

Phytochemical screening and biological activities of *Hypotrigena squamuligera* raw honey

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ABSTRACT

Aims: To evaluate phytochemical content and biological activities of *Hypotrigena squamuligera* honey.

Study design: Standard chemical tests, DPPH antiradical activity assay and agar diffusion methods

Place and Duration of Study: Department of Chemistry (Natural product section) and Biological Science department November 2011 and March 2012.

Methodology: Standard chemical tests, DPPH antiradical activity and agar diffusion methods were adopted to determine phytochemical compounds present, antioxidant and antibacterial activity respectively. The following bacterial strains were employed in the screening studies: *Staphylococcus aureus* and *Escherichia coli*, all from stock cultures of Bindura University biological sciences department.

Results: Phytochemical screening revealed presence of tannins, alkaloids, flavonoids, terpenoids, anthraquinones and cardiac glycosides. *H. Squamuligera* honey exhibited marked antioxidant and antibacterial activity. Its antiradical activity was lower than that of ascorbic acid and quercetin at low concentrations 0,0125 and 0,025mg/ml however as from 0,05mg/ml methanolic extract exhibited significantly higher antioxidant activity. *H. squamuligera* honey (100%) produced a greater inhibition ($24 \pm 0,09$ mm) than amoxicillin ($19 \pm 0,01$ mm) for *S. aureus*. For *E. coli* amoxicillin produced greater inhibition than *H. squamuligera* honey. Compared to *Apis mellifera* honey at a concentration of 100%, *H. squamuligera* honey revealed better inhibitory activity. Minimum inhibitory values ranged from 10 - 25 µg/ml. The lowest MIC value (15 µg/ml) for *H. squamuligera* honey was recorded for *S. aureus*.

Conclusion: The results suggest that *H. squamuligera* honey could potentially be used as an alternative pharmaceutical agent against certain microorganisms and source of natural antioxidants for promotion of health however there is a need to perform structural elucidation to identify the structures of phytochemical compounds present in the honey.

Keywords: *Hypotrigena squamuligera*, phytochemicals, antioxidant activity, antibacterial activity

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1. INTRODUCTION

Resistance of pathogenic micro-organism to current medicines is on the rise prompting a need to search for alternative medicines (Al-Jasser 2006; Sundqvist and Kahlmeter, 2005; Gulluce et al., 2006; Halama and van Haluwin, 2004; Rankovic et al., 2008). Honey offers attractive options which include wound healing and antibacterial activity (Mavric et al 2008; Blair and Carter 2005; Church et al 2006; Ainul Hafiza et al 2005). Although composition and antimicrobial activity of honey has been effectively established it differs according to the type and source (Tumin et al 2005; Lusby et al 2005; Basson and Grobler 2008). Currently no extensive research on phytochemical composition and antibacterial activity of *Hypotrigona squamuligera* honey has been conducted. The objectives of this study were to conduct such studies so as to authenticate its use in folk medicine. Information obtained from social gathering indicates that infection on wounds is rapidly cleared, swelling and inflammation quickly disappears when *Hypotrigona squamuligera* honey is applied. Serious burns, stubborn wounds and cervical ulceration are said to disappear completely. Some traditionalist confessed cancer healing, analgesic and soothing ability of *Hypotrigona squamuligera* honey. It is also believed to shorten the duration of diarrhoea in patients. *Hypotrigona squamuligera* honey is widely used as medicine and food in many African countries such as Zimbabwe, Zambia, Mozambique, Botswana, Cameroon, Niger and Senegal (Eardley 2004). The insect *Hypotrigona squamuligera* and its honey are shown in Fig 1 below



Fig 1. *Hypotrigona squamuligera*, its honey and hive entrance

2. MATERIAL AND METHODS

2.1 Collection of Honey

The honey used in this study was obtained from Chinyani village in Murewa, Mashonaland East, Zimbabwe in November 2011. Samples were stored in a freezer before use. Samples were collected from ten different hives and mixed into one composite sample.

2.2 Extraction

For extraction, 50g of honey (*Hypotrigona squamuligera* and *Apis mellifera*) was taken and added to 125mL of methanol in a conical flask. Later the solution was mixed well by vortexing and centrifuged at $1532 \times g$ for 10 minutes at 25°C. The extracts were filtered over Whatman No.1 filter paper into stoppard test tubes. The extract was dried at reduced pressure at 40°C to a constant mass of $17,34 \pm 0,07$ g/Kg for *H. squamuligera* and 14.58g/Kg for *Apis mellifera*. The extraction was performed six times using different samples at each time. The extract was kept in a freezer (0-5 °C) before use.

