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Antibacterial and Antibiofilm Efficacy of the Stingless Bee Honey from Dakshina Kannada District Regions against Gram Positive and Negative Strains

G. B. Anil[1,2*], Sudhakar Malla[3] and M. S. Reddy[1]

[1]Centre for Apiculture Studies, Department of Zoology, Bangalore University, Jnana Bharathi, Bengaluru - 560 056, India

[1,2]Department of Zoology, MES College of Arts, Commerce & Science, Malleswaram, Bengaluru -03, India*

[3]Director. R&D. Insearch Biotech Laboratory, Bengaluru, India

**Corresponding author*

Abstract

Stingless bee honey (SBH) is produced by tropical Meliponini bees. Beneficial qualities such as antibacterial, bacteriostatic, anti-inflammatory, neurotherapeutic, neuroprotective, and wound and sunburn healing effects have been demonstrated in studies. The advantages of SBH come from high quantities of phenolic acid and flavonoids. This study aims to assess the antibacterial efficacy SBH collected from six different regions of Dakshina Kannada district. The activity was studied against six bacterial strains; *Klebsiella pneumoniae, Escherichia coli, Helicobacter pylori, Bacillus subtilis, Staphylococcus aureus* and *Streptococcus mutans.* The antibacterial activity of honey samples was evaluated using agar well diffusion, broth dilution assay and biofilm inhibition assay. About 20 samples were collected from each area and the results are expressed as mean. The antibacterial efficacy of each examined sample varied, with inhibition zones ranging from 10 to 30 mm. When compared to other samples, Belthangady samples had the strongest antibacterial activity. Antibacterial activity was lower in Buntwal samples. We found that activity was higher against Staphylococcus aureus and Bacillus subtilis. SBH showed antibacterial activity against all the strains tested. Further study needs to be confirmed with more samples and assays.

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Keywords

Dakshina kannada, Belthangady, Biofilm inhibition, Stingless bees, Honey

Introduction

The stingless bee species T. *bengalensis, Tetragonula iridipennis, Tetragonula ruficornis, Tetragonula laeviceps, Tetragonula praeterita,* and *L. terminata* were discovered in India (Rahman *et al.,* 2013). Meliponiculture is the practise of raising stingless bees, or T. sapiens, in Indonesia. Through the practise of meliponiculture, researchers can learn more about the food supplies necessary for the expansion of stingless bee populations. Additionally, this knowledge is crucial for enhancing our understanding of how bees and plants interact. The kinds of pollen that bees collect from plants can be a guide for developing stingless bee food.

The world's tropical and subtropical regions, including Central and South America, Africa, Asia, and northern Australia, are home to stingless bees (Apidae, Meliponini) (Cortopassi-Laurino, M, 2006). All stingless bees construct intricate nests that frequently feature characteristics that are specific to the species or higher taxa (Michener, 1900). The tribe Meliponini has two

genera, Lisotrigona and Trigona, which are distributed in Asia but have received little research (Michener, 1900). Tetragonula iridipennis, often known as the dammar bee or Indian stingless bee, is primarily found in India. Numerous investigations have been made on *T. iridipennis* colonies found in the states of Karnataka, Kerala, and Tamil Nadu (Da Silva P.M, 2016). Stingless bees are abundant and widely distributed due to the tropical temperature, diverse physiographic surroundings, higher altitudes, and lush vegetation.

The majority of stingless bee species make honey, just like Apis species. As a food source, stingless bee honey is highly regarded. Stingless bees are extremely versatile, exhibit variety in nest building, and are simple to domesticate (Patel, V 2020). The primary source of protein for bees is pollen, which is one of their main dietary sources along with honey. These bees' legs feature thick plumose hair that makes it easier to capture pollen. With the assistance of the plumose legs, resin, a crucial component of nest formation, is also obtained (Alvarez-Suarez, J.M, 2010).

