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Microbial profile of *Tella* and the role of gesho (*Rhamnus prinoides*) as bittering and antimicrobial agent in traditional *Tella* (Beer) production

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Abstract

Tella is widely brewed in Ethiopia. The quality may be deteriorated by microbial contaminants. Determination of profile of spoilage microorganisms in *tella*, bittering agents and antimicrobial activity of gesho are important. Therefore, the objective was to determine microbial dynamics of *tella* phases, bittering agents and the antimicrobial activity of gesho. *Tella* fermentation was carried out using traditional methods. Enumeration of microorganisms were carried out using Malt, MaconKey, Man Rogosa Sharpe (MRS), dextrose, nutrient and Seed Culture Medium agar. Essential oil, total, hard and soft resins as well as Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined. The microbial count was significantly ($P \leq 0.05$) decreased towards the end of fermentation phases. Particularly, molds and bacteria were almost disappeared at the end of fermentation phase. Yeasts were dominated at the middle of phases. This phenomenon may be due to the synergic effect of gesho antibacterial substance, alcohol concentration, reduction of pH (7.7 to 3.43) and nutrient content of *tella*. Total resin, soft resin, hard resin and essential oil of gesho were found to be comparable with values of varieties of hops. Therefore, gesho can substitute hops for beer production. The MIC and MBC of gesho extract against standard pathogenic and clinical bacteria were ranged from 97.5 to 780 mg/ml and 195 to 780 mg/ml, respectively. So, gesho extract during *tella* brewing can inhibit bacteria growth and thereby help to extend the self-life of the product. In conclusion, brewing of *tella* should be carried out in aseptic conditions in order to avoid risks of having pathogenic bacteria.

Introduction

Tella is widely brewed and consumed in both rural and urban part of Ethiopia. It is well known as local beer (Getahun, 1976) since it is malt based beverage like that of commercial beer (Shale and Gashe, 1991; Kunze, 1996). *Tella* brewing requires largely malted barley (*Hordeum vulgars*), *Enkuro*, gesho (*Rhamnus prinoides*) leaves and stems chopped to small pieces, kita (from different grains) or *enkuro* and *derekot* (Shale and Gashe, 1991). Generally, *tella* is brewing from substrates such as barley, wheat, maize, millet, sorghum, teff or other cereals. Commercial beer is mostly made from malted barley and adjunct like corn, rice or wheat provide the carbohydrate substrates for ethanol production by *Saccharomyces carlsbergensis* or *Saccharomyces cerevisiae* (Kunze, 1996). With regard to the substrate, there is no as such basic difference between *tella* and beer. The only difference is that the physical environment for fermentation process, which is not as such controlled as commercial beer and the techniques used in brewing to standard quantify of gradients and aseptic method is not well developed. Prior to follow aseptic technique and use of regular art and craft for *tella* brewing like that of beer production, determination of microbial profile in each *tella* fermentation phases is significant. The data generated about microbial profile of each phases of fermentation is important to develop aseptic method during brewing process. The quality of *tella* is variable from local to local, from individual to individual. Even within the same individual, the quality is variable from time to time.

The quality of *tella* may be deteriorated and affected by contaminants such as fungi and different types of bacteria. These organisms can produce different undesired byproducts. To avoid such types of undesired metabolic products of microorganisms' breweries make continuous efforts to ensure the highest quality of their goods by using aseptic methods. To guarantee the consistency of product quality, the different stages of *tella* production should be monitored for the presence of spoilage microorganisms as that of beer (Weber *et al*., 2008). *Tella* brewing process is traditionally divided into three phases, namely *tejet*, *tenses* and *difedef*. At first phase, the milled *bikil* and the pounded gesho (stems and leaves) are added to the water and covered

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with net cloth and then allowed to ferment for 1 - 2 days in big earthen pot. After the first phase of fermentation (*tejet*), either *enkuro* or *kita* will be added and ferment for about 5-7 days depending on environmental conditions, which is commonly known as *tenses*. At the third phase (*difedef*), the *derekot* flour will be mixed with the entire content of *tenses* and can be kept for 10 - 12 or more days depending on the use. To prepare *tella* for the use of extended period of time, the amount of water and malt should be reduced while the amount of pounded gesho should be increased but for immediate use the reverse is true. In conclusion, *tella* with high quality can be prepared relatively using small quantity of water. Though there are several recipes for making *tella* and even it appears as if every housewife has her own version of the recipe, the procedure used in this project is taken from one of the most known *tella* brewing area, Debre-Markos, Ethiopia.

