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Evaluation of Antibacterial Activity of some Indian and Yemeni Honey against Few Bacterial Isolates from Human Patients

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THE PRESENT study is aimed to evaluate the antibacterial effect of four types of Indian and Yemeni honey at 80% and 50% w/v concentrations against different pathogens including Methicillin-Resistant *Staphylococcus aureus* (MRSA), Methicillin-Sensitive *Staphylococcus aureus* (MSSA), *Escherichia coli,* and *Pseudomonas aeruginosa.* Agar well diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were used in this evaluation. All the examined honey samples recorded antibacterial activity in a dose-dependent manner. Yemeni honey were more efficient than Indian honey in producing the inhibitory growth impact. The average MICs values of Sider Daowaney and Somer honey from Yemen, Punjabi and Kashmiri honey from India against all tested bacteria were 15%, 20%, 30% and 30% (v/v), respectively. Tested honey samples had significant results ($P< 0.05$) against different tested pathogens, the difference in antibacterial activity attributes to Physico-chemical properties, total phenols contents, geographic area as well as botanical resource. The findings reveal that Indian and Yemeni honey may potentially be used as antibacterial agents, where there is a promising future to use these types of honey in many medical applications against multidrug-resistant and foodborne bacteria.

Keywords: Antibacterial activity, Honey, India, Pathogenic bacteria, Yemen.

Introduction

Honey is the natural sweet substance produced by honeybees from the nectar of blossoms or from the secretion of living parts of plants or excretions of plant sucking insects on the living parts of plants, which honeybees collect, transform and combine with specific substances of their own, store and leave in the honey comb to ripen and mature. This is the general definition of honey in the Codex Alimentarius (1989). The utilization of honey as a medication for the treatment of illness goes back to 2100-2000 BC. For example, pale honey was portrayed by Aristotle (384-322 BC) as being ‘‘good for sore eyes and wounds” (Vallianou et al., 2014). The antimicrobial properties of honey have been very much archived, and honey has been utilized from antiquated occasions as a technique for quickening wound recuperating. Its capability to help wound mending has been shown over and over (Molan, 1999; Vallianou et al., 2014). A potential purpose for its action depends on its capacity to produce hydrogen peroxide by the bee-inferred enzyme glucose oxidase (Jing et al., 2014). Another plausibility is the structure of honey, which has in excess of 181 constituents (Mandal & Mandal, 2011; Vallianou et al., 2014). Hydrogen peroxide and phenolic components are the significant supporters of the antimicrobial action of honey, and the difference in quantities of these components in various honey bring about their ability as antimicrobial agents (Almasaudi et al., 2017). As of late, numerous investigations have detailed the antibacterial activity of honey from various origins against *Shigella dysenteries*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella*

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pneumoniae, *Proteus mirabilis* (Beno-Costa et al., 2016; Wasihun & Kasa, 2016; Deng et al, 2018; Ghramh et al, 2019). Over and aimless utilization of antibiotics agents has prompted the rise of multidrug-resistant bacterial strains, a worldwide general medical issue (Kacaniova et al. 2011; Wasihun & Kasa, 2016). To manage this matter, alternate antimicrobial procedures like plants and plant-based items, for example, honey have presently got more consideration (Wasihun & Kasa, 2016).

Because of the absence of research and documentation the medical properties of Yemeni honey still remain insufficient. The purpose of the present study was to investigate the Physico-chemical properties and differentiate the *in vitro* inhibitory effect of four types of Indian and Yemeni honey against some pathogenic microorganisms isolated from human patients.

Materials and Methods

Honey Samples

Four types of Indian and Yemeni honey were used in antibacterial susceptibility testing including Sidr Doawany, Somer Shabwah, Punjabi, and Kashmiri honey were taken from beekeepers of different areas of India and Yemen (Table 1).

Preparation of honey concentration

Two different concentrations (80% and 50%) of each honey sample were prepared using sterile distilled water to test antibacterial activity. Water and honey quantities required for different concentrations were calculated using the formula: C1 × V1= C2 × V2.