Honey's antibacterial properties have long been studied scientifically and utilised medically. Up until recently, relatively little attention was given to honey produced by stingless bees; instead, the majority of research has concentrated on honey produced by the European honeybee *Apis mellifera*. The Aboriginal people of northern Australia highly regard stingless bee honey as a food source, and it is also culturally significant, contributing to the people's social traditions and ceremonies (Kimoto-Nira, H, 2008). According to Layek, U., aurino *et al.,* (2018), stingless bee honey has also been employed in traditional medicine in Central and South America, as well as Africa. This suggests that stingless bee honey may possess therapeutic properties similar to those of currently popular medicinal honeys like manuka honey from New Zealand (Adams *et al.,* 2008).

Nowadays, it is understood that the majority of honey varieties include antibacterial properties, and that these properties depend on both physical and chemical aspects. Honey has a viscosity that is high enough to physically form a barrel that prevents airborne pathogenic organisms from contaminating the wound (Berhanu Andualem, 2013). Due to its high sugar content, honey osmotically kills the majority of microorganisms. The honey's acidity, the presence of phytochemicals like flavonoids and phthalic acids and most importantly, the action of oxygen peroxide, which is produced in honey as a result of the presence of the glucose oxidase enzyme secreted by honeybees' hypopharyngeal glands, can all be considered contributing factors to the antibacterial activity (Snow MJ, 2004).

Studying honey bees in Karnataka is significant for understanding pollination's role in rich biodiversity (especially in Kodagu), identifying unique local bee genetic diversity (*Apis cerana* subspecies), managing bee health challenges (pests, climate change), and promoting sustainable livelihoods through apiculture, supporting economic growth in regions like Uttara Kannada, Shimoga, and Hyderabad-Karnataka by conserving native flora, boosting crop yields, and developing local beekeeping knowledge.

We aimed to study the antibacterial efficacy of the honey samples collected from various regions from Dakshina kannada district of Karnataka, India. The study included 6 strains (both gram positive and negative).

Materials and Methods

Sample Collection

In the present study, total of one hundred twenty samples were collected from the different locations of five taluks of Dakshina Kannada district namely Belthangady, Buntwal, Mangalore, Puttur and Sullia taluk and studied. Honey was collected in sterile centrifuge tubes and was later filtered with a muslin cloth to remove debris and then streaked onto agar plates to check the sterility of the samples. The samples were then stored at 2–8°C until further use.

Honey Solution Preparation

The honey obtained after filtration was considered to be hundred percent pure (100% v/v) and sample was further diluted to dilutions like 16, 32, 64, 126 and 256µg/ml with sterilized distilled water. This was used for the antibacterial activity assays. Pure honey was used for the disc diffusion assay and dilutions were used for broth dilution assays.

Bacterial Cultures

For the study, three Gram positive *viz., Bacillus subtilis, Staphylococcus aureus, Streptococcus mutans*, and three gram negative bacteria *viz., Escherichia coli, Klebsiella pneumoniae, Helicobacter pylori* were used. Bacteria was cultured in LB broth and incubated at 37°C for 24

hours. After 24 hours, they were sub-cultured on their respective medium and incubated again at 37°C for another 24 hours before being processed for long storage at -80° in cryogenic vials.

Table.1 List of the strains and the positive controls used in the study.

Strain	Gram Strain	Positive control	Concentration
Klebsiella pneumoniae	Negative	Amoxicillin	5mg/ml
E.coli	Negative	Ampicillin	10mg/ml
H.pylori	Negative	Amoxicillin	5mg/ml
Bacillus subtilis	Positive	Streptomycin	20mg/ml
Staphylococcus aureus	Positive	Erythromycin	10mg/ml
S.mutans	Positive	Ampicillin	10mg/ml

Antibacterial Activity

Agar well diffusion assay

The antibacterial testing was done on LB agar plates using the agar diffusion method. In brief, about 100µL of the bacterial culture was spread on the LB agar plates. Wells (about 3mm in diameter) were cut with a sterile glass capillary tube at respective positions. The wells were loaded with 50µL of samples (pure honey, 100%). Respective positive controls at different concentrations were added in the plates. The plates were then incubated at 37°C for about 24-48hr. Following incubation, the mean diameter of the inhibition zone (mm) was measured and recorded. All the tests were done in triplicates and done under the same experimental conditions. All the six strains were studied separately for all the six samples.

