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Comparative antibacterial activity of stingless bee honey and standard antibiotics against common eye pathogens

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ABSTRACT

Isolates of pathogens collected from infected eyes and their sensitivity to eight standard antibiotics used in the treatment of eye infections-and to stingless bee honey (SBH), used for treating eye infections in Ghana, was compared. Pure Meliponula bucandei honey and concentrations of 80%, 60%, 40% and 20% in distilled water were prepared. Twenty four patients with ocular infections who attended the Central Regional hospital and Christian Eye Hospital were selected for this study. Cultures of the collected specimens collectively revealed that Pseudomonas aeruginosa was the most frequently isolated organism, representing 50% of the isolates, followed by Staphylococci aureus (31.25%). The least frequently isolated was Staphylococcus epidermidis (18.75%). In a disc diffusion method, pure SBH and concentrations of ≥60% produced the strongest activity on all three isolated pathogens. The isolate Pseudomonas aeruginosa was totally resistant to the 20% and 40% concentrations of SBH. The standard antibiotics; Ampicillin , Tetracycline , Gentamycin , Erythromycin , Penicillin , Cloxacillin , Cefuroxime and Cotrimoxazole , used in concentrations of 10 µg/ml varied in their activity against the test microbes but was generally lower than the antibacterial activity of the pure SBH and its ≥60% concentrations ($p < 0.05$). SBH had more inhibitory effect on the test microbes than commonly used antibiotics although the activity against the Gram negative bacterium (Pseudomonas aeruginosa) was limited. SBH therefore, can offer a suitable and better alternative in managing common eye infections in the event of therapeutic failure with standard antibiotic compounds.

Keywords*:* infections, antimicrobial effect, microbial organisms, isolates, pathogens, test microbes

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INTRODUCTION

Streptococcus aureus, *Streptococcus epidermis* and *Pseudomonas aeruginosa* are opportunistic pathogens which owe their clinical significance in ophthalmology to the fact that they are causes of most eye infections [1]. *Staphylococcus aureus* and Staphylococcus *epidermidis* are gram positive innocuous microbial flora of lid and conjunctiva which most commonly cause bacterial conjunctivitis and blepharo-conjunctivitis while *Pseudomonas aeruginosa* is a gram negative virulent organism responsible for a broad spectrum of eye infections which includes corneal ulcers, endophthalmitis, sclera abscess, blephero-conjonctivitis, and ophthalmia neonatorum in children [20].

Gentamycin, Erythromycin, Tetracycline, and Ampicillin are well established first line antibiotics for ocular infections, and have been shown to possess broad spectrum of activity against Gram-positive and few Gram-negative organisms at concentrations of 10µg/ml [3]. Similarly, Cefuroxime and Cotrimoxazole have recently

attracted clinical significance owing to their high potency against gonococcal and Chlamydia infections of the eye respectively [3]. However, resistance to these antibiotics is increasing. For example, resistance to topical aminoglycoside (Gentamycin) therapy may be encountered in as many as 80% to 100% of ocular infections caused by *Pseudomonas aeruginosa* [4] Resistance appears to be even greater in ocular infections caused by Gram positive organisms [5]. Adverse drug effects such as punctuate epithelial keratitis have been encountered in some patients.

Stingless bee honey (SBH) is a valuable natural product from a diverse group of highly eusocial bees (meliponines) comprising the tribe Meliponini in the family Apidae. The honey is a sour and bitter flowery liquid, with a long consumption tradition to which several medical uses are attributed [6-8]. The chemical composition and antimicrobial activities attributed to these specific chemicals have also been extensively studied [9-11].

Comparative studies have however identified SBH as a more effective remedy than some antimicrobial compounds [12-13]. In one study, samples of SBH were collected from four colonies and tested against five pure strains of bacteria; *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*. The honey exhibited highest inhibitory effect on all gram positive strains compared to known antibiotic agents [14].

