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Antioxidant Capacity and Microbial Attributes of Raw Cow Milk Fortified with *Hypotrigona Squamuligera* Honey

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Abstract - Diseases such as diabetes, cancers, hypertension and obesity are problematic diseases in the adult population. The use of functional foods that consists of phytocompounds such as antioxidants that can act as prophylactics against such diseases has received considerable attention by the academia, food industry and the general public. The present study aimed at investigating antioxidant capacity and microbial attributes of cow milk fortified with Hypotrigona squamuligera honey. Antioxidant capacity was assayed using 2.2-diphenyl- 1-picrylhydrazyl (DPPH) antiradical activity and peroxide value method. Pour plate assay was used to investigate microbial attributes. The fortified milk revealed a significant DPPH antiradical activity than unfortified milk $p > 0.05$. A higher percentage inhibition of 98.38 ± 0.40 was achieved with fortified milk as compared to 47.27 ± 1.00. Fortified milk also exhibited significantly lower peroxide values and microbial attributes than unfortified milk. The present study results reveals that fortifying milk with H. squamuligera honey improves its antioxidant capacity and microbial inhibitory activity. This means that fortifying milk with H. squamulingera honey preserve milk and maintains its health promoting effects.

Keywords : *antioxidant capacity, microbial attributes, hypotrigona squamuligera, fortified milk, honey.*

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I. Introduction

Studies in alternative methods of counteracting adult long standing diseases such as type 2 diabetes, obesity, hypertension, and cancers has increased over the years (Ariyoshi et al., 2004). Diet management is one of the most effective ways of lowering the occurrence of such diseases in the general public (Praveeshi et al., 2011). Milk plays an important role in the diet (Chirlaque, 2011). Drinking raw milk is believed to have many advantages (Chirlaque, 2011). It can act as an immune system booster. This is because it consists of natural lymphocytes, antibodies, hormones and growth factors (Chirlaque, 2011). It can also help in fighting diseases such as gout, kidney stones, breast cancer, rheumatoid arthritis and migraine headaches. Raw milk has been used successfully to cure diseases in the past. Raw milk is a wholesome food source of many essential nutrients such as vitamin B, C and antioxidants (Krushna et al., 2007). Antioxidants inhibit the accumulation of free radicals in the body (Francois et al., 2010). Free radicals have been implicated in the pathology of diseases such as cancers, diabetes, obesity and cardiovascular diseases. Vitamins help in preventing breast cancer, colorectal cancer and pancreatic cancer. Although raw milk is beneficial unfortunately it is not free from microbial contamination (Tasci, 2011). Contaminating bacterial can be pathogenic and therefore resulting in serious illness. Heating and sealing is the widely used method. Although this is an effective method unfortunately it destroys useful milk components such as enzymes and antioxidants that are crucial in maintaining health in humans. Other methods of ensuring that milk is free from harmful microorganisms include the use of H_2O_2 and lactoperoxidase system however their acceptance by the public is low. *Apis mellifera* honey has also been tried and interesting results were reported (Krushna et al., 2006). The advantage of using honey is that it is a food substance and it consists of many other useful components such as antioxidants which have been reported to help in preventing current problematic adult diseases. This study aimed at investigating antioxidant capacity and microbial attributes of raw cow milk fortified with *Hypotrigona squamuligera honey. A study conducted by (Dzomba et al., 2012) revealed that H. squamuligera* Fig 1 honey Fig 1 consists of invitro antibacterial and antioxidant activity. *H. squamuligera* honey is used to treat various diseases in folk medicine therefore addition of its extract to milk may help in increasing the prophylactic activity of milk.

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II. Materials and Methods

a) Honey Collection

H. squamulingera honey used in this study was collected from Chinyani village forests in Murewa, Mashonaland East, Zimbabwe in November 2012. Samples were stored in a freezer before use.

b) Extraction

Two grams of honey was added to 20ml of absolute ethanol in a conical flask. The contents were mixed by vortexing and centrifuged at 3000rpm for 10 minutes. The supernatants were then collected, filtered using a Whatman filter paper No. 1 into stoppard test tubes and then dried under vacuum at 40oC. The yield was recorded. The extract was dissolved in ethanol/water (2:3) mixture and stored in a freezer at between 0-5oC.

c) Collection of Milk

Raw milk was collected from farms around Bindura. The milk was divided into two. One lot was quickly fortified with *H. squamuligera* honey extracts at different concentrations. Both the fortified and unfortified milk was placed in a cooler box and transported straight to the laboratory.

