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Evaluation of Extracted Honey Bee Propolis as a Natural Preservative of Raw Cow's Milk in Awi Zone, North western Ethiopia

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Research Article

Keywords: Alcohol test, Microbiological Examination, Raw Milk, Propolis, propolis extract

Posted Date: May 14th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9591829/v1

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Additional Declarations: No competing interests reported.

Abstract

This study aimed to evaluate the effectiveness of honey bee propolis as a natural preservative for raw cow's milk. Propolis was collected from three agro-ecologies in the Awi zone Banja, Dangla, and Chagni, and extracted using 70% ethanol. Raw milk samples were obtained from the Injibara University dairy farm and divided into five groups, with different concentrations (0ml, 5ml, 10ml, 15ml, and 20ml) of propolis added to 100ml of milk in sterilized bottles.

The study analyzed the milk's quality through clot-on-boiling, alcohol, and methylene blue reduction tests, and microbiological parameters, including aerobic plate count (ABC), coliform count (CC), and yeast and mold counts (YMC). Data analysis was performed using SAS version 9.4. Results indicated that both agro-ecology and propolis concentration significantly impacted raw milk quality ($P < 0.001$), with interaction effects on coagulation and microbial counts ($P < 0.05$). The control group showed the shortest coagulation time, while propolis-treated milk exhibited the longest.

Furthermore, microbial quality was influenced by both the agro-ecology and propolis concentration, with the lowest microbial load found in milk treated with 5ml and 10ml of propolis from the Guangua Woreda (lowland). In conclusion, the study demonstrated that propolis, particularly at 5ml and 10ml concentrations from Guangua Woreda, effectively extended the shelf life of raw milk by reducing microbial load.

Introduction

In Ethiopia, there are about 15 million dairy cows that collectively producing approximately 4.69 billion liters of milk annually, on with an average of 1.48 liters per cow per day during lactation period of 210 days (CSA, 2021b, a). Milk is defined as a fresh, clean and normal secretion from mammary glands, secretion obtained through the milking of properly fed and cared dairy cows (Mbuk et al., 2016). As an essential source of nutrition, milk consumption is steadily increasing alongside the growth of human population (Kassa et al., 2020).

In Ethiopia, and specifically in the Awi zone of our study area, milk and dairy products play significant role in both nutrition of consumers and income of producers. currently, the estimated annual total milk production at national level is 4.7 billion liters, with Amhara region contributing 820,094,328 liters and Awi zone producing 65,557,551liters (CSA, 2021b). Despite the high milk production in the study area and the country as a whole, milk is prone to spoilage, due to its favorable conditions for the growth of various microbes(Adugna Eshetu, 2021).

To prevent milk spoilage and foodborne health risks, various preservation methods, such as smoking with herbal plants and using chemicals like Sorbic acid (E200), benzoic acid (E210), sodium benzoate and potassium sorbate have been widely applied around the world (Owusu-Kwarteng et al., 2020). However, synthetic chemicals can cause undesirable side effects for consumers (Johnson et al., 2021). Moreover, the growing consumer preference for natural preservatives has had a significant impact on the food industry (Mesías et al., 2021). Researchers worldwide have sought neutral food preservatives that avoid contamination and harm to consumers (Basa et al., 2016, 2020, Hadi Koohsari, 2020). Among Natural preservatives, honey bee propolis has been shown to be effective in preserving various foods, including milk and dairy products products(El-Deeb, 2017). Despite the potential for both beekeeping and dairy production in the study area, as noted by several scholars, propolis is not widely used in local communities due to a lack of awareness about its antimicrobial properties and its role as a natural preservative.

The antimicrobial and antioxidant properties of propolis make it a promising natural preservative for the food industry (Essawy, 2007). Therefore, the objectives of this research was to assess the effects of adding different volumes of ethanol-extracted honey bee propolis as a natural preservative of raw cow's milk.

Materials and Methods

Propolis Sample Collection Site

Propolis was collected from different agro-ecologies of the Awi zone. From a total of 12 districts in the zone, it was stratified into 3 categories based on the agro-ecologies of administrative woredas, which are high, medium, and low land districts in order to harvest propolis from different botanical sources to see the preservative roles of propolis on raw cow's milk. One district from each agro-ecology was selected purposively based on the potential of beekeeping to collect enough amount honey bee propolis. Then Banja, Dangla, and Guangua woreda were selected from high, middle, and low lands respectively.

Banja Shekudad district

is bordered on the south by Ankesha, on the West by Guangua, on the North by Fageta Lekoma, and on the East by the West Gojam zone. The district consists of 26 kebeles, of which 25 are rural kebeles and one urban kebele. One urban kebele(02) was selected to harvest honey bee propolis. The Apiary site of which propolis collected was established by Injibara University in Liwi Mountain for demonstration purposes in injibara town. The town is both the capital of Awi Zone and Banja district, which is located about 450 km northwest of Addis Ababa and 118 km south of Bahir Dar. The district is comprised of Dega (80 percent) and Winadega (20 percent) agro-ecologies, and the altitude ranges from 1900 to 2750 meters above sea level. The annual temperature is 26°C at the maximum and 16°C at the minimum.

