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Antioxidant potential of *Moringa stenopetala* leaf extract on laagered beer stored at room temperature

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Abstract

Controlling beer oxidation using natural antioxidants for better shelf-life stability is the prominent objective of modern breweries. Flavour instability resulting from beer storage and oxidations are the most important quality problems in the brewing industry. This study evaluated the influence of the addition of 80% ethanol leaf extract of *Moringa stenopetala* to lager beer at 400, 600 and 800 ppm concentrations for 30-, 60- and 90-days storage at room temperature. The total phenolic and total flavonoid contents were determined by Folin-Ciocalteu and aluminium chloride method respectively. Antioxidant activity of each treatment was evaluated and compared by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and total antioxidant using phosphomolybdate assay. The addition of the extract in beer showed a linear increment in the total phenolic content from 46.79 up to 88.30 milligrams of gallic acid equivalent per litre of beer (mg GAE/L) and total flavonoid contents from 123.36 up to 167.09 mg of catechin equivalent per litre of beer (mg CE/L). A similar increment was observed within the DPPH scavenging potential, from 46.55up to 67.16% and total antioxidant power from 139.12 up to 216.67 milligrams of butylated hydroxytoluene equivalent per litre of beer (mg BHTE/L). The result indicates a promising use of leaf extract of *M. stenopetala* as a functional ingredient in beer to reduce beer oxidation probability and keep its freshness for a period of storage time.

1. Introduction

Beer is one of the oldest and most widely consumed alcoholic beverages in the world for its freshness, taste, low calories and nutritional value (Arnold, 2005). It is a good source of phenolic compounds (Oñate-jaén *et al*., 2006) and contains potassium, magnesium, calcium and sodium (Styburski *et al*., 2018). The production of beer involves extremely complicated processes of chemical and biochemical reactions. Thus, the final beer contains various compounds with antioxidant activity mainly originated from yeast, malt and hops or formed during processing (Quifer-Rada *et al*., 2015). The types and concentrations of these antioxidants in final beer vary largely because of brewing technology, raw materials, and yeast applied in the brewing. Slight changes in the structural composition of these compounds can cause significant changes in the antioxidant activity, which alters the overall oxidative or flavour stability of beer. Nowadays, flavour stability has become the most important factor in determining the shelf-life of packaged beer and prolonging shelf-life by delaying flavour staling. Shelf life problems of beer have become a very important issue for most breweries due to losses of freshness and quality of beer over time by change of its chemical composition during storage (Aron *et al*., 2011). Although the flavour stability of beer depends primarily on the oxygen content of the packed beer, the brewing process and the raw materials used can also influence the flavour stability. Flavour instability resulting from beer storage also remains one of the most important quality problems in the brewing industry (Aron *et al*., 2011). Therefore, attention is now increasingly shifting towards increasing the antioxidant activity of beer itself since oxidative staling of beer is still noticeable even if the level of oxygen might be as low as 0.1 mg/L (Bamforth *et al*., 2018). Scientists are searching for different mechanisms to decrease beer oxidation using synthetic antioxidants. However, current researches recommend not using synthetic antioxidants in food and beverage industries due to prolonged health

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effect and the evolution of food laws and regulations which forbids synthetic antioxidants in food (Arnold, 2005). Therefore, there is a growing interest in the use of natural antioxidants in the food industry, not only for the application of quality but also for the potential benefits to human health. Therefore, the replacement of synthetic antioxidants with natural antioxidants is the issue of modern breweries to tackle such antagonists and succeed their business with customer satisfaction and respect government regulations.

Moringa is one of the most powerful sources of natural antioxidants by supplying bioactive compounds and mitigate the effect of free radicals. The study conducted by Nadeem *et al*. (2013) showed that *M. stenopetala* contains a high concentration of phenolic and flavonoid compounds, like cryptochlorogenic acid, astragalin, glucosinolates and isothiocyanates. Also, the leaves are rich in flavonoids such as isoquercetin and rutin, and the beta carotene present in *M. stenopetala* leaves also acts as antioxidants (Tesfaye and Solomon, 2014). It also showed antimicrobial effects for shelf life extension of alcoholic beverages by suppressing lactic acid bacteria(Florence *et al*., 2016). This study focused on the possibility of the extension of the shelf life of beer using 80% ethanol leaf extract of *M. stenopetala*.

2. Materials and methods

2.1 Chemicals and reagents

All chemicals and solvents used in this experiment were of analytical grade. Gallic acid, butylated hydroxytoluene (BHT), catechin, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, sodium carbonate, and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The rest reagents and solvents were of analytical grade.

2.2 Raw materials

Moringa stenopetala leaf was obtained from the Hawassa teachers training centre where it was botanically classified and planted for research purposes by the institute. The malt was collected from the Assela malt factory through BGI Ethiopia. Newly propagated *Saccharomyces cerevisiae* yeast (S-189 type) and brewing liquor (water) treated for brewing standard were collected from BGI Ethiopia.