Biofilm inhibition assay

Bacterial biofilm inhibition assay was done (Asal Mohseni Sajadi Araghi, 2016). In brief, about 180µL of LB broth was added to all the wells of a 96 well of a microtiter plate and 10µL of respective bacterial culture was inoculated into all the wells except the last column which serves as sterile control (containing only 180µL of LB broth). Respective positive controls were added with their sensitive culture.

About 20µL of test samples were added into their respective labelled wells. The microtiter plates were incubated at 37°C for 24-48 h and following incubation, the medium was discarded and the wells were washed thrice with Phosphate Buffered Saline (PBS). The cells which are supposed to be adhered to the wells were stained with 0.1% crystal violet and then washed thrice with PBS (pH 7.4). The cell-bound dye was then eluted with about 200µL of ethanol: acetone (80:20) and the absorbance of the eluted solution was read at 595 nm by using Plate reader (Genetix Ltd.).

Broth dilution assay

Broth dilution assay was performed to calculate the MIC of the samples (Panda *et al.,* 2011). The MIC of the honey samples were studied using the microdilution method with 96 multi-well microtiter plates as described by Panda *et al.,* (2011).

About 180µL of LB broth was added to all the wells in the study. To each labelled tube, 10µL of the diluted honey samples of each region were pipetted. Lastly, 10µL of the respective bacterial suspensions were added to all the wells except for the negative control. Respective positive controls were used as positive controls. The plates were incubated at 37°C for about 18-24 hours. Sterile distilled water served as negative control. Following incubation, the plates were read for optical density under PLATE reader (Genetix Ltd) at 600nm. The MIC was considered as the lowest concentration which inhibited the growth of the respective microorganisms. All assays were performed in triplicate.

