constituents of extracellular matrix, thereby stimulating cancer progression. Hence, inhibition of gelatinases provides an effective strategy in preventing spread of the disease serving as an effective therapeutic strategy [46]. Present study employed gelatin zymography to assess inhibition of MMP-2 and 9 activity against fibrosarcoma cells (HT1080) by *Ajwain* honey. AJ-3 seems the most potent inhibitor of both MMP-2 and MMP-9 activity. Therefore, the present study indicated that *Ajwain* honey possesses anti-MMP activity and might be a potential natural source of MMP inhibitor. Role of MMPs have been closely associated as modulators of inflammation in many chronic diseases [47]. Studies on inflammatory diseases such as rheumatoid arthritis have emphasized the role of MMP-2 or MMP-9 in synovial fibroblast survival, proliferation, migration and invasion overall leading to degradation of cartilage and may directly contribute to joint destruction in rheumatoid arthritis disease progression [48]. Potent modulators of MMP-2 and 9 may pave a way for treatment or control of inflammatory diseases. Our present study demonstrates possible modulation of MMP-2 and MMP-9 activity; hence, *Ajwain* honey may be proposed as an oral nutritive supplement or adjuvant to patients of chronic diseases.

Amongst *Ajwain* honey, the AJ-3 sample had a higher concentration of flavonoids and corresponded to higher MMP inhibition, which indicates that phytochemical concentration, geographical region, and floral source are determining factors of the bioactivity. Assessment of the true potential of the honey sample needs further exploration into various mechanisms of inhibitions, including expression studies.

5. Conclusion

Ajwain honey, enclosing a perfect mix of synergistically active ingredients, offers a promising therapeutic intervention that can be used to prevent and manage human diseases. The result suggests that unifloral *Ajwain* honey collected from the Western Ghats, displayed the presence of polyphenols and flavonoids. This may aid in preventing cancer progression by inhibition of pro-inflammatory markers like MMP-2 and MMP-9. Further, *Ajwain* honey with such biological properties might be associated with an excellent nutritional value. However, a study with more number of samples and other biological aspects of this indigenous honey has to be explored.

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Conflict of interest

None.

Author Contributions

Shruti Kulkarni: Methodology, Writing - Original draft preparation. Sanjay Mishra: Conceptualization, Supervision, Investigation, Data analysis, **Sadanand Patil:** Supervision, Editing. **Jyotsana Nambiar and Avinash Math**: Observation, Writing and Reviewing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaim.2021.05.012.

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