Hop is one of the ingredients used for bittering, flavoring and aroma imparting agent for beer brewing. It is also used as bacteriostatic activity to inhibit the growth of most microorganisms (Simpson and Smith, 1992). In Ethiopia, gesho (*Rhamnus prinoides*) is particularly used to provide a special aroma and flavor (Shale and Gashe, 1991; Berhanu Abegaz *et al*., 1999). The chemical substances such as emodin, physcion, rhamnazin, prinoidin, and many other emodin-derived compounds were reported from *Rhamnus prinoides* (Abegaz and Kebede, 1995). Among different chemical substances found in *Rhamnus prinoides*, naphthalenic glucoside, geshoidin (Van Staden and Drewes, 1994) is the basic bittering agent for beverages. Although gesho may have antibacterial effect against some groups of bacteria, its main purpose in the process is believed to impart the typical bitter taste to *tella* (Ashenafi, 2006). However, there is no any *in vivo* test of gesho extract against pathogenic bacteria to determine antimicrobial activity of gesho. So there is a need to assess the antimicrobial activity of gesho.

Hops currently used for flavoring of commercial beer are cultivated flowers (*Humulus lupulus*) with the characteristics of green in color with yellow lupulin glands down between the petals. The bittering characteristics of hop are widely used to balance the sweetness of the malt in one hand and its essential oils help to impart special flavor/aroma to beer in another hand. The hop resins (bitter) are the most valuable and most characteristic components of hops. They give beer its bitter taste, improve foam stability and act as antiseptics towards microorganisms. Based on degree of solubility, hop resins are further subdivided into as hard and soft resins. Soft resins contribute a lot as flavoring agent and also help to preserve beer for relatively long period of time in comparison with that of hard resins. Soft resins are hexane soluble while the totals resin is soluble in cold methanol. The most important property of the resin is, of course, its bittering value (Kunze, 1996).

Apart from bittering and flavoring properties of hops, they are widely used for their oils (Tinseth, 1994) to impart characteristic aroma for beer production (Goldammer, 2000). In brief, essential oil with the range of 0.5 to 5% and the volatile resins are together responsible for the aroma in hops (Versele and Keukeleire, 1991). One of the raw materials (as mentioned in the text) that are widely used as bittering agent in *tella* preparation is *Rhamnus prinoides.* It is completely different from hops and largely cultivated in Ethiopia and currently available in dried form in the local market. Although gesho may have antibacterial effect against some groups of bacteria, its main purpose in the process is to impart the typical bitter taste to *tella* (Ashenafi, 2006). Even it was reported in such a way, the presence and the quantity of total resins, soft and hard resins as well as total oil and essential oil content of *Rhamnus prinoides* is not yet determined. Therefore, the main objective of this investigation is to determine the major chemical compositions that contribute(s) a lot for *tella* preparation and to study the antimicrobial activity of the extracted substance(s) from *Rhamnus prinoides* in comparison with that of hops. It is also designed to evaluate the antimicrobial profile in each phases of *tella* fermentation. The data generated at the end of this study is significant to transform local *tella* production into industrial commercial product using standard methods.

Materials and Methods

Tella brewing process

Tella ingredients such as gesho-malt mix, *enkuro* and *derekot* were used in the ratio of 25:25:100, respectively. Three phases of fermentation were carried out according to the traditional methods of *tella* brewing. The first phase (*tejet*) was carried out by mixing 8:10 ratio of malt flour and gesho powder, respectively and then three fold water (v/w) was mixed to the entire content. After 96 h fermentation, *enkuro* (27% of the entire *tella* ingredients) was mixed with the content of *tejet* and fermented for another 96 h. This phase is rationally referred as *tenses.* At last, *derekot* was mixed the entire content of *tenses* and fermented for another 96 h. The last phase which is thick slurry in nature is commonly known as *difedif* by the local society. During each fermentation phases

of *tella*, the pH change was measured by taking 3 gm of sample aseptically and diluted with 5 ml of distilled water using pH meter.

Determination of enumeration of microorganisms in different phases of tella fermentation

From each of the three phases of *tella* fermentation, 1 gm was aseptically taken and diluted to 10^7 dilution factor. From each 10^{-7} dilution, 1 ml was cultured using plate spread techniques on Malt agar containing 100 mg/l chloramphenicol, nutrient agar, MaconKey agar and MRS agar. Nutrient agar was used for general microbial count. In this case, 0.25gm samples was taken and diluted to 10^{13} dilution factor. The plates were then incubated at 30 ± 1ºC for 3 days and the resulting colonies examined for purity. Seed culture medium was also used to isolate acetic acid bacteria from the last phase of *tella* fermentation. The composition of the medium was 0.5% glucose, 0.5% yeast extract, 0.5% peptone, 0.5% glycerol and 1.5% agar powder dissolved in 100 ml of distilled water (Sudsakda *et al*., 2007). Moulds and yeasts were enumerated on Sabouraud dextrose agar (SDA, Fluka 84088) supplemented with chloramphenicol (100 mg/l) incubated aerobically at 25°C for 3 - 5 days (Omafuvbe and Akanbi, 2009).