Results and Discussion

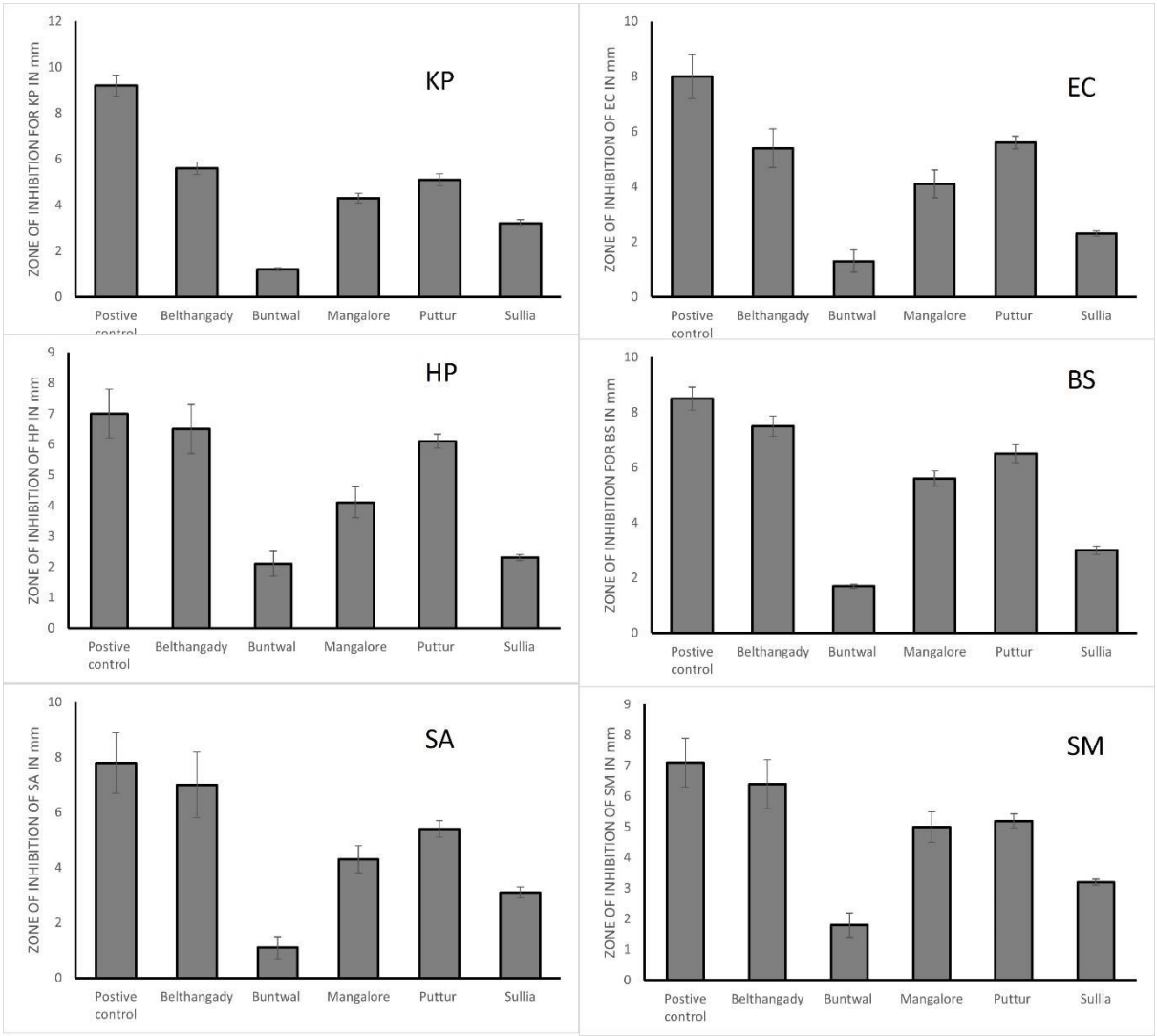
Agar well diffusion

In the study, *Klebsiella pneumoniae* showed maximum inhibition of 15.94mm with Belthangady honey and minimum inhibition of 3.85mm with Buntwal honey. *Escherichia coli* showed maximum inhibition of 16.28mm with Belthangady honey and minimum inhibition of 3.85mm with Buntwal honey. *Helicobacter pylori* showed maximum inhibition of 8.66mm with

Belthangady honey and minimum inhibition of 2.15mm with Buntwal honey. *Bacillus subtilis* showed maximum inhibition of 16.28mm with Belthangady honey and minimum inhibition of 5.95mm with Buntwal honey. *Staphylococcus aureus* showed maximum inhibition of 9.4mm with Mangalore honey and minimum inhibition of 4.6mm with Buntwal honey. *Streptococcus mutans* showed maximum inhibition of 15.51mm with Belthangady honey and minimum inhibition of 3.85mm with Buntwal honey.

Throughout the study, significant antibacterial activity was observed between the strains for the samples across the regions remembered at P<0.05 Belthangady to be highly significant in antibacterial activity especially to *Escherichia coli* followed by *Streptococcus mutans* and then *Klebsiella pneumoniae*.