Dangila district

which is found in Awi zone, Amhara Regional State, in north-western Ethiopia, along the main road between the cities of Addis Ababa and Bahir Dar, is about 472 km from Addis Ababa, the capital of Ethiopia. From the regional capital, Bahir Dar, Dangila district is about 72 km. astronomically; Dangila is located at 11° 18' N latitude and 36° 57' E longitude. Geographically, it is located at an elevation of 2200 m above sea level with area coverage of 9486.4 hectares and a Woina Dega (temperature) climate. The annual average rainfall and temperature are 1576 mm and 17°C, respectively. From the total 29 kebeles of Dangla Woreda, East Zelesa was selected based on bee-keeping production potential to harvest honey bee propolis for extraction.

Guangua District

is a town in north-western Ethiopia. Located in the Agew Awi Zone of the Amhara Region, this town has a longitude and latitude of 10°57'N, 36°30'E and an elevation of 1583 meters above sea level. The average annual temperature of the Guangua district is 360°C, and the rainfall is about 950 mm (Agricultural Office of Chagni, 2018).Semen Degela Kebele was selected purposively based on the beekeeping production potential of a total of 27 kebeles to harvest honey bee propolis. The study area listed in below figure(1)

Harvesting and Preparation of Propolis Extract

Propolis from three different agro-ecologies were collected using plastic propolis traps which were placed on top of the hive for up to four weeks then the plastic propolis trap was put for this reason after trap enough amount of propolis. The propolis sample was cut into small pieces after freeze-drying and in order to obtain ethanol extracts, 20 grams of propolis for each 200 ml volume of 70% ethanol were added for each three agro-ecologies and mixed well and shaker for 72 hours at the room temperature. Then, the solution of ethanol extracts was filtered with filter paper using the conical flasks. After purified the extracts were stored in a dark place at 4°C in a dark bottle until used(Said, 2012).

Preparation of Treatments

The milk sample was collected from Injibara University dairy farm morning milking with holding pre-sterilized, properly labeled, stoppered bottle transported to the microbiology laboratory of Injibara University in an ice-packed cooler box. The sample milk was divided into three parts as used to evaluate adding extracted honey bee propolis as natural preservative of raw cow's milk. Then from each parts of milk, One hundred milliliters of the raw cow's milk sample were placed into five pre-sterilized sample bottles and then T1(control), T2(5ml), T3(10ml), T4(15ml) and T5(20ml) volume of extracted honey bee

propolis was measured by measuring cylinder as a supplement to each pre-measured one hundred milliliters raw cow's sample milk.

Experimental Design

A Factorial Completely Randomized block Design was used involving two factors. Agro-ecologies effect for the source of propolis collected (dega, weyna dega, and low land) and volumes of the propolis extracts (T1 = control, T2 = 5ml, T3 = 10ml, T4 = 15ml, T5 = 20ml) and each observation was done in three replication. The total sample size consists of N = 3×5×3 = 45 outcome variables. Samples were examined for Total Aerobic Bacterial Count (TABC), Total Coliform Count (TCC), Total Yeast and Mold Count (TYMC), clot on boiling test, alcohol test, and methylene blue reduction test. The laboratory analysis was done in Injibara University microbiology laboratory, Injibara University, Ethiopia.

Table 1
Lay out of the Experimental design

Factors	**Propolis concentration**				
Agro- ecologies	**T1**	**T2**	**T3**	**T4**	**T5**
Banja(B)	BT1	BT2	BT3	BT4	BT5
Dangla(D)	DT1	DT2	DT3	DT4	DT5
Guangua(G)	GT1	GT2	GT3	GT4	GT5

Method of Laboratory Analysis

The raw data was summarized using Microsoft Excel and analyzed using Statistical Analysis Software (SAS) version 9.4. Both descriptive and exploratory analysis was performed using the General Linear Model (GLM) with least square means using least significance difference(LSD) tests to see the effects of agro-ecologies and concentration level of propolis on the parameters of physiochemical, microbial test, sensory attribute, and organoleptic of raw cow's milk.

The following model was used to analyze the microbiological quality of milk:

$Yij = \mu + Ai + Bj + ABij + Eijk$

Where = μ is the overall mean,

Ai= agro-ecologies effect of propolis harvested

Bj= Volume of propolis added per 100ml of milk

ABij=interaction effect of i level agro-ecologies and j level volume of propolis added

Eijk=random error

Microbiological tests:

The determination of microbial load was performed at the initial time, 24 and 72-hour storage period.

Aerobic Total Bacterial Count (ATBC)

ATBC was determined using plate count agar (PCA) following the ISO standard (ISO 17025:1999). 10 ml of the sample was homogenized in 100 ml of peptone water which gave the first dilution (10^{-1} dilution) and up to 10^{-5} serial dilutions were

prepared in a sterile test tube containing 9 ml of peptone water. 1 ml of each serial diluted sample was poured into sterile Petri dishes in triplicate and mixed thoroughly by adding 15–20 mL of molten PCA (Oxoid, UK) tempered to 44 0C. The plates were allowed to solidify at room temperature and then incubated at 32 ºC for 48 hours(Murphy et al., 1992). The plates with colonies ranging from 30–300 colony forming units (cfu) per plate were selected and counted for determination of TABC(Alemu et al., 2023). The TABC was determined as cfu per ml of milk sample calculated by the formula provided by (Chipilev *et al.*, 2016) and IDF (2004).

$$N = \frac{\sum C}{[(1xn1) + (0.1xn2)]d} \qquad (1)$$

Where = N is the number of cfu per milliliter of milk sample

ΣC = is the sum of colonies on all plates counted;

n1 = is the number of plates in the first dilution counted;

n2 = is the number of plates in the second dilution counted; and

d = is the dilution from which the first counts were obtained.