Bacterial strains

Cultures of various human pathogenic strains were obtained from the Microbiology Department, Yenepoya University, Mangalore, India. Species included Methicillin-Resistant *Staphylococcus aureus* (MRSA), Methicillin-Sensitive *Staphylococcus aureus* (MSSA), *E. coli* and *P. aeruginosa*. These strains were isolated from human specimens. The isolated bacteria were sub-cultured on Nutrient agar and incubated aerobically at 37°C for 24hrs. Organisms were maintained in the laboratory on nutrient agar slants at 4°C**.**

Physico-chemical properties of honey Samples

Moisture

Water content was determined using an Atago HHR-2N refractometer. The samples were prepared according to the International Honey Commission guidelines (Bogdanov, 2009). Representative samples of each honey were transferred to sterile universal containers, sealed and incubated in a shaking waterbath at 50ºC for 30min. After incubation the samples were allowed to cool to 20ºC in an airconditioned laboratory. Before testing the sample was thoroughly mixed. A drop of honey was placed on the lens of the refractometer, and the lid closed carefully to ensure an even spread of the sample with no air bubbles on the lens. Then the refractometer was held towards the light and the position of the interface was recorded. Between each sample, the refractometer was cleaned and dried.

Determination of pH

The pH of the honey samples was determined following the method described by the International Honey Commission (Bogdanov, 2009). Ten Grams of honey that had been equilibrated to 20ºC for 24hrs in air-conditioned laboratory was dissolved in 10ml ultrapure water, making a 50% (w/v) solution and mixing using a magnetic stirrer. The pH final solution was measured by using a Chem lab instrument pH 1000meter previously calibrated using buffer of known pH (Fisher).

TABLE 1. Types of Indian and Yemeni honey used in the study.

Honey identity	Local name	Honey sample code	Origin of honey	Floral source
Sidr Doawany	Bagaih	SDH	Doawan-Hadramout, Yemen	*Ziziphus spina Christi*
Somer Shabwah	Somer	SSH	Shabowah, Yemen	*Acacia tortilis*
Punjabi	Punjabi	P	Punjab, India	*Litchi chinensis* (Litchi)
Kashmiri	Kashmiri	K	Kashmir, India	Multi wild types of Flowers

Sugar content

Sugar content was determined using a Billingham & Stanley 40-85% sugar refractometer (Bogdanov, 2009). The undiluted honey samples were allowed to equilibrate to 20ºC for 24hrs then thoroughly mixed before testing. A drop of honey was placed on the lens of the refractometer, and the lid closed carefully to ensure an even spread to the sample with on air bubbles on the lens. Then the refractometer held towards the light and position of the interface was recorded, which gives% readout, equivalent to the % sugars present in the sample. Between each sample, the refractometer was cleaned and dried.

Total phenolic content (TPC)

The total phenolic content (TPC) of honey samples was analysed by using Folin-Ciocalteu reagent, based on the method described by Singleton et al. (1999) with some modification. Honey solution (0.5ml) was mixed with 2.5ml of Folin-Ciocalteau reagent (2N) and incubated for 5min. Subsequently, 2ml of sodium carbonate solution (75g/L) was added into the honey solution and incubated for another 2hrs at room temperature. After incubation, the absorbance of the solution was measured at 760nm by using a UV-Visible spectrophotometer (LMS-UV1900, Labman scientific instruments, India). Gallic acid (0–1000mg/L) was used as a standard chemical for calibration curve preparation, and the results were expressed as mg of gallic acid equivalents (GAEs) per kg of honey.

Antibacterial activity assay

Antibacterial activity of various concentrations of honey samples was determined by agar well diffusion assay. Bacterial isolates were inoculated in 10 mL nutrient broth and placed overnight in shaking incubator at 37°C. Mueller Hinton Agar (MHA) plates were prepared according to the manufacturer's instructions. Using a sterile 6mm cork borer, wells were cut in the agar. Before making wells, each bacterial suspension (~10^8 colony-forming unit (cfu)/ml) was spread on a single agar plate with a sterile cotton swab. One hundred microliters of each honey sample were deposited into a separate well on the MHA agar plate. These Petri plates were incubated aerobically at 37°C for 24hrs in an incubator. The diameter of the zone of inhibition around the outer surface of the well was measured (Barry & Thornsberry, 1985).