61 2.3 Phytochemical screening

62 Phytochemical tests were carried out on pure honey using standard chemical test reported
63 by Sofowora, (1994) with slight modifications. Two millimeters of honey was mixed with
64 aqueous sulphuric acid and benzene to test for presence of anthraquinone glycosides. Also,
65 Mayer and Dragendorff's reagents were employed to test for presence of alkaloids. With the
66 addition of gelatin and ferric chloride solution, test for tannins was conducted. Keller-keliani
67 and Legal tests were carried out for cardiac glycosides, frothing tests for saponins and 1%
68 aluminum chloride solution in methanol was used to investigate flavonoids. Lastly,
69 Benedict's and Fehling's solutions were added to determine presence of reducing sugars.

70 2.4 Determination of antioxidant activity

71 The radical scavenging activities of *Hypotrigena squamuligera* honey against 2,2-Diphenyl-
72 1-picryl hydrazyl radical (DPPH) (Sigma-Aldrich) were determined by UV spectrophotometry
73 at 517 nm (Hitachi UV vis spectrophotometer model U-200(Japan). The following
74 concentrations of honey extract/ quercetin/ ascorbic acid were prepared, 0.0125, 0.025, 0.05
75 and 0.1 mg/ml in methanol. Both the solutions were prepared from a stock solution of
76 5mg/ml prepared by mixing 0.25g in 50ml methanol. One milliliter of the extract was placed
77 in a test tube, followed by 0.5 ml of 1 mM DPPH in methanol. The radical scavenging
78 activity was calculated using the following formula:

79 % radical scavenging effect =
$$= \frac{A_c - A_s}{A_c} \times 100$$

80 where A_c is the absorption of the control sample (methanol + DPPH) and A_s is the absorption
81 of the sample, methanol + DPPH. The blank consisted of the sample + methanol.

82 2.5 Antibacterial Activity of Methanolic Extract

83 The extracts were diluted with absolute methanol to produce the following concentrations:
84 10, 30, 50 and 100% (v/v). Nutrient agar was melted and then kept in a steam bath set at
85 30°C to prevent solidification. Six Petri dishes were pre-inoculated with *E.coli* (ATCC25922)
86 and another six with *S. aureus* (ATCC29213) in the following manner. One milliliter of the
87 bacterial suspension was pipetted into the appropriately labeled Petri dish to which 25ml of
88 molten agar was then added followed by thorough mixing of the bacteria and molten agar.
89 The agar was allowed to set for 1hr. Holes of 3 x 4mm wide were then made in the agar
90 using a borer. Extracts (25µl) of a specific concentration was introduced into each of the
91 holes in an appropriately labeled Petri dish using a sterile micropipette. Amoxicillin (10µg/ml)
92 was used as a positive control and absolute methanol as a negative control. The dishes
93 were then incubated at 25°C for 24hours after which zones of inhibition were measured and
94 recorded. The zone of inhibition was taken to be the diameter of the zone visibly showing the
95 absence of bacterial growth including the 4mm hole. Where there was no inhibition, the
96 value of 0 mm was assigned to the test sample.

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2.6 Minimal Inhibitory Concentration (MIC) Determination

The Minimum Inhibitory Concentration (MIC) was determined and defined as the lowest concentrations of the solution of the *Hypotrigena squamuligera*, amoxicillin and *Apis mellifera* honey at which the microorganisms tested do not show visible growth. The MICs were determined by micro-dilution techniques. A suspension of each sample to be tested was diluted from 1 to 50µg/ml. The plates were incubated at 37°C and the MICs were determined after 24 hours of incubation.

2.6 Statistical Analyses

Results for antioxidant and antibacterial activity are presented as mean value $\pm$ standard deviation for six determinations). ANOVA was applied to compare antioxidant activity values between the honey and standard antioxidants followed by multiple comparisons using the least significant difference (LSD) test. Differences were considered statistically significant at $p = 0.05$. Student t test was used to compare antibacterial activity of the *Hypotrigena squamuligera* honey, amoxicillin and *Apis mellifera* honey. All statistical analyses were performed using SPSS 16 software (SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

Since ancient times *Apis mellifera* honey has been used for its medicinal properties in many societies. Information on the use of honey for the treatment of many human ailments can be found in magazines, beekeeping journals and natural products leaflets however little is known about *Hypotrigena squamuligera* honey. The phytochemical analysis carried out on methanolic extract showed the presence of tannins, flavonoids, alkaloids, terpenoids, anthraquinones and cardiac glycosides as shown in Table 1.