Essential oil extraction

In this study, 10 gram of the sample was taken in 1000 ml round bottom flask and then 750 ml of distilled water was added. The mixture was agitated by swirling the flask. A Clevenger apparatus was mounted and fitted on to the round bottom flask and then connected to a tap water source. The set up was held tight with a retort stand and the mixture was placed on a suitable electric heating mantle. When the water boils, the steam rises through the stockings, thus extracting the essential oil. The delivery from the condenser was connected to the separating funnel to receive the mixture of steam and oil on condensation. After 120 min, the set up was switched-off and allowed to cool. The water-oil mixture was decanted to separate the oil from the water at the water oil interface. After drying in an electric oven the mass was recorded and calculated as a percentage from the original sample.

Analysis of bitterness of gesho (Rhamnus prinoides)

Total resin determination

For resin analysis 20 g of the sample was dissolved in 100 ml of cold methanol in a conical bottom flask and the mixture was vigorously agitated by swirling the flask. Thereafter, the solution was filtered. The filtrate containing the resin was then dried and the total resin was calculated as a percentage of the original sample weight.

Soft resin determination

With regard to resin determination, 20 grams of each sample was dissolved in 20 ml of n-hexane thoroughly stirred and filtered using filter paper. Filtrate was dried to a constant weight at 50 degree centigrade. The soft resin was calculated as the percentage of the original weight of sample dissolved in the n-hexane. Hard resin was determined by subtracting soft resin from total resin.

Extraction and preparation of plant extracts for antibiotic test

The fresh plant leaves and shoots of *Rhamnus prinoides* which was brought from University of Gondar gust house were dried in an open air protected from direct exposure to sunlight. The dried materials were powdered using blender. Ethanol and methanol were used as solvents for the extraction of antibiotic substance (Silva *et al*., 1996). Twenty gram of each sample was soaked in 100 ml of 95% ethanol and methanol separately for a period of about 120 hours. The extracts were filtered through Whatman No. 2 filter paper under vacuum. Extracts were concentrated to dryness at 37°C for 48 h and kept at 0°C until the determination of antimicrobial antibiotic activity, MIC and MBC analysis.

Inoculum preparation

Standard bacteria such as *Escherichea coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella dysenterae* (clinical isolate), *Staphylococcus aureus* (ATCC 25923), *Shigella flexneri* (ATCC 12022), *Listeria moncytogenes* (ATCC 19116), *Streptococcus pneumoniae* (ATCC 63), *Staphylococcus* sp. (clinical isolate) and *Salmonella* (NCTC 8385) were obtained from Ethiopian Health and Nutrition Research Institution (EHNRI). McFarland standard No. 0.5 was prepared according to Andrews (2006). Standard and clinical isolates of pathogenic bacteria were inoculated and spread over on Muller-Hinton (MH) agar and incubated for 24 h. From 2 - 3 colonies were peaked up by wire loop aseptically into sterile saline solution and the turbidity was adjusted to the 0.5 McFarland's standard solution (a concentration of 1.5 x 10^8 cfu/ml) (Andrews (2006).

Determination of antibiotic activity using well-diffusion method

Antibacterial activity was measured using a

well diffusion method according to the National Committee for Clinical Laboratory Standard (NCCLS, 1993). Pathogenic bacteria suspension was adjusted to be the proper density as the McFarland 0.5 by adding sterile saline or more bacterial growth. Petri plates containing approximately 25 - 30 ml of Mueller Hinton agar medium were swabbed with bacterial suspension using cotton applicator. Wells (6 mm diameter) were punched using cork bore on the agar and filled with 35 µl of *Rhamnus prinoides* extracts. On the other hand, commercial chloramphenicol filter paper discs were placed on surface of each inoculated plate as positive control (Ayandele and Adebiyi, 2007). The plates were incubated at 37°C for 24 h. The antibacterial activity was assessed by measuring the inhibition zone diameter (mm) around the well (Ahmad and Aqil, 2007).

Determination of minimum inhibitory concentration (MIC)

The determination of the minimum inhibitory concentration (MIC) by the extract of the leaves and shoot part of the plant at the dose levels of 97.5, 195, 390 and 780 mg/ml was carried out by the method as described by NCCLS, (1990). Briefly, 50 µl of test standard bacterial suspensions containing 1 x 10^8 cfu cells were introduced into the test tubes containing 3 ml of nutrient broth. The test tubes were incubated at 37°C for 24 h. Controls were set up with the test organisms using distilled water instead of the plant extract. The minimum inhibitory concentration was taken as the tube with the least concentration of the extract with no visible growth after incubation.