Fig. 1 : H. squamulingera insect and its honey

d) DPPH Antiradical Assay

A volume of 1 ml of fortified/unfortified milk was added to 2 ml of DPPH (100µM) dissolved in ethanol. Absorbance of the samples was measured at 517nm after standing for 30 minutes. The blank consisted of ethanol/water mixture. Percentage antiradical activity was calculated as follows;

$$\% \text{ antiradical activity} = \frac{Ac - AS}{Ac} X\ 100$$

Where A_c is the absorbance of DPPH solution without milk, A_s the absorbance of milk and DPPH solution.

e) Peroxide Value Determination

This was performed following a method reported by (Lafka et al., 2007) with some modifications. Two millimeters of milk (fortified or unfortified, 10ml of chloroform, 15ml of acetic acid and 1 ml potassium iodide (10%) were added to a stoppard conical flask. The flask was shaken for 2 minutes and left standing for 10 minutes in the dark. The contents were then titrated with 0.01M sodium thiosulphate and 1% starch as the indicator. A blank run was also performed. Peroxide value expressed as mmoles of active oxygen per Kilogram was calculated as follows.

$$\text{PV (mM/Kg)} = \frac{V - Vo\ X\ C\ X\ 1000}{m}$$

Where V is the volume of sodium thiosulphate for the sample, Vo is volume of sodium thiosulphate of blank, C is the concentration of sodium thiosulphate and m the mass of the sample in g, All assay were repeated five times.

f) Microbial attributes

Enumeration of total viable counts was performed by tenfold dilution of milk samples using ringer solution. Total Viable Count (TVC) was determined using the pour plate technique. Each dilution 0.1 was transferred using a sterile pipette into Petri dishes followed by nutrient agar. The plates were incubated at 37oC for 48 hours. Following incubation plates exhibiting 30-300 colonies were counted. The average number of colonies in a particular dilution was multiplied by the dilution factor to obtain TVC. TVC was expressed as the number of organisms of Colony Forming Units (CFU) of samples according to (ISO, 1995).

III. Statistical Analysis

The data is presented as mean ± Standard deviation of five determinations. T-test, $p = 0.05$ was used to compare results of fortified and unfortified milk.

IV. Results and Discussion

a) DPPH antiradical activity

Fortified milk exhibited the highest DPPH antiradical activity. Antioxidant activity remained constant from day 1 to day 4 Table 1. From day 4 percentage inhibitory activity decreased to 77.45 ± 0.80% Table 1. This shows that antioxidants were used to counteract free radicals. Unfortified milk antiradical activity was significantly lower than that for fortified milk in all days. Values started to decrease from day 1 Table 1. Milk consists of appreciable levels of lipids. During storage lipids are easily attacked by oxygen forming peroxyl radicals which are dangerous to health. Even though milk consists of natural antioxidants these are quickly depreciated. This is shown by a quick decrease in antiradical activity. Adding functional foods fortify the milk such that its integrity is maintained. The present results show that fortifying milk with *H. squamuligera* honey maintains its antioxidant properties for some time therefore its health promoting effect.

b) Peroxide Value Determination

The primary products of lipid oxidation are hydroperoxides. Determination of peroxide value gives an indication of how far the milk has gone bad. Peroxidation value for the fortified milk was significantly

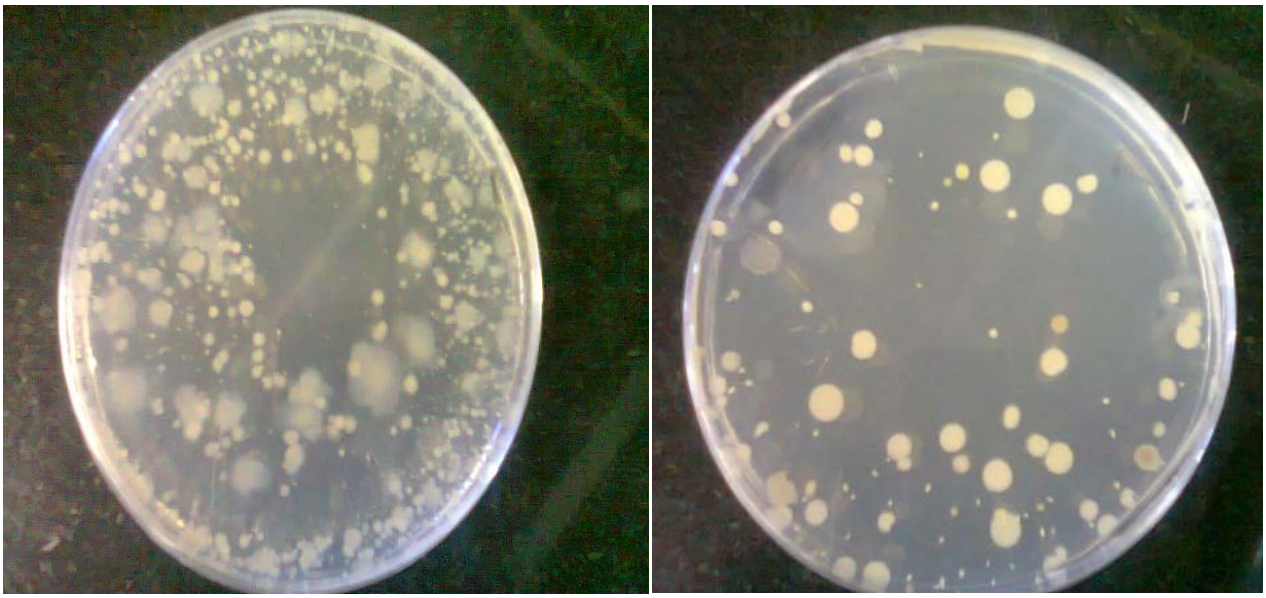
lower than that for the unfortified milk. Peroxidation values for the fortified milk were almost constant Table 2 revealing stabilization. Peroxidation values for unfortified milk increased from day 1 showing quick deterioration.