152 Table 1: Phytochemical components of the extracts in *Hypotrigena squamuligera* honey
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Phytochemical component	Remarks
Alkaloids	+
Flavonoids	+
Tannins	+
Saponins	+
Terpenoids	+
Cardiac glycosides	+
Anthraquinones	+

154 + = present, - = absent

155 The results of antioxidant activity of different concentrations of methanolic extract and
156 standard antioxidant (quercetin and ascorbic acid) are shown in Table 2. The extract
157 exhibited marked antioxidant activity by scavenging DPPH (free radical) and converting it
158 into DPPHH. The extract has shown concentration dependent radical scavenging activity.
159 The scavenging activity of standards was higher than that of methanol extract at low
160 concentrations however after a concentration of 0,05mg/ml methanolic extract exhibited
161 significantly higher antioxidant activity (ANOVA analysis, $p > 0,05$). Phytochemicals such as
162 flavonoids, tannins and saponins Aliyu et al (2008) constitute a major group of compounds
163 that act as antioxidants. They consist of high redox potentials which enable them to act as
164 reducing agents, hydrogen donors and singlet oxygen quenchers (Kähkönen et al., 1999).
165 Delocalization of electrons over the phenolic ring and stabilization by resonance effect of the
166 aromatic nucleus makes the antioxidant radical unreactive (Tsao and Akhtar, 2005). The
167 antioxidant effect of *Hypotrigena squamuligera* honey may be rationalized in terms of the
168 large number of phytocompounds present. (Lachman et al. (2010a, 2010b); Phillips et al
169 2009; Gheldof and Engeseth, 2002) carried out studies and found out that honey consisted
170 of various phenolic compounds which differ according to origin of the honey. The antioxidant
171 capacity of honey was as a result of the combined activity of a wide range of compounds
172 including phenolics, peptides, organic acids and enzymes.

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176 Table 2: Antioxidant activity of methanolic extract of *Hypotrigona squamuligera* honey,
177 ascorbic acid and quercetin by DPPH free radical scavenging assay

% radical scavenging activity $\pm$ SD, n = 6			
Concentration(mg/ml)	Methanolic extract	Ascorbic acid	Quercetin
0.0125	7.35 $\pm$ 0,05	83.80 $\pm$ 0,05	80.70 $\pm$ 0,04
0.025	76.4 $\pm$ 0,04	85.80 $\pm$ 0,03	82.60 $\pm$ 0,04
0.05	100 $\pm$ 0,02	86.20 $\pm$ 0,04	85.10 $\pm$ 0,05
0.1	100 $\pm$ 0,05	99.00 $\pm$ 0,05	98.40 $\pm$ 0,05

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179 The results of antibacterial activity of methanolic extract of *Hypotrigona squamuligera* honey
180 are shown in Table 3. The results were recorded as presence or absence of zones of
181 inhibition around the well. The inhibitory zone around the well indicated the absence of
182 bacterial growth.

183 Table 3: Antibacterial activity of methanolic extract of *Hypotrigona squamuligera* honey for
184 extract concentration 0, 10, 30, 50, 100% and *Apis mellifera* 100% (v/v).

Test organism	Diameter of zone of inhibition (mm), $\pm$ SD, n = 6						
	0% (-control)	10%	30%	50%	100%	Amoxicillin (10 μ g/ml)	<i>Apis Mellifera</i> (100%v/v methanol)
<i>E. coli</i>	0	8 ^a	13 ^b	15 ^a	19 ^b	21 ^b	17 ^b
<i>S. aureus</i>	0	11 ^a	12 ^b	15 ^c	24 ^c	19 ^a	15 ^b

185 a = $\pm$ 0,01; b = $\pm$ 0,06; c = $\pm$ 0,09

186 The data obtained showed a dose dependent inhibitory action of *H. squamuligera* honey.
187 The results suggest that *S. aureus* is more susceptible to *H. squamuligera* honey than *E. coli*
188 with diameter zone of inhibition of 24 and 19mm respectively. *H. squamuligera* honey