Coliform Counts (CC)

The coliform was determined using the ISO standard by plate count method (ISO 17025:1999). 1 ml of each serially diluted sample was poured into sterile Petri dishes in duplicates and mixed thoroughly by adding 15–20 ml of molten Violet red bile agar (VRBA, Oxoid, UK) tempered to 44 0C. The plates were allowed to solidify at room temperature and then incubated at 32 ºC for 24–48 hours(Murphy et al., 1992). The plates containing red or violet colonies ranging from 15–150 colony-forming units (cfu) were considered coliform and counted using colony counters(Stannard, 1984). The coliform was calculated as cfu per ml which is mentioned earlier formula IDF (1991(Chipilev *et al.*, 2016)).

Determination of Yeast and Mold Counts

Yeast and mold count (YMC) were determined using sterile Potato Dextrose Agar (PDA)(Oxoid, UK). Serial diluted up to 10^{-7} samples of 0.1ml, each serial diluted sample was added to sulfurized PDA plates in triplicate and spread- plate using sterile bent-glass rod. The plates were incubated at 25 ^{0} C for 5 days(Andrews et al., 1983, Roberts Greenwood, 2008). Creamy to white/gray colonies were counted as yeasts whereas, filamentous (fuzzy) colonies of various colors (yellow, green, light brown) were counted as molds(Hassen Amentie, 2022). Plates with 10–150 colonies were used for determining yeast and mold counts ((Chipilev *et al.*, 2016).

Milk quality test

Clot-On-Boiling Test

Five ml of milk in a sterile test tube was placed in a boiling water bath for five minutes. Then, the test tube was carefully removed from the water bath and examined for the presence of curds for the freshness of milk. This was done in one hour of time intervals and the hours sample milk started to form curd was scored as a shelf life of sample raw cow's milk.

Alcohol Test

Milk was sampled at the interval of an hour; five ml of milk and 5 ml of 68 percent ethanol were placed in a test tube. The test tube was inverted several times with the thumb held tightly over the open end of the tube. Then the tube was examined for the

formation of curd particles for a freshness checkup of the milk(O'Connor, 1994). An hour's sample milk started to form curd and was scored as a shelf life of sample raw cow's milk.

Methylene Blue Reduction Test

The methylene blue test was performed according to the method described by (O'Connor, 1994). The methylene blue decolonization test is a good measure of its bacterial content and hence of its hygienic quality. 1 ml of methylene blue was added to 10 ml of milk in test tubes. The test tube was inverted 4–5 times tightening the mouth with stoppers, and then putting it in a water bath at 37C^{o}. The quality of the milk is determined by observing the color. The time taken by the dye to get reduced determines the number of microbes in the milk and the quality of the milk (Mayra et al., 2020).

Results

Effects of honey bee propolis on alcohol and clot on boiling test

The agro-ecology effect on clotting and alcohol tests of raw cow's milk was highly significant ($p < 0.0001$). Mean comparisons revealed that the longest negative results (12 hours) in both tests occurred in Guangua (lowland) with T5 treatment. In contrast, the shortest negative results (2 hours) were observed in the control group (T1) across all agro-ecologies. This indicates that lowland agro-ecologies, particularly in Guangua, allowed milk to stay fresh longer, which is beneficial for rural areas with limited infrastructure for milk preservation.

Propolis concentration also showed a significant effect ($p < 0.0001$) on clotting and alcohol tests. Results indicated that higher concentrations of propolis led to gradually increased coagulation times (hours). Interaction effects revealed that increasing propolis concentration lengthened coagulation times, with the highest times recorded in Guangua (T5). The variation in coagulation time may be attributed to the volume differences in propolis extracts and the distinct agro-ecologies where the propolis was harvested.

Effects of honey bee propolis on Milk methylene blue reduction test

The methylene blue reduction test was highly significantly influenced ($P < 0.001$) by both the agro-ecologies of propolis harvested and the concentrations of propolis extracts. The interaction effect also significantly influenced the methylene blue reduction test ($P < 0.05$). As shown in Table 2, the highest methylene blue color disappearance times were recorded at GT5, DT5, and BT5, while the lowest times were observed at GT1, DT1, and BT1 in the evaluated methylene blue reduction test of raw cow's milk. The results indicate that the addition of extracted honey bee propolis improves the methylene blue color disappearance time in raw cow's milk, with the effect directly proportional to the dose of propolis supplementation. Milk treated with lowland-harvested honey bee propolis exhibited the highest color disappearance times, followed by midland and highland-harvested propolis.

Table 2
Main effects of agro-ecologies of propolis harvested and propolis concentration on shelf life of raw cow's milk (hours/hrs)

Agro ecologies	**Parameters**		
	C.O.B TEST	**Alcohol test**	**MBRT**
Banja	6^{c}	7^{c}	6.50^{c}
Dangla	7^{b}	7.30^{b}	7.40^{b}
Guangua	8^{a}	8^{a}	8^{a}
P-values	***	***	***
Concentration T1	2^{e}	2^{e}	2^{e}
T2	5^{d}	6.30^{d}	5.30^{d}
T3	7.20^{c}	8.30^{c}	7.50^{c}
T4	9.30^{b}	9.10^{b}	9^{b}
T5	10.50^{a}	11^{a}	10.30^{a}
P-values	***	***	***

Where; * = Significant ($P < 0.05$); ** = highly significant ($P < 0.01$); *** = very highly significant ($P < 0.001$); T1 = control sample raw milk, T2 = sample raw cow's milk supplemented 5 milliliter propolis; T3 = sample raw cow's milk supplemented 10 milileter propolis; T4 = sample raw cow's milk supplemented 15 milileter propolis; T5 = sample raw cow's milk supplemented 20 milileter propolis; and; means followed by the different letter (s) within a column are significantly different.