2.3 Sample preparation and extraction

The green leaves of *M. stenopetala* were collected carefully, wrapped up with aluminium foil and taken to Hawassa university food science and post-harvest technology laboratory. It was then washed with distilled water, air-dried under a shed and grounded using an electrical grinder(Nadeem *et al*., 2013). The fine powder was mixed with 80% ethanol with a 1 g to 10 mL ratio using a triplicate Pyrex beaker of 1000 mL. The beakers were tightly closed with a cover bush and macerated using an electrical shaker for 18 hours. The extract was then separated and filtered using Whatman No.1 filter paper and evaporated to dryness under vacuum at 40°C by using a rotary evaporator (Buchi, 3000 series, Switzerland). A stock solution was prepared and stored in a refrigerator at 4°C for subsequent usage (Siddhuraju and Becker, 2003).

2.4 Experimental design and treatments

Using a factorial design, leaf extract concentrations of 400, 600, and 800 ppm, as well as storage times of 1, 30, 60, and 90 days were investigated on total phenolic and flavonoid content and antioxidant levels. A beer containing 12 ppm potassium metabisulphite (KMS) was used as a positive control, and a beer without any antioxidant was used as a negative control (control). Three concentration of treatment were chosen based on the trial experiment: 400, 600, and 800 ppm.

2.5 Beer preparation

The preparation of beer was carried out according to the method developed by Pires and Brányik (2015) using a dry milling system. The mash was prepared from malt which was milled 2.25 mm diameter sieve size and mixed with water at 55°C with 2.3 L/kg water to grist ratio in a commercial brewing plant. The mash was heated to 64°C with 20 mins rest time and 74°C with 15 mins rest time. After saccharification of the mash, the temperature was raised to 78°C and the mash was filtered using a mash filter. The filtered wort was then boiled with 0.12 kg CO_2 extract hop per hectoliter for 60 mins. Hot trub was separated using a wort settling tank after 20 mins rest and the hot wort was cooled to 10°C and aerated with 18 ppm of oxygen. The wort was then left for primary fermentation using a conical fermenter vessel at 12°C until the original gravity decreased from 18°P to 8°P and secondary fermentation at 16°C until the vicinal diketones (VDK) reaches less than 0.18 ppm. The beer was cooled to -2°C when fermentation was completed, it was then kept for 2 days laagering period for maturation in the fermenter tank at 0.5 bar counter pressure using carbon dioxide. The matured beer was then purged and filtered using a candle filter with help of filter aids. The filtered beer was diluted to 11.05°P using de-aerated water and carbonated to 5.8 g/L CO_2 which was then packed aseptically using 330 mL sanitised amber bottles and crowned with

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manual crowner after antioxidant dosing at different concentrations. All samples were labelled and pasteurised using a tunnel pasteuriser at 60°C for about 20 mins and stored at room temperature. The physicochemical properties and sensory properties of each sample were analysed every 30 days for three consecutive months starting from the first day of sample preparation.

2.6 Total phenolic and flavonoid contents

2.6.1 Total phenolic content

The total phenolic content of each sample beer was determined according to the Folin-Ciocalteu spectrophotometric method described by Zhao *et al*. (2010) with slight modifications. The beer sample was diluted to five-folds, out of which 0.1 mL of the diluted sample beer was taken and 1 mL of Folin-Ciocalteu reagent (diluted ten times) was added and the mixture was left for 5 mins. Then 1 mL sodium carbonate (7.5% w/w) was added and the mixture was incubated for 90 mins at room temperature. After incubation was completed, the absorbance of the solution was measured at 765 nm with a UV-visible double beam spectrophotometer. The total phenolic content was then estimated from the gallic acid calibration curve ($y = 0.023x + 0.014$, $R^2 = 0.996$) and the result was expressed as milligram gallic acid equivalent per litre of beer (mg GAE/L).

2.6.2 Total flavonoid content

The total flavonoid content of each sample beer was determined based on the method described by Pai *et al*. (2015). The beer sample was diluted to five folds and 1 mL of the diluted beer sample was diluted with 1.25 mL of distilled water. Then, 75 µL of $NaNO_2$ (5%) was added to the mixture and after 6 mins 150 $AlCl_3$ (10%) was added. After another 5 mins, 1 mL of NaOH (1M) was added and immediately the absorbance of the mixture was measured at 510 nm. Simultaneously, a blank solution was prepared using distilled water in place of the beer samples. All the results were expressed as milligram catechin equivalents per litre of beer (mg CE/L) obtained from standard calibration curves, $y = 0.011x + 0.132$, $R^2 = 0.973$.