Determination of minimal inhibitory concentration (MIC) minimum bactericidal concentration (MBC)

The MIC is defined as the lowest concentration of honey that can inhibit the growth of bacteria. The MIC of honey samples was determined by following the method of (Wasihun & Kasa, 2016) with some modifications. Eight clean test tubes (18- 27mm) were placed in a stand. Muller Hinton Broth was prepared according to manufacturer instructions and employed for the preparation of serial dilution test tubes. Two mL of pure honey (100%) was added to a test tube which served as a positive control. While another test tube received only 2ml of nutrient broth but no bacterial suspension (negative control). For the remaining six test tubes, serial dilutions of the honey sample were made that contained 2ml final volume of Muller Hinton Broth to give the concentrations of 80%, 60%, 40%, 20%, 10%, and 5% (v/v). Each tube except negative control was inoculated with 20ml of bacterial suspension (~108cfu/ml) and then incubated at 37°C for 24hrs. The whole process was repeated for each honey sample in triplicate against all the bacteria. The MIC was observed by visual inspections for the presence and absence of growth (turbidity). From the tubes showing no visible sign of growth or turbidity in MIC determination, test microorganisms were inoculated onto sterile nutrient agar plates by streak plate method. The plates were then incubated at 37°C for 24hrs. The least concentration that did not show growth of test organisms was considered as the MBC.

Statistical analysis

All results were expressed as the means of three replicates ± standard deviation (SD). The analysis was made using the IBM SPSS Statistics 23 software. The significant differences represented by letters were obtained by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) *post hoc* test ($P< 0.05$). The dendrogram was constructed using Ward's Linkage method with Euclidean distances.

Results and Discussion

Physico-chemical properties of honey samples

Moisture

The average of moisture contents of the tested honey samples were 13.7± 0.03% /100g for SDH honey, 16.2± 0.03% /100g for SSH honey, 24.2± 0.03% /100g for P honey and 23.2±

0.00% /100g for K honey, respectively and the differences were significant (P< 0.05). The moisture content of Yemeni honey was within the range of 13.5–19.5% recommended by Codex Alimentarius (<20%) as shown in Table 2, while Indian honey samples, which were not accepted by codex range (Codex Alimentations, 2001). Nevertheless, there are no significant differences (P> 0.05) between Indian honey in terms of water content. Moniruzzaman et al. (2013) stated that moisture content was affected by climate, season, and moisture content of original plant nectar and was considered unripened at the moisture content higher than 20%. The moisture content of honey samples is important as it contributes to its ability to resist fermentation and granulation during storage (Boussaid et al., 2018). Low moisture content also helps to promote longer shelf life during storage (Boussaid et al., 2018). However, moisture content depends on the temperature and relative humidity in the geographical origin during honey producing in honey-colonies.

pH

All honey samples were light acidic (Table 2). The average of pH values ranged from 4.5 to 5.2. The Somer Shabowah honey (SSH) has lower pH value of 4.5±0.45. The pH values of the Indian and Yemeni honey samples were very similar to those reported for Saudi, Malaysian, Bangladeshi, and Tunisian honey between pH 3.6 and 6.0 (Almasaudi et al., 2017; Moniruzzaman et al., 2013; Islam et al., 2012; Boussaid et al., 2018). Bogdanov (2009) reported that the high acidity of honey is attributed to the fermentation of sugar into organic acid, which has been reported to be responsible for honey's flavor and stability against microbial spoilage. In general, the pH values of the studied honey samples were within the limit that indicated the freshness of the honey.

Sugar content

The sugar content of honey samples ranged from 75.4± 0.07% to 82.4± 0.14% (Table 2) there were significant differences among the honey samples (P< 0.05). The highest sugar content showed in Sidr Yemeni (SDH) honey. Indian honey recorded the lower percentage of sugar content values 75.4– 75.6%. These results are similar to those reported for Indian honey, which ranged from 43.3 to 66.7% (Saxena et al., 2010; El Sohaimy et al., 2015). The lower total sugar content can be caused by the conversion of sugar into inorganic acid or overheating of honey during processing or storage for very long periods in high temperature can lead to the conversion a part of sugars to Hydroxymethylfurfural (HMF).

Total phenolic content (TPC)

The total phenolic content (mg GAE/kg of honey) of Indian and Yemeni honey was found in the range of 111.2 to 212.4, which was determined using gallic acid as standard. A similar level of phenolic content was also observed for Saudi honey for which the phenolic content varied from 81.3 to 96.0mg GAE/ kg (Almasaudi et al., 2017). For Malaysian and Bangladeshi honey, the phenolic content ranged from 144.5 to 508.1 and 152.4 to 688.5mg GAE/ kg, respectively (Moniruzzaman et al., 2013; Islam et al., 2012). The concentration and type of polyphenolic substances in honey is variable and depends on the floral origin of honey (Deng et al., 2018).