Table 3
Interaction effects of agro-ecologies of propolis harvested and propolis concentration on the shelf life of raw cow's milk (hours/hrs).

Ag*con	Parameters		
	Clot on boiling test	**Alcohol test**	**Methylene blue taste**
BT1	2^{g}	2^{j}	2^{i}
BT2	4^{f}	5.50^{i}	4^{h}
BT3	6.50^{d}	7.50^{fgh}	7.50^{ef}
BT4	8^{c}	8^{efg}	7.50^{ef}
BT5	9.50^{b}	9.50^{bcd}	9^{cd}
DT1	2^{g}	2^{j}	2^{i}
DT2	5^{ef}	6.50^{hi}	5.50^{g}
DT3	7^{cd}	8.50^{def}	7.50^{ef}
DT4	9.50^{b}	9.50^{bcd}	8.50^{de}
DT5	10^{b}	10.50^{b}	10.50ab
GT1	2^{g}	2^{j}	2i
GT2	6^{de}	7^{gh}	6.50^{fg}
GT3	8^{c}	9^{cde}	8.50^{de}
GT4	10.50b	10^{bc}	10^{bc}
GT5	12^{a}	12^{a}	11.50^{a}
P-values	*0.05*	*0.05*	*0.05*

T1 = control sample raw milk, T2 = sample raw cow's milk supplemented 5-milliliter propolis; sample raw cow's milk supplemented 10-milliliter propolis; sample raw cow's milk supplemented 15-milliliter propolis; sample raw cow's milk supplemented 20-milliliter propolis; and; means followed by the different letter (s) within a column are significantly different.

Microbial Quality of Raw Cow’s Milk

The microbial quality of raw cow’s milk was significantly influenced by the agro-ecologies of propolis harvested and the concentration of propolis extract ($p < 0.001$), as shown in Table 4.

Total Bacterial Count (TBC)

The TBC results of raw cow’s milk treated with ethanol-extracted honey bee propolis harvested from highland, midland, and lowland areas were evaluated at initial, 24 hours, and 72 hours. The lowest total bacterial count was observed in raw cow’s milk treated with lowland propolis, while the highest counts were recorded in milk treated with highland and midland propolis. Across all study districts, the highest TBC was recorded at the initial time, with a gradual decrease in bacterial counts observed at 24 and 72 hours.

The TBC results revealed that the control group (T1) had significantly higher TBC than all propolis-treated groups. Moreover, the TBC in the control group increased progressively from the initial time to 72 hours. In contrast, raw cow’s milk

supplemented with various concentrations of ethanol-extracted honey bee propolis (EEP) exhibited a steady decrease in TBC values from the initial to the 72-hour storage period.

Total Coliform Count (TCC)

The analysis of variance showed no significant effect ($P > 0.05$) of agro-ecologies on total coliform counts at the initial evaluation time. However, at 24 and 72 hours, a very highly significant effect ($P < 0.001$) was observed. Specifically, raw cow's milk treated with propolis exhibited a significant reduction in total coliform counts compared to the untreated control sample. The untreated (control) samples showed an increase in total coliform counts over time, while the propolis-treated samples (T2, T3, T4, T5) demonstrated a gradual decrease in coliform bacteria up to 72 hours of storage.

Furthermore, the effect of agro-ecologies and propolis concentration on coliform bacteria was significant ($p < 0.05$) at the 72-hour evaluation time. However, no significant interaction effect between agro-ecologies and propolis concentration was observed at the initial or 24-hour evaluation times ($p > 0.05$). The lowest total coliform count was recorded in raw cow’s milk treated with a high concentration of ethanol-extracted honey bee propolis, as indicated in Table 5. The following Fig. 3 showed that result after incubation of coliform in treated raw cows milk during I count it.

Total yeast and mold count (TYMC)

The total yeast and mold count (TYMC) of raw cow's milk was significantly influenced by both the agro-ecologies of propolis harvested and the concentration of extracted honey bee propolis. As shown in Table 4, the lowest total yeast and mold count was recorded in milk from Gangua (lowland) woredas, while the highest counts were observed in Dangla and Banja woredas, located in the midland and highland regions, respectively.

Furthermore, the total yeast and mold count decreased progressively from the initial to 24 and 72 hours of storage in all study districts. This reduction followed a similar trend across all areas, indicating that storage time has an impact on yeast and mold proliferation in raw cow's milk, regardless of agro-ecology.

The effect of propolis concentration on total yeast and mold counts was highly significant ($p < 0.001$), as indicated in Table 4. The control (T1) group, which did not receive propolis treatment, showed a substantial increase in yeast and mold counts over the storage period, suggesting that raw cow's milk without propolis is more prone to fungal contamination. In contrast, milk supplemented with higher concentrations of honey bee propolis exhibited a marked reduction in yeast and mold counts, with the most significant decrease observed at 72 hours of storage. These reductions occurred gradually at all evaluation points (initial, 24, and 72 hours), demonstrating that higher concentrations of propolis are more effective in inhibiting fungal growth in raw cow's milk. Figure 4 showed that incubated yeast and mold results during counting via counter.