Minimum bactericidal concentration (MBC)

A loop full dilutions showing no visible growth for the MIC was subcultured onto a fresh Mueller Hinton agar plate and incubated at 37°C for 24 h. The lowest concentration of the extracts yielding no growth on the MH plate was recorded as the minimal bactericidal concentration (MBC) (Patel *et al*., 2011).

Determination of risk assessment of pathogenic contamination

In this study, 100 ml of *difedef* was mixed with 200 ml of water and filtered after mixing with Whatsman paper. From the filtered *tella* sample, 5 ml was taken in separate caped test tubes and autoclaved. From each test standard bacterium, a loop full was inoculated into autoclaved filtered *tella* aseptically and incubated at 37°C. After 48 h of incubation, a loop full sample was taken and streaked over Muller-Hinton agar medium to detect the growth of tested standard bacterium in *tella*.

Data analysis

The data were analyzed using SPSS version 16.0. Means and standard deviations of the triplicate analysis were calculated using two way analysis of variance (ANOVA) to determine the significance differences among variables ($p < 0.05$) when the F-test demonstrated significance. The statistically significant difference was defined as $p < 0.05$.

Results and Discussions

Determination of dynamics of microorganisms in *tella* fermentation, the amount of bittering agents in comparison with commercial hops and antimicrobial activity of gesho against pathogenic bacteria are important to design aseptic condition for *tella* brewing process. In consideration with these significant points, this study was attributed using profound scientific methods. As the result, profile of microorganisms, quantity of bittering agents and antimicrobial activates of *Rhamnus prinoides* were determined in this section.

Evaluation of variations among different phases fermentation in tella with gesho and without gesho

The preparation of many indigenous or traditional fermented foods and beverages is still a household art. Such types of fermented products are fermented in non-aseptic conditions. Of those locally produced alcoholic beverages produced in non-aseptic condition, *tella* is the one. In this study the dynamics of microorganisms were investigated along with different *tella* fermentation phases using different types of media. Many different media have been designed for use to isolate and identify microorganisms in brewing process (EBC, 1998; IOB, 1997; ASBC, 1992; Bridson, 1998). Some of the media were used to study the microbial dynamics in *tella* brewing process (Table 1). *Tella* was brewed with gesho and without gesho in order to evaluate the role of gesho as potential aseptic agent. Malt agar is used for the isolation, cultivation and enumeration of yeasts and molds from foods and beverages (Bridson, 1998). In this study, the colony count of yeast and molds from samples taken in different phases of *tella* with gesho and without gesho fermentation using malt gar was shown on Table 1. In this case *tella* with gesho, the colony count was statistically reduced from *tejet* phase (53 ± 1) to *difedef* phase (14 ± 2) after 192 h of fermentation at 107 dilution factor and after 288 h of fermentation, there was no any growth of yeast and molds on malt agar. The same type of

Table 1. Determination of enumeration of microorganisms in different phases of *tella* fermentation (with gesho and without gesho)

Type of media agar	*Tella* fermentation phases	Sample taken	Dilution factor 10^7	*Tella* with gesho (Colony count)	*Tella* without gesho (Colony count)	Incubation period (h)
Malt	*Tejet* phase (96 h)	1 gm	10^7	53 ± 1^a	51 ± 1^a	48
	Tenses phase (96 h)	1 gm	10^7	48 ± 2^b	41 ± 3^b	24
	Difedef phase (96 h)	1 gm	10^7	7 ± 1^d	9 ± 1^c	48
	Difedef phase (192 h)	1 gm	10^7	14 ± 2^c	30 ± 2^d	48
	Difedef phase (288 h)	1 gm	1 gm	0 ± 0	0 ± 0	48
MacConkey	*Tejet* phase (96 h)	1 gm	10^7	174 ± 1^a	110 ± 1^a	48
	Tenses phase (96 h)	1 gm	10^7	18 ± 1^b	28 ± 1^b	48
	Difedef phase (96 h)	1 gm	10^7	10 ± 1^c	27 ± 1^c	48
	Difedef phase (192 h)	1 gm	10^7	2 ± 1^d	4 ± 1^d	48
	Difedef phase (288 h)	1 gm	1 gm	0 ± 0^e	3 ± 1^e	48
MRS	*Tejet* phase (96 h)	1 gm	10^7	$16^a \pm 1$	11 ± 3^b	48
	Tenses phase (96 h)	1 gm	10^7	15 ± 3^b	12 ± 1^a	48
	Difedef phase (96 h)	1 gm	10^7	8 ± 2^c	9 ± 2^c	48
	Difedef phase (192 h)	1 gm	10^7	2 ± 1^d	5 ± 2^e	48
	Difedef phase (288 h)	1 gm	10^7	2 ± 1^d	7 ± 1^d	48
Dextrose	*Tejet* phase (96 h)	1 gm	10^7	120 ± 2^c	112 ± 1^d	24
	Tenses phase (96 h)	1 gm	10^7	127 ± 1^b	120 ± 3^b	24
	Difedef phase (96 h)	1 gm	10^7	132 ± 2^a	167 ± 1^a	24
	Difedef phase (192 h)	1 gm	10^7	72 ± 1^d	114 ± 3^c	24
	Difedef phase (288 h)	1 gm	10^7	4 ± 2^e	13 ± 1^e	24
Nutrient	*Tejet* phase (96 h)	0.25gm	10^{13}	153 ± 2^a	187 ± 3^b	24
	Tenses phase (96 h)	0.25gm	10^{13}	128 ± 3^b	130 ± 2^d	24
	Difedef phase (96 h)	0.25gm	10^{13}	123 ± 2^c	299 ± 1^a	24
	Difedef phase (192 h)	0.25gm	10^{13}	97 ± 1^d	250 ± 2^c	24
	Difedef phase (288 h)	0.25gm	10^{13}	53 ± 1^e	91 ± 1^e	24
Seed Culture Medium	*Difedef* phase (288 h)	1 gm	10^7	21	37	24