(100%) produced significantly greater inhibition than amoxicillin for *S. aureus* Student t test p > 0,05. For *E. coli* amoxicillin produced greater inhibition than *H. squamuligera* honey. Compared to *Apis mellifera* honey at a concentration of 100%, *H. squamuligera* honey revealed better inhibitory activity Student t test p > 0,05. Differences in antibacterial activity of *H. squamuligera* can be as a result of differences in differential susceptibility of bacterial cell walls, which is a result of slight differences inherent in the cell wall structure (Zhao et al. 2001). Gram-positive and gram-negative microorganisms differ in many features other than the structure of their cell walls, for example the presence of lipoproteins and lipopolysaccharides in gram-negative bacteria form a barrier to hydrophobic compounds Mazutti et al. (2008). Antimicrobial activity of honey has been attributed to hydrogen peroxide, which is produced by naturally occurring glucose oxidase, and phenolic compounds (Taormina et al 2001). When honey is used in wound dressing hydrogen peroxide is produced by dilution of the honey with body fluids resulting in it being released slowly and acts as an antiseptic. Honey chelates and deactivates free iron, which would otherwise catalyze the formation of oxygen free radicals from hydrogen peroxide, leading to inflammation. Also, the antioxidant constituents in honey help clean up oxygen free radicals present. The Minimum inhibitory concentration (MIC) values are shown in (Table 4). They ranged from 10 - 25 µg/ml. The lowest MIC value (15 µg/ml) for *H. squamuligera* honey was recorded for *S. aureus*.

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Table 4. Minimum inhibitory concentration (MIC) of *Hypotrigona squamuligera*, *Apis mellifera* honey and amoxicillin

Test organism		concentration (µg/ml)								
		1	2	5	10	15	20	25	30	50
<i>H. squamuligera</i>	<i>S. aureus</i>	-	-	-	-	+	+	+	+	+
	<i>E. coli</i>	-	-	-	-	-	+	+	+	+
<i>A. mellifera</i>	<i>S. aureus</i>	-	-	-	-	-	-	+	+	+
	<i>E. coli</i>	-	-	-	-	-	+	+	+	+
Amoxicillin	<i>S. aureus</i>	-	-	-	-	+	+	+	+	+
	<i>E. coli</i>	-	-	-	+	+	+	+	+	+

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+ has effect and there is a zone of inhibition

- has no effect and there is no observable zone of inhibition

CONCLUSION

The results of the present research show that *H. squamuligera* honey consist of many bioactive compounds, significant antioxidant and antibacterial activity. It showed better antioxidant activity than standard antioxidants, ascorbic acid and quercetin. Compared with *Apis mellifera* honey, *H. squamuligera* honey showed superior antibacterial activity. Significant potency of *H. squamuligera* honey against certain microorganisms suggests its potential to be used as an alternative therapeutic agent for a number of medical conditions, particularly bacterial infections and wound healing. The study scientifically authenticate the use of *H. squamuligera* honey in folk medicine

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

Author A designed the study, performed the statistical analysis, wrote the protocols, and wrote the first and final draft of the manuscript. 'Author B, C and D' checked the analyses and managed literature searches of the study. All authors read and approved the final manuscript.

REFERENCES

1. Ainul Hafiza, A.H., Yusof, N., Maimon, A. (2005). Potential of Malaysian local honey as an antibacterial agent. *Sains Malaysiana*, 34(1),17-20.
2. Aliyu, A.B., Musa, A.M., Abdullahi, M.S., Oyewale, A.O. (2008). Phytochemical and antibacterial properties of *Ludwigia suffruticosa* (Willd.) Oliv. ex. O. Ktze (Onagraceae). *Int. J. Appl. Sci*, 2(4), 1-5. (IJPAS Online).
3. Al-Jasser, A.M. (2006). *Stenotrophomonas maltophilia* resistant to trimethoprim-sulfamethoxazole: An increasing problem. *Ann Clin Microbiol Antimicrob*, 5, 23.
4. Basson, N.J., Grobler, S.R. (2008). Antimicrobial activity of two South African honeys produced from indigenous *Leucospermum cordifolium* and *Erica* species on selected micro-organisms. *BMC Complement Altern Med*, 8, 41.
5. Blair, S.E., Carter, D.A. (2005) The potential for honey in the management of wounds and infections. *J Australian Infect Control*, 10(1), 24-31.
6. Church, D., Elsayed, S., Reid, O., Winston, B., Lindsay R. (2006) Burn wound infections. *Clin Microbiol. Rev*, 19(2), 403-434.
7. Eardley, C.D. (2004). Taxonomic revision of the African stingless bees. *African plaant protection*. 10, 63-96.
8. Gheldof, N., Engeseth, N.J. (2002). Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. *J Agric Food Chemistry*, 50, 3050–3055
9. Gulluce, M., Asian, A., Sokmen, M., Sahin, F., Adigüzel, A., Agar, G., Sokmen, A. (2006). Screening the Antioxidant and Antimicrobial Properties of the Lichens *Parmelia saxatilis*, *Parmelia glauca*, *Ramalina pollinaria*, *Ramalina polymorpha* and *Umbilicaria nylanderina*. *Phytomedicine*, 13(7), 515-521.