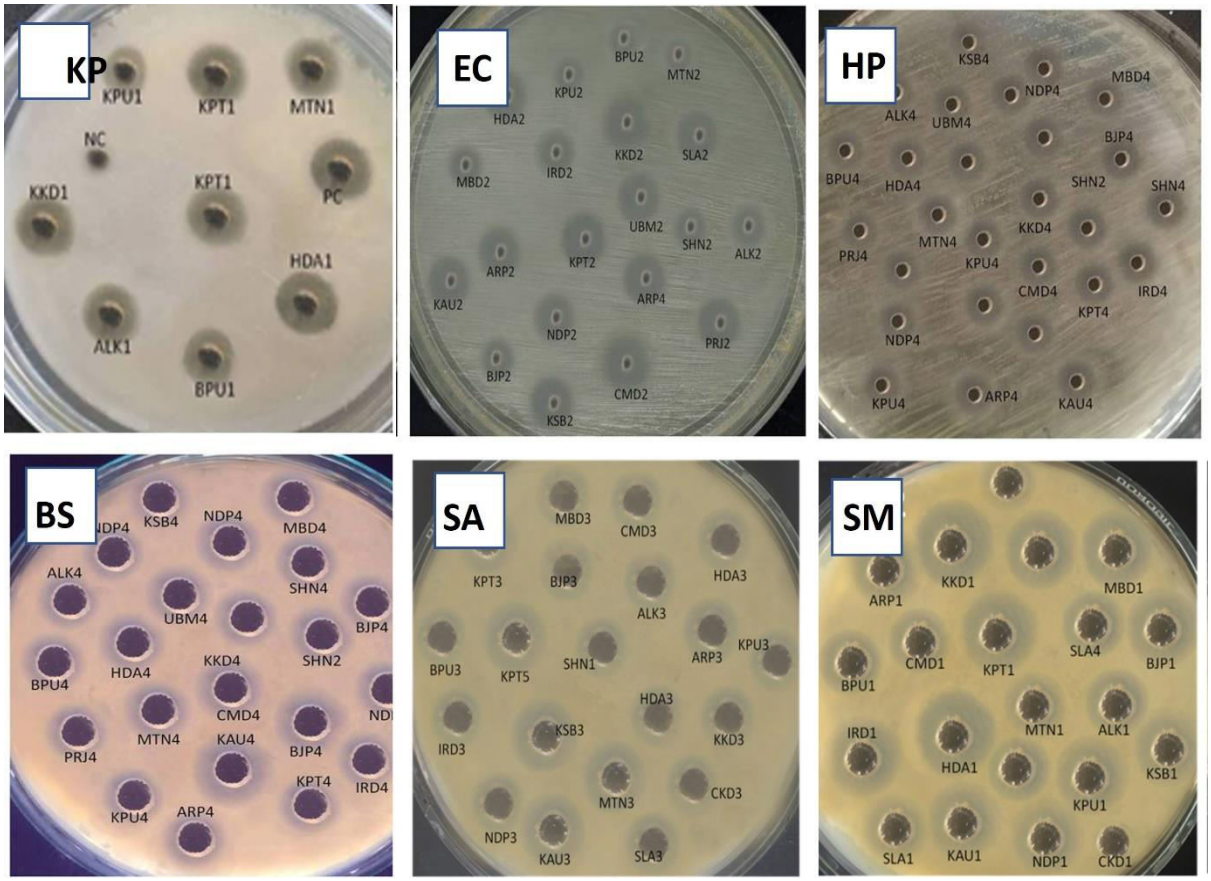
Figure.1 Graph showing zone of inhibition in mm of the samples collected from different regions against both gram positive and gram negative strains.



All the values are average of triplicates. KP: *Klebsiella pneumoniae*; EC: *Escherichia coli*; HP: *Helicobacter pylori*; BS: *Bacillus subtilis*; SA: *Staphylococcus aureus*; SM: *Streptococcus mutans.*

From the study, we found Belthangady to be highly significant in antibacterial activity especially to *Escherichia coli* followed by *Streptococcus mutans* and then *Klebsiella pneumoniae*. All the samples showed less antibacterial activity against *Helicobacter pylori* and this was observed across the regions when compared to positive control (P<0.05).

Figure.2 Plates showing zone of inhibition in mm of the samples collected from different regions against both gram positive and gram negative strains.



All the values are average of triplicates. KP: *Klebsiella pneumoniae*; EC: *Escherichia coli*; HP: *Helicobacter pylori*; BS: *Bacillus subtilis*; SA: *Staphylococcus aureus*; SM: *Streptococcus mutans.*