Values are means of triplicate determinations; Values within the same row followed by different superscripts are significantly different at ($p < 0.05$).

trend was also observed on the sample taken from *tella* fermentation phases without gesho.

MacConkey Agar is widely used to inhibit most species of gram-positive bacteria and gram-negative bacteria usually grow well on this medium (Briggs *et al*., 2004). The colony count of microorganisms from different phases of *tella* fermentation with gesho 10^7 dilution factors were shown on Table 1. Using MacConkey agar, the colony count of gram-negative bacteria was significantly ($P \leq 0.05$) reduced from *tejet* (174) to *difedef* phase (2) after 192 h of ferenentation and even after 288 h fermentation of *difedef* phase, colony of gram-negative bacteria was not found on MacConkey agar. One the other hand, the colony count of gram-negative bacteria from *tella* without gesho on MacConkey agar was significantly ($P \leq 0.05$) greater in *tenses* (28) and *difedef* (27) phases after 96 h of fermentation than *difedef* after 196 and 288 h of fermentation but less than that of *tejet* (110) phases of *tella* fermentation.

MRS medium suppresses the growth of non-beer spoiling *Lactobacillus* strains but permits the growth of those capable of spoilage (Simpson and Smith, 1992). Using MRS agar medium, the growth of microorganisms from samples of different phases of *tella* fermentation after 10^7 dilution factors were investigated (Table 1). The colony count of microorganisms on MRS agar from *tella* sample with gesho was statistically ($P \leq 0.05$) reduced from phase of *tejet* (16) to both 192 h and 288 h of fermentation of *difedef* (2) phase. With regard to *tella* without gesho, the colony count of microorganisms in MRS agar plate was significantly ($P \leq 0.05$) decreased from *tejet* and *tenses* phase (12) to *difedef* (5) phase after 192 h of fermentation but the colony count of *difedef* phase after 288 h of fermentation was significantly ($P \leq 0.05$) increased (7) in comparison with *difedef* phase after 192 h of fermentation of *tella.*

Dextrose agar is mostly used for yeasts cultivation (Bridson, 1998). In this study, colony count dynamics of yeast along with different fermentation phases of *tella* was investigated using dextrose agar (Table 1). The colony number of yeast on dextrose agar from the sample of *tella* with gesho was significantly ($P \leq 0.05$) increased from *tejet* (120) to *difedef* phase (132) of 96 h fermentation but statistically ($P \leq 0.05$) reduced starting after 196 h of fermentation (72) to 288 h of fermentation (4) of *difedef.* The colony count of yeast on dextrose agar from the sample of *tella* without gesho was significantly ($P \leq 0.05$) increased from *tejet* (112) to 96 h of fermentation of *difedef* (167) phase but the colony number was reduced after from 192 h (114) of fermentation to 288 h (13) fermentation of *difedef.*

Nutrient agar is widely used for general-purpose to culture both bacteria and yeasts (Bridson, 1998). Nutrient agar was also used to evaluate the total microbial profile in different phases of *tella* fermentation using 10^7 dilution factor (Table 1). The colony of microorganisms on nutrient agar of *tella* with gesho was statistically decreased from phases of *tejet* (153) to *difedef* phase (53 ± 1 after 288 h

of fermentation. The same trend of dynamics of microbial profile was observed on the fermentation of *tella* made from without gesho.