10. Halama, P., van Haluwin, C. (2004). Antifungal Activity of Lichen Extracts and Lichenic Acids. *BioControl*, 49(1), 95-107.
11. Kähkönen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.P., Pihlaja, K., Kujala, T.S. (1999). Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.* 47, 3954-3962.
12. Lachman, J., Hejtmánková, A., Sýkora, J., Karban, J., Orsák, M., Rygerová, B. (2010). Content of major phenolic and flavonoid antioxidants in selected Czech honey. *Czech Journal of Food Sciences*, 28(5), 412-426.
13. Lachman, J., Orsák, M., Hejtmánková, A., Kovářová, E. (2010). Evaluation of antioxidant activity and total phenolics of selected Czech honeys. *LWT – Food Science and Technology*, 43(1), 52-58.
14. Lusby, P.E., Coombes, A.L., Wilkinson, J.M. (2005). Bactericidal activity of different honeys against pathogenic bacteria. *Arch Med Res*, 36(5), 464-467.
15. Mavric, E., Wittmann, S., Barth, G., Henle, T. (2008). Identification and quantification of methylglyoxal as the dominant antibacterial constituent of manuka (*Leptospermum scoparium*) honeys from New Zealand. *Mol Nutr Foods Res*, 52(4), 483-489.
16. Mazutti, M., Mossi, A.J., Cansian, R.L., Corazza, M.L., Dariva, C.J., Oliveira, V. (2008). Chemical profile and antimicrobial activity of Boldo (*Peumus boldus* Molina) extracts obtained by compressed carbon dioxide extraction. *Braz. J. Chem. Eng.* 25 (02) , 427- 434, April – June.
17. Phillips, K.M, Carlsen, M.H, Blomhoff, R. (2009). Total antioxidant content of alternatives to refined sugar. *J. Am Diet Assoc.* 109(1), 64-71
18. Rankovic, B., Mišić, M., Sukdolak, S. (2008). The Antimicrobial Activity of Substances Derived from the Lichen *Physcia aipolia*, *Umbilicaria polyphylla*, *Parmelia caperata* and *Hypogymnia physodes*. *World J. Microbiol. Biotechnol.* 24(7), 1239-1242.
19. Sofowora, E.A. (1994). Medical Plant and Traditional Medicine in Africa. University of Ife Press. Nigeria.1, 1-23.
20. Sundqvist, M., Kahlmeter, G. (2005). *E. coli* and *S. aureus*– antimicrobial resistance surveillance and the effect of excluding duplicate isolates in a consecutive database 1990–2003.15th European Congress of Clinical Microbiology and Infectious Diseases. Copenhagen / Denmark, April 2-5.
21. Taormina, P.J., Niemira, B.A., Beuchat, L.R. (2001). Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power, *Int. J Food Microbiol.* 69(3), 217-25.
22. Tsao, R. and Akhtar. M.H. (2005). Nutraceuticals and functional foods I: Current trend in phytochemical antioxidant research. *Agric. Food Chem. Environ.*, 3,10-17.

- 332 23. Tumin, N., Halim, N.A., Shahjahan, M., Noor Izani, N.J., Sattar, M.A., Khan, A.H.,
333 Mohsin, S.S.J. (2005). Antibacterial activity of local Malaysian honey. Malaysian
334 Journal of Pharmaceutical Sciences , 3(2),1-10.
335
- 336 24. Zhao, W.H., Hu, Z.O., Okubo, S., Hara, Y., Shimamura, T. (2001). Mechanism of
337 synergy between epigallocatechin gallate and β lactams against methicillin-resistant
338 *Staphylococcus aureus*. Antimicrob. Agents Chemother. 45, 1737- 1742.
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340