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Full Length Research Paper

Evaluation of starter culture fermented sweet potato flour using FTIR spectra and GCMS Chromatogram

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Starch is the major component of cereal grains and starchy foods, and changes in its biophysical and biochemical properties (such as, amylose, amylopectin, pasting, gelatinization, viscosity) will have a direct effect on its end use properties (such as, bread, malt, polymers). *Lactobacillus brevis* and *Debaromyces polymorphous* earlier obtained from fermented sweet poatao broth were used to ferment sweet potato and these starter cultures broke down the carbohydrate (starch) to produce alcohol, organic acid and carbon dioixde (CO_2). The study identified that starter cultures *L. brevis* and *D. polymorphous* fermented the sweet potato thereby breaking down the carbohydrate (starch) to produce alcohol, organic acid and CO_2 hence lactic acid fermentation occurred. Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography Mass Specrometry (GCMS) were used to identify the chemical properties of starter culture fermented sweet potato flour. The FTIR spectra showed peaks at 3322.15, 3298.87, 3292.59, 3279.59 and 3274.59 cm^{-1} for the raw sweet potato, starter culture fermented sweet potato flour at various periods (24, 48 and 72 h) and spontaneous fermented sweet potato (control) respectively. The peaks at 2930, 2928.10, 2930.33, 2929.48, 2929.31 and 2927.29 cm^{-1} are attributed to C–H bond stretching. Functional groups such as hydroxyl, aldehydes, alcohol and carboxyl were detected in the fermented samples. The GCMS analysis detected the presence of alcohol such as ethanol, butanol etc., and carboxylic acid such as hexadecanoic acid, octadecadienoic acid etc. They were produced *in situ* from the fermentation process and this can serve as antioxidants, help inhibit spoilage organisms and serve as preservatives, thereby increasing shelf life of the product.

Key words: Sweet potato starch, fermentation, FTIR, GCMS.

INTRODUCTION

Carbohydrate is a class of chemical compounds that consists of carbon, oxygen and H_2 (Kim et al., 2007). It

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includes sugars, starch and cellulose. These compounds are classified as monosaccharides (such as, glucose, fructose), disaccharides (such as, sucrose, lactose) or polysaccharides (such as, starch, cellulose) (Kim et al., 2007). All carbohydrate compounds have been used as a source of biomass and a large number of microorganisms use them as energy source as a result, carbohydrates are essential for maintaining life (Kim et al., 2007). Glucose is the key compound as most life systems are built around it.

Starch is a carbohydrate consisting of glucose compounds joined to form a polysaccharide (Dias et al., 2008). It is a plant natural energy source that is most abundant and valuable which needs to be converted to simple sugars before it can be utilized as a carbon source (Yoo and Jane, 2002; Mosier et al., 2005; Yang et al., 2006; Gray et al., 2006).

The starch stored in the seeds and tubers of various agricultural crops including maize, wheat, rice, barley, potato and cassava provide the main sources of energy in the human diet (Evers et al., 1999; Perez et al., 2009; Perez and Bretoft 2010, Schwartz and Whistler, 2009). Starch is the major component of cereal grains, and changes in its biophysical and biochemical properties are related to the amount and ratio of amylose and amylopectin, which influences and affect properties such as viscosity, gelatinization, that will determine its end use properties (such as, bread, malt, beer, polymers) (Evers et al.,1999; Schwartz and Whistler, 2009; Willett, 2009).

Starch functions mainly as a carbohydrate source for the growing plant (such as, for germinating seeds and leaf tissue development) and is consequently the primary source of stored energy in the plant. Depending on the plant, starch can be found in a variety of tissues, including leaves, tubers, fruits, and seeds. It is the primary source of stored energy in cereal grains. Although the amount of starch contained in grains varies, it is generally between 60 and 75% of the weight of the grain and provides 70 to 80% of the calories consumed by humans worldwide.

It consists of two α-glycan bipolymers, namely, amylose and amylopectin (Yang et al., 2006; Dias et al., 2008; Shariffa et al., 2009). Amylose is a more linear glucose polymer consisting of 200 to 20000 glucose units forming a helix shape; while amylopectin is a highly branched molecule of 10-15 nm in diameter and 200-400 nm long (Yoo and Jane, 2002; Yang et al., 2006; Shariffa et al., 2009).

Amylopectin consist of D-glucopyranose monomers linked to either α-(1,4) or α-(1,6) glucosidic bonds (Yang et al., 2006). The joined monomers of α-(1,4) results in a linear chain; however, α-(1,6) bond serves as a glue that joins together the linear chains (Yang et al., 2006). Amyloses consist of linear glucan connected via α-(1,4) bonds (Lesmes et al., 2009). Starches contain about 17 to 28% of amylose (Matveev et al., 2001). Microbial enzymes (Wang et al., 2008) easily hydrolyze these bonds.

Root vegetables are plant roots used as vegetable, they are generally storage organs enlarged to store energy in form of carbohydrates, starch root vegetable are important staple food particularly in tropical regions overshadowing cereals throughout much of west Africa, Central Africa, they are used directly or mashed to make fufu or poi. Storage roots can be categorised in bulb, rhizome and tubers. Examples of tuberous root include desert yam (*Ipeoma costata),* sweet potato (*Ipeoma batatas*), cassava (*Manihot esculenta*), etc. Sweet potato carbohydrate has been reported to contain pectin substances, lignin, cellulose and hemicellulose, which are all converted to simple sugars when fermented (Yokoi et al., 2001).

Sweet potato has been processed into prickles and consumed as lacto-juices by processing it with lactic acid bacteria as the fermenting organism and the juice produced has been reported to be very rich in minerals and vitamins (Smita et al., 2007; Panda and Ray, 2007). Sweet potato has also been processed into chips in much the same way as Irish potato (Brigato et al., 2010; Hagenimana and Owori, 1998). It can also be eaten boiled, fried and in roasted form. In addition, it can be sliced, dried in the sun and ground to give flour that remains in good condition for a long time (Wheatly, 2009). Sweet potato can be fermented, dried and milled into flour. Fermentation is the conversion of carbohydrate into alcohols and short chain fatty acids by enzymes of microorganisms (Silva et al., 2008; Yuan et al., 2008). It is a basis of many biological products which involves a process of chemical reactions with the use of microbes such as bacteria, yeast and filamentous fungi (Huang and Tang, 2007; Fortman et al., 2008). The primary benefit of fermentation is the conversion of sugars and other carbohydrates to usable products. As stated earlier, not all bacteria can readily use starch as their energy and carbon source (Nigam and Singh, 1995). This means that some starches need to be broken down to simple fermentable sugars so they can be utilized by bacteria (Nigam and Singh, 1995). On the other hand, sweet potato is rich in β-amylase, which converts long chained starch into readily used maltose units making it a good energy and carbon source for bacteria (Yoshida et al., 1992; Brena et al., 1993; Cudney and McPherson, 1993; Nigam and Singh, 1995). Starter cultures are living microorganisms of defined combination used for fermentation purposes. They help to elicit specific changes in the chemical composition, nutritional value and sensorial properties of the substrate (Opere et al., 2012) and they are generally recognised as safe (Aguirre and Collins, 1993). Moreover, their properties are as follows: they are harmless, initiate and control the fermentation process, typical for product, help in rapid acid formation, and help protect against spoilage organisms. Starter cultures are cheaply reproducible in large amount; they also help provide desirable sensory properties and assist in reducing fermentation period. Ajayi et al. (2016) have done work on the fermentation of sweet potato into flour using starter culture.

Fourier transforms infrared (FTIR) spectroscopy is one of the most important and emerging tool used for analysing functional groups present in test samples. This technique is rapid and sensitive with a great variety of sampling techniques. FTIR is a rapid, non-

destructive, time saving method that can detect a range of functional groups and is sensitive to changes in molecular structure. FTIR provides information on the basis of chemical composition and physical state of the whole sample (Cocchi et al., 2004). In addition the sensitivity and accuracy of FTIR detectors along with wide variety of software algorithms have dramatically increased the practical use of infrared for quantitative analysis (Dowell et al., 2006). FTIR works because of functional groups and provide information in the form of peaks.

GCMS combines the features of gas chromatography and mass spectrometry to identify different substances within a test sample. Gas chromatography portion separates the chemical mixture into pulses of pure chemicals and the mass spectrometer identifies and quantifies the chemicals. It reveals the compounds eluted at different retention times with mass spectra corresponding to compounds present (Siong et al., 2014).

This study aimed at investigating the functional groups of the Starter culture fermented sweet potato flour using Fourier transform infrared (FTIR) spectroscopy as well as reporting the effect of the functional group on the products, the chemical compounds present in the starter culture fermented sweet potato flour will be detected using the GCMS.

MATERIALS AND METHODS

Sourcing of raw materials

Yellow-fleshed sweet potatoes were obtained from Oshodi market (Oshodi), Lagos, Nigeria. The samples were transported to the biotechnology department of the Federal Institute of Industrial Research for immediate use.

Starter cultures

The starter cultures used were obtained from the biotechnology department of the Federal Institute of Industrial Research Oshodi.

Potato dextrose agar and De man Rogosa Sharpe (MRS) agar were prepared using manufacturers specification and sterilized using the autoclave at 121°C for 15 min. *L. brevis* and *D. polymorphous* stored in MRS and PDA slants were subcultured into freshly prepared MRS and PDA agar plates.

Preparation of innoculum

This was carried out using the method of Asmahan et al. (2009). *Lactobacillus brevis* were cultivated by streaking on MRS agar plates (Oxoid) and incubated anaerobically at 37°C for 24 h. A colony was picked from each pure culture plate, grown successively in MRS broth before centrifugation at 5000 rpm for 15 min. The pellet was washed in sterile distilled water centrifuged again and redistributed in distilled water. This procedure achieved a culture preparation containing about 10^9 colony forming units cfu/ml, checked as viable count on MRS agar. Pure cultures of *D. polymorphous* were cultivated by streaking on Potato dextrose agar (Oxoid), incubated at 37°C for 24 h and the picked colony was inoculated into yeast extract peptone dextrose broth (YEPD) and incubated at 28°C for 24 h. These cultures were centrifuged and washed as described above. This procedure achieved a culture preparation containing 10^7 cfu/ml, as viable count on potato dextrose agar.

Preparation of starter culture fermented sweet potato flour

The sweet potatoes were washed to remove adhering soil particles and peeled. The peeled tubers were chipped into slices (4-5 mm).Starter cultures were prepared and inoculated into the sweet potato, then left to ferment for a period of two days (48 h).

After this period has elapsed, the fermented chips were drained and dried in a cabinet drier (Mitchel, Model SM220H) at 55°C for 9 h and milled into flour (≤ 250 µm) using the method of Ajayi et al. (2016) and the starter culture fermented sweet potato flour was produced.

Preparation of fermented sweet potato flour (control)

The sweet potatoes were washed to remove adhering soil particles and peeled. The peeled tubers were chipped into slices (4-5 mm) and soaked in potable water for a period of two days (48 h).

After this period has elapsed, the fermented chips were drained and dried in a cabinet drier (Mitchel, Model SM220H) at 55°C for 9 h and milled into flour (≤ 250 µm) (Oluwole et al., 2012).

Fourier Transform Infrared (FTIR)

FTIR spectra illustrate absorption bands with characteristic frequency attributed to different functional groups and all spectra were obtained, using a Bruker FTIR CLASS 1 ALPHA. The spectra were collected at a resolution of 4 cm^{-1} in the range of 500- 4000cm^{-1}. Each spectrum was rationed against a fresh background spectrum recorded from the bare crystal. Prior to collection of each background spectrum, the crystal was cleaned with absolute ethanol to remove any residual. Each sample was scanned in triplicate.

Gas chromatography mass spectrometry (GCMS)

Ten grams of samples were dissolved in 15ml of ethanol. The sample was analysed on a Shimadzu GC-MS system model QP2010, with a medium polarity capillary column SLB-5ms supelco column (length 30.0m x thickness 0.20 mm × Diameter 0.20 mm), with helium as the carrier gas. Column oven temperature was at 40°C, injection temperature was at 250°C, injection mode split (10:1), temperature program was 40°C (Hold 3 min) 9°C/min to 290°C (Hold 6mins), MS ion source temperature at 200° C interface temperature at 250°C. Detector voltage = Relative to the tuning result solvent cut time 4min, Acqusition mode-scan, scanning range 40-550 mz. One microlitre of the sample was injected using splitless injection with injector temperature 300°C according to the following scheme: 50°C for 2min with 10°C/min up to 300°C. The final temperature was held for 10 min. The total runtime for each sample was 37min. For MS detection, electron ionization with 70 eV was applied and mass fragments were detected between 40 and 500 m/z. The ion source temperature and transfer line temperature were 200°C and 300°C, respectively. The detector was activated after 5min.

RESULTS

The FTIR Spectra were recorded in regions below 800 cm^{-1}, 500 cm^{-1} (the fingerprint region), the region between 2,800 and 3,000 cm^{-1} (C-H stretch region), and finally the region between 3,000 and 3,600 cm^{-1} (O-H stretch region) (Table 1).

The O-H $_{stretching}$ for the raw sweet potato occurred at 3322.15 cm^{-1}.The peaks at 2928.10 cm^{-1} was observed as a result of C–H bond$_{stretching.}$ The peaks at 1097 cm^{-1}

Table 1. GCMS Peak report for raw sweet potato (G).