Evaluation of variations between tella with gesho and without gesho at phases of fermentation

The colony count of yeast and molds from *tella* samples made from gesho and without gesho was evaluated using malt agar (Table 1). At *tejet* and *tenses* phase of *tella* made from gesho and without, the number of colonies was not shown significant ($P \geq 0.05$) difference. However, after 192 h of fermentation of *difedef* phase, the colony count of yeasts and molds in *tella* without gesho (30) was statistically ($P \leq 0.05$) greater than *difedef* made with gesho (14). But after 288 h of fermentation period, there was no found any colony of yeast and mold in both *difedef* with and without gesho.

The colony count of gram-negative bacteria from *tella* samples made from gesho and without gesho was compared using MacConkey agar (Table 1). At *tejet* phase of *tella* made with gesho, the number of colonies on MacConkey agar was significantly ($P \leq 0.05$) greater (174) than *tella* without gesho (110). The colony count of bacteria was significantly ($P \leq 0.05$) greater in *tella* without gesho than *tella* with gesho after *tejet* phase. At the end of *tella* fermentation (*difedef* after 288 h of fermentation), there was no any colony of gram-negative bacteria on MacConkey agar cultured from *tella* made with gesho. Samples of different phase of *tella* with gesho and without gesho were taken and cultured on MRS, dextrose and nutrient agars. The colony counts of microorganisms grown were shown the same trend as shown on MacConkey agar.

As it has been seen from MacConkey, MRS and nutrient agar plates, the colony count of bacteria and molds were reduced as the time of fermentation of *tella* increased in both *tella* with gesho and without gesho. However, the colony count of bacteria were significantly ($P \leq 0.05$) reduced in *tella* brewed with gesho than *tella* without gesho. This was may be due to the presence of antimicrobial substance found in gesho. In both cases (*tella* with gesho and without gesho), bacteria may be affected due to the increment of alcohol concentration and reduction of pH value of *tella* after fermentation. As oppose to the report of Shale and Gashe, 1991), the microbial count of this study was decreased markedly towards the end of the phases of *tella* fermentation. This was may be due to reduction of nutrient content of *tella* and the increment of gesho extracts in addition to the effect of pH and alcohol concentration. Generally, the microbial flora such as molds and bacteria were almost disappeared at the end of *tella* fermentation phase, especially in *tella* made with gesho. Yeasts were dominated at the middle of *tella* fermentation phases but at the last phase they were dramatically reduced in colony count. The possible reason for this phenomenon may be due to the synergic effect of both *Rhamnus prinoides* antibacterial substance, high alcohol concentration, reduction of pH as fermentation time increases and reduction of nutrient content of *tella.*

Table 2. The pH of *tella* at different fermentation phases

Samples	pH value
pH of *tejet* without gesho	6.7
pH of *tejet* with gesho	6.6
pH of *tensese* without gesho	3.7
pH of *tensese* with gesho	3.6
pH of *difedef* with gesho	4.02
pH of *difedef* without gesho	4.12
pH of *difedef* with gesho after 2 weeks	3. 43
pH of *difedef* without gesho after 2 weeks	3. 96

Acetic acid bacteria are well known obligate aerobes and produce acetic acid from ethanol. They are tolerant of ethanol, hop resins and low pH. Typically, acetic acid bacteria spoil beer where some oxygen is present in the fermentation system. Spoilage becomes evident in the form of surface pellicles, turbidity and ropiness. The latter refers to the formation of extracellular polysaccharide material, which can be seen suspended as slime in the infected beer. As the result of contamination of acetic acid bacteria, infected beer becomes acid and off-flavours develop (Briggs *et al.*, 2004). In this study, at the end of *tella* fermentation (*difedef* after 288 h of fermentation), acetic acid bacteria species were isolated using seed culture medium agar. These spices of bacteria may play a great role for the acidification of *tella*. According to Shale and Gashe, 1991) report, after ten days of fermentation, *tella* becomes too sour to consume due to the growth of *Actobacter* spp., which can convert ethanol to acetic acid under aerobic conditions. During survey of the process of *tella* brewing with local society, they extend the shelf-life and quality of *tella* by avoiding free air circulation (cover the brewing container with tight cloth) and they also increased the quantity of gesho with reduction of water and malt used for brewing of *tella.*

The pH of tella at different fermentation phases

The pH of *tella* along with different fermentation phase was presented on table 2. The pH of *tella* with gesho was reduced from 6.7 to 3.43 starting from *tejet* to *difedef* after 288 h of fermentation and from 6.6 to 3.96 in *tella* without gesho on the same types of fermentation phases. The pH was slightly increased at

Table 3. Determination of essential oil, total, soft and hard resins from fresh leaf and stem powder of *Rhamnus prinoides*

Samples	Chemical compositions (%)			
	Essential oils %	Soft resin (%)	Hard resin (%)	Total resin %
Rhamnus prinoides leaf	1.13±0.02[a]	15.73±0.03[a]	2.73± 0.01[c]	18.46±0.03[b]
Rhamnus prinoides stem	0.60± 0.01[c]	12.40±0.02[c]	4.74±0.02[b]	17.16± 0.01[c]
Humulus lupulus control	1.0 ±0.03[d]	13.20± 0.01[b]	6.33±0.02[a]	19.52±0.02[a]

Values are means of triplicate determinations; Values within the same row followed by different superscripts are significantly different at ($p < 0.05$).