Broth dilution assay

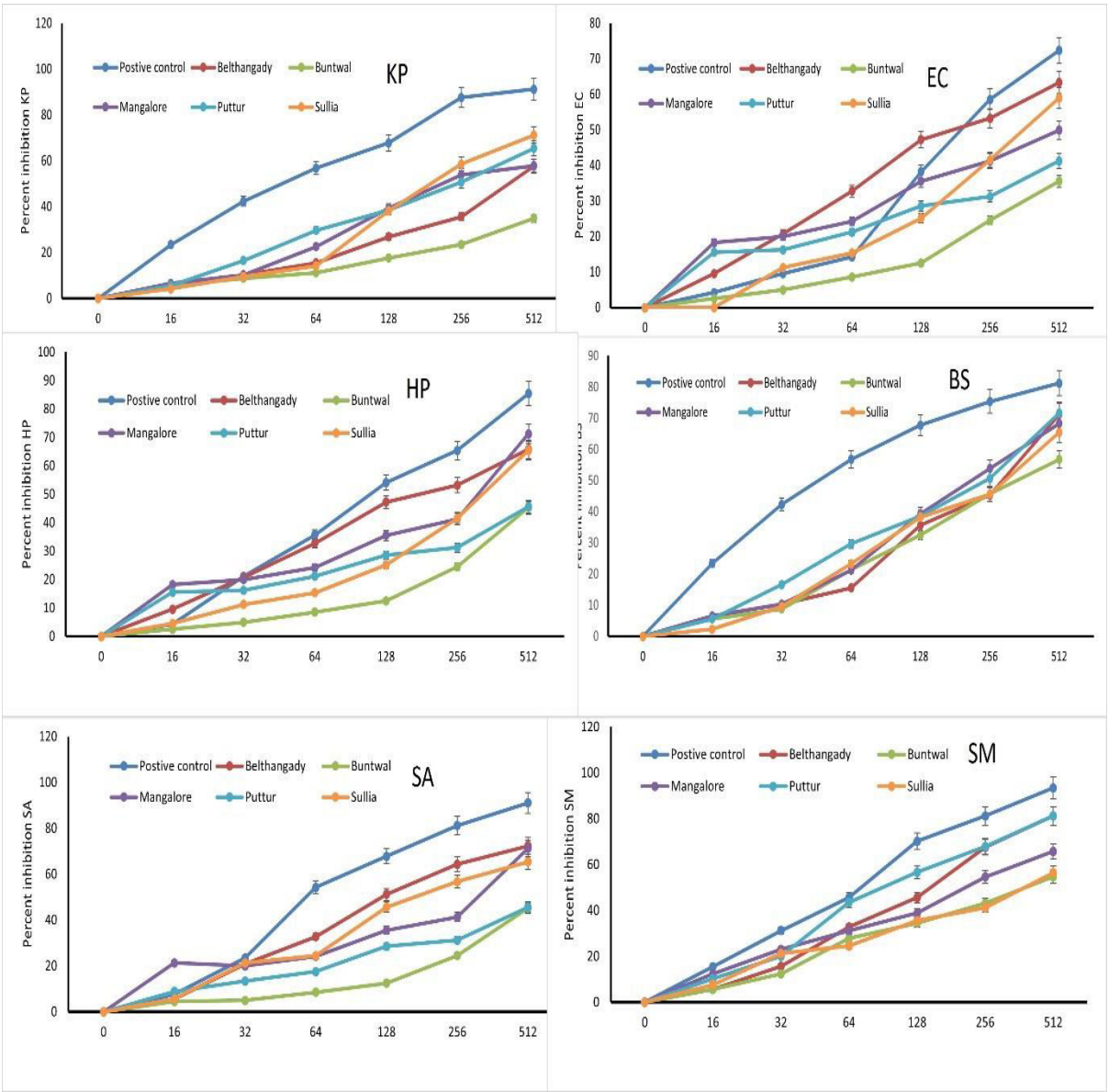
In all the cases, Belthangady (BG) showed lowest MIC values when compared to their respective controls (positive and negative). Buntwal (BT) region showed less MIC values and these values are in accordance to our previous well diffusion assay. All the strains responded equally for all the regions and MIC values were less for *Helicobacter pylori* which is again an observation which is similar to our previous section result.

MIC values ranged from 20±2.15 (BG) to 75±1.03 (BT) for *Klebsiella pneumonia* when compared to positive control (5±1.23). MIC values ranged from 15±2.23 (BG) to 65±1.11 (BT) for *Escherichia coli* when compared to positive control (10±2.14). MIC values ranged from 34±1.01 (BG) to 120±1.21 (MG) for *Helicobacter pylori* when compared to positive control (15±0.95). MIC values ranged from 17±1.05 (BG) to 70±1.81 (MG) for *Bacillus subtilis* when compared to positive control (12±1.12). MIC values ranged from 26±1.41 (BG) to 35±1.03 (BT) for *Staphylococcus aureus* when compared to positive control (8±1.06). MIC values ranged from 38±1.23 (BG) to 60±1.1 (SL) for *Streptococcus mutans* when compared to positive control (10±1.11).