Peak #	R Time	Area	Area %	Height	Height (%)	A/H	Name
1	5.720	1027541	0.27	220079	0.64	4.67	Pyrimidine -2,4(1H,3H) -dione, 5-amino-6-nitro
2	6.268	3010757	0.80	1092925	3.19	2.73	2 -Furanmethanol
3	6.556	677120	0.18	262700	0.77	2.58	Propanoic acid, 2- oxo
4	6.802	2180121	0.58	454271	1.32	4.80	Cycloserine
5	7.150	1176657	0.31	484808	1.41	2.43	Dihyroxyacetone
6	7.406	881086	0.24	487437	1.42	1.81	Glyceraldehyde
7	7.440	2679476	0.72	565822	1.65	4.74	Ethanamine, N- ethyl- N- (1-methylethoxy)meth
8	7.726	112304	0.30	540485	1.58	2.08	6-Oxa-bicyclo[3.1.0] hexan-3-one
9	8.793	324740	0.09	178905	0.52	1.82	4H-pyran-4-one, 2,3- dihydro-3, 5- dihydroxy -6-
10	9.175	8645942	2.31	923865	2.69	9.36	2 -Hydroxy -gamma- butyrolactone
11	9.518	1320939	0.35	234913	0.69	5.62	2-propanol, 1- chloro- 3- (1- methylethoxy
12	9.734	482881	0.13	157144	0.46	3.07	Tetrahydro-4H- pyran-4-ol
13	10.015	492607	0.13	245929	0.72	2.00	Esprocarb
14	10.198	2912455	0.78	707736	2.06	4.12	1, 3-Dioxol-2-one
15	10.390	555860	0.15	167491	0.49	3.32	(3-Methyl-oxiran-2-yl)-methanol
16	10.498	2298739	0.61	666881	1.95	3.45	2,5-Dimethyl-4-hydroxy-3(2H)-furanone
17	10.552	557914	0.15	225743	0.66	2.47	6,7-Dioxabicyclo[3.2.2] nonane
18	10.818	2540952	0.68	662644	1.93	3.80	Cyclopentane, 1-acetyl-1,2-epoxy-
19	11.049	5259236	1.41	816451	2.38	6.44	Cyclopropyl carbinol
20	11.432	253776	0.07	82742	0.24	3.07	Homopiperazine
21	11.592	542159	0.14	161569	0.47	3.36	Hexane, 1, 1- oxybis
22	11.725	2355125	0.63	971901	2.83	2.42	Pentanoic acid, 4- oxo-
23	11.938	7737745	2.07	2812686	8.20	2.75	4H- Pyran-4- one,-2,3- dihydro-3,5- dihydroxy
24	12.203	3353258	0.90	475206	1.39	7.06	2(3H) Furanone, dihydro-4- hydroxy-
25	12.759	1974174	0.53	565909	1.65	3.49	Catechol
26	12.863	1769021	0.47	479487	1.40	3.65	Butanenitrile, 2,3- dioxo-, dioxime, O, O - diacet
27	13.168	1356627	0.36	311275	0.91	4.36	2- Furanmethanol,tetrahydro-5-methyl-
28	13.306	10515375	2.81	3280635	9.57	3.20	5-Hydroxymethylfurfural
29	13.596	1615272	0.43	388747	1.13	4.16	1,2,3- Propanetriol, 1- acetate
30	13.793	1298808	0.35	374957	1.09	3.46	Dimethylmucanoic acid
31	14.936	67094767	17.93	2900347	8.46	23.13	Sec - Butylnitrite
32	17.641	76203093	20.37	2020814	5.89	37.71	Sucrose
33	18.306	2901986	0.78	295502	0.86	9.82	Butyl 2- acetoxyacetate
34	17.597	51846108	13.86	3099727	9.04	16.73	1,6-Anhydro-2,4-dideoxy-beta-D-ribohepoxy
35	20.113	87200037	23.30	2246302	6.55	38.75	3- Deoxy- d- mannoic acid
36	23.010	5199882	1.39	1042633	3.04	4.99	n - Hexadecannoic acid
37	23.151	703283	0.19	425723	1.24	1.65	Scopoletin
38	23.344	465000	0.12	310018	0.90	1.50	Hexadecanoic acid, ethyl ester
39	24.832	4985304	1.33	913118	2.66	5.44	9, 12- Octadecadienoic acid (Z, Z) -
40	25.102	605346	0.16	264178	0.77	2.29	12 - Methyl- E- 2, 13-octadecadien-1- ol
41	26.656	4610547	1.23	1353688	3.95	3.41	1-Benzoyl-2-t-butyl-3-methyl-5-vinylimidazoli
42	28.399	304422	0.08	105130	0.31	2.90	6H - Pyrazolo [1, 2-a] [1,2,4,5] tetra-zine, hexahydr
43	28.557	188310	0.05	98405	0.29	1.91	Hexanol
44	29.915	715744	0.19	131722	0.38	5.43	Z, Z- 10, 12- Hexadecadien- 1- ol acetate
45	34.066	238334	0.06	76775	0.22	3.10	alpha- Tocopheryl acetate
		374181566	100.00	34285425	100.00		

and 1019 cm^{-1} were assigned as the C–O bond $_{stretching}$ (Table 2). This indicates that compounds belonging to hydroxyl group, hydrocarbon and aldehydes group are present in the raw sweet potato.

GCMS peak report reveals the compounds eluted at different retention times with mass spectra corresponding to compounds present. Figure 1 shows the GCMS chromatogram of raw sweet potato sample from 5.72 to 34.066 s. It showed 45 peaks, compounds such as pyrimidine-2,4(1H,3H) dione was detected with a retention time of 5.72 s and an area percentage of 0.27%, propanoic acid had the retention time of 66.556

Table 2. GCMS Peak report for starter culture fermented sweet potato flour 24 h (H).

Peak #	R. Time	Area	Area %	Height	Height %	A/H	Name
1	5.043	407371	0.54	253520	2.70	1.61	Fornamide, N - mmet hoxy-
2	5.245	316923	0.42	178489	1.90	1.78	(S)-(+)-1, 2- Propanediol
3	7.118	23036722	30.71	1491815	15.87	15.22	L - Lactic acid
4	9.032	2009458	2.68	228988	2.44	8.78	1,2,3,4 - Butanetetrol, [S-(R*,R*)]-
5	11.789	402293	0.54	175872	1.87	2.29	4H- Pyran-4-one, 2,3-dihydro-3,5- dihydroxy-6-
6	13.238	416022	0.55	197832	2.10	2.09	5- Hydroxymethylfurfural
7	14.241	20559512	27.40	1087754	11.57	18.90	Oxirane,(propoxymethyl)-
8	15.175	189501	0.25	94701	1.01	2.00	Phenol,2,6- dimethixy
9	16.340	322572	0.43	161965	1.72	1.99	Benzeneethanol
10	16.679	3480912	4.64	375027	3.99	9.28	1,3-propanediol, 2-(hydroxymethyl) -2-nitro-
11	17.373	85920	0.11	43809	0.47	1.96	Propanediol acid, propyl-
12	17.789	354609	0.47	50080	0.53	7.08	Erythritol
13	18.162	294498	0.39	104520	1.11	2.82	n- Decanoic acid
14	18.558	1777004	2.37	285223	3.03	6.23	Diethyl Phthalate
15	18.999	1142058	1.52	201352	2.14	5.56	D -erythro - Pentose, 2- deoxy-
16	23.025	6648837	8.86	1508499	16.05	4.16	n -Hexadecanoic acid
17	24.357	170486	0.23	83154	0.88	2.05	Decanoic acid, ethylester
18	24.848	6196913	8.26	1082209	11.51	5.93	9,12 - Octadecadienoic acid (Z,Z)-
19	25.123	2650278	3.53	364339	3.88	6.86	Octadecanoic acid
20	26.295	79665	0.11	40134	0.43	1.98	Pentanal
21	26.632	319090	0.43	125222	1.33	2.55	2,4-Di-tert- butyl phenyl benzoate
22	26.730	327804	0.44	98670	1.05	3.32	Oxirane, dodecyl-
23	28.406	838178	1.12	308627	3.28	2.72	Hexadec anoic acid, 2- hydroxy-1- (hydroxymeth
24	28.560	414349	0.55	1522901	1.62	2.72	trans-2- Dodecene-1-ol
25	29.928	1497026	2.00	316816	3.37	4.73	Z,Z- 3, 13- Octadecedien-1-ol
26	30.867	710752	0.95	320106	3.41	2.23	Squalene
27	33.517	374032	0.50	67679	0.72	5.53	Carbamic acid, N-[10, 11- dihydro-5- (2 methyla
		75022785	100.00	9398692	100.00		

s and an area percentage of 0.018% and sucrose had a retention time of 17.641s and an area percentage 20.37%.

Various functional groups such as hydroxyl group, carboxyl group were also observed in Figure 2. The peak at 3298.87 cm^{-1} is attributed to O–H $_{stretching}$. The O-H $_{stretching}$ was also observed and after 24h fermentation using starter cultures it reduced to 3298.87cm^{-1}. The 2930.33 cm^{-1} peak observed is attributed to C–H bond $_{stretching.}$ The peaks at 1412.94 cm^{-1} is attributed to the bending modes of O–H.

The GC/MS analysis of the starter culture fermented sweet potato for 24 h of wheat extract showed the presence of 27 compounds corresponding with retention time from 5.043 to 33517 s. The most intensive peak was the lactic acid with an area percentage of 30% and a retention time of 7.118 s. The chromatogram had the following organic acids identified: octadecanoic acid at 25.123 s with an area percentage of 3.53%. n-Hexadecanoic acid at 23.025 s with an area percentage of 8.86% and oxirane had an area percentage of 27.40% and retention time of 14.241 s. The presence of lactic acid in the starter culture fermented sweet potato flour will help serve as a biopreservative, extend shelf life, and give a tart and tangy flavour (Schnurer et al., 2005; Cizeikiene et al., 2013).

The absorbance at 3292.59 cm^{-1} is attributed to O–H $_{stretching}$; while 2929.48 cm^{-1} is attributed to C–H bond $_{stretching}$. The peaks at 1412.83 cm^{-1} is attributed to the bending modes of O–H. The various functional groups detected are similar to those obtained in the 24 h fermented sweet potato flour.

Table 3 shows the chromatogram of starter culture fermented sweet potato flour after 48 h with 24 peaks. Lactic acid was detected and it also had the most intensive peak after 48 h fermentation using starter cultures (Figure 3), it had an area percentage of 55.53% and a retention time of 7.378 s, oxirane was detected at 14.117 s with an area percentage of 11.70%. Other compounds such as 9,12-octadecanoic acid (Z,Z) are polysaturated fatty acid which are anti-inflammatory, hypocholestrolemic, and cancer preventive according to Adeoye-Issijola et al. (2018) were detected with an area percentage of 10.265% and a retention time of 24.850 s. At 23.026 s, n-Hexadecanoic acid was detected with an area percentage of 11.87%.

The absorbance spectra obtained from Figure 4 shows O–H $_{stretching}$,C–H bond $_{stretching}$ and O–H$_{bending.}$

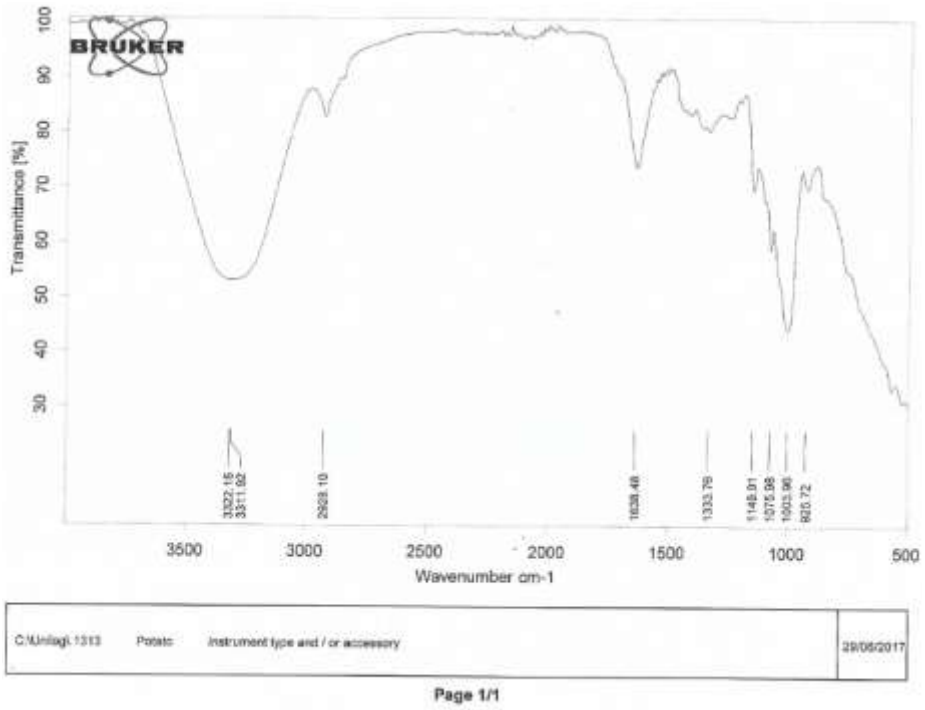

Figure 1. FTIR absorbance spectra for raw sweet potato (G).

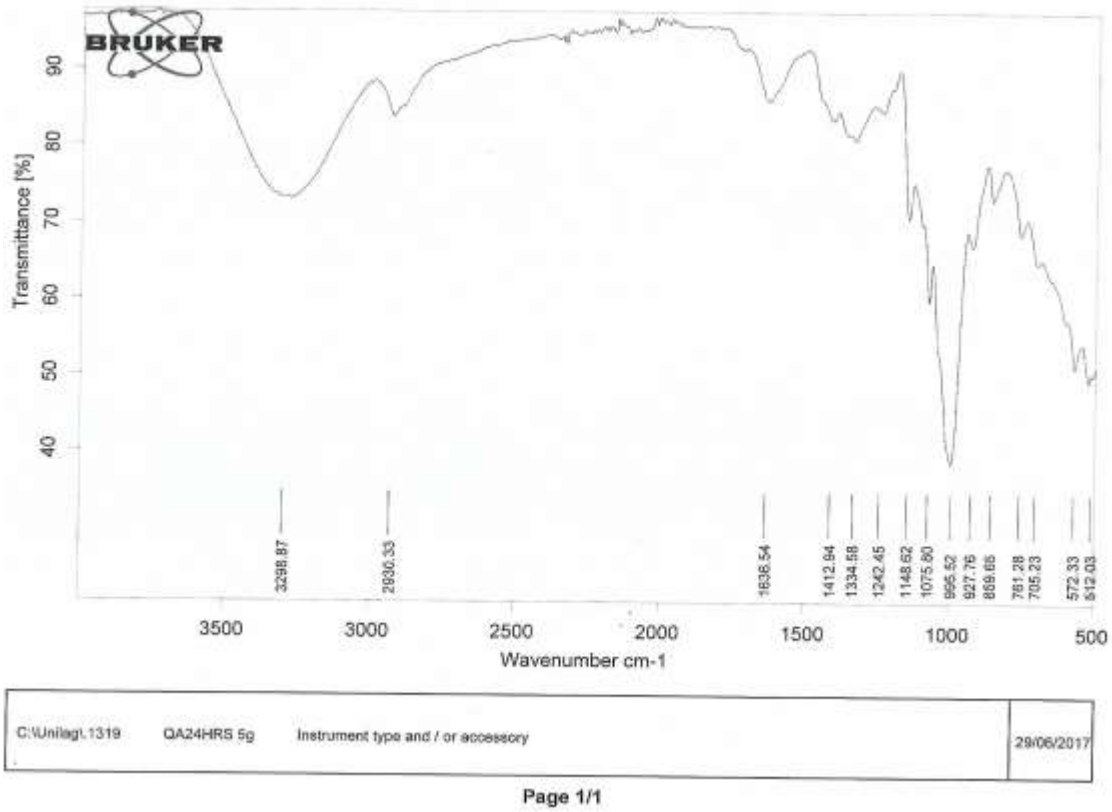

Figure 2. FTIR absorbance spectra for Starter culture fermented sweet potato flour 24 h (h).

The absorbance at 3279.59 cm^{-1} is attributed to O–H $_{stretching}$ while C–$H_{stretching}$ occurred at 2929.31 cm^{-1} (Table 4). The peaks at 1414.11 cm^{-1} is attributed to the bending modes of O–H.

The chromatogram for 72 h starter culture fermented sweet potato flour is showed in Table 5 and Figure 5. Compounds such as n-Hexadecanoic acid with area percentage of 11.875% with a retention time of 23.026

Table 3. GCMS Peak report for starter culture fermented sweet potato flour 48 h (H).