Antibacterial activity

The agar well diffusion method was performed for the initial screening of antibacterial activity of Indian and Yemeni honey samples at different concentrations. The inhibition zone diameters of honey samples against gram-positive bacteria (MRSA and MSSA) and gram-negative bacteria (*E. coli*, and *P. aeruginosa*) were shown in (Table 3) and the results were represented graphically in Fig. 1. Both Indian and Yemeni tested honey inhibited the growth of tested bacteria at all examined concentration, and there were significant differences (P< 0.05) between most of the samples. Sidr Doawany (SDH) honey consistently gave zones of inhibition against all four pathogens strains at the two concentrations and it was found to be the most effective one. The inhibition increased with increasing concentration in a dose-dependent manner. The zone of inhibition (ZOI) for Sidr Doawany (SDH) honey at 80% (w/v) was 16.0, 15.0, 16.0 and 14.0mm against MRSA, MSSA, *E. coli*, and *P. aeruginosa* respectively. Somer Shabwah (SSH) honey came in the second rank after Sidr honey. Kashmiri (K) honey showed the least antibacterial activity against the four tested bacteria. The antiracial activity for all tested honey decreased with decreasing the concentration till 50 % (w/v). These finding were in line with (Dash et al., 2016) who reported that Sider and Acacia honey collected from UAE showing the best activity against *S. aureus*, *E. coli* and *P. aeruginosa.* and with (Almasaudi et al., 2017) who tested Sdir Saudi honey against MRSA and MSSA bacteria.

TABLE 2. Physico-chemical parameters of Indian and Yemeni honey samples (average ± standard deviation).

Parameter	SDH	SSH	P	K
Moisture % /100g	13.7 ^{d} ± 0.03	16.2^{c} ± 0.00	24.2 ^{a} ± 0.03	23.2^{b} ± 0.00
pH	5.2 ^{a} ± 0.65	4.5 ^{b} ± 0.45	5.0 ^{a} ± 0.65	4.9^{a} ± 0.45
Sugar content % /100g	82.4 ^{a} ± 0.14	81.5 ^{b} ± 0.35	75.4 ^{c} ± 0.07	75.6 ^{c} ± 0.10
Total phenols (mg GAEs/kg)	212.4 ^{a} ± 0.28	171.4 ^{b} ± 0.21	158.3 ^{c} ± 0.28	111.2 ^{d} ± 0.14

(i) SDH: Sidr Dowany honey; Yemen, SSH: Somer Shabowah honey; Yemen, P: Punjabi honey; India, K: Kashmiri honey; India.

(ii) Results are reported as a means± standard deviation. Means are compared by using one-way ANOVA-HSD test, the same row with different letters are significantly different at P< 0.05.

TABLE 3. Antibacterial activity of Indian and Yemeni honey samples.

Bacterial Strain	Zone of inhibition (ZOI) diameter "mm" ± standard deviation of honey samples at different concentrations							
	Honey concentration (80% w/v)				Honey concentration (50% w/v)			
	SDH	SSH	P	K	SDH	SSH	P	K
MRSA	16.± 0.45**a**	12 ± 0.62**c**	12 ± 0.62**c**	11± 0.26**d**	13 ± 0.22**b**	10± 0.06**e**	10 ± 0.06**e**	10± 0.19**e**
MSSA	15 ± 0.08**a**	12± 0.01**b**	11± 0.01**c**	11.± 0.59**c**	12 ± 0.25**b**	9± 0.50**d**	10 ± 0.50**d**	10± 0.31**d**
E. coli	16± 0.06**a**	14± 0.20**b**	10± 0.45**d**	10± 0.35**d**	12± 0.21**c**	11± 0.40**d**	8± 0.60**e**	8± 0.47**e**
P. aeruginosa	14 ± 0.15**a**	13± 0.50**b**	10± 0.49**d**	10± 0.17**d**	11± 0.12**c**	10± 0.40**d**	8± 0.51**e**	8± 0.49**e**

(i) Means are compared by using one-way ANOVA-HSD test, the same row with different letters are significantly different at (P< 0.05).

(ii) MRSA: Methicillin-Resistant *Staphylococcus aureus*, MSSA: Methicillin-Sensitive *Staphylococcus aureus*.

(iii) SDH: Sidr Dowany honey; Yemen, SSH: Somer Shabowah honey; Yemen, P: Punjabi honey; India, K: Kashmiri honey; India.