In all the cases, MIC values were found to be significantly high for BG samples when compared to positive controls and other samples. This is in accordance to our agar well diffusion study ($P<0.05$). Throughout the study, we observed there was a significant effect of samples between the regions and on strains ($p<0.05$).

There was a significant effect of varying concentrations on the strain inhibition ($p<0.05$). The effect was completely dose dependant in all the cases ($p<0.05$).

Figure.3 Graph showing % inhibition by broth dilution assay of the samples collected from different regions against both gram positive and gram negative strains.



All the values are average of triplicates. KP: *Klebsiella pneumoniae*; EC: *Escherichia coli*; HP: *Helicobacter pylori*; BS: *Bacillus subtilis*; SA: *Staphylococcus aureus*; SM: *Streptococcus mutans.*

A two-way ANOVA between the regions and concentration of samples was conducted to compare the percent inhibition on strains. There was a significant effect of the inhibition both between the regions and concentrations remembered at the $p<0.05$ level.

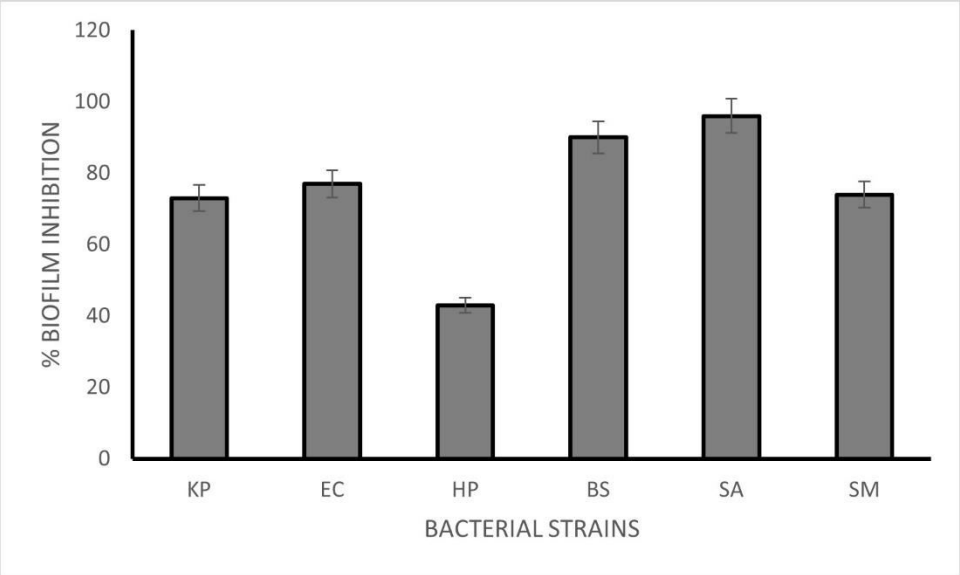
The significance effect of the regions on the strains was reported to be [$F(5,30) = 1160.298$, $p = 1.75E-07$]. There was also a significant effect of concentrations on strains inhibition [$F(6,30) = 3187.854$, $p = 2.79E-13$].

Biofilm inhibition

We found % biofilm inhibition to be 90 and 96% for Belthangady sample against *Bacillus subtilis* and *Staphylococcus aureus* respectively ($P<0.05$).

Biofilm inhibition was very low against *Helicobacter pylori* (43%). On the other hand, KP, EC and SM showed % inhibition of 73, 77 and 74% respectively ($P<0.05$).

Figure.4 Graph showing % inhibition in biofilm of the samples collected from belthangady region against both gram positive and gram negative strains.



All the values are average of triplicates. KP: *Klebsiella pneumoniae*; EC: *Escherichia coli*; HP: *Helicobacter pylori*; BS: *Bacillus subtilis*; SA: *Staphylococcus aureus*; SM: *Streptococcus mutans.*

From the biofilm inhibition assays, we could confirm similar results to that of broth dilution assay in the previous sections. The activity was time dependant across all the samples and all the strains ($p<0.05$).

Throughout the study, we observed similar findings on the all the samples of regions. There was a significant effect of sample between the strains ($p<0.05$). There was a significant effect of incubation times on the biofilm inhibition ($p<0.05$).

Throughout the study, significant antibacterial activity was observed between the regions remembered at $P<0.05$. Belthangady region samples showed high antibacterial activity when compared to other regions. Buntwal samples were found to show low antibacterial activity, when compared to both positive and other region samples. Throughout the study, summer and winter did have high antibacterial values but this depended mostly on the region and not between the seasons. Belthangady samples showed high antibacterial activity during both summer and winter ($p<0.05$).

Since the values are having high degree of variations between the regions in one season and the next season, average could not be taken for analysis. Hence, study was done individually. Throughout the study, significant antibacterial activity was observed between the strains for the samples across the regions remembered at $P<0.05$ Belthangady to be highly significant in antibacterial activity especially to *Escherichia coli* followed by *Streptococcus mutans* and then *Klebsiella pneumoniae*.

Stingless bee honey has broad-spectrum antibacterial activity although activity against Candida was limited. Stingless bee honey samples varied in activity and the basis for this remains to be determined (Boorn *et al.,* 2010). Two recent publications have described the antimicrobial activity of honey derived from the Australian native stingless bee T. carbonaria (Kimoto-Nira and Amano 2008). *Homotrigona fimbriata* honey showed the highest antimicrobial activity with inhibition against five bacteria viz; *Serratia marcescens, Escherichia coli*, *Bacillus subtilis*, *Alcaligenes faecalis and Staphylococcus aureus* (Rosli, 2020).

Studies done by Berhanu Andualem (2013) also confirmed of the potential antibacterial activity of stingless bee honey against *Salmonella* (NCTC 8385), *Staphylococcus aureus* (ATCC 25923), *Lyesria moncytogenes* (ATCC 19116) and *Streptococcus pneumonia* (ATCC 63) and they also reported an enhanced activity when combined with garlic extract.

To the best of our knowledge, we report the antibacterial activity of Stingless bee honey against six bacterial strains and also these samples were from Karnataka region. The works reported so far from Karnataka region were too general and with less sample number.

We studied antibacterial activity of almost 120 samples from different regions of Dakshina Kannada district. Our diversity and sequencing results confirm the species to be *Tetragonula iridipennis*. We found high and significant antimicrobial activity against both gram positive and negative strains.

In conclusion, one of the most helpful insects is the stingless bee, which makes both outstanding pollinators and high-quality honey. They are quite versatile, display a variety of nesting patterns and nest building, and are simple to domesticate. These stingless bees are seen all around India. There is still much to learn about bee systematics, distribution, ecology, and ecosystem services. Stingless bee species level identification across the Indian subcontinent becomes challenging utilising global and published identification keys due to the highly complex nature of their variety.

Stingless bee honey (SBH) is a remarkable "wonder liquid" with a wide range of medical benefits for conditions like gastroenteritis, cataracts, and wound healing. However, information on it is still relatively limited. Therefore, it is exciting for us to think about the less-researched stingless bee in general and its honey. In order to further demonstrate the health benefits of honey, 120 study locations were examined for their antibacterial capacity.

The honey samples from the Belthangady region exhibited the strongest antibacterial activity, inhibiting all six of the study's microorganisms. Results of antibacterial susceptibility of the samples in the study displayed a potent antimicrobial activity against both Gram-positive and Gram-negative bacteria ($p<0.05$).

Throughout the study we found Belthangady samples to show high antibacterial activity. One more striking feature we found was, *Helicobcater pylori* was little resistant to all of the samples when compared to other bacterial strains susceptibility ($P<0.05$). Belthangady samples were found to possess high antibacterial activity against both Gram positive and gram negative strains. Buntwal samples were found to show low antibacterial activity, when compared to both positive and other region samples. Belthangady was found to be highly significant in antibacterial activity especially to *Escherichia coli* followed by *Streptococcus mutans* and then *Klebsiella pneumoniae*. *Helicobacter pylori* was least susceptible to all of the samples across all the five regions.

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