Peak #	R. Time	Area	Area %	Height	Height %	A/H	Name
1	5.232	932784	0.86	473537	3.70	1.97	(S)-(+)-1,2 - Propanediol
2	7.378	60375505	55.53	2912673	22.76	20.68	L- Lactic acid
3	9.052	1980434	1.82	170548	1.33	11.61	1,2,3,4- Butanetetrol,[S-(R*,R*)]-
4	14.117	12717260	11.70	874627	6.83	14.54	Oxirane, (Propoxymethyl)-
5	15.180	196411	0.18	100516	0.79	1.95	Phenol, 2,6- dimethoxy
6	15.514	196211	0.18	54044	0.42	3.63	Butanal, 3- hydroxy
7	16.336	188766	0.17	87901	0.69	2.15	Methanimidane, N, N- dimethyl-N'-phenyl
8	16.586	1416787	1.30	240524	1.88	5.89	1,3- Propanediol, 2- (hydroxmethyl) -2- nitro
9	17.712	260612	0.24	78974	0.62	3.30	1- Imidazolidine carboxaldehyde, 5- hydroxy-2,4
10	18.165	464043	0.43	147730	1.15	3.14	undecanoic acid
11	18.563	842982	0.78	191940	1.50	4.39	Diehtyl Phthalate
12	18.954	369712	0.34	87169	0.68	4.24	Butanal, 3-methyl -
13	22.132	185140	0.17	89378	0.70	2.07	1- Undecanol
14	23.031	7858036	7.23	211210	16.50	3.72	n- Hexadecanoic acid
15	23.346	590259	0.54	222690	1.74	2.65	Ethyl tridecanoate
16	24.854	9832442	9.04	2098048	16.39	4.69	9, 12 -Octadecadienoic acid (Z, Z)-
17	25.107	3374356	3.10	509313	3.98	6.63	Z,Z- 8, 10 Hexadecadien-1-ol
18	26.614	507526	0.47	259537	2.03	1.96	1-(2- Tetrahydrofurylmethyl)piperidine
19	28.032	352962	0.32	128807	1.01	2.74	9,12- Octadecadienal
20	28.405	1081626	0.99	407600	3.19	2.65	Hexadec anoic acid, 2-hydroxy-1- (hydrometh)
21	28.561	596971	0.55	188941	1.48	3.16	Tridecanal
22	29.925	2653622	2.44	606240	4.74	4.38	9,12- Octadecadienoic acid (Z,Z)-, 2- hydroxy-1
23	30.866	1446910	1.33	697038	5.45	2.08	Squalene
24	33.512	297818	0.27	58459	0.46	5.09	Carbamic acid, N-[10,11- dihydro-5-(2-methyla
		108719175	100.00	12797444	100.00		

s was detected.9,12 octadecanoic acid (Z,Z) had a retention time of 24.850 s and an area percentage of 10.26% was detected. The chromatogram had 26 peaks and the most intensive peak was lactic acid with an area percentage of 58.645% and a retention time of 7.295 s. n-Hexadeacanoic acid (palmitic acid) is a fatty acid which is are antioxidant, antibacterial, anti-inflammatory, cancer preventive amongst other (Adeoye-Isijola et al., 2018).

In Figure 5 3274.59 cm^{-1} could be attributed to O–H $_{stretching}$. 2927.29 cm^{-1} are attributed to C–H bond $_{stretching.}$ The peaks at 1410.41 cm^{-1} was attributed to the bending modes of O–H.

The chromatogram of the control (spontaneously fermented sweet potato flour) was observed in Figure 5 with 22 peaks. It also had lactic acid has the most intensive peak with an area percentage of 28.71% at 6.92 s retention time, octadecanoic acid (Z,Z) was also detected at 24.843 s with an area percentage of 10.64%. n-Hexadecanoic acid was also detected with an area percentage of 11.8755% at a retention time of 23.026 s (Table 6).

DISCUSSION

The peaks obtained were at 3322.15, 3311.92 , 2928, 1638.48 cm^{-1} for the raw sweet potato, 3298.87, 2930.33,1636.54, 1412.94 and 995.52 cm^{-1} for the starter culture fermented sweet potato flour for 24 h. Then, 3292.59, 2929.48,1637.64, 1412.83 and 994.41 cm^{-1} for starter culture fermented sweet potato flour for 48 h 3279.59, 2929.31, 1636.20, 1414.11, 994.75 cm^{-1} the starter culture fermented sweet potato flour for 72 h. Also, 3274.59, 2927.29, 1635.65, 1414.41 and 995.42 cm^{-1} for the spontaneously fermented sweet potato flour for 72 h. This is in line with earlier works carried out on starch contents spectra of starch were recorded using FT-IR, key bands by Belton et al. (1991). Spectra were recorded in regions below 800 cm^{-1}, 500 cm^{-1} (thefingerprint region), the region between 2,800 and 3,000 cm^{-1} (C-H stretch region), and finally the region between 3,000 and 3,600 cm^{-1} (O-H stretch region). Fourier transform infrared (FTIR) spectroscopy is a tool used to differentiate between patterns of amylose in different granule. Peaks near 3500, 3000, 1600, 1400, 1000, 800, described the IR spectrum of starch samples and Manley et al. (2002) to determine the presence of moisture used 500 cm^{-1} as seen in the study by Zeng et al. (2011) and it. Moisture contents were also on mid infrared range. Peaks for water were observed on 1,640 and 3,300 cm^{-1}. The absorption is done on the base of functional groups H and OH. The absorption spectra of starter culture fermented sweet potato flour and the control shows strong peaks in the same region indicating the presence of moisture in the

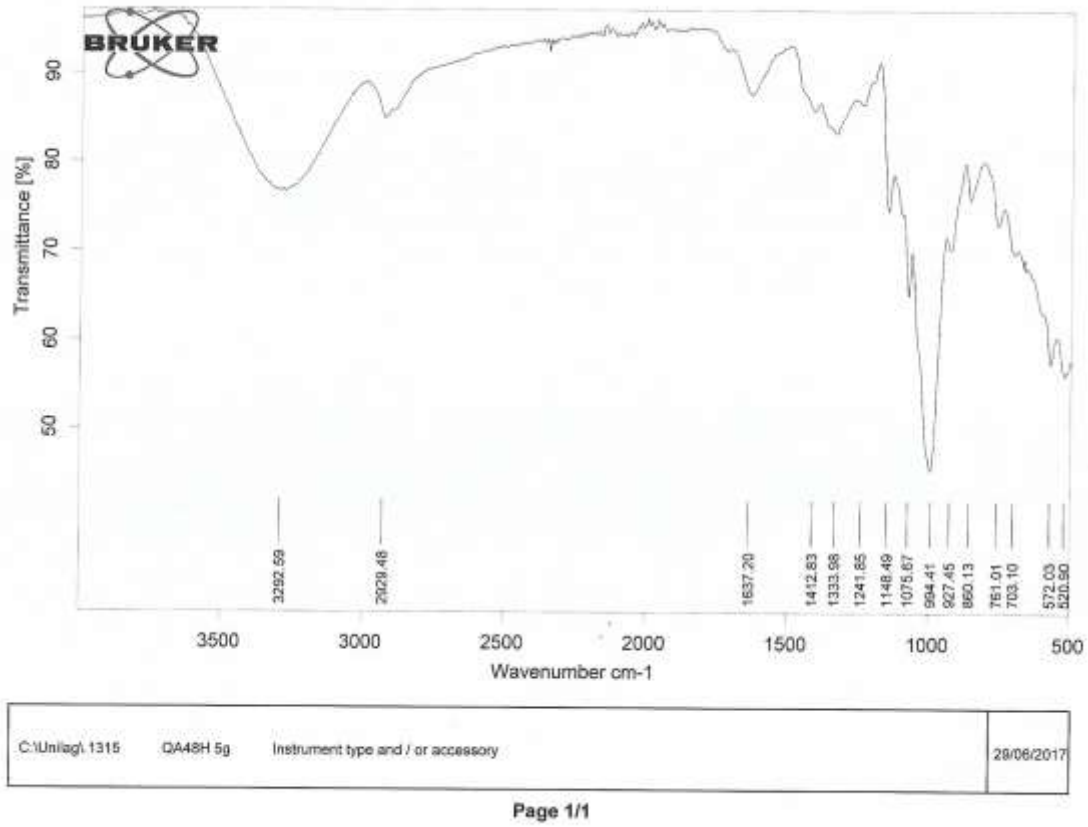

Figure 3. FTIR absorbance spectra for starter culture fermented sweet potato flour 48 h (I).

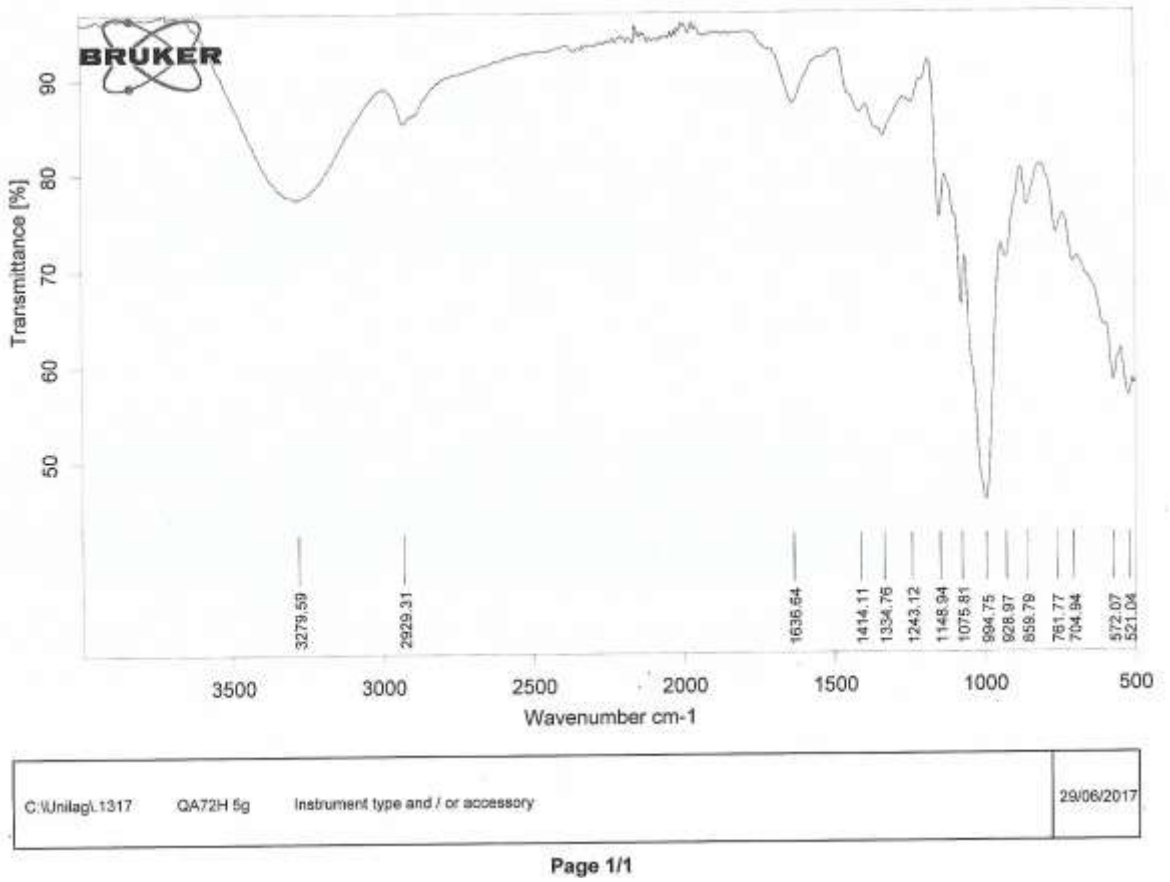

Figure 4. FTIR absorbance spectra for starter culture fermented sweet potato flour for 72 h (J).

Table 4. GCMS Peak Report for starter culture fermented sweet potato flour 72 h (J).

Peak #	R. Time	Area	Area %	Height	Height %	A/H	Name
1	5.235	663461	0.86	351595	3.60	1.89	(S)-(+)- 1,2- Propanediol
2	7.295	44982527	58.64	2582353	26.45	17.42	L- Lactic acid
3	8.298	78983	0.10	51596	0.53	1.53	Ethanamine, 2-propoxy-
4	9.048	1711842	2.23	182475	1.87	9.38	1,2,3,4- Butanetetrol, [S-(R*,R*)]-
5	9.848	271735	0.35	69837	0.72	3.89	Butanoic acid, 2- hydroxy-3- methyl-
6	10.011	58890	0.08	35416	0.36	1.66	Benzeneacetic acid 1- methyl ethylester
7	10.151	81200	0.11	36343	0.37	2.23	Oxirane, 2,3- dimethyl-, cis-
8	13.937	4303934	5.61	559557	5.73	7.69	2- furanol, tetrahydro-2,3- dimethyl -, trans-
9	15.780	68755	0.09	37857	0.39	1.82	2 - Heptanamine, 5- methyl-
10	15.900	58065	0.08	34379	0.35	1.69	3,4 - Hexanedione, 2,2,5- trimethyl-
11	16.332	148684	0.19	65630	0.67	2.27	2- propenoic acid, 3 - phenyl
12	17.690	417392	0.54	128830	1.32	3.24	1,2,3,4- Cyclopentanetetrol, (1.alpha., 2beta., 3.1
13	18.057	31594	0.04	17255	0.18	1.83	Cyclopropyl carbinol
14	18.160	210811	0.27	71411	0.73	2.95	Propanedioic acid propyl-
15	18.564	337334	0.44	115687	1.19	2.92	Diethyl Phthalate
16	18.674	61127	0.08	34664	0.36	1.76	Pentanal
17	23.026	9108301	11.87	2018266	20.68	4.35	n - Hexadecanoic acid
18	23.347	197817	0.26	112642	1.15	1.76	Ethyl tridecanoate
19	24.343	261728	0.34	88641	0.91	2.95	Oxalic acid, allyl pentadecyl ester
20	24.850	7871474	10.26	1756535	17.99	4.48	9, 12- Octadecadienoic acid (Z, Z)-
21	25.109	2179835	2.84	388434	3.98	5.61	9, 9- Dimethoxybicyclo[3. 3. 1] nona- 2,4- dione
22	28.404	507649	0.66	175857	1.80	2.89	Hexadecanoic acid, 2 -hydroxy-1-(hydroxymeth
23	28.562	390981	0.51	129992	1.33	3.01	Heptanal
24	29.562	1156839	1.51	206547	2.12	5.60	2- Methyl- Z,Z-3,13- octadecadienol
25	30.865	982993	1.28	419254	4.29	3.34	2,6,10,14,18- Pentamethyl-2,6,10,14,18- eicosap
26	33.516	569698	0.74	90453	0.93	6.30	Carbamic acid, N -[10, 11- dihydro-5-(2-methyla
		76713649	100.00	9761506	100.00		

Table 5. GCMS Peak Report for starter culture fermented sweet potato flour 72 h (J).