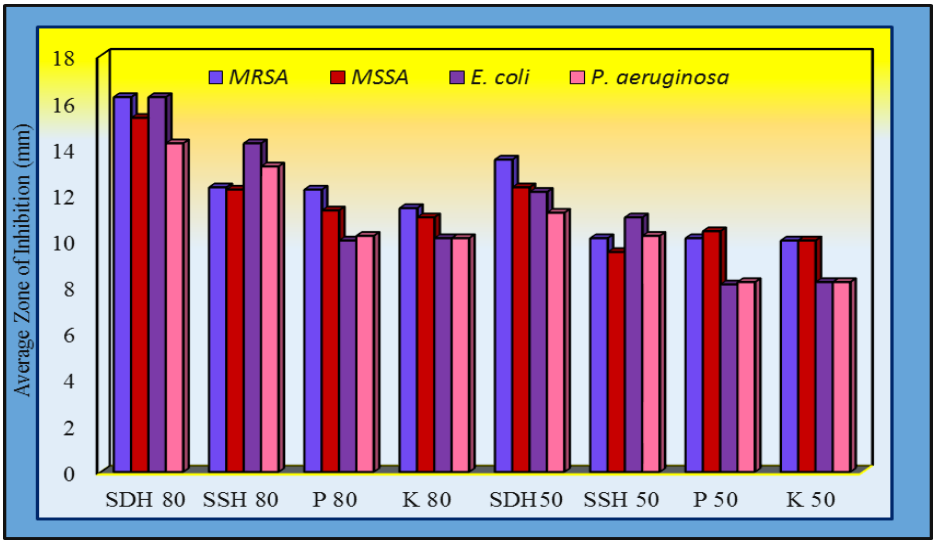

Fig. 1. Graphic of inhibition zone diameter ''mm" of Indian and Yemeni honey samples at different concentrations (80% and 50%) against four bacterial pathogens, where SDH= Sidr Doawany honey; Yemen, SSH= Somer Shabowah honey; Yemen, P= Panjabi honey; India and K= Kashmiri honey; India.

The MICs of (SDH) honey was noticed at 10%, 20%, 10%, 20%, (v/v); for (SSH) honey sample at 10%, 10%, 40%, 40% (v/v); for (P) and (K) honey samples were 20%, 20%, 40%, 40% (v/v) against MRSA, MSSA, *E. coli*, and *P. aeruginosa*, respectively. Likewise, the average MICs of each honey samples, i.e. SDH, SSH, P, and K honey against all four tested bacteria was 15%, 20%, 30%, and 30 % (v/v), respectively. While the average of MBCs of SDH, SSH, P, and K honey was 45%, 50%, 55, 55% (v/v) against MRSA, MSSA, *E. coli*, and *P. aeruginosa,* respectively

(Table 4). These MICs and MRCs values obtained in this study demonstrate that Yemeni honey were more effect in inhibiting than Indian honey this due to the nature of Sidr and Acacia honey and its high phenolic content compared to Indian honey (Abalaka et al., 2010; Alqurashi et al., 2013; Othman, 2014; Dash et al., 2016). The data of cluster analysis of honey samples at different concentrates against tested bacteria classified examined honey samples into two major groups. The first group was more than 10 included SDH, SSH honey at 80% w/v and SDH honey at 50% w/v. The second group was less than 10 presented by five honey samples divided in two subgroups, sub-group-I formed P, and K honey at 80% and SSH honey at 50% w/v concentration while sub-group-II formed of P and K honey (50% w/v). (Fig. 2).

TABLE 4. MIC & MBC (%v/v) of different Indian and Yemeni honey samples against bacterial pathogens.

Bacterial Strain	Honey Dilutions % (v/v)								Honey Sample Code	MIC value % (v/v)	MBC value % (v/v)
	100 control	80	60	40	20	10	5	0 Control			
MRSA	-	-	-	-	-	+	++	+++	SDH	10	40
MSSA	-	-	-	-	+	++	++	+++	SDH	20	40
E. coli	-	-	-	-	-	+	++	+++	SDH	10	40
P. aeruginosa	-	-	-	-	+	++	++	+++	SDH	20	60
Mean MIC & MBC values for SDH honey						**15**					**45**
MRSA	-	-	-	-	-	+	++	+++	SSH	10	40
MSSA	-	-	-	-	-	+	++	+++	SSH	10	40
E. coli	-	-	-	+	++	++	+++	+++	SSH	40	60
P. aeruginosa	-	-	-	+	++	+++	+++	+++	SSH	40	60
Mean MIC & MBC values e for SSH honey					**20**						**50**
MRSA	-	-	-	-	+	++	+++	+++	P	20	40
MSSA	-	-	-	-	+	++	+++	+++	P	20	60
E. coli	-	-	-	+	++	++	+++	+++	P	40	60
P. aeruginosa	-	-	-	+	++	+++	+++	+++	P	40	60
Mean MIC & MBC values for P honey				**30**							**55**
MRSA	-	-	-	-	+	++	+++	+++	K	20	40
MSSA	-	-	-	-	+	++	+++	+++	K	20	60
E. coli	-	-	-	+	++	+++	+++	+++	K	40	60
P. aeruginosa	-	-	-	+	++	+++	+++	+++	K	40	60
Mean MIC & MBC values for K honey				**30**							**55**