Table 4
Main effects of agro-ecologies of propolis harvested and propolis concentration on microbial quality of raw cow's milk at 0, 24, and 72 hours

Agro ecologies	**TBC_{0hr}**	**TBC_{24hr}**	**TBC_{72hr}**	**$TCFC_{0hr}$**	**$TCFC_{24hr}$**	**$TCFC_{72hr}$**	**$TYMC_{0hr}$**	**$TYMC_{24hr}$**	**$TYMC_{72hr}$**
Banja	5.83^{a}	5.73^{a}	5.51^{a}	4.34^{a}	4.51^{a}	4.16^{a}	2.94^{a}	2.87^{a}	2.85^{a}
Dangla	5.65^{b}	5.55^{b}	5.03^{b}	4.34^{a}	4.27^{b}	4.23^{a}	2.80^{b}	2.78^{a}	2.67^{ab}
Guangua	5.58^{b}	5.48^{b}	4.83^{c}	4.20^{a}	4.1^{b}	3.88^{b}	2.70^{c}	2.67^{b}	2.5^{b}
P-values	***	***	***	*ns*	***	***	***	***	***
LSD(0.05)	0.136	0.12	0.0198	0.228	0.1984	0.2596	0.0815	0.0862	0.2131
Concentration T1	7.42^{a}	7.46^{a}	7.48^{a}	5.99^{a}	6.05^{a}	6.3^{a}	3.17^{a}	3.26^{a}	3.5^{a}
T2	6.30^{b}	6.28^{b}	5.15^{b}	5.05^{b}	4.86^{b}	4.55^{b}	3.04^{b}	2.95^{b}	2.84^{b}
T3	5.67^{c}	5.56^{c}	4.9^{c}	3.88^{c}	3.95^{d}	3.87^{c}	2.88^{c}	2.78^{c}	2.62^{c}
T4	5.49^{d}	5.35^{c}	4.7^{d}	3.34^{d}	3.57^{d}	3.15^{d}	2.64^{d}	2.57^{d}	2.3^{c}
T5	3.54^{e}	3.44^{d}	3.3^{e}	3.2^{d}	2.9^{e}	2.83^{d}	2.36^{e}	2.3^{e}	2.1^{c}
P-values	***	***	***	***	***	**	***	***	**
LSD(0.05)	0.1755	0.1755	0.0256	0.2944	0.2562	0.3352	0.1053	0.1113	0.2751
CV (%)	2.4	2.49	0.4	5.5	4.8	6.6	3.01	3.2	8.2

Where; * = Significant ($P < 0.05$); ** = highly significant ($P < 0.01$); *** = very highly significant ($P < 0.001$); T1 = control sample raw milk, T2 = sample raw cow's milk supplemented 5 mililiter propolis; T3 = sample raw cow's milk supplemented 10 mililiter propolis; T4 = sample raw cow's milk supplemented 15 mililiter propolis; T5 = sample raw cow's milk supplemented 20 mililiter propolis; and; means followed by the different letter (s) within a column are significantly different

Table 5
Interaction effects of agro-ecologies of propolis harvested and propolis concentration on microbial quality of raw cow's milk

Agro*con	TBC_0	TBC_{24}	TBC_{72hr}	$TCFC_0$	$TCFC_{24hr}$	$TCFC_{72}$	$TYMC_0$	$TYMC_{24}$	$TYMC_{72}$
BT1	7.38^{a}	7.50^{a}	7.46^{a}	6.04^{a}	6.13^{a}	6.18^{a}	3.23^{a}	3.29^{a}	3.97^{a}
BT2	6.65^{b}	6.15^{b}	6.02^{b}	4.98^{b}	4.80^{b}	4.58^{b}	2.97^{bc}	2.84^{de}	2.69^{cd}
BT3	5.82^{de}	5.35^{cde}	5.47^{c}	4.01^{c}	3.91^{cd}	4.21^{bc}	2.73^{de}	2.58^{fgh}	2.45^{def}
BT4	5.66^{ef}	5.10^{e}	5.17^{d}	3.43^{def}	3.39^{ef}	3.17^{def}	2.53^{fg}	2.47^{gh}	2.14^{ef}
BT5	3.64^{g}	3.49^{f}	3.43^{j}	3.23^{ef}	3.11^{fg}	3.07^{ef}	2.15^{h}	2.15^{i}	2.0^{f}
DT1	7.44^{a}	7.50^{a}	7.46^{a}	6.00^{a}	6.02^{a}	6.18^{a}	3.23^{a}	3.28^{a}	3.97^{a}
DT2	6.17^{c}	5.90^{bcd}	4.63^{g}	5.14^{b}	4.9^{b}	4.56^{b}	3.02^{bc}	2.95^{cd}	2.84^{bcd}
DT3	5.70^{ef}	5.60^{bcde}	4.48^{h}	3.89^{cd}	4.29^{c}	4.21^{bc}	2.86^{cd}	2.77^{def}	2.65^{cde}
DT4	5.45^{f}	5.34^{ced}	4.39^{i}	3.36^{def}	4.27^{c}	3.51^{de}	2.60^{ef}	2.55^{fg}	2.34^{def}
DT5	3.49^{g}	3.49^{f}	3.19^{l}	3.27^{ef}	3.07^{fg}	2.84^{f}	2.38^{g}	2.18^{h}	2.15^{ef}
GT1	7.44^{a}	7.50^{a}	7.46^{a}	6.04^{a}	6.01^{a}	6.18^{a}	3.23^{a}	3.22^{ab}	3.97^{a}
GT2	6.08^{cd}	6.03^{bc}	4.88^{e}	5.03^{b}	4.87^{b}	4.50^{b}	3.11^{ab}	3.06^{bc}	3.0^{bc}
GT3	5.49^{f}	5.44^{cde}	4.78^{f}	3.73^{cde}	3.69^{de}	3.60^{de}	3.05^{abc}	2.97^{cd}	2.77^{cd}
GT4	5.38f	5.25^{de}	4.74f	3.23^{ef}	3.04^{fg}	2.77^{f}	2.77^{de}	2.65^{efg}	2.38^{def}
GT5	3.50^{g}	3.36^{f}	3.27^{k}	3.07^{f}	2.8^{g}	2.56^{f}	2.53^{fg}	2.38^{h}	2.13^{ef}
P-values	*0.1983*	*0.6494*	*< .0001*	*0.9928*	*0.0174*	*0.4979*	*0.4674*	*0.1852*	*0.6385*