Peak #	R. Time	Area	Area %	Height	Height %	A/H	Name
1	5.059	537269	2.06	117343	2.34	4.58	Ethanol, 2-nitro-
2	5.264	140986	0.54	67247	1.34	2.07	Formamide
3	6.920	7496208	28.71	617319	12.33	11.93	L- Lactic acid
4	8.773	212749	0.81	52524	1.05	4.05	Propanedioic acid, propyl-
5	8.849	671411	2.57	102578	2.05	6.55	1,2,3,4 - Butanetetrol, [S-(R*, R*)]-
6	11.331	115365	0.44	53716	1.07	2.15	Phenylethyl Alcohol
7	13.932	4338523	16.62	454674	9.08	9.29	(S)-(-)-1,2,4- Butanetriol, 2- acetate
8	15.177	84153	0.32	60439	1.21	1.39	Phenol, 2,6- dimethoxy-
9	18.156	15142	0.59	50058	1.00	3.08	Propanedioic acid, propyl-
10	23.026	3099692	11.87	1051581	21.00	2.95	n - Hexadecanoic acid
11	23.346	308180	1.18	126965	2.54	2.43	Decanoic acid
12	24.343	116249	0.45	63494	1.27	1.83	2- Heptanamine, 5-methyl -
13	24.843	2779010	10.64	524660	10.48	5.30	9,12- Octadecadienoic acid (Z,Z)-
14	25.102	1304627	5.00	263292	5.26	4.96	9- Octadecyonic acid
15	25.336	183668	0.70	57224	1.14	3.21	Hexanal
16	26.429	150043	0.57	65403	1.31	2.29	2- Heptanamine, 5 -methyl-
17	26.961	173089	0.66	87917	1.76	1.97	Cyclooctyl alcohol
18	28.032	534373	2.05	130463	2.61	4.10	Z, -1,9- Hexadecadiene
19	28.405	751722	2.88	284641	5.69	2.64	Hexadecanoic acid, 2- hydroxy-1-(hydroxymeth
20	29.931	1550434	5.94	288336	5.76	5.38	Z,Z -3, 13- Octadecedien-1-ol
21	30.867	985028	3.77	403555	8.06	2.44	Squalene
22	31.417	419765	1.61	82964	1.66	5.06	Carbamic acid, N -[10,11-dihydro-5-(2-methyla
		26106596	100.00	5006393	100.00		

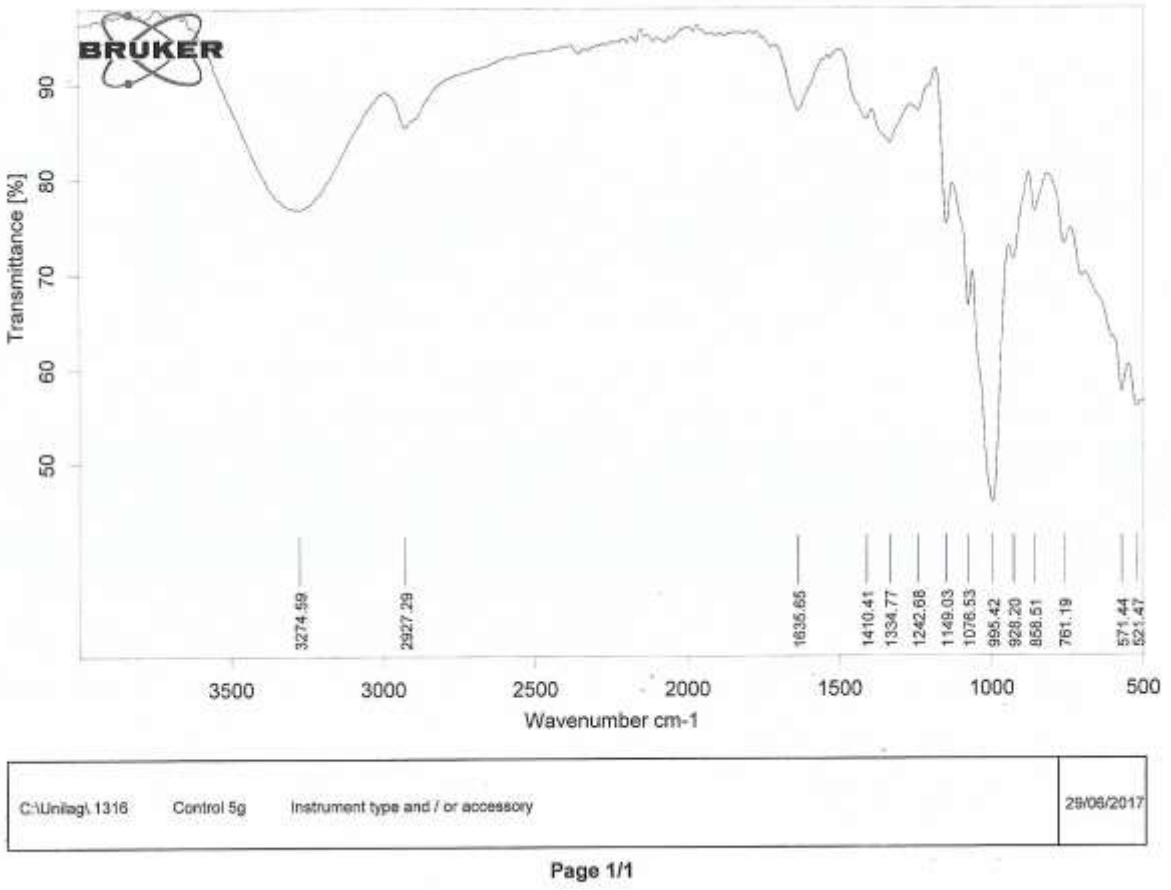

Figure 5. FTIR absorbance spectra for starter culture fermented sweet potato flour for 72 h control (K).

flour. FTIR is one of the most elusive methods for the analysis of moisture. Water absorbs strongly in the infrared region of the spectrum due to its O-H stretching and H bending vibrations however its quantization is frequently complicated by spectral interferences from other OH containing constituents such as alcohols, phenols and hydroperoxides and confounded further by hydrogen bonding effects (Dong et al., 2000).

The O-H $_{stretching}$ for the raw sweet potato occurred at 3322.15 cm^{-1} and after 24h fermentation using starter cultures, it reduced to 3298.87cm^{-1}; at 48 h it was 3279.59 cm^{-1} and at 72h it was 3279.59 $cm^{-1.}$ The decrease in the wavelength with increase in time depicts dilution of the crystalline (amylose) region, leading to the breakage of α- 1,4- glycosidic linkage. Consequently, the hydrophilic O-H group will contribute to the increasing amorphous fraction.

The peaks at 2928.10, 2930.33, 2929.48, 2929.31 and 2927.29 cm^{-1} are attributed to C–H bond $_{stretching.}$ The C-H $_{stretching}$ for raw potato, starter culture fermented sweet potato flour at 24 h, 48 h, 72 h were 2928.10, 2930.33, 2929.48 and 2929.31 cm^{-1} respectively. These values fall within the same range. This indicates that there was a change in the functional group and this could be the oxidation of an aldehyde group (CHO) during the fermentation.

Supriya et al. (2015) observed that Crude fat of flour samples had peaks at 1,600 cm^{-1} to 1,700 cm^{-1} and 1,550 to 1,570 cm^{-1}.The absorption peaks are determined on the basis of C-H bonds. The absorption spectra of starter culture fermented sweet potato flour also show strong peaks in the same region, which indicates the presence of fat in flour. It has been reported that FTIR spectroscopy could be utilized as a quality control method for fat and moisture determination in butter and high-fat products (Van de Voort et al., 1992). Che-Man and Setiowaty (1999) and Rai et al. (2013) also reported similar results.

Supriya et al. (2015) observed absorption bands that are two primary features of the protein, amide I and amide II bands at approximately 1,660 cm^{-1} and 1,550 cm^{-1}, respectively. Amide I arises from the stretch of C=O of the peptide group in the protein. Peaks were also observed around this region for the starter culture fermented sweet potato flour as well as the control.

A cursory look at the spectra shows a slight decrease in the wavelength of the carboxyl ($C=O_{stretching}$) of the α 1,4- and α1,6- glycosidic linkeage (1638.48 cm^{-1} for raw sweet potato,1636.54 cm^{-1} after 24 h,1637.20 cm^{-1} after 48 h and 1636.64 cm^{-1} after 72 h. The slight decrease in wavenumber indicates arial oxidation to carboxylic acid such as, acetic acid /ascorbic acid which can serve as preservative to the flour.

The stretching vibration for α 1,4- glycosidic linage at the amylose region decreased from 1003.96 cm^{-1} for raw sweet potato to 994.75 cm^{-1} after 72 h, this will

Table 6. Summary of some compounds obtained from GC-MS analysis.

Duration	Compounds	Area (%)
0 h (G)	3- Deoxy-d-mannoic acid	23.3
	Sucrose	20.37
	Sec- Butylnitirte	17.93
	1,6- Anhdr-2,4-dieoxy-beta-D-ribo-hexopy	13.86
	5-Hydroxymethylfufura	2.81
24 h (H)	L-Lactic acid	30.71
	Oxirane ,(Propoxymethyl)	27.4
	n-Hexadecanoic acid	8.86
	9,12-Octadecadienoic acid (Z, Z)	8.26
	1,3-Propanediol,2-(hydroxymethyl)-2-nitro	4.64
	Octadecanoic acid	3.53
	1,2,3,4-Buatanetetrol	2.68
	Diethyl phthalate	2.37
	L-Lactic acid	55.53
48 h (I)	Oxirane	11.7
	9,12-Octadecanoic acid	9.04
	n-Heaxdecanoic acid	7.23
	Z, Z-8,10-Hexadecadien-1-ol	3.1
	9,12-Octadeacadienoic acid	2.44
	1,2,3,4-Butanetetrol	1.82
72 h (J)	L-Lactic acid	58.64
	n-Hexadecanoic acid	11.87
	9,12-Octadecadienoic acid	10.26
	2-Furanol,tetrahydro-2,3-dimethyl-trans-9,9-	5.61
	Dimethoxybicyclo(3.3.1)nona-2,4-dione	2.84
	1,2,3,4-Butanetetrol	2.23

further release more glucose unit into the amorphous region thus enhancing the swelling properties of the flour.

The peaks at 1412 .94, 1412.83, 1414.11 and 1414.41 cm^{-1} were attributed to the bending modes of O–H.

The peaks at 1097 and 1019 cm^{-1} were assigned as the C–O bond stretching. The bands at 1047 and 1022 cm^{-1} were associated with the ordered and amorphous structures of starch respectively.

The starter culture fermented sweet potato flour had more peaks than the control after 72 h fermentation. It also had higher concentration of lactic acid, which indicates a faster rate of fermentation with the use of starter cultures. The presence of high concentration of lactic acid will also help inhibit the presence of spoilage organisms, the elimination of spoilage organisms and it will serve as preservatives.

Some of the compounds detected by the GCMS include 9,12-octadecadienoic acid; hexadecanoic acid which are antioxidant; ethanol which is used as food preservative and propanoic acid which is widely used as an antifungal agent amongst others Supriya et al. (2015).

Generally, the chemical components were identified in each sample. The purpose of the study was identify the chemical components and compare presence of components in the various sample. Similar chromatograms were also obtained for all the starter culture fermented sweet potato samples. This study has shown that it is possible to compare the chemical compounds of samples with GCMS chromatography.

Conclusion

The study reveals that sweet potato contain starch (amylose and amylopectin) and sucrose as sugar.

Starter cultures *L. brevis* and *D. polymorphous* fermented the sweet potato thereby breaking down the carbohydrate (starch) to produce alcohol, organic acid and CO_2 hence lactic acid fermentation occurred.

Functional groups such as hydroxyl, aldehydes, alcohol and carboxyl are present in the fermented samples. Lactic acid fermentation occurred and it caused a shift in some of the functional groups.

The chemical shift indicates that the starter culture fermented sweet potato flour containing compounds

such as carboxylic acids, alcohols, aldehydes, hydroxyl and alkenes.

Starter culture fermented sweet potato flour had a higher concentration of carboxylic acids, alcohols, aldehydes etc. Alcohol and carboxylic acid was produced in situ from the fermentation process, which will help inhibit spoilage organisms moreover, it will also serve as preservatives thereby increasing shelf life of the product.

The FTIR showed a similar spectrum for all the fermented sweet potato flour and it was possible to verify the main organic functions associated, the results corroborate the wide variety of volatile organic compounds identified by the CGMS. The presence of OH group detected by FTIR which was further emphasized by the presence of butanol detected by the GCMS, and the presence of the C=O group detected by FTIR which was also emphasized.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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In vitro antibacterial activity of *Rumex nervosus* and *Clematis simensis* plants against some bacterial human pathogens

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Due to quick growth of resistance and high cost of new generation antibiotics, lots of efforts were made to discover new antimicrobial agents from various sources. So, current study was assessed antibacterial activity of ethanol, methanol, acetone, diethyl ether and hexane leave extracts of *Rumex nervosus* and *Clematis simensis by* used paper disc diffusion and broth dilution procedures against six human pathogenic bacterial strains. The pathogenic bacteria were *Shigella dysenteriae, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella* Typhi and *Klebsiella pneumoniae* were susceptible to ethanol, methanol and acetone extracts of the leaves of *R. nervosus* followed by *C. simensis,* but hexane extract didn't displayed any activity. The extreme inhibition zone of 16.3±0.57 mm was detected against *E. coli* by ethanol extract of *R. nervosus* and MIC of 3.125 mg/ml against *E. coli* and *S. dysenteriae* by methanol extract. The methanol extract of *C. simensis* formed a marked inhibition of 13.1±0.37 mm against *E. coli* and ethanol extract of *C. simensis* displayed activity against *S. dysenteriae* 14.4±0.45 mm and MIC of 6.25 mg/ml against *S.* Typhi. Four dissimilar antibiotics like ciprofloxin, tetracyclin, kanamycin and chloramphenicol were used as standard for tested antibacterial activity against six different human pathogens. The activities were recognized the presence of some secondary metabolites existed in the tested floras which have related with antibacterial activities.

Key words: Antibacterial activity, *Clematis simensis,* Human pathogens, *Rumex nervosus*.

INTRODUCTION

Traditional medicine is a popular form therapy in developing countries and its use broadly recognized in numerous literatures. The improving emergence of antimicrobial resistance deteriorates the impact (Mulu et al., 2006; Olivier et al., 2010). It has been shown that risk of negative clinical consequences, mortality, and high treatment costs with drug-resistant bacteria is generally higher compared to patients infected with the same non-resistant bacteria (WHO, 1991). Improved prevalence of resistant bacteria, together with lack and high cost of new generation drugs has escalated infection-related morbidity and mortality particularly in developing countries like

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Ethiopia (Mulu et al., 2006; Borkotoky et al., 2013). This proliferation endorsed to undifferentiating use of broad-spectrum antibiotics, immunosuppressive agent, intravenous catheters and ongoing epidemics of HIV infection (Dean and Burchard, 1996; Gonzalez et al., 1996). However, the progress of new antibiotics should continue as the primary significance to retain the usefulness of antimicrobial treatment (Marchese and Shito, 2001). The potential of floras are bases for modern medicine to achieve new values (Evans et al., 2002).