96 h of fermentation of *difedef* in both *tella* with and without gesho. This was may be due to the addition of *derekot* to the fermentation system. In summary, the reduction of pH along with length of fermentation time may help to reduce microbial contamination with synergic effect of alcohol concentration and gesho extraction in the fermentation process. The pH of *tella* was within the range of 4.5 - 4.8 (Shale and Gashe, 1991). It is assumed that gesho (*Rhamnus prenoids*) maintains acidic pH during *tella* fermentation so as to modify the nature of the mash and inhibits the growth of undesirable microorganism (Kebede, 1994.

Most of the brewing value of the hop is found in the resins and essential oils which are only slightly soluble in water (Briggs *et al.*, 2004). Even gesho is widely used as bittering agent in *tella* brewing; there is no quantitative study of essential oils and resins. The essential oils, resin component of fresh leaf and stem powder of *Rhamnus prinoides* was shown on Table 3. The total resin of *Rhamnus prinoides* leaf powder (18.46%) was significantly ($P \geq 0.05$) similar with the total resin of *Humulus lupulus* Control (19.52 ± 0.02) and *Humulus lupulus* (18.5 ± 0.01) (Kunze, 1996). The total resin of stem of *Rhamnus prinoides* (17.16%) was significantly ($P \leq 0.05$) greater than the total resin of *Garcinia cola* (8.24 ± 0.03) and *Azadirachta indica* (15.07 ± 0.01) (Ajebesone and Aina, 2004) but statistically ($P \leq 0.05$) less than bittering agents *Humulus lupulus* Control (19.52 ± 0.02) presented on Table 3. According to Kunze, 1996), the amount of hop resin was ranged from 14 - 21% with the mean value of 18.5%. In this case, the total resin of leaf (18.46%) and stem powder (17.16%) of *Rhamnus prinoides* was found to be within the range. Resins are the most valuable and most characteristic components for beer production. They give beer its bitter taste, improve foam stability and act as antiseptics against microorganisms (Kunze, 1996). This is also true that during local (*tella*) beer production, to improve the quality of *tella* and to have an extended shelf life, brewers use high amount of *Rhamnus prinoides*. The antimicrobial characteristics of *Rhamnus prinoides* was also reported by Abegaz *et al.*, 1999). In this respect, *Rhamnus prinoides* can inhibit the growth of microorganisms in *tella*.

Total resins are further divided into as soft and hard resins. Soft resin is normally hexane soluble and easily separated from hard resin (which is insoluble in hexane) (Kunze, 1996). In this study, soft resin (15.73%) of leaf powder of *Rhamnus prinoides* was significantly ($P \leq 0.05$) greater than *Humulus lupulus* used as control (Table 3). On the other hand, soft resin (12.4%) of stem powder of *Rhamnus prinoides* was statistically ($P \leq 0.05$) greater than *Garcinia cola* (6.07%) and *Azadirachta indica* (10.28%). In this investigation, quantity soft resin in leaf of *Rhamnus prinoides* was comparable with that of hops (*Humulus lupulus*) used for beer production. The hard resin (4.74%) of *Rhamnus prinoides* was significantly ($P \leq 0.05$) greater than *Garcinia cola* (2.17%) and *Humulus lupulus* (2.03%) hard resins but less than the control *Humulus lupulus*.

At the same time, the hard resin (4.74%) of *Rhamnus prinoides* stem powder was statistically ($P \leq 0.05$) greater than *Grongonema latifollum* (4.35%) *Garcinia cola* (2.17%) and *Humulus lupulus* (2.03%) but less than the rest bittering agents on table 3. In this study, the quantity of hard resin in the leaf of *Rhamnus prinoides* was very low in comparison with other bitter source substances found in the literature (Adama *et al*., 2011, Kunze, 1996, Ajebesone and Aina, 2004).

The amount of essential oil (1.13 ± 0.02 %) of leaf powder of *Rhamnus prinoides* was statistically ($P \geq 0.05$) in line with the quantity of essential oil found in bitter substances in the investigated bittering substances (Adama *et al.*, 2011, Kunze, 1996; Ajebesone and Aina, 2004) but greater than the essential oil of *Rhamnus prinoides* stem powder. Essential oil is the best source of aroma and flavor during beer production (Briggs *et al*., 1981). In this study, the quantity of essential oil from leaf (1.13%) and stem (0.60%) powder of *Rhamnus prinoides* was within the range of dry hops contains 0.5 – 2% of essential oil (Kovacevic and Kac, 2001).