Where; T1 = control sample raw milk, T2 = sample raw cow's milk supplemented 5 milileter propolis; T3 = sample raw cow's milk supplemented 10 milileter propolis; T4 = sample raw cow's milk supplemented 15 milileter propolis; T5 = sample raw cow's milk supplemented 20 milileter propolis; and; means followed by the different letter (s) within a column are significantly different.

Discussions

Effects of honey bee propolis on alcohol and clot on boiling test

The agro-ecology effect on clotting and alcohol tests of raw cow's milk was highly significant ($p < 0.0001$). Mean comparison tests showed that the longest coagulation times (12 hours) in both tests were recorded in the lowland area of Guangua with T5 treatment. The shortest coagulation times (2 hours) were observed in the control treatment (T1) across all agro-ecologies. This could be attributed to the influence of propolis' chemical composition, which varies based on the botanical sources from which it is harvested. This is consistent with the findings of Bankova et al. (2016), who reported that the chemical composition of propolis is influenced by plant sources, geographical location, climate, and environmental factors. The longer coagulation times in Guangua may offer significant benefits for rural areas with limited infrastructure to maintain milk quality. This result aligns with Przybyłek and Karpiński (2019), who noted that the geographical location of propolis collection affects its antibacterial properties, which are crucial in preventing contamination in raw milk.

The concentration of propolis also showed a significant effect ($p < 0.0001$) on both clotting and alcohol tests. Higher concentrations of propolis led to progressively increased coagulation times in raw cow's milk, suggesting the antimicrobial properties of propolis play a role in preserving milk freshness. This finding is in agreement with El-Deeb (2017), who reported that supplementing raw milk with 2% ethanol-extracted honey bee propolis (20% extract) extended the milk's freshness for up to 12 hours at 30°C and 48 hours at 5 ± 1°C. Similarly, MA ElSherif and Wahba (2012) observed that milk samples treated with honey bee propolis showed delayed coagulation times, indicating preserved freshness, possibly due to reduced acidity.

The interaction between agro-ecology and propolis concentration resulted in increased coagulation times with higher propolis concentrations across the three study woredas. The highest coagulation times were recorded in Guangua with the T5 treatment level, suggesting that both the volume of propolis extract and the agro-ecological factors where the propolis is harvested play significant roles in determining its effectiveness. This finding is consistent with El-Deeb (2017), who demonstrated that adding water-extracted honey bee propolis to raw milk enhanced freshness for extended periods. Furthermore, the results align with the work of Przybyłek and Karpiński (2019), confirming that the antibacterial potential of propolis is influenced by its geographical origin.

Effects of honey bee propolis on Milk methylene blue reduction test

The methylene blue reduction test results showed a significant influence of both the agro-ecology of propolis harvesting and the concentration of propolis extracts. The interaction effect between these factors also had a marked impact on the color disappearance time. The addition of honey bee propolis to raw milk resulted in a longer methylene blue reduction time, suggesting that propolis, particularly in higher concentrations, plays a role in improving the preservation quality of milk.

The results also revealed that propolis harvested from lowland areas led to the most significant improvements in the methylene blue reduction test. This could be attributed to the unique chemical composition of propolis from different geographical regions, which may exhibit varying levels of antimicrobial activity, as supported by previous research (Przybyłek & Karpiński, 2019). These findings underscore the importance of both the concentration of propolis and the agro-ecology of its harvest in enhancing the preservation properties of raw cow's milk.

Total Bacterial Count (TBC)

The lowest TBC was observed in raw milk treated with propolis harvested from lowland areas, while higher TBCs were found in milk treated with propolis from midland and highland regions. This variation may be attributed to differences in the chemical composition of propolis from different geographical regions, which influences its antimicrobial efficacy. The findings suggest that propolis from lowland areas may be particularly effective in reducing microbial contamination in raw cow's milk. The results revealed a consistent pattern of a high initial TBC, which gradually decreased over 24 and 72 hours in propolis-treated milk. This is consistent with previous studies (Hadi Koohsari, 2020), which suggested that antimicrobial activity of propolis becomes more pronounced after 24, 48, and 72 hours, as propolis needs time to activate its bioactive compounds that serve as preservatives in food. The gradual reduction in bacterial counts highlights the role of propolis as a natural preservative, which acts over time to inhibit microbial growth. Furthermore, the control group (T1), which was not treated with propolis, exhibited an increase in TBC over the storage period, emphasizing the antimicrobial efficacy of propolis extracts. This is in agreement with studies by Hendi et al. (2011) and Bankova et al. (2005), which noted the influence of geographical origin, plant sources, and vegetation on the antimicrobial properties of propolis. The use of propolis extracts, particularly from lowland regions, shows promising potential for improving the microbial quality and shelf-life of raw cow's milk.