In recent years, pharmacological enterprises consumed a lot of time and money in developing natural products extracted from plants, to harvest extra cost real medicines that are reasonable to the population (Doughari, 2006). Today, many commercially confirmed drugs used in modern medicine were firstly used in crude form in traditional or folk healing performs, or for other purposes that suggested potentially useful biological activity. The therapeutic floras around the world contain various compounds with antibacterial activity (Marjorie, 1999). So, orderly screening them may result in the detection of novel real antimicrobial compounds (Costa et al., 2008). The screening of plant extracts and plant products for antimicrobial activity has shown that floras represent a potential source of new anti-infective agents (Amani et al., 1998; Costa et al., 2008). Many researches have carried out to screen natural products for antimicrobial property (Nair and Chanda, 2006). Therapeutic floras possess immune modulatory and antioxidant properties, leading to antibacterial activities. They have versatile immune modulatory activity by stimulating both non-specific and specific immunity (Pandey and Chowdhry, 2006).

Rumex nervosus mostly originated high altitude areas (above 1000 m) and continue about 200 species. The leaves of this plant are edibles in Ethiopia. In Ethiopia, the leaves and stem of this herb are used for purifying the body by women traditionally as substituent of olive tree, to do this, the leaves are put on fire then they cover the patient body with that hot leaves and blanket so that the vapors and smoke surround all the body. *Rumex* species contains anthrax Editorial Office derivatives like chrysophanol, physician, emodin, aloe-emodin, rhein; which are the main biologically active compounds responsible for anti-cancer, cytotoxic, genotoxic and mutagenicity properties (Wegiera et al., 2012). Traditionally in Ethiopia, the leaves, stems and roots of *R. nervosus* were used as traditional medicines, for the eye disease, taeniacapitis, hemorrhoids, infected wounds, arthritis, eczema, abscess and gynecological disorders.

Clematis simensis is woody climber that escalates up to 10m or more, occasionally with long branches lying on the ground. The stem is pubescent; leaves are pinnate while the leaflets are ovate. The superior of the leaves have disseminated hairs while the inferior one is to Mentos. The inflorescence was various flowered, the flowers being pale yellow to white in color (Edwards et al., 2000). Traditionally in Ethiopian the plants leaves were used for dress wounds and also for the treatment of eczema, *tinea capitis* and tropical ulcers *and also the* seeds of this plant were used for rheumatic pain while the sap was used as a febrifuge and against bloat in animals. A recent study reported that the leaves of *C. simensis* used in combination with another plant from the same family (Addis et al., 2001; Gedif et al., 2001). Traditionally plants used for the treatment of gonorrhea, syphilis and sore throat. The leaves have also been used for the treatment of leprosy, fever and various skin diseases and headaches (Iwu, 1993; Kakwaro, 1976). The extracts leaves of *C. simensis* by aqueous and methanol are exhibit activity against certain bacteria like *Staphylococcus aureus*, *Pseudomonas aeruginosa* and fungi *Candida albicans* (Desta et al., 1993; Cos et al., 2002).

In Ethiopia, medicinal plants are still the most important and occasionally the only bases of therapeutics for nearly 80% of human and more than 90% in livestock population. Estimated floras of 6,500 to 7,000 species of higher plants are originated in Ethiopia and about 12% are endemic to the country (Tadeg et al., 2005). Despite their vital role in providing for the health of human and livestock population, large part of the knowledge of ethno medicinal plants is irreversible loss and declining to deterioration due the oral passage of herbal heritage from generation to generation rather than in writings (Mesfin et al., 2009). Ecological degradation, farming growths, cultivation of marginal lands and suburbanization are also posing a significant threat to the future wellbeing of human and animal populations that have relied on these resources to fight several ailments for generations (Lulekal et al., 2008; Devi et al., 2009).

MATERIALS AND METHODS

Location of the study area

The study was conducted on selected medicinal plants composed from Sinana and Agarfa districts of Bale zone, Oromia Regional State, South Eastern Ethiopia. Sinana district was found at 430 km southeast of Addis Ababa. The area was situated at 7°7’ N and 40°10’ E and 2,400 masl. The mean average rainfall of the area was 353 mm. For the same period, average annual maximum temperature was 21.2°C and minimum temperature was 9.4°C. The dominant soil type was pellic vertisol and slightly acidic (pH = 6). Agricultural production system of the study area was mixed farming. Agarfa district was located at 464 km south east of Addis Ababa. The area was situated at 6°11’ N and 40°3’ E and 2,350 masl. The mean average rainfall of the area was 880 mm and bimodal. The average annual maximum temperature was 24.75°C and minimum temperature was 7.1°C. The dominant soil type was clay soil and slightly acidic (pH = 5.8). Agricultural production system of the study area was mixed farming (Figure 1).

Collection and identification of plant materials

Two medicinal plants *R. nervosus* and *C. simensis* were collected

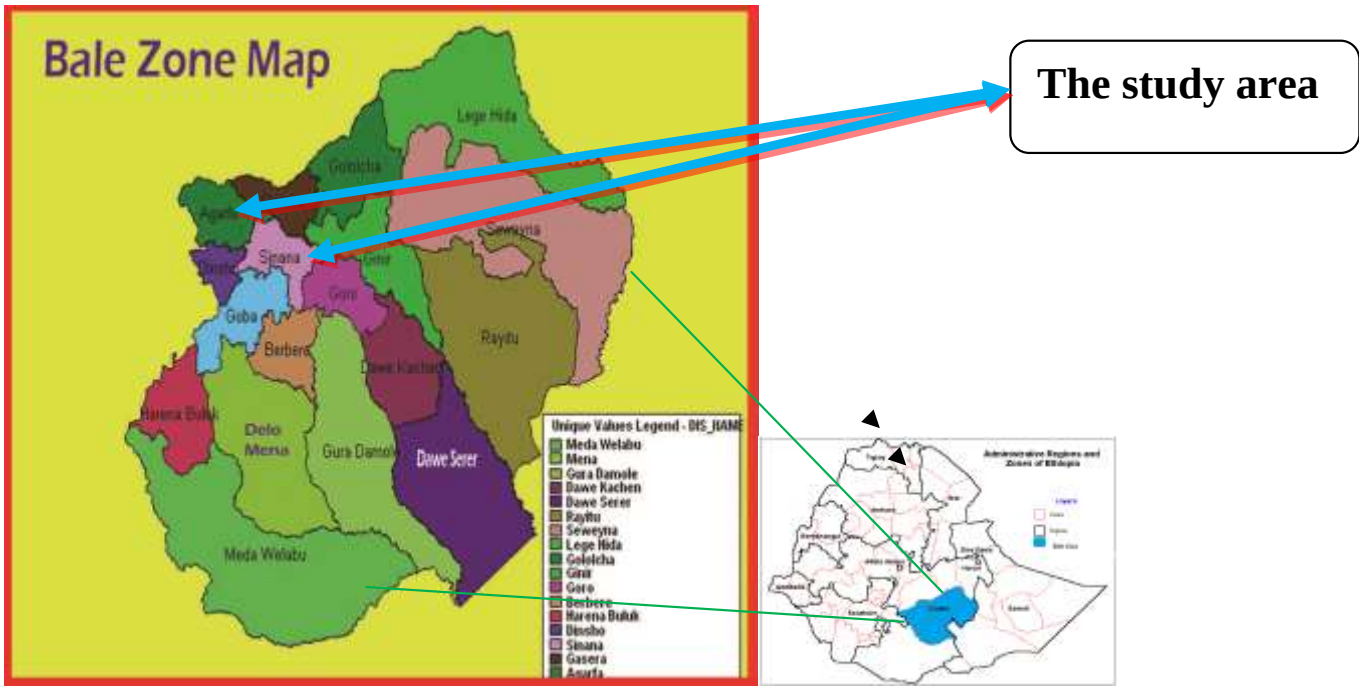

Figure 1. Map of study area.

from Bale Zone, Sinana and Agarfa district Oromia region, Ethiopia. The taxonomic situation of the plants was identified and authenticated by plant experts from National Herbarium in Addis Ababa University. Leaves from the study plants were taken in a large quantity and repeatedly washed under tap water to remove any debris and were air dried under shade for fifteen days.

Preparation of plant's crude extracts

The preparation of crude extracts of plants under this study was conducted followed the methods used by Tadeg et al. (2005) used different solvents. Five hundred grams of leaves from each plant was taken for extraction procedure and ground in a mortar and pestle separately under aseptic condition. Twenty grams of each powdered plant material were extracted with apparatus with 250 ml of ethanol, methanol, diethyl ether, hexane and acetone separately by maceration for 48 h with frequent agitation on orbital shaker for continuous two days and the resulted liquid was filtered (Whatman No. 3 filter paper, Whatman Ltd., England). Extraction was repeated five times and the filtrates of all portions were combined in one vessel. The organic solvent was removed by evaporation used Rota vapor (BU¨CHI Rota-vapor R-205, Switzerland) at 40°C. The resulted dehydrated mass was then crushed, packed into a glass vial until used. Finally, the gram yield of dried residue of each plant extracts were calculated. The concentrated extracts were stored at 4°C for the next antimicrobial study. Dried residues were dissolved in 100% dimethyl sulfoxide (DMSO) to obtain a stock concentration of 100 mg/ml, which was kept at 4°C until used.

Preparation of tested microorganisms

The tested microorganisms included *Escherichia coli, Salmonella typhi, Shigella dysenteriae, P. aeruginosa, S. aureus* and *K. pneumoniae* were obtained from Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia. These microorganisms were suspended in nutrient broth and subcultured into fresh nutrient agar medium and kept at 4°C until used. The inoculated preparation was standardized by inoculated bacterial strains from the exponential phase and standardized with 0.5 McFarland turbidity standard prepared by added a 0.5 mL aliquot of 1.175% w/v $BaCl_2.2H_2O$, added to 99.5 mL of 0.18 mol/L H_2SO_4 (1% v/v).

Antimicrobial assay

Antibacterial sensitivity tested used disc diffusion method

The antibiotic susceptibility tested, stock concentrations of (100mg/ml) plant crude extracts were prepared in DMSO. A circular antibiotic assay disc of 6 mm diameter was prepared from the Whatman filter paper No. 3 and sterilized by autoclave for 15 min at 121°C. The sterile discs were impregnated with 50 µl of the reconstructed extract and were dried completely at 37°C overnight. A sterile cotton swab was dipped into a homogenous suspension of tested microorganism with adjusted 0.5 McFarland turbidity standards. The tested pathogenic microorganisms were swabbed gently by cotton swab onto Muller Hinton Agar (MHA) and were then allowed to dry for half an hour. The discs were aseptically placed over plates of Muller Hinton Agar (MHA) (Haniyeh et al., 2010). The plates were incubated in an upright position at 37°C for 24 h and the zone of inhibition was measured (in mm diameter). Inhibition zones with diameter less than 12 mm was considered as had low antibacterial activity. Diameters between 12 and 16 mm was considered moderately active, and these with >16 mm was considered highly active (Indu et al., 2006). The tested microorganisms were tested for their sensitivity against the standard antibiotics, Ciprofloxacin (35 µg), Chloramphenicol (30 µg) Tetracycline (30 µg) and Kanamycin (20 µg) by the disc diffusion method (Bauer et al., 1966).

Minimum inhibitory concentration (MIC) assay methods

The MIC was determined by compared the various concentrations of plant extracts which have different inhibitory effect and selected the lowest concentration of extract showed inhibition (Agatemor, 2009). The MIC was determined for extracts that showed inhibition zone of≥7 mm diameter and for extract that inhibited the growth of all tested bacteria at concentration of 200 mg/ml. The tested was performed by used standard tube dilution (serial dilution) method used nutrient broth as diluents. Accordingly, the plant extract was prepared by double serial dilution from 200 mg/ml to obtain 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 in order to get 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 mg/ml concentration of extract, respectively using 50% DMSO. 1 ml of each extracts was dissolved in sterile test tubes which contained 9 ml of nutrient broth. Then, 0.1 ml of the tested microorganism was inoculated to the each tube. One tube was used as the control (broth + extract). The tubes were incubated at 37°C for 24 h and the existence of growth was assessed by compared the optical density (OD) of each well before and after incubation. When the difference of OD value (after incubation-before incubation) of the test (broth + extract + organism) was greater than that of the control (broth + extract) at each concentration, it was considered as presence of turbidity or growth of bacteria. The lowest concentration, at which there was no turbidity, was also regarded as MIC value of the extract.

Data analysis

Data on mean inhibition zone formed by each plant extract and MIC on various bacteria were entered in to Microsoft excels spreadsheet and SPSS (Statistical Package Software for Social Science version 16). Values were given as mean± standard deviation (SD).

RESULTS

Antibacterial activity of the plant extracts

The crude extracts study plant such as *R. nervosus* and *C. simensis* were tested for antibacterial activity on six human pathogens. The solvents that were used in this study produced an overall yield of plant crude extracts that were ranging from 0.6 to 2.4 gm from different plants (Table 1).

In-vitro antimicrobial activity of crude extracts of plants under this study was evaluated against human pathogenic bacteria of *E. coli, S.* Typhi*, S. dysenteriae, P. aeruginosa, S. aureus* and *K. pneumoniae.* The results obtained in the present study revealed that the tested two medicinal plants (*R. nervosus* and *C. simensis)* extracts possess a potential antibacterial activity.

The antibacterial activity of R. nervosus crude extracts

The antibacterial activity of *R. nervosus* crude extracts was assayed by disc diffusion method. The methanol and ethanol leaves extract of *R. nervosus* showed considerably a higher mean antibacterial activity as compared to other solvents. The highest antibacterial activity was exhibited on *E. coli* (16.3±0.57 mm) by ethanol extract, followed by *S. dysenteriae* (12.5±0.5 mm) and a moderate inhibition of *Klebsiella pneumoniae* (10±1.0 mm) and the least activity against *Salmonella typhi* (6.1±0.76 mm). The methanol extracts showed a strong inhibitory activity against *S.* Typhi (14.8±0.76 mm), followed by *S. dysenteriae* with a zone of inhibition 11±0.57 mm and a moderate inhibition against *S. aureus* (9.8±0.28 mm) and *P. aeruginosa* (8.8±0.76 mm). With methanol, a minimum zone of inhibition of *R. nervosus* (6.5±0.5 mm) was exhibited by *E. coli*.

Acetone extracts of *R. nervosus* were exhibited a maximum zone of inhibition against *S.* Typhi (11.9±0.35 mm) followed by *S. aureus* (10.5±0.5 mm) and minimum activity against *P. aeruginosa* (5.4±0.5 mm). Diethyl ether extracts showed inhibitory activity against only three pathogens. The maximum inhibition was detected on *S.* Typhi (6.2±0.68 mm) followed by *K. pneumoniae* (7.9±0.17 mm) and least activity against *E. coli* (4.8±0.76 mm). Hexane extract didn't show any antibacterial activity against tested pathogenic bacteria (Table 2).