There are very few reports on the use of SBH in ophthalmology. In these reports, conjunctivitis, cataract and pterygium were other benefits suggested for the topical application of SBH in the eyes [15-16]. In Ghana, it is a common practice to see traditionalists instilling few drops of the honey on the eyes of patients presenting with conjunctival redness, sometimes associated with varying severity of ocular exudates. However, no work has been done to compare the efficacy of SBH to standard ophthalmic preparations on common eye pathogens. The present study aims to evaluate the antimicrobial effect of SBH on organisms isolated from infected eyes in comparison to established antibiotic compounds used in treatment of eye infections.

The specific objectives were:
1. To collect swab of patients with various eye conditions
2. To isolate the causative organisms of such eye conditions and culture them
3. To run a bioassay of the isolates using SBH and standard antibiotics
4. To compare the effectiveness of SBH and standard antibiotics in inhibiting growth of eye pathogens.

MATERIALS AND METHODS

Collection of Stingless Bee Honey and Measurement of pH

A matured honey sealed in pots within stingless bee (*Meliponula boncadei* Spinola.*)* hives located at the International Stingless Bee Centre (ISBC) at Abrafo, near Kakum national park, Ghana, was used for this study. The collection was done with disposable syringes. During collection, the sealed portion of the pot was carefully broken with a sterile needle. The tip of the sterile disposable syringe was dipped into the opening of the honey pot. The honey was carefully sucked with the syringe, transferred into a sterile empty container and covered immediately. This was kept in a cool place against high temperature.

The pH of the SBH collected was determined using a pH meter. This was done by placing a drop of honey on the pH meter. The color change created in the process was compared with standard values on the pH chart.

Collection of Swabs

Twenty four patients with infected eyes subsequent to treatment at the Regional Hospital and Christian Eye Hospital, both in Cape Coast, Central Region of Ghana, were selected for this study. All ethical principles governing the conduct of research with human subjects were taken into consideration [17]. Ocular sites were sampled using calcium alginate swabs. This method has been shown to enable good recovery of organisms from ocular sites [18]. Samples were taken from the upper bulbar conjunctiva, avoiding contact with the lids, lashes, and tarsal conjunctiva. A second swab was passed along the lower lid margin, avoiding contact with the bulbar conjunctiva and lashes. Swabs were immediately placed-into 2 ml of phosphate-buffered saline (PBS) containing 1% (w/v) $(NaPO_3)_6$ and vortexed for 30 s until the swab fibres were finely dispersed. Each culture swab was placed in a sterile tube with 10 ml of transport-dissolving buffer, and was delivered to the microbiological laboratory of the Regional Hospital within 4 hours of collection.

Culturing of Bacteria and Characterization of Microorganisms

All bacteria strains were cultured on mac-conkey agar for 24 h at 37◦C. Culturing of the specimen was done in the hood to ensure a sterile environment. Single colonies of the mixed culture obtained were further sub-cultured to obtain a pure culture. Colonies were enumerated and identified using gram stain, standard biochemical methods [19], and, API strips (Vitek BioMerieux, Sydney, Australia) and Biolog GN strips (manufactured by Special Diagnostics, Sydney, Australia but obtained from Lynx medical services, Accra)

Preparation of Honey into Honey Disc Concentrations

A sterile filter paper was cut into small, circular and equal sizes by using a perforator. Using the pipette, pure SBH and concentrations of 80%, 60%, 40% and 20% were constituted by adding distilled water. The various concentrations was embedded into the filter paper aseptically and incubated for 24 hours to enable full adsorption of the honey into the filter paper.

Bioassay

The disc diffusion technique was employed as described in the literature [20-22] in determining the susceptibility of the bacteria to the SBH and standard antibiotics. Density of bacterial cells was measured with McFarland's standard solution. The size was adjusted to 0.5 McFarland standard turbidity/ approximately 10^8 colony forming units (CFU/ml). The bacteria cells were introduced into the agar plates and spread thinly on the plates using a glass spreader. Discs of 6mm diameter were impregnated with 25µl of SBH. The discs were then placed on inoculated agar plates. The plates were incubated at 37^oC for 24 hours under aerobic conditions. Considerations for the sensitivity and resistance of bacteria were based on the extent of the presence or absence of zones of growth inhibition around the disc after 24 hours and 48 hours respectively. A 15 centimeter rule was used to measure the diameter of the inhibited zones in millimeters. The inhibition zone was measured from the edge of the disc to the inner margin of bacterial colony. Two measurements were made at two different directions (90^o and 180^o) and the mean diameter of the inhibited zone for each group was recorded. The test was repeated using 10 ug/ml of Ampicillin (AMP), Tetracycline (TET), Gentamycin (GEN), Erythromycin (ERY), Penicillin (PEN), Cloxacillin (CXC), Cefuroxime (CXM) and Cotrimoxazole (COT), (manufactured by Abitek Diagnostic Company, UK but obtained from Lynch Medical services in Accra, Ghana). The experiment was performed in duplicate.