In this study, different physicochemical characteristics of *Rhamnus prinoides* leaf and stem powder were determined in comparison with reference standard commercial hops. The results obtained from *Rhamnus prinoides* for total resin, soft resin, hard resin and essential oil were found to be comparable with that of the values of different commercial varieties of hops obtained from the literature and with that of commercial hop used as control in the laboratory. Thus, *Rhamnus prinoides* can substitute the standard commercial hops for even beers brewed for commercial purpose.

Antimicrobial activity of gesho extract was determined using standard methods. Bothe ethanol

Table 4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of gesho against tested pathogenic standard and clinical isolate bacteria

Test pathogenic bacteria	MIC (mg/ml)	MBC (mg/ml)
1. *Escherichea coli* (ATCC 25922)	97.5	195
3. *Lyesria moncytogenes* (ATCC 19116)	390	390
4. Staphylococcus aureus (ATCC 25923)	97.5	195
5. *Staphylococcus sp.* (clinical isolate)	390	780
6. *Shigella flexneri* (ATCC 12022)	97.5	195
8. *Shigella dysenterae* (clinical isolate)	780	780
9. *P. vulgaris* (ATCC 881)	390	780
10. *Salmonella* NCTC 8385	195	195
11. *Streptococcus pneumoniae* (ATCC 63)	390	390

Table 5. Risk of having pathogenic bacteria in local *tella* (Positive = +, Negative = - for tested standard bacteria)

Code of test organisms	Filtered *tella* with Gesho	Filtered *tella* without Gesho
1. *Escherichea coli* (ATCC 25922)	-	+
3. *Lyesria moncytogenes* (ATCC 19116)	+	+
4. Staphylococcus aureus (ATCC 25923)	-	+
5. *Staphylococcus sp.* (clinical isolate)	-	+
6. *Shigella flexneri* (ATCC 12022)	-	+
8. *Shigella dysenterae* (clinical isolate)	-	+
9. *P. vulgaris* (ATCC 881)	-	+
10. *Salmonella* NCTC 8385	-	+
12. *Streptococcus pneumoniae* (ATCC 63)	+	+

and methanol extracts have been shown antimicrobial activity against different species of pathogenic bacteria using well assay and the clear zone diameter of the wells were greater than 10 mm in all pathogenic bacteria. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of gesho against tested pathogenic standard and clinical isolate bacteria was shown on Table 4. The MIC and MBC of gesho extract against tested pathogenic standard and clinical isolate bacteria were ranged from 97.5 to 780 mg/ml and 195 to 780 mg/ml, respectively. From this study, it is possible to conclude that gesho extract during *tella* brewing can inhibit bacteria growth and thereby help to extend the self-life of the product.

The contamination possibility of *tella* with pathogenic bacteria was determined by deliberately inoculation of pathogenic bacteria to *tella* with gesho and without gesho (Table 5). Except *Listeria moncytogenes* (ATCC 19116) and *Streptococcus pneumoniae* (ATCC 63), any of the others were not grown and detected in *tella* brewing with gesho but in case of *tella* without gesho all test pathogenic bacteria were grown. This result indicated that gesho extracts during *tella* brewing process can inhibit most bacterial growth. Any way brewing of *tella* should be carried out in aseptic conditions in order to avoid risks of having pathogenic bacteria.

Conclusion

In this study, the microbial flora such as molds and bacteria were almost disappeared at the end of *tella* fermentation phase, especially in *tella* made with gesho. The possible reason for this phenomenon may be due to the synergic effect of both *Rhamnus prinoides* antibacterial substance, high alcohol concentration, reduction of pH as fermentation time increases and reduction of nutrient content of *tella.* Yeasts were dominated at the middle of *tella* fermentation phases but at the last phase, they were dramatically reduced in colony count.

According to this finding, total resin, soft resin, hard resin and essential oil of *Rhamnus prinoides* were found to be comparable with that of the values of different commercial varieties of hops obtained from the literature and with that of commercial hop used as control in the laboratory. Therefore, *Rhamnus prinoides* can substitute the standard commercial hops for even beers brewed for commercial purpose. Based on the data generated to determine MIC and MBC of gesho extract, it is possible to conclude that gesho extract during *tella* brewing can inhibit bacteria growth and thereby help to extend the self-life of the product.

At this moment, the large numbers of people consume *tella* almost in all part of Ethiopia. However, the quality of *tella* is variable from local to local, from individual to individual and even within the single individual the quality of *tella* is variable from time to time. So there is a need for standardization of this microbial product by biotechnological process in order to deliver a consistent and uniform product. To do in such a way, the data generated this investigation can play great role and can serve as base line for optimization and standardization of local beer (*tella*) production.

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