The antibacterial activity of C. simensis crude extracts

The methanol extract of *C. simensis* formed a marked inhibition zone of 13.1±0.37 mm in diameter against *E. coli*, followed by *K. pneumoniae* (10.9±0.3 mm) and *S.* Typhi (9.7±0.64 mm). The methanolic extracts exhibited the least inhibitory activity against *S. dysenteriae* and *S. aureus* with mean inhibition zone of 7.2±0.46 and 7.7±0.45 mm, respectively. The prominent zone of inhibition from the ethanol extract of *C. simensis* against *S. dysenteriae* was 14.4±0.45 mm followed by *K. pneumoniae* (13.9±0.35 mm), *S.* Typhi (12.9±0.51 mm) and 11.5±0.51 mm against *E. coli*. Moderate inhibitory activity was noticed against *S. aureus* (10±0.15 mm) followed by 12.1±0.3 mm against *P. aeruginosa* and a moderate activity of 8±0.2 mm against *S. dysenteriae* and 7.9±0.35 mm against *K. pneumoniae* and minimum inhibitory activity against *E. coli* with a zone size of 5.6±0.52 mm. Acetone extract of *C. simensis* inhibited *S. aureus* with a highest zone of inhibition 11.9±0.25 mm and minimal inhibition was 6.8±0.2 and 5.7±0.32 mm against *S. dysenteriae* and *P. aeruginosa*. No good antibacterial activity was excreted by the Hexane extracts (Table 3).

Inhibitory zones of test pathogens with standard antibiotics (positive control)

Four dissimilar antibiotics, ciprofloxin, tetracyclin, kanamycin and chloramphenical were used as standard and as positive control for the testing of antibacterial activity of six different human pathogens. Ciprofloxin displayed maximum zone of inhibition ranging from 20 to

Table 1. The yield of plant crude extracts by using different solvents.

Plant species	Parts used (gm)	Extraction type	Yield in grams (Mean in mm)
Rumex nervosus	20 g Leaves	Methanol	1.6
		Ethanol	1.35
		Diethyl ether	0.6
		Acetone	1.6
		Hexane	1
Clematis simensis	20 g Leaves	Methanol	2
		Ethanol	2.4
		Diethyl ether	1.3
		Acetone	2.1
		Hexane	1.2

Table 2. The effect of the different extracts of the leaves of *Rumex nervosus* against tested pathogenic bacteria (Zones of inhibition in mm; mean±SD).

Test organism	Mean Inhibition zone of leaves extract of **R. nervosus in mm* (Mean±SD)				
	Methanol	Ethanol	Diethyl Ether	Acetone	Hexane
Escherichia coli	6.5±0.5	16.3±0.57	4.8±0.76	-	-
Salmonella typhi	14.8±0.76	6.1±0.76	-	11.9±0.35	-
Shigella dysenteriae	11±0.57	12.5±0.5	6.2±0.68	7.3±0.57	-
Staphylococcus aureus	9.8±0.28	8.6±0.52	-	10.5±0.5	-
Pseudomonas aeruginosa	8.8±0.76	6.1±0.36	-	5.4±0.5	-
Klebsiella pneumoniae	8.8±0.28	10±1.0	7.9±0.17	5.8±0.28	-

-= implies no inhibition zone detected; ∗ = a crude extract at concentration of 100 mg/ml was used for assay.

35 against all pathogens; Kanamycin exhibited average zone of inhibition 20mm, Tetracycline exhibited ranging from 8 to 20 mm and Chlomphenicol showed least inhibition against all test pathogens.

MIC of plant extracts

MIC assay was employed to evaluate the effectiveness of the plant extracts to inhibit the growth of bacterial tested microorganisms. The extracts of the two medicinal plants were exposed to the concentrations ranged from 0.78 to 100 mg/ml. In the antibacterial activity tested, five different solvents were used for their *in vitro* antibacterial tested among which only best three solvents methanol, ethanol and acetone had selected for MIC test.

MIC of R. nervosus leaf extracts against tested pathogenic bacteria (in mg/ml)

The methanol extract of *R. nervosus* exhibited the lowest MIC at 3.12 mg/ml against *E. coli* and *S. dysenteriae* followed by *S. typhi* and *P. aeruginosa* at a concentration of 6.25 mg/ml. The ethanol extract exhibited MIC at 3.12 mg/ml concentration against *S. dysenteriae* and *K. pneumoniae* and at concentration of 6.25 mg/ml against *E. coli.* The ethanol extract also displayed its MIC at concentration of 12.5 mg/ml against *S.* Typhi and *S. aureus.* The MIC of acetone extract of *Rumex nervosus* was 6.25 mg/ml against the *E. coli* and *S.* Typhi followed by *S. dysenteriae* at 25 mg/ml and *S. aureus* at 50 mg/ml (Table.5).

MIC of C. simensis leaf extracts against tested pathogenic bacteria in mg/ml

The methanol extract of *C. simensis* showed MIC activity at 6.25 mg/ml concentration against *E. coli* and *S. typhi* followed by *S. dysenteriae* and *K. pneumoniae* at 12.5 mg/ml concentration. The ethanol extracts showed strong MIC activity at 1.56 mg/ml against *S. dysenteriae* and against *S.* Typhi at 6.25 mg/ml concentration followed by *S. aureus* and *P. aeruginosa* at 12.5 mg/ml. The acetone extract of *C. simensis* exhibited a MIC at 12.5 mg/ml against *S. dysenteriae* followed by *S. aureus* at 25 mg/ml and at 50 mg/ml against *P. aerugenosa* and *K. pneumoniae* (Table 6).

Table 3. The effect of the different extracts of the leaves of *Clematis simensis* tested pathogenic bacteria (Zones of inhibition; mean±SD mm).

Test organism	Mean Inhibition zone of leaves extract* *Clematis simensis* (mean±SD mm)				
	Methanol	Ethanol	D/ Ether	Acetone	Hexane
Escherichia coli	13.1±0.37	11.5±0.51	5.6±0.52	-	-
Salmonella typhi	9.7±0.64	12.9±0.36	6.2±0.62	-	3.7±0.26
Shigella dysenteriae	7.2±0.46	14.4±0.45	8±0.2	6.8 ±0.2	-
Staphylococcus aureus	7.7±0.45	10±0.15	-	11.9±0.25	-
Pseudomonas aeruginosa	-	8.5±0.55	12.1±0.32	5.7±0.32	-
Klebsiella pneumoniae	10.9±0.3	13.9±0.35	7.9±0.35	6±0.2	-

-= implies no inhibition zone detected; ∗ = a crude extract at concentration of 100 mg/ml was used for assay.

Table 4. The inhibition zone of antibiotics against human pathogens.

Test organism	Zone of inhibition in mm			
	Ciprofloxin	Kanamycin	Tetracycline	Chlromphenicol
Escherichia coli	30	20	15	10
Salmonella typhi	35	20	15	10
Shigella dysenteriae	32	20	13	10
Staphylococcus aureus	31	20	10	5
Pseudomonas aeruginosa	30	15	8	5
Klebsiella pneumonia	20	15	20	11

DISCUSSION

Ethno botanical investigations have been found to offer significant evidences in the identification and development of traditionally used therapeutic florae into modern drugs. Involvement of the field has also reflected in the current study. The first step towards this goal was the in vitro antibacterial activity assay (Samy and Ignacimuthu, 2000). Many reports were available on the antiviral, antibacterial, antifungal, anthelminthic, and anti-inflammatory properties of plants (Palombo and Semple, 2001; Kumarasamy et al., 2002).

In the present study, *R. nervosus* and *C. simensis* was extracted by used different solvents such as methanol, diethyl ether, ethanol, acetone and hexane. The results of current study were an indication of such understandings. The yield of the extract that was obtained by different solvents considerably differs in two of the medicinal plants (Table 1).

In the present study, among the solvents used to extract the biologically active substances from two medicinal plants, ethanol and methanol were the best solvents, followed by acetone and least by diethyl ether and hexane (Tables 2 to 5). This specified that the extraction of medicinal plants with different solvents may produce different *in vitro* inhibitory result which based on the potential of the solvents used to extract the biologically active constituents (George et al., 2010). The methanol and ethanol leaf extracts of *R. nervosus* showed significant antibacterial activity against most of bacterial human pathogens evaluated in the present study. The highest antibacterial activity exhibited was against *E. coli* (16.3±0.57 mm) by ethanol extract, followed by *Shigella dysentriae* (12.5±0.5 mm) and a moderate inhibition against *K. pneumoniae* (10±1.0 mm). In the present study, the methanol extract exhibited the second with inhibition zone of 14.8±0.76 mm against *S.* Typhi, followed by *S. dysentriae* with a zone of inhibition of 11±0.57mm. A different study reported that the antibacterial activity of the methanolic extracts of *R. nervosus* leaves against *E. coli, P. aeruginosa, S. aureus, Streptococcus mutans* and *C. albicans*, with zones of inhibition of 38, 36, 15, 38 and 32 mm, respectively (Mariam et al.,1993).

Pavithra et al. (2011) reported that the methanol extracts of *Mollugo cerviana* inhibited the growth of *S. aureus* and *E. coli* with zones of 7.33±0.57 and 11±1 mm, respectively while chloroform extracts were ineffective against these bacterial strains. Current study showed that the methanolic extract of *Rumex nervosus* to have a strong inhibitory activity against tested pathogens which were in concordance with other studies. The decrease of antibacterial activity of *R. nervosus* against tested pathogens in the current study may be attributed to the difference in the initial plant extract used and extraction

Table 5. Minimum Inhibitory Concentration (MIC) of *Rumex nervosus* leaf extracts against bacterial tested microorganism in mg/ml.

Rumex nervosus	Conc. (mg/ml)	*Escherichia coli*	*Salmonella typhi*	*Shigella dysenteriae*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
Methanol	1.56	-	-	-	-	-	-
	3.12	**	-	**	-	-	-
	6.25	+	**	+	-	**	-
	12.5	+	+	+	-	+	-
	25	+	+	+	**	+	-
	50	+	+	+	+	+	-
Ethanol	1.56	-	-	-	-	-	-
	3.12	-	-	**	-	-	**
	6.25	**	-	+	-	-	+
	12.5	+	**	+	**	-	+
	25	+	+	+	+		+
	50	+	+	+	+	**	+
Acetone	1.56	-	-	-	-	-	-
	3.12	-	-	-	-	-	-
	6.25	**	**	-	-	-	-
	12.5	+	+	-	-	-	-
	25	+	+	**	-	-	-
	50	+	+	+	**	-	-

**Minimum Inhibitory concentration, + = Positive inhibition observed, - = No activities.

Table 6. Minimum Inhibitory Concentration (MIC) of *Clematis simensis* leaf extracts against bacterial tested microorganism in mg/ml.

Clematis simensis	Conc. (mg/ml)	*Escherichia coli*	*Salmonella typhi*	*Shigella dysenteriae*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
Methanol	1.56	-	-	-	-	-	-
	3.12	-	-	-	-	-	-
	6.25	**	**	-	-	-	-
	12.5	+	+	**	-	-	**
	25	+	+	+	-	-	+
	50	+	+	+	**	-	+
Ethanol	1.56	-	-	**	-	-	-
	3.12	-	-	+	-	-	-
	6.25	-	**	+	-	-	-
	12.5	**	+	+	-	-	**
	25	+	+	+	**	**	+
	50	+	+	+	+	+	+
Acetone	1.56	-	-	-	-	-	-
	3.12	-	-	-	-	-	-
	6.25	-	-	-	-	-	-
	12.5	-	-	**	-	-	-
	25	-	-	+	**	-	-
	50	-	-	+	+	**	**

**Minimum Inhibitory concentration, + = Positive inhibition observed, - = No activities (bacterial growth observed).

method used the difference in the strains of tested pathogens or due to unexplained reasons.

The acetone extracts of *R. nervosus* exhibited the maximum zone of inhibition against *S. typhi* (11.9±0.35 mm) followed by *S. aureus* (10.5±0.5 mm) and minimum activity against *P. aeruginosa* (5.4±0.5 mm). Related investigations have reported where acetone extracts showed a marked inhibitory effect on the growth of pathogenic bacteria (Abdullahi et al., 2010). The methanol and Ethanol extract of *R. nervosus* exhibited the lowest MIC at 3.21mg/ml concentration against *E. coli* and *S. dysentriae* and *K. pneumoniae.* The result of the present study showed that the plant extracts of *Clematis simensis* exhibited antibacterial activity against some of the common pathogenic bacteria. The prominent zone of inhibition from the ethanol extract of *C. simensis* against *S. dysenteriae* was 14.4±0.45 mm and against *K. pneumoniae* was 13.9±0.35 mm followed by *Salmonella typhi* 12.9±0.51 mm. Previous study showed that ethanolic extract of *C. simensis* exhibited a highest zone of inhibition (28.33 mm) against *S. aureus* with MIC 12.5 µg/ml (Mariam et al., 1993) a result higher than the size of inhibition zone in current study. The results of this study showed that the extracts from *C. simensis* was found to have significant antibacterial activity against both the selected Gram positive and Gram negative bacteria.

The methanol extract of *C. simensis* produced a pronounced inhibition zone of 13.1±0.37mm against *E. coli*, followed by *K. pneumoniae* with a zone of inhibition of 10.9±0.3 mm and *S.* Typhi *9.7*±0.64 mm. In current study, the result clearly shows that this plant was effective against *E. coli*. The possible explanation for this difference in inhibitory activity might be the ecological difference on their distribution plants which might have contributed to variations in the concentration of the active ingredients. The methanol extract of *C. simensis* showed MIC activity at 6.25 mg/ml concentration against *E. coli* and *S.* Typhi which was supported by work of (Mariam et al.,1993) where the MIC of isolated compounds from *Clematis simensis* against *S. aureus, Enterococcus faecalis, E. coli* and *P. aeruginosa* was found to be varied from 16 µg/ml to more than 250 µg/ml. Ethanol extract showed a very minimal MIC of 1.56 mg/ml against *S. dysenteriae* and *S.* Typhi which was strongly supported by the results of Tegenu (2011) where the Acetone and ethyl acetate extracts of the leaves of *Zehneria scabra* showed best activity against *S. aureus* exhibited an MIC of 1.56 and 0.781 mg/ml, respectively. The least inhibition zone was observed for hexane extract against *S.* Typhi according to Tsuchiya et al. (1996).

Conclusion

From the aforementioned results it could be determined that the crude extracts of the two plants especially the ethanol and methanol revealed the fact that they have higher potential to produce broad spectral antibacterial activity with minimal concentration against a wide range of human pathogens. The extracts were good in inhibited *E. coli, S.* Typhi*, S. dysenteriae, P. aeruginosa* and in some instances *K. pneumoniae.* The results of this study provided an insight into the antimicrobial properties of the extracts of *C. simensis* and *R. nervosus.* As well as it formed an opportunity for selection of bioactive extracts for initial fractionation and further studies of these two medicinal plants in the antibacterial assays. This *in vitro* study demonstrated that these two folklore medicinal plants have good potential. This study gives a suggestion of the efficacy of the plants acquired from the traditional healers. The results of study initiate basis for further studies of the powerful plants so as to segregate the compounds responsible for the antimicrobial activity. Numerous modern drugs were extracted from traditional therapeutic floras through the use of plant material succeeding the ethno botanical leads from indigenous cures used by traditional remedial systems.

CONFLICT OF INTERESTS

The authors have not declared conflict interests.