The findings also suggest that the concentration of ethanol-extracted honey bee propolis plays a critical role in reducing microbial load. Previous studies (Hadi Koohsari, 2020; Said, 2012) have shown that higher concentrations of propolis extract, such as 3% and 5%, exhibit more significant antimicrobial activity compared to lower concentrations. This further supports the conclusion that propolis supplementation can significantly reduce microbial contamination in raw cow's milk, particularly when higher concentrations are used. In conclusion, propolis, especially from specific agro-ecological zones, has

demonstrated significant antimicrobial properties that can enhance the microbial quality and preservation of raw cow's milk, offering a natural alternative to synthetic preservatives.

Total coliform count (TCC)

The total coliform count (TCC) in raw cow's milk was significantly influenced by the agro-ecologies of propolis harvested and its concentration. At the initial evaluation time, the agro-ecologies of propolis had no significant effect ($P > 0.05$) on the TCC. However, at the 24 and 72-hour evaluation periods, the analysis of variance revealed a very highly significant effect ($P < 0.001$) on the TCC, as indicated in Table 4. The results showed that untreated (control) samples exhibited a progressive increase in TCC, while the treated samples (T2, T3, T4, and T5) demonstrated a gradual decrease in coliform bacteria over the 72-hour storage period. This finding suggests that propolis requires time to exert its antimicrobial effects on the microbes present in milk, and the varying concentrations of ethanol-extracted honey bee propolis used in the treatment may contribute to this effect.

The observed gradual decrease in coliform bacteria in treated milk is consistent with the findings of Mbuk et al. (2016) and Shaban et al. (2021), who reported a similar trend where untreated samples showed a gradual increase in TCC, while treated samples exhibited a delayed increase up to 20 hours of storage. This suggests that propolis' antimicrobial activity takes time to manifest, which could be due to the activation of its bioactive compounds during storage.

Regarding the interaction between agro-ecologies and propolis concentration, the results showed a significant ($p < 0.05$) effect on coliform bacteria at the 72-hour evaluation time. However, no significant interaction was observed at the initial or 24-hour evaluations ($p > 0.05$). As indicated in Table 5, the lowest total coliform count was consistently recorded in milk treated with high concentrations of propolis, regardless of the agro-ecological region from which it was harvested. This suggests that the concentration of propolis plays a crucial role in determining its antimicrobial effectiveness. Higher concentrations of propolis appear to be more effective in reducing microbial load in raw cow's milk.

These results are consistent with the studies by Hadi Koohsari (2020) and Owusu-Kwarteng et al. (2020), which reported significant differences in the antimicrobial effectiveness of propolis extracts at concentrations of 3% and 5%, compared to other lower concentrations. This further supports the notion that higher concentrations of ethanol-extracted honey bee propolis are more effective at inhibiting microbial growth in dairy products.

Total yeast and mold count (TYMC)

The total yeast and mold count (TYMC) in raw cow's milk was significantly influenced by both the agro-ecology of propolis harvested and its concentration. These findings are in agreement with Hadi Koohsari (2020), who reported that the antimicrobial activity of propolis extracts, particularly against yeast and mold in raw cow's milk, becomes more evident after 24, 48, and 72 hours of storage. Koohsari also noted that the compounds responsible for the biological activity of propolis vary depending on the geographical origin of the propolis, which could explain the differences observed in TYMC across the different agro-ecologies in this study.

The effect of extracted honey bee propolis concentration on TYMC was found to be highly significant ($p < 0.001$), as indicated in Table 4. The control group (T1) showed a substantial increase in yeast and mold counts, suggesting that raw cow's milk without propolis is more susceptible to fungal contamination during storage. In contrast, raw cow's milk supplemented with higher concentrations of propolis exhibited a marked reduction in yeast and mold counts at all evaluation points (initial, 24, and 72 hours). These reductions were more pronounced in milk treated with higher concentrations of propolis, supporting the potential of propolis as an effective antifungal agent.

These results are consistent with those of Elkassas *et al*. (2023), who reported that the initial total yeast and mold counts in control and propolis-treated milk samples differed significantly. At the 72-hour evaluation, the total yeast and mold count in control samples increased to 4.34 log10 CFU/ml, while the counts in the 1%, 2%, and 3% ethanol-extracted propolis (EEP) groups were 3.10, 2.85, and 2.67 log10 CFU/ml, respectively. This further supports the notion that higher concentrations of

propolis are more effective in reducing fungal contamination in raw cow's milk, as propolis exerts a stronger antifungal effect at higher concentrations.

Conclusions and recommendations

This study aimed to assess the effectiveness of ethanol-extracted honey bee propolis collected from three different agro-ecologies in Awi zone, specifically Banja, Dangla, and Guangua woredas. Milk quality was evaluated through tests such as clot on boiling, alcohol, methylene blue reduction, and microbial quality, including total bacterial, total yeast and mold, and total coliform counts in raw cow's milk. The results revealed that both the agro-ecologies from which the propolis was harvested and the concentration of propolis had a highly significant effect ($P < 0.001$) on the main parameters, and a significant interaction effect ($P < 0.05$) on the clot on boiling, alcohol, and methylene blue reduction tests of cow's raw milk.