c) Microbial Attributes

Total Viable Count results reveal that fortify milk with H. squamuligera honey reduces multiplication of bacteria in milk Fig 2.

Table 1 : DPPH free radical scavenging assay

Percentage inhibition ± SD n = 5, All results significantly different T-test, p = 0.05					
	Day 1	Day 2	Day 3	Day 4	Day 5
Fortified	98.00 ± 0.26	97.50 ± 0.50	98.38 ± 0.40	77.45 ± 0.80	73.23 ± 0.50
Unfortified	47.27 ± 1.00	34 .50 ± 0.93	28.86±1.06	18.32 ± 1.30	2.00 ± 0.10



A. B.

Figure 2 : Antibacterial activity of (A) unfortified raw milk (B) raw milk fortified with ethanolic extract of *H. squamuligera* honey

Table 2 : Mean peroxide value for fortified and unfortified milk

% Peroxide value (mM/Kg) ± n = 5 All results significantly different T-test, p = 0.05					
	Day 1	Day 2	Day 3	Day 4	Day 5
Fortified	5.57 ± 0.18	7.05 ± 0.54	7.13 ± 0.15	8.40 ±1.15	8.30 ± 0.34
Unfortified	25.10 ± 0.72	38.20 ± 0.17	55.20 ± 0.07	56.2 ± 0.10	55.20 ± 1.31

Table 3 : Antibacterial activity of ethanolic extract of H. squamuligera honey for extract concentration 10, 20, 50, 100% and unfortified raw milk

Time	Bacterial count cfu/ml n =5, * = significantly different T-test, p = 0.05				
	10%	20%	50%	100%	Unfortied Milk
Day 1	4.5×10^{2}	4.2×10^{2}	3.8×10^{2}	3.2×10^{2}	5.5×10^{2}
Day 2	1.32×10^{6}	1.28×10^{6}	1.01×10^{6}	$4.5\times10^{2*}$	1.41×10^{7}*
Day 3	1.20×10^{7}	1.16×10^{6}	1.11×10^{6}	$8.7\times10^{2*}$	1.70×10^{7}*
Day 4	1.10×10^{7}	1.09×10^{7}	8.50×10^{6}	$1.67\times10^{2*}$	1.50×10^{7}*

Samples that were treated with H. squamuligera honey extracts significantly inhibited the bacteria growth, T-test p > 0.05 as compared to unfortified raw milk. The mean bacterial count of fortified milk was 7.13 X 105 at a concentration of 100% (v/v) and for unfortified was 1.17 X107. Bacterial multiplication inhibition was concentration dependent. Low inhibition was observed at 50% and less. According to (Chambers 2002) when raw milk is of good quality the bacterial load should be around 103/ml. Bacterial load of 107/ml show poor

quality milk. Present results Table 2 show that raw milk collected from the farms bacterial load falls within 103/ml showing quality milk. However milk quality deteriorated as days went by except that for 100% extract concentration. Even on the fourth day its bacterial load was far below 103/ml. The antibacterial activity of honey can be attributed to peroxide and non peroxide components. H2O2 is the major antibacterial factor in honey (Krushna et al., 2007). It is produced during oxidation of glucose and other monosaccharides by glucose oxidase. Non peroxide components include phenolic acids and flavonoids. Phenolic acids and flavonoids are important phytocompounds that have been shown to promote health in humans (Francois et al., 2010).

V. Conclusion

Results of the present study are interesting. Fortifying milk with H. squamuligera honey extracts improves its antioxidant capacity and its microbial attributes. Quality raw milk is favoured by the public because it consists of many nutrients that are believed to promote health. It consists of micronutrients that can act as prophylactics against most adult diseases such as middle age cancers and diabetes type 2. Honey is an attractive option for use in preserving milk because it is a food substance and has been shown to consist of antioxidants.

VI. Acknowledgement

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