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Study on bovine mastitis with isolation of bacterial and fungal causal agents and assessing antimicrobial resistance patterns of isolated *Staphylococcus* species in and around Sebeta town, Ethiopia

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A cross-sectional study was conducted from December 2016 to May 2017 in and around Sebeta town with the aim of assessing the prevalence of mastitis, isolation of aerobic bacterial and fungal causal agents and assessing antimicrobial resistance pattern of isolated *Staphylococcus* species in dairy cows. From a total of 383 dairy cows, 220 (57.4%) were found to be positive for mastitis of which 10.4% were affected by clinical mastitis and 47% by subclinical mastitis. Mastitis was more likely to occur in cows above 8 years of age (OR = 16.9, 95% CI = 7.8 - 36.00) and in those cows washed by hand before milking (OR =7.8, 95% CI = 4.2-14.6) as compared to those subjected to udder washing and drying using towels. *Staphylococcus aureus* was the most frequently isolated bacterial species (25%) followed by *Streptococcus agalactiae* (12.3%) and coagulase negative *Staphylococcus species* (10.5%). *Yarrowia lipolytica* (10.9%) and *Candida etchellsii* (7.3%) were the major yeast species isolated, while *Aspergillus* (6.8%), *Mucor* (5.9%), *Penicillium* (3.6%) and *Fusarium* (3.6%) were the major filamentous fungi species identified from the cultured milk samples. The results of antimicrobial susceptibility testing revealed that the isolated *Staphylococcus* species were highly resistant to penicillin G (93.1%) and oxytetracycline (79.3%) but were susceptible to vancomycin (100%), sulphamethoxazole/trimethoprim (96.6%), ampicillin (89.7%) and erythromycin (86.2%). It could be concluded that bovine mastitis is a major challenge to the dairy producers in and around Sebeta towns. Appropriate control and preventative measures must be instituted and dairy farmers and workers must be trained on proper milking and hygiene practices in order to reduce the prevalence of mastitis in this region. The penicillin resistant *S. aureus* could be a source of serious infection in humans as well and hence comprehensive studies including molecular characteristics of drug resistance gene of *S. aureus* especially of methicillin-resistant should be conducted as farm animals, primarily dairy cattle might serves as a reservoir of infection for humans. In 2% of the cases, only fungal species were identified as causes of mastitis, hence further investigation regarding their pathogenicity and contribution to bovine mastitis is needed.

Key words: Antimicrobial resistance, bovine mastitis, bacteria, Ethiopia, fungus, prevalence, Sebeta.

INTRODUCTION

Ethiopia has the largest cattle population in Africa with an estimated population of 57.83 million (CSA, 2016). Development of the dairy sector in the country can contribute significantly to poverty alleviation and

nutrition (Shapiro et al., 2015). However, currently milk production do not satisfy the countries milk requirement due to multitude of factors such as mastitis and other diseases that lead to significant loss in production (Biffa et al., 2005).

Mastitis occurs worldwide among dairy animals and it has been described to have an extreme economic impact (Al-majali et al., 2008). According to Bhikane and Kawitkar (2000), dairy cattle mastitis contributes up to 70% of reduced milk production, 9% of discarded milk after treatment, 7% of the cost of veterinary services and 14% of premature culling. The disease can be classified as clinical or subclinical (Eriskine, 2001).

The disease is caused by a multitude of etiological agents that includes bacteria, virus, fungi and algae (Wellenberg et al., 2002; Kivaria et al., 2004). The most common bacterial pathogens are *Staphylococcus aureus, Streptococcus agalactiae,* other *Streptococcus* species and coliforms (Sumathi et al., 2008). Other organisms may also include *Arcanobacterium pyogenes*, *Pseudomonas aeruginosa*, *Nocardia asteroides*, *Clostridium perfringens, Mycobacterium*, *Mycoplasma*, *Pastuerella* and *Prototheca* species and yeasts (Radostits et al., 2007). Fungal infections account for up to 2 to 13% of all cases of mastitis in cows (Krukowski et al., 2006). Usually mycotic mastitis is unnoticed by clinician in the first attempt of treatment and administration of antibiotics may aggravate fungal mastitis as some of the antibiotics like penicillin and tetracycline act as a source of nitrogen for various species of fungi (Tarfarosh and Purohit, 2008).

In Ethiopia, available information indicates that bovine mastitis is a serious challenge in dairy farms. Based on one meta-analysis report that evaluated 39 different studies, the overall prevalence of bovine mastitis was 47.0% (95% confidence interval [CI] = 42.0-52.0) of which 8.3% (95% CI = 6.5-10.3) was clinical mastitis and 37% (95% CI = 32.9-40.7) was subclinical mastitis (Getaneh and Gebremedhin, 2017).

Prevalence of bovine mastitis and predisposing factors were assessed 12 years ago in Sebeta towns (Sori et al., 2005). Since then, many dairy farms have been introduced into the area. Moreover, there are many complaints on poor response to treatment of mastitis using common antimicrobial drugs. Furthermore, to the author's knowledge, there are no published literatures on major fungal pathogens isolated from bovine mastitis in Ethiopia. Based on the aforementioned facts, this study was conducted to assess risk factors, causative agents and to determine antimicrobial resistance patterns of isolated *Staphylococcus* species isolated from bovine mastitis in and around Sebeta town.

MATERIALS AND METHODS

Study area and population

Sebeta town is located around 25 km from Addis Ababa city at 8°55′N latitude and 38°37′E longitude and 2,356 m above sea level. The climate is warm with the average annual temperature of 17.4°C and 1073 mm averages annual rainfall.

The study population comprises of lactating dairy cows that are managed under extensive, semi intensive and intensive farming systems. A total of 383 lactating dairy cows in and around Sebeta town were examined to determine the overall prevalence of mastitis in the area.

Study design and sample size determination

A cross-sectional study was conducted from December 2016 to May 2017 and study cows were randomly selected from extensive, semi intensive and intensive dairy farms in the area. The sample size was determined based on the formula given by Thrufield (2007) considering 5% absolute precision, 95% confidence interval and 52.78% expected prevalence from previous studies in the area (Sori et al., 2005). Therefore, the calculated sample size was 383.

Study methodology

Physical examination of the udder and milk

The udders were first examined visually and then palpated to detect any possible fibrosis, inflammatory swelling and atrophy of the tissue. The size and consistency of the mammary quarter was inspected for the presence of any abnormalities such as disproportional symmetry, swelling, firmness and blindness of the teat canal. In addition, two streaks of milk from each quarter in a strip cup were inspected visually for the presence of any flakes, clots, pus, watery appearance, blood and color change (Radostits et al., 2007).

California mastitis test (CMT) screening

After physical examination, milk samples were tested by California mastitis test (CMT Kit Lot number 67467, ImmuCell, USA). Briefly, a squirt of milk from each quarter of the udder was placed in each of four shallow cups in the CMT paddle and an equal amount of the reagent was added. A gentle circular motion was applied in a horizontal plane. Positive samples show gel formation within a few seconds. The California mastitis test was conducted to diagnose the presence of subclinical mastitis and based on the thickness of the gel formed by CMT reagent and milk mixture 1:1 test results

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were scored as 0 (negative), 1 (weak positive), 2 (distinct positive) and 3 (strong positive). Milk samples with test result of CMT 1 to 3, were classified as evidence of subclinical mastitis (Radostits et al., 2007). If at least one quarter was positive by the CMT then the cow was considered positive for mastitis.

Data and sample collection

A semi-structured questionnaire was used to collect data on risk factors which include age, parity and hygiene of udder, farm owners and milker's. The age of the animals was determined from birth records and asked from owner and categorized as young adult (3 to 5 year), adult (6 to 8 year) and old (8 years and above); parity as few (1 to 2 parities), moderate (3 to 4 parities), and many (4 and above) (Umer et al., 2015). Udder hygiene was evaluated by observing the presence of any cow dung stain or spot on the udder and hind legs and through asking milker's. The practice for keeping the hygiene of udder was divided into four categories; those that do not practice udder washing at all wash by hand without drying, wash using towel without drying, and wash and dry by using towels.

Milk samples were collected following the standard procedures by the national mastitis council (NMC, 2004). After a quarter had been washed with tap water and dried (in cases when there was a considerable amount of dirt), the teat end was swabbed with cotton soaked in 70% ethyl alcohol. Approximately 10 ml of milk sample was then collected aseptically from clinically and sub clinically (CMT positive) mastitic cows into sterile universal bottles after discarding the first milking streams (squirt). The bottles were labeled with permanent marker and transported on packed ice box to National Animal Health and Diagnostic Investigation Center (NAHDIC), where they were immediately cultured or stored at 4°C until processed for bacterial and fungal isolation.

Laboratory work

Bacterial identification

Collected milk samples were inoculated on sheep blood agar (OXOID) and MacConkey agar (OXOID) and incubated aerobically at 37°C and cultured plates were examined after 24 to 48 h of incubation for any visible growth. The colonies were identified using primary and secondary biochemical tests at least to determine the genus of the suspected isolate. Then, OMNILOG/BIOLOG (fully automated coated microplate based bacterial identification system) using GEN III micro plate (Lot number 3003241, BIOLOG, USA) with protocol A method was used to further confirm the species of suspected colonies. A single colony grown in Biolog Universal growth (BUG) agar medium was selected and emulsified into 'inoculating fluid A' (IF A). According to the manufacturer's instructions, cell density of the bacterial inoculum was measured and adjusted for a specified transmittance (90 to 98%) using a turbidiometer. For each isolate, 100 µl of the bacterial suspension was inoculated into each of the 96 well coated micro plates, using 8 channel pipette and incubated aerobically at 33°C for 22 h. The Omni Log identification system automatically reads each microplate and provides species/sub-species identification (ID), and then the results were printed out (OMNILOG, 2010).

Fungal identification

Milk samples were also simultaneously inoculated onto sabouraud dextrose agar (SDA) and cultured at 26°C for up to 4 weeks (if no visible fungal growth was observed within this period, no growth was recorded). Isolates were examined macroscopically and identified based on colony shape, size, color and growing pattern. For filamentous fungi, slides were prepared from each colony using scotch tape method where transparent scotch tape was lightly pressed to colony and then the tape was fixed to slide that had a drop of lactophenol cotton blue stain. The slides were observed under microscope in X10 and X40 magnification power and were identified at genus level using fungal identification key (Quinn et al., 2002). Yeast colonies were examined microscopically using Gram staining and species level identification was conducted by OMNILOG identification system using yeast microplates.

Antimicrobial susceptibility test

Three to five well isolated colonies of isolated *Staphylococcus* species were transferred to 5 ml nutrient broth and incubated at 35°C for 4 h. The turbidity of the broth culture was adjusted to obtain turbidity optically comparable to 0.5 McFarland standard solution. After adjusting the turbidity, sterile cotton swab was dipped into the suspension and then Mueller Hinton agar plate was inoculated by rotating 60°. Antimicrobials disc were applied on the media using disc dispenser and then incubated for 24 h. Measurement of zone of inhibition was done by using digital caliper. Six antimicrobials were used: Ampicillin, erythromycin, penicillin G, oxytetracycline, vancomycin, sulphamethoxazole/trimethoprim (OXOID discs) (CLSI, 2010).

Data analysis

Statistical analysis was performed using 'STATA, version 12. Mastitis prevalence was calculated by dividing the number of CMT positive cows by the total number of cows tested. Multivariate logistic regression was used to see the association of the potential risk factors with occurrence of mastitis. The strength of the association was measured using odds ratio (OR). Factors with odds ratio greater than one were considered as risk factors and those with odds ratio less than one were protective factor. In all the analysis, P- value lower than 0.05 was considered as significant.

RESULTS

Prevalence of clinical and subclinical mastitis

From a total of 383 dairy cows examined for mastitis, 220 (57.4%) of them were found positive. The details of the types of mastitis and quarter level mastitis were indicated in Table 1. From the total of 1532 quarters examined, 60 (3.9%) were blind (inactive quarters) and 619 (42.1%) were affected by mastitis (Table 1).

The association of risk factors with mastitis

The prevalence of mastitis was higher in older cows than young and adults and the difference was statistically

Table 1. Prevalence of clinical and subclinical mastitis.

Form of mastitis	Positive cows (%)	Quarters affected (%)
Clinical	40/383 (10.4%)	73/1472 (4.9%)
Subclinical	180/383 (47%)	546/1472 (37.1%)
Total	**220/383 (57.4%)**	**619/1472 (42.1%)**

Table 2. Multivariable logistic regression analysis of the association of different potential risk factors associated with mastitis.

Risk factor	Total examined	No. of positive (%)	Adjusted OR and 95% CI	P-value
Age				
2-5	132	37(28.0%)	1	
6-8	167	110(65.9%)	5.0(2.9 -8.6)	0.000
>8	84	73(86.9%)	16.9(7.8-36.00	0.000
Parity				
1-2	237	102(43.0%)		
3-4	118	91(77.1%)	1.9(.96-3.91)	0.066
>4	28	27(96.4%)	8.3(.92-74.88)	0.060
Udder preparation hygiene				
No washing	26	12(46.2%)	1	
Washing by hand	117	93(79.5%)	7.8(4.2-14.6)	0.009
Washing by cloth	116	80(69.0%)	5.1(2.8-9.4)	0.062
Wash and dry	124	35(28.2%)	.4(.12-1.12)	0.079

OR=Odds ratio; CI=Confidence interval.

significant (P<0.05). The disease was more likely to occur in cows above 8 years of age in comparison to younger animals (OR = 16.9, 95% CI = 7.8- 36.00). Similarly, the prevalence was statistically higher (P<0.05) in cows which their udders were washed by hand only before milking (OR =7.8, 95% CI = 4.2-14.6) as compared to those cows which their udders were washed and dried using towels. The details of factors considered and association with mastitis are summarized in Table 2.

Bacteria and fungi agents isolated from mastitic milk

From 220 milk samples cultured for bacterial and fungal species identification, 41% were positive for bacterial isolates and 2% for fungal species. Mixed bacterial and fungal isolates were observed in 56% of the samples (Figure 1).

The predominant bacterial isolates were *Staphylococcus aureus* with isolation rate of 25% followed by *Streptococcus agalactiae* (12.3%) and coagulase negative *Staphylococcus species* (10.5%). *Yarrowia lipolytica* (10.9%) and *Candida etchellsii* (7.3%) were the major yeast species observed while *Aspergillus* (6.8%), *Mucor* (5.9%), *Penicillium* (3.6%) and *Fusarium* (3.6%) were filamentous fungi species identified from the cultured milk samples (Table 3).

In vitro antimicrobial susceptibility testing

Antimicrobial susceptibility test was carried on 29 randomly selected *S. species* isolates*. Staphylococcus species* were found to be resistant to penicillin G (93.1%) and oxytetracycline (79.3%) but were highly susceptible to vancomycin (100%), sulphamethoxazole/Trimethoprim (96.6%), ampicillin (89.7%) and erythromycin (86.2%) (Tables 4 and 5).