In the microbial quality test, a gradual reduction in total bacterial count (TBC), total coliform count (TCFC), yeast, and mold counts was observed in raw cow's milk treated with higher concentration of propolis, compared to the control group. Significantly lower TBC, TCFC, and total yeast and mold counts (TYMC) were recorded in milk treated with propolis harvested from Guangua woreda. These results suggest that the use of extracted honey bee propolis in raw cow's milk can significantly improve its chemical composition, shelf life, and microbial quality. Furthermore, the application of 5ml and 10ml propolis concentrations was found to extend the shelf life of raw cow's milk by 8 to 9 hours.

Based on the findings of this study, Further research should be focus on

- Analyzing the Chemical components of propolis that contribute to its antimicrobial properties for preserving raw cow's milk.
- Conducting pollen analysis of propolis to identify plants with chemical components that possess strong antimicrobial activities.
- Investigating the antimicrobial effectiveness of propolis at various preservation temperatures to determine the optimal conditions for preserving dairy products using honey bee propolis extracts.

Declarations

Acknowledgments

The authors would like to thank Injibara University for allowing different resources to accomplish this study and also Special thanks go to my colleague, Animal science department for their moral and technical support.

Disclosure statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

A-M: conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing – original draft, writing – review and editing, visualization, supervision; **T-A**: methodology, software, validation, formal analysis, writing – review and editing; **T-A**: writing – review and editing, validation; **G-S**: conceptualization, methodology, software, formal analysis, writing – review and editing, visualization, supervision, project administration, funding acquisition. All authors reviewed the results

Funding

The author(s) declare that no financial support was received for the research and/or publication of this article.

Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without uploading anyrepository dataset. I have the data, and if you request, I am willing to share it with you via this email address: abebem59@gmail.com. You can request without hesitating, when it is needed.

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Figures

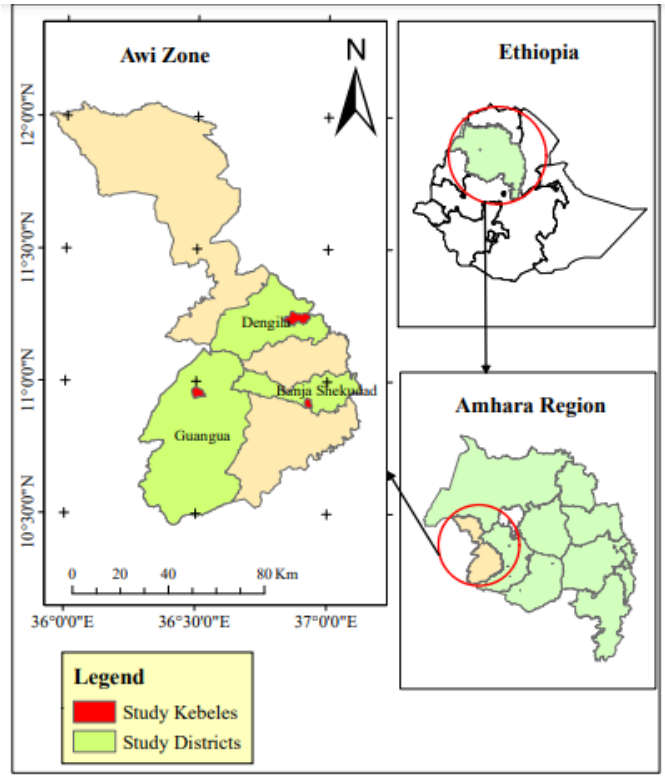


Figure 1

Map of the study area



Figure 2

Total coliform count of raw cow's milk

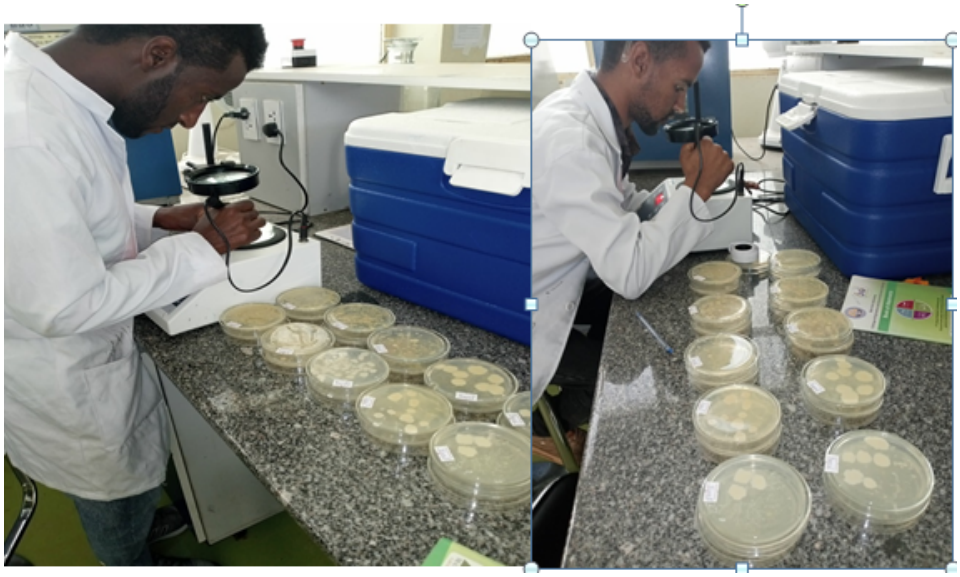


Figure 3

Total yeast and mold count of raw cow’s milk