(i) -: No growth, +: Minimum growth, ++: Mild growth, +++: Dense growth.

(ii) Grey color indicates the MIC.

(iii) MRSA: Methicillin-Resistant *Staphylococcus aureus*, MSSA: Methicillin-Sensitive *Staphylococcus aureus.*

(iv) SDH: Sidr Dowany honey; Yemen, SSH: Somer Shabowah honey; Yemen, P: Punjabi honey; India, K: Kashmiri honey; India.

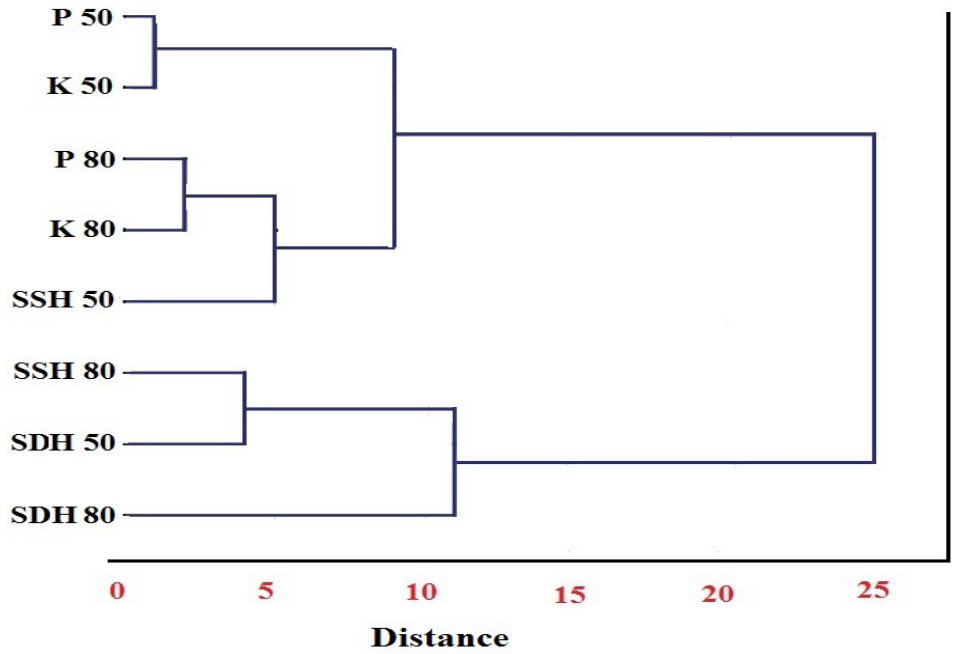

Fig. 2. The dendrogram of Indian and Yemeni honey samples at different concentrations (80% and 50%) against four bacterial pathogens (Methicillin-Resistant *Staphylococcus aureus* (MRSA), Methicillin-Sensitive *Staphylococcus aureus* (MSSA), *E. coli* and *Pseudomonas aeruginosa*), where SDH= Sidr Doawany honey; Yemen, SSH= Somer Shabowah honey; Yemen, P= Panjabi honey; India and K = Kashmiri honey; India.

Conclusion

This study has provided a broad overview of the antibacterial activity of some Indian and Yemeni honey and shown both honey have potential for therapeutic use as antibacterial agents. Yemeni honeys have exceptional levels of antibacterial activity comparable to Indian honey. These findings indicate that there is an opportunity for Indian and Yemeni apiarists to share in the lucrative medicinal honey market. However, the factors affecting antibacterial activity in honey are complex, numerous, and not solely dependent on the floral source. We suggest of our finding the antibacterial activity of tested honey attributed to the content of the phenol compounds.

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