DISCUSSION

The overall prevalence of mastitis observed in the present study (57.4%) was higher than the reported prevalence 12 years ago (52.78%) from the same study area (Sori et al., 2005). Some eight years ago, Mekibib et

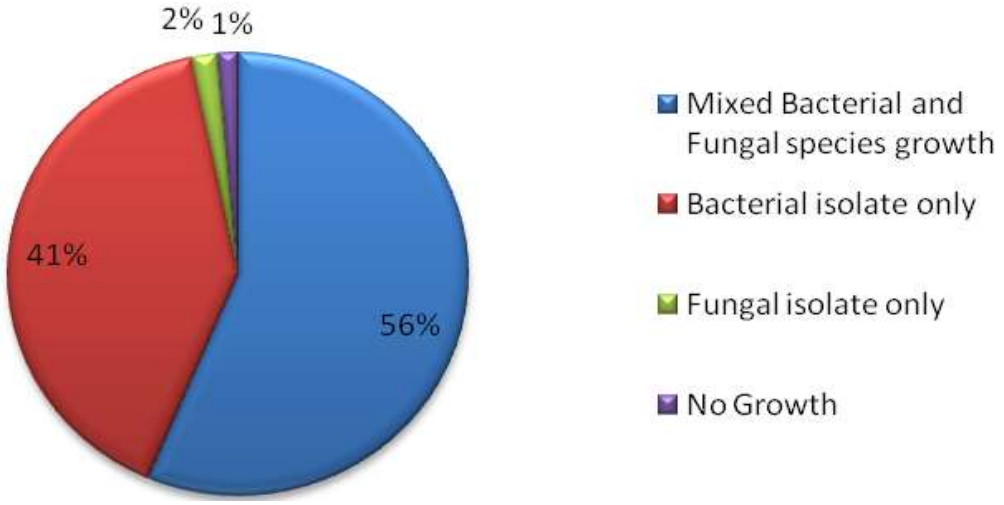

Figure 1. Culture result of milk samples collected from mastitic cows.

al. (2009) reported 71.1% prevalence of bovine mastitis from Holeta, a town which was located close to the present study site. This indicated that bovine mastitis remains a serious problem to the dairy producers in and around these neighborhood towns which costs the farmers from losses associated with reduced production, increased replacement cows, drug costs, veterinary fees and labour costs. It might be due to lack of coordinated actions on prevention and control of bovine mastitis. The numbers of dairy farms has increased in the study areas as compared to previous years. However, most of these farms have poor housing facilities and this might contribute to the contamination and exposure of teats to environmental pathogens and could be reason for increased prevalence of bovine mastitis.

Region to region variations on prevalence of both clinical and subclinical mastitis were wide in Ethiopia. Lakew *et al*. (2009) reported 64.6% overall prevalence from Assela, a town which has similar agro-ecology with the present study site. Mungube et al. (2004) and Delelesse (2010) reported 46.6 and 44.1% overall prevalence while Tolosa *et al*. (2009) reported 9.5%, Bedada and Hiko (2011) 12.1%, Sori et al. (2005) 16.11% and Workineh et al. (2002) 25.1% subclinical mastitis from different localities in Ethiopia. This similarities and differences might be due to complex nature of the disease involving interactions of various factors such as management and husbandry, environmental conditions, animal risk factors, and causative agents (Radostits et al., 2007). The variation in the prevalence of mastitis might also be due to management differences like hygienic condition during milking process practiced by each farm, or individual cow's defense mechanism (Suriyasathaporn et al., 2000).

The higher prevalence of subclinical mastitis than that of clinical mastitis in the present as well previous studies could be attributed to the little attention given to subclinical mastitis as subclinical mastitis is not clinically visible while treating clinical cases. Moreover, dairy farmers might not be well informed about the silent nature of subclinical mastitis (Karimuribo et al., 2006; Almaw et al., 2008). Likewise, the predominance of subclinical mastitis and its serious economic relevance compared to clinical mastitis was underscored elsewhere out of Ethiopia (Kaliwal and Kurjogi, 2011; Awale et al., 2012; Shittu et al., 2012; Elbably et al., 2013; Katsande et al. , 2013).

Quarter level prevalence of mastitis (42.14%) was lower than finding of Kifle and Tolosa (2008) who reported prevalence rate of 63.1%, but higher than the report made by Zelalem (2001) in Ethiopia. The teat canal is the first barrier against invading pathogens, and the efficiency of teat defense mechanisms depends on the integrity of teat tissue; its impairment leads to an increase in the risk of intra-mammary infection. The observed high number of inactive quarter 60(3.9%) may be an indication of a serious mastitis problem on the respective farms and the absence of culling chronically infected cows that can serve as a means to prevent and control the disease within a farm.

In the present study the prevalence of mastitis was higher in old adult cows than young adults. This might be due to older cows have largest teats and more relaxed sphincter muscles, which increase the accessibility of infectious agent in the cow's udder (Radostits et al., 2007). Cows with many parities were also at greater risk

Table 3. Bacterial and fungal species identified from milk of cows with clinical and subclinical mastitis.

Identified bacteria		Identified yeasts		Identified filamentous fungi	
Species	**Number (%)**	**Species**	**Number (%)**	**Species**	**Number (%)**
Staphylococcus aureus	55 (25%)	*Candida etchellsii*	16 (7.3%)	*Aspergillus species*	15 (6.8%)
Staphylococcus intermedius	23 (10.5%)	*Candida edax*	8 (3.6%)	*Mucor species*	13 (5.9%)
Staphylococcus epidermidis	12 (5.5%)	*Candida heamulonii*	2 (0.9%)	*Penicillium species*	8 (3.6%)
Staphylococcus hyicus	11 (5%)	*Yarrowia lipolytica*	24 (10.9%)	*Fusarium species*	8 (3.6%)
CNS	23 (10.5%)	*Rhodotorula graminis*	5 (2.3%)	No growth	176 (80%)
Streptococcus agalactiae	27 (12.3%)	*Rhodotorula glutinis*	2 (0.9%)	**Total number of samples**	**220**
Streptococcus dysgalactiae	12 (5.5%)	*Rhodosporidium diobovatum*	5 (2.3%)		
Streptococcus uberis	3 (1.4%)	*Galactomyces geotrichum*	8 (3.6%)		
Bacillus species	11 (5%)	*Geotrichum terreste*	3 (1.4%)		
Micrococcus species	6(2.7%)	*Trichosporon species*	7 (3.2%)		
Pseudomonas species	6(2.7%)	*Saccharomyces species*	4 (1.8%)		
Corynebacterium species	6(2.7%)	No growth	136 (61.8)		
E.coli	11(5%)	**Total number of samples**	**220**		
Klebsiella species	3(1.4%)				
Pasteurella species	4(1.8%)				
No growth	7 (3.2%)				
Total number of samples	**220**				

CNS = Coagulase negative *Staphylococcus species* other than *S. epidermidis.*

than moderate and few parities which might be due to physical alterations of udder, and is in line with findings reported by other authors (Carlen et al., 2004; Zwald et al., 2004; Abdel-Rady and Sayed, 2009; Belayneh et al., 2013; Katsande et al., 2013, Abrahmsen et al., 2014; Mureithi and Njuguna, 2016).

Cows in farms with poor milking hygiene were severely affected than those with good milking hygiene practices. Similar findings were reported by Sori et al. (2005); Lakew et al. (2009) and Moges et al. (2011). The reason most probably might be due to cross contamination from infected teat to others, or from infected to non infected cows during milking. The milkers' hand and washing towels might also facilitate pathogens spread. It was also documented that udder preparation both before and after milking influence the prevalence of mastitis.

In this study, the dominant bacterial pathogens isolated from milk samples were *Staphylococcus* species (25%) however; this was lower than that of the 42.1% reported by Abera et al. (2010). Similarly, *S. aureus* was isolated as main etiological agent of mastitis in cattle in many African and Asian countries (FAO, 2014). *S. aureus* is considered as typical contagious pathogen causing bovine mastitis. Accordingly, the wide spread *S. aureus* mastitis might be cows positive in herd which act as primary reservoir and infected others especially during milking. Radostits et al. (2007) asserted that *S. aureus* is well adapted to survive in the udder and usually establishes a mild sub clinical infection of long duration from which it shed in milk facilitating transmission to healthy animals mainly during

Table 4. Summary of antimicrobial susceptibility test result for *Staphylococcus species* (number of isolates =29).

Antimicrobial disc	Number of fully susceptible isolates (%)	Number intermediately susceptible (%)	Number of resistant isolates (%)
Vancomycin (30 µg)	29 (100%)		
Ampicillin (10 µg)	26 (89.7%)	1 (3.4%)	2 (6.9%)
Erythromycin (15 µg)	25 (86.2%)	4 (13.8%)	-
Penicillin G (10 Unit)	1 (3.4%)	1 (3.4%)	27 (93.1%)
Oxytetracycline (30 µg)	6 (20.7)	-	23 (79.3%)
Sulphamethoxazole/Trimethoprim (25 µg)	28 (96.6%)	-	1 (3.4%)

Table 5. Penicillin and oxytetracycline resistance patterns of *Staphylococcus species.*

Isolated *Staphylococcus species*	Number of tested isolates	Penicillin G			Oxytetracycline	
		S	I	R	S	R
Staphylococcus aureus	13	-	-	13	-	13
Staphylococcus scuiri	3	-	1	2	3	0
Staphylococcus lentus	2	1	-	1	1	1
Staphylococcus xylosus	4	-	-	4	2	2
Staphylococcus intermedius	4	-	-	4	-	4
Staphylococcus haemolyticus	1	-	-	1	-	1
Staphylococcus epidermidis	2	-	-	2	-	2

S= Susceptible; I= Intermediate; R= Resistance.

milking. Generally, *S. aureus* has been designated as a causative agent of both clinical and subclinical mastitis.

The isolation rate of *S. agalactiae* (12.3%) in this finding coincides with that of Bitew et al. (2010) at Bahir Dar who reported 13.9%. However, the finding was higher than the 4% report by Lakew et al. (2009) from Asella, and 6.4% isolation rate reported by Sori et al. (2005) in and around Sebeta town.

E. coli was identified from 5% of the samples in this study and this proportion was lower than reports by Sori et al. (2005), Mekebib et al. (2009), Bitew et al. (2010) who reported an isolation rate of 26.57, 43.13 and 20.3%, respectively. This lower isolation rate of environmental mastitis causal agents might be partly associated with effective and good sanitation of the barns with immediate removal of feaces practices. Moreover, the proportion of *Micrococcus* species in this study was lower than the finding of Mekonnen et al. (2005) and Bedada and Hiko (2011), who reported 10.2 and 5.6%, respectively. It was also reported that *Micrococcus* species causes mastitis only occasionally.

Mixed (fungal and bacterial) and fungal infection alone in this study represented 56 and 2% respectively. The overall 56% mixed fungal infection were comparable with the result of Pachauri et al. (2013) who found 64%. However, this was lower than the results of Al-Ameed (2013) in Iraq who reported 80% and was higher than the 13% fungal mastitis prevalence reported by Sukumar and James (2012). This might be due to unhygienic condition of the animal sheds and high humidity along with favorable environmental conditions supporting growth of fungal spores. Hence favorable conditions increase the chances of fungal spore to enter into the udder which provide suitable environment to these fungi (Williamson and Di Menna, 2007). Under immunosuppressive conditions, the dynamics of microorganisms may be disrupted, and the fungi together with the other microorganisms are able to overcome the udder defense mechanisms.

The overall isolation rate of yeast from the current study was 38.18% of which *Candida* species accounted for 11.8%, *Yarrowia lipolytica* for 10.9%, *Rhodotorula* species for 3.2%, *Rhodosporidium diobovatum* (2.3%), *Galactomyces geotrichum* (3.6%), *Geotrichum terreste* (1.4%), *Trichosporon species (3.2%)* and *Saccharomyces species* (1.8%). This isolation rate was lower than that reported by Andreia et al. (2008) on *Candida* (37.9%), *Cryptococcus* (10.3%) and *Rhodotorula* (10.3%). Although

the distribution of *Candida* species shows diversity in several countries, it is important to note the increase in number of mammary gland infections caused by *Candida* species in the recent years (Krukowski et al., 2001). Filamentous fungi was isolated from 20% of the tested samples, the isolated fungal species were *Aspergillus* (6.8%), *Mucor* (5.9%), *Penicillium* (3.6%) and *Fusarium* (3.6%). The isolation rate of *Aspergillus species* was lower than the 38% reported by Mdegela et al. (2005) and Blowey and Edmondson (2010). The management practices adopted on dairy cows , like discarding first few strips of milk on ground while milking of animals as well as during treatment of mastitic animals and reluctance to disinfect hand between milking by milkers may contribute as potent source of lateral transmission of fungal and yeast infections (Pachauri et al., 2013). There are also reports in which yeasts like *Candida* spp. utilizes nitrogen from penicillin and tetracycline antibiotics, antibiotic therapy leads to perturbation in udder homeostasis, inhibition of T cells and neutrophil activity and in consequence this may also stimulates yeast growth (Corti et al., 2003; Noris et al., 2007).

In the present study, *Staphylococcus* species were found resistant to penicillin G (93.1%) and oxytetracycline (79.3%) and this is comparable with many previous reports in the country. The resistance of S*taphylococcus* species to penicillin may be attributed to the production of beta lactamase, an enzyme that inactivates penicillin and closely related antibiotics. The development of antibiotic resistance probably is a result of repeated therapeutic use or indiscriminate use of these antibiotics (Jaims et al., 2002). The uses of antimicrobials have, overtime, increased the number of antimicrobial-resistant microbes globally, and any use of antimicrobial agents will to some extent facilitate the development of resistant strains (Williams, 2000). The majority of authors have noted the development of antimicrobial resistance by *Staphylococcus* species isolated from mastitis cases (Pitkala et al., 2004; Turutoglu et al., 2006; Pyorala and Taponen, 2009; Sori et al., 2011).

Conclusion

It could be concluded that bovine mastitis is a major challenge to the dairy producers in and around Sebeta towns. Large numbers of microorganism were isolated from milk of CMT positive cows with *Staphylococcus, Streptococcus,* C*andida* species and *Y. lipolytica* being the predominant. The demonstrated resistance pattern of *Staphylococcus* species to penicillin and oxytetracycline may alarm on the repeated use of these drugs for mastitis treatment in the country. Hence, comprehensive studies including molecular characteristics of drug resistance gene of *S. aureus* especially of methicillin-resistant should be conducted in farm animals. In 2% of the cases, fungal species were identified as causes of mastitis, hence further investigation regarding their pathogenicity and contribution to bovine mastitis is needed.

ABBREVIATIONS

CI, Confidence interval; **CLSI,** Clinical Laboratory Standards Institute (CLSI); **CMT,** California mastitis test; **FAO,** Food and Agriculture Organization; **NAHDIC,** National Animal Health Diagnostic and Investigation Center; **OR,** odds ratio.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work is a part of sub-thematic research “Bovine mastitis: Udder morphometrical traits, common bacterial isolates, histopathological changes and predisposing factors to clinical and subclinical mastitis in local zebu and crossbreed dairy cattle in central Ethiopia “RD/LT-038/15” for which the investigators have received ethical clearance referenced with ***VM/ERC/005/08/2015*** from ethical clearance and animal welfare committee of Addis Ababa University college of Veterinary Medicine and Agriculture. After briefing the purpose of the study consent was requested from all participating dairy farm owners for collecting samples. All the procedures used were non invasive and in addition all the results were communicated to animal owners.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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