2.7 Determination of antioxidant potential

2.7.1 DPPH radical scavenging activity

DPPH radical scavenging activity of each sample beer was determined according to the method described by Tafulo *et al*. (2010). The beer samples were diluted five-fold and 1.0 mL of the sample was mixed with 2.0 mL freshly prepared DPPH solution (0.06%, w/v) in ethanol. The reaction mixture and the reference standard (ascorbic acid) were vortexed and left at room temperature in the dark for 30 mins. Then the absorbance of the solution was measured using a double beam UV-visible spectrophotometer (JENWAY-9500, UK) at a wavelength of 520 nm. Free radical scavenging activity was then calculated by the discolouration of DPPH expressed as a percentage using the equation,

$$\text{DPPH scavenging (\%)} = \left[\left(1 - \frac{As}{Ac}\right)\right] \times 100$$

Where *A*c is the absorbance of the control and *A*s is the absorbance in presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration (in mg/mL) of extracts that scavenges the DPPH radical by 50%.

2.7.2 Total antioxidant activity using phosphomolybdate assay

The total antioxidant capacity of each sample beer was evaluated by phosphomolybdenum assay as per the methodology applied by (Huda-Faujan *et al*., 2009). The method was based on the reduction of Mo (VI) to Mo (V) by the antioxidants and subsequent formation of green Mo (V) complexes with maximal absorption at 695 nm at an acidic medium. Each sample was diluted five-fold using distilled water from which 0.3 mL was taken and mixed with 3 mL of phosphomolybdenum reagent (28 mM sodium phosphate, 4 mM ammonium molybdate, and 0.6 M sulphuric acid) in a capped test tube. Then the solutions were incubated for 90 mins in water at 95°C and cooled to room temperature. Finally, the absorbance of each solution was measured using a spectrophotometer (JENWAY-6300, UK) at a wavelength of 695 nm against the blank which contains 3 mL ethanol without the beer sample. The total antioxidant activity of the sample beer was expressed as milligram butylated hydroxytoluene equivalents per litre of beer based on the calibration curve, $y = 0.432x + 0.078$, $R^2 = 0.99$.

2.8 Statistical analysis

The experimental data were subjected to analysis of variance (ANOVA) and Duncan's multiple range tests were used to detect a difference ($p \leq 0.05$) between the mean values. Statistical analyses were performed with the statistical program SAS 9.0 (SAS Inc. Cary, USA) and origin 8 software package and the data were presented as mean ± standard deviation.

3. Results and discussion

3.1 Effect of leaf extract of Moringa stenopetala on total phenolic content of laagered Beer

The effect of leaf extract at different concentrations

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and storage times on the total phenolic content of beer is presented in Table 1. The total phenolic content of extract-treated beer samples was significantly affected by their concentration. The total phenolic content of untreated beer was found 46.79 mg GAE/L and the addition of 400 ppm extract raised total phenolic content to 70.51 mg GAE/L which revealed a 33% increment. Increasing the concentration of the extract to 800 ppm, linearly increased the concentration of total phenolic content to 88.60 mg GAE/L. This finding was supported by a study conducted by Ulloa *et al*. (2017) that bioactive compounds increased with the concentration of propolis extract. This significant increment of phenolic content in beer samples enriched with 80% ethanol leaf extract might come from the potentially high accumulation of bioactive compounds in *M. stenopetala* leaf. A study conducted by Tesfaye and Solomon. (2014) on the antioxidant potential of *M. stenopetala* found up to 92.8 mg GAE/100 g of dry weight which supports our finding that probably increased phenolic content in beer samples enriched with the extract. The total phenolic content of beer sample treated with potassium metabisulphite (66.79 mg GAE/L) was found with lower total phenolic content when compared with beer sample treated with 400 ppm of extract. This potential of leaf extract reveals that higher probability of replacement of the potassium metabisulphite (commercial beer antioxidant).

Table 1. Effect of 80% ethanol *M. stenopetala* leaf extract and storage time on TPC and TFC of laagered beer

Storage Time	Treatment	TPC (mg GAE/L)	TFC (mg CE/L)
Day 1	S01	46.79±1.26^{i}	123.36±1.81D^{h}
	S02	66.79±1.15^{fe}	143.99±1.28^{f}
	S03	70.51±1.22^{e}	147.51±1.75^{e}
	S04	82.75±1.40^{cb}	159.82±1.10^{dc}
	S05	88.60±0.92^{a}	167.09±1.63^{a}
Day 30	S01	44.13±1.30^{j}	109.22±1.32^{j}
	S02	66.18±1.45^{f}	142.23±1.48^{fe}
	S03	69.55±1.49^{ed}	146.32±1.51^{e}
	S04	82.75±1.23^{cb}	156.74±1.71^{dc}
	S05	88.60±0.92^{a}	165.99±1.61^{b}
Day 60	S01	35.71±1.04^{k}	101.80±1.20^{k}
	S02	61.38±1.73^{g}	134.20±1.04^{g}
	S03	66.01±1.05^{f}	141.36±1.08^{f}
	S04	78.56±0.89^{c}	154.10±1.63^{dc}
	S05	88.09±1.15^{a}	162.20±1.57^{b}
Day 90	S01	26.16±1.37^{l}	85.71±1.62^{l}
	S02	56.78±1.45^{h}	120.02±1.68^{i}
	S03	61.73±1.10^{g}	125.69±1.15^{h}
	S04	73.08±1.60^{d}	141.81±1.20^{f}
	S05	84.34±1.26^{b}	150.93±1.66^{d}