Statistical Analysis

Results were analyzed using Analysis of Variance (ANOVA) with the probability p= 0.05 as the critical value for all test. Tukey's post-hoc test was used for separation of statistically significant means.

RESULTS

The pH of the undiluted (100%) SBH was found to be 3.8. Of the 24 swabs sampled, sixteen swabs had pathogens present, indicating 66.7% infection rate (Table 1). The most frequently isolated organism was *Pseudomonas aeruginosa*, representing 50% of the isolates, followed by *Staphylococci aureus* (31.25%). The least frequently isolated was *Staphylococcus epidermidis* (18.75%). The isolation rate was approximately 35% in conjunctivitis, 50% in corneal ulcer and 15% in others (Table 1).

Table 1: Frequency of Occurrence of Eye Pathogens present in Swabs of Patients

Pathogens	No. of Swabs	% occurrence
Staphylococci aureus	5	31.25
Staphylococcus epidermidis	3	18.75
Pseudomonas aeruginosa	8	50.00

Sensitivity of *Staphylococcus aureus* to Antibiotics and Stingless Bee Honey

From Table 2, the mean inhibition zone of pure (undiluted) SBH (11.46±0.2) against *Staphylococcus aureus* was significantly higher than that of antibiotics (PEN, CXC, GENT, AMP, ($p<0.05$) and COT ($p<0.01$)). Cefuroxime did not exert any inhibitory effect on *Staphylococcus aureus*.

Table 2: Sensitivity of Pathogens to Pure SBH and Standard antibiotics

Source of Anitibiontics	*Staphylococci aureus*	*Staphylococcus epidermidis*	*Pseudomonas aeruginosa*
Pure SBH	11.46±0.2	15.19±0.4	11.50±0.1
Erythromycin	10.50	5.50	8.50
Penicillin	8.50	6.00	8.00
Tetracycline	10.00	3.00	9.00
Cotrimoxazole	3.50	9.00	8.00
Gentamicin	8.00	ND	3.50
Ampicillin	7.50	ND	3.00
Cloxacillin	8.50	ND	1.5
Cefuroxime	ND	ND	ND

SBH – Stingless Bee Honey; ND-No inhibition was detected.

Sensitivity of *Staphylococcus epidermidis* to Antibiotics and Stingless Bee Honey

The mean inhibition zone of SBH (15.19±0.4) against *Staphylococcus epidermidis* was significantly higher than that of the eight antibiotics ($p<0.01$). No inhibitory effect of GEN, AMP, CXC and CFM on *Staphylococcus epidermis* was noted.

Sensitivity of *Pseudomonas aeruginosa* to Antibiotics and Stingless Bee Honey

SBH had the highest inhibitory effect against *Pseudomonas aeruginosa* compared to the standard antibiotics. Among the standard antibiotics, Tetracycline showed the highest inhibitory effect on growth of the Gram-negative bacterium, although the zone of inhibition was not statistically different to that observed for Erythromycin, Penicillin and Cotrimoxazole ($p>0.05$). The Gram–negative bacterium *Pseudomonas aeruginosa* was found to be totally resistant to Cefruroxime.

Table 3 shows the inhibition zones of various concentrations of SBH on the isolated organisms. Pure (100%) SBH exerted highest inhibitory effect on the *Staphylococcus epidermidis* microbe compared to the other test microbes; *Staphylococci aureus* and *Pseudomonas aeruginosa* ($F=196.65$; df 2,6; $p=0.00$). The inhibition on *Staphylococcus aureus* was not statistically different to that observed for the *Pseudomonas aeruginosa* ($p>0.05$). Zones of inhibitions increased with increasing concentration of SBH, with highest inhibitory effect on the Gram positive bacteria. Similarly, the 80% and 60% concentrations had significantly higher inhibitory effects compared to the 40% and 20% concentrations ($p=0.01$). However, the Gram-negative bacterium (*Pseudomonas aeruginosa*) was totally resistant to the 20% and 40% concentrations of SBH while the Gram-positive bacteria were sensitive to all concentrations of SBH.

Table 3: Zones of inhibition (mean ± SD) of varying concentrations of SBH against *Staphylococcus aureus* (SA), *Staphylococcus epidermidis* (SE) and *Pseudomonas aeruginosa* (PS)

Bacteria	Zones of inhibition of SBH (mean ± SD) in mm				
	20%	40%	60%	80%	100%
SA	0.36±0.2	4.50±0.1	8.46±0.18	10.68±0.2	11.46±0.2
SE	2.38±2.7	7.5±0.98	10.44±0.4	13.56±0.3	15.19±0.4
PS	ND	ND	9.50±0.00	10.25±0.0	11.50±0.1

ND-No inhibition was detected.

Table 4: Zones of inhibition (mean) of 10ug standard antibiotic compounds against *Staphylococcus aureus* (SA), *Staphylococcus epidermidis* (SE) and *Pseudomonas aeruginosa* (PS)

Bacteria	Tet	Gen	Ery	Cxc	Amp	Pen	Cxm	Cot
SA	10.0	8.0	10.5	8.5	7.5	8.5	0	3.5
SE	3.0	0.0	5.5	0.0	0.0	6.0	0	9.0
PS	9.0	3.5	8.5	1.5	3.0	8.0	0	8.0

The 10 µg/ml concentration of the standard antibiotics exhibited varying levels of antibacterial activity against the bacterial cultures tested as indicated by the zones of growth inhibition in Tables 4. Erythromycin exhibited the strongest activity on all three isolated pathogens. *Staphylococcus epidermis* was resistant to Gentamycin,

Ampicillin, Cefuroxime and Cotrimoxazole. Only Tetracycline, Erythromycin, Penicillin and Cotrimoxazole recorded a broad spectrum activity on the growth of the three isolates, with the strongest activity against *Staphylococcus aureus*. The three isolates tested were Cefuroxime-resistant.

From Table 5, the mean inhibition zones (in mm) produced by the pure (100%) SBH when applied to all the isolates was significantly higher ($p<0.05$) than the 10 µg/ml of each of the standard antibiotics. Similarly, at ≥ 60% concentration, the SBH exhibited broad spectrum inhibitory effect which was significantly higher than the 10 µg/ml of the standard antibiotics (($F= 5.63$; df 15, 24; $p=0.01$).

Table 5; Relative mean zones of inhibition of *Staphylococcus aureus* (SA), *Staphylococcus epidermidis* (SE) and *Pseudomonas aeruginosa* (PS) to SBH and Standard antibiotics.

Bacteria	SBH					Tet	Gen	Ery	Cxc	Amp	Pen	Cxm	Cot
	20%	40%	60%	80%	100%								
SA	0.36±0.2	4.50±0.1	8.46±0.18	10.68±0.2	11.46±0.2	10	8	10.5	8.5	7.5	8.5	0	3.5
SE	2.38±2.7	7.5±0.98	10.44±0.4	13.56±0.3	15.19±0.4	3	0	5.5	0	0	6	0	9
PS	0.00±0.0	0.00±0.0	9.50±0.00	10.25±0.0	11.50±0.1	9	3.5	8.5	1.5	3	8	0	8

DISCUSSION

Infection of the eye leads to conjunctivitis, keratitis, endopthalmitis and other infections which are responsible for increased incidence of morbidity and blindness worldwide [23]. These infections are a common occurrence in resource poor African countries due to poor hygiene and environmental contaminants. Furthermore, the problem of resistance, adverse responses and high cost of established antibiotic compounds have given rise to the search for new anti-infective agents [4-5]. To provide scientific support on the use of SBH in the treatment of eye infections, we tested various concentrations on some common eye pathogens and compared with 10 µg/ml of standard antibiotics commonly used in eye infections.

In this study, *Pseudomonas aeruginosa* was the organism most frequently isolated from the subjects with infected eye, representing 50% of the isolates, followed by *Staphylococci aureus* (31.25%) and *Staphylococcus epidermis* (18.75%). Several studies stated that *Pseudomonas aeruginosa* continued to be the organism responsible both for the majority and for the most serious eye infections, and that all recent efforts were directed to solve this problem [24-25]. However, this finding is not well correlated with a previous study in Ghana [26] which reported that *Streptococcus* species was the commonest isolate (20%), followed by *Staphylococcus aureus* (10%), among corneal ulcers. Continuous surveillance and epidemiological characterization of ocular infections are important in Ghana.
This study observed that SBH concentrations of ≥60% were more effective than the standard antibiotics, and in particular on *Pseudomonas aeruginosa*, which is responsible for a broad spectrum of eye infections. The result tends to agree with similar studies [6,12]. The antimicrobial activity of bee honey has been attributed to several properties of honey, including its osmotic effect, acidity, hydrogen peroxide, phytochemical factors, and seven tetracycline derivatives [6-7]. The high antimicrobial activity found support in this study against the two Gram-positive bacteria tested and one Gram-negative bacterium. This was evident in the mean zones of inhibition of bacterial growth with the pure SBH - as high as 15.19 mm for Staphylococcus epidermis, 11.46mm for *Staphylococcus aureus* and 11.50mm for *Pseudomonas aeruginosa*.

However, there was a differential sensitivity pattern to the SBH between the Gram-positive and Gram-negative bacteria as the lower concentrations (20% and 40%) did not inhibit the growth of the gram negative isolate, *Pseudomonas aeruginosa*. These variations were consistent with the reports of [14, 27-28] and have been attributed to the outer phospholipids membrane with structural lipopolysaccaride components, which make the gram negative cell wall impenetrable to antimicrobial agents while the Gram positive bacteria is more susceptible having only an outer peptidogycan, which is not an effective permeability barrier. With appropriate standardization of minimum inhibition concentration, the therapeutic application of honey in eye infections could effectively complement standard antibiotics with beneficial healing effects.

Also of interest is the finding that *Staphylococcus epidermis*, a major opportunist pathogen in the eye was totally resistant to the activity of 10 µg/ml gentamicin, ampicillin, cloaxicillin and cefuroxime; four standard antibiotics

originally noted for their strong activity against common eye pathogens, but was sensitive to all aqueous dilutions of SBH. This result confirms earlier reports of honey and standard antibiotics in the treatment of burn wounds[29].

The pH of the SBH was 3.8. Our results are in the range reported by other studies [30] which mentioned that honey is characteristically quite acidic, its pH being between 3.2 and 4.5. Acidification has been shown to promote healing by causing oxygen release from haemoglobin [31]

Collectively, our findings indicate that SBH from the International Stingless Bee centre in Ghana had a higher antimicrobial activity against the isolated microbes compared to the standard antibiotics and therefore, can offer a suitable and better alternative in managing common eye infections in the event of therapeutic failure with standard antibiotic compounds.

Conclusion and Recommendations

In this study we investigated the antimicrobial activity of different concentrations of the stingless bee (*Meliponula bucandei*) honey collected from the International Stingless Bee Centre at Abrafo in the Central Region of Ghana against three bacteria isolates representing two Gram positives (*Staphylococcus aureus* , *Staphylococcus epidermidis* and one Gram negative (*Pseudomonas aeruginosa*) from infected ocular sites, and compared with 8 standard antibiotic agents commonly used in ophthalmology.

The standard antibiotics used in concentrations of 10µg/ml varied in their activity against the isolates (*Staphylococcus aureus*, *Staphylococcus epidymidis*, and *Pseudomonas aeruginosa*) but was generally lower than the antibacterial activity of the SBH at concentrations ≥ 60%. These differences were statistically significant.
This study therefore justifies the claimed uses of the SBH in the traditional system of managing various infectious diseases caused by microorganisms. The result of the study is encouraging to the certain degree that it can reinforce the concept of ethno-botanical approaches to drug discovery through screening of other traditional agents as potential source of bioactive substances

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