Beer treated with different concentrations of 80% ethanol leaf extracts of *M. stenopetala*, S01: untreated, S02: treated with 12 ppm potassium metabisulphite, S03: treated with 400 ppm extract, S04: treated with 600 ppm extract, S05: treated with 800 ppm extract. Values are average of duplicated experiment (mean±SD). Values of the same concentration with different letter in the histogram bar are significantly different at $p<0.05$.

There was a drastic decline in the total phenolic content of the controlled beer up to 26.16 mg GAE/L within 90 storage days. There was a 44% reduction in total phenolic content while beer samples enriched with potassium metabisulphite had only a 15% reduction in total phenolic content (66.79-56.78 mg GAE/L). This reduction of total phenolic content would have been due to the reaction of phenolic groups with free proteins. Vanderhaegen *et al*. (2006) found that during beer storage, phenolic compounds react with proteins and form high molecular weight species and hazes. Besides, 80% ethanol leaf extract of *M. stenopetala* treated beer samples showed a slight reduction of total phenolic content (70.51-61.73 for 400 ppm, 82.75-73.08 mg GAE/L for 600 ppm and 88.60-84.34 mg GAE/L for 800 ppm) with a storage time of 90 days. The slight reduction of total phenolic content in the extract-treated beer sample might be because the extract contained a great number of bioactive compounds which are considered biologically active (Abuye *et al*., 2003). Moreover, phenols in beer easily react with ROS and free radicals along with beer ageing (Vanderhaegen *et al*., 2006), which could be the reason for total phenolic content reduction for untreated beer samples. According to their findings, phenolic compounds polymerise to high molecular weight species either by acid catalysts or by oxidative mechanism.

3.2 Effect of leaf extract of Moringa stenopetala on total flavonoid content of Laagered beer

The effect of extract and storage time on the total flavonoid content of beer enriched with leaf extract of *M. stenopetala* is illustrated in Table 1. The total flavonoid content of beer samples enriched with extracts increased linearly relative to the control beer. Increasing the concentration of the extract from 400 ppm to 600 ppm raised the total flavonoid content from 147.51 up to 159.82 mg CE/L and further increment to 800 ppm increased its total flavonoid content to167.09 mg CE/L. The beer sample enriched with 12 ppm potassium metabisulphite had also raised the concentration to 143.99 mg CE/L.

The total flavonoid content for the untreated beer sample was significantly affected by storage time with a drastic reduction of total flavonoid content.

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The result disclosed that flavonoids concentration had been reduced linearly from 123.36 mg CE/L (at the first date of storage) to 85.71 mg CE/L (after 90 days of storage) which implies a 30% reduction. On the contrary, only 15% flavonoid content reduction was observed for potassium metabisulfite treated beer sample (143.99-120.02 mg CE/L) within the same storage time. The beer samples enriched with leaf extracts of *M. stenopetala* showed a slight reduction of total flavonoid content from starting date to 90 days of storage (147.51-125.69 mg CE/L for 400 ppm, 159.82-141.81 mg CE/L for 600 ppm and 167.09-150.93 mg CE/L for 800 ppm) which is in an average of only 10% reduction.

In all cases, linear reduction of total flavonoid content had been observed and the slight reduction for leaf extract treated beer might be due to the nature of the high antioxidant potential of the extract. According to the study held by Dadi *et al*. (2019) on the flavonoids composition of *M. stenopetala*, there was an abundant amount of total flavonoids content on the leaf extract (up to 20.40 mg/g) which might be the cause of slight reduction over a period of storage time.

3.3 Effect of leaf extract of Moringa stenopetala on DPPH scavenging activity of beer

DPPH scavenging activity of beer enriched with leaf extract of *M. stenopetala* with storage time is demonstrated in Figure 1. The result for untreated beer was 46.55%, but its potential was decreasing with increasing storage time from 46.55 up to 30.91% which might be due to the reduction of endogenous antioxidants that happened by reacting with hydroxyl radicals through time (Dennis *et al*., 2004). However, the beer sample enriched with Potassium metabisulfite showed 61.80% DPPH scavenging activity which is significantly higher than the untreated beer and there was a relatively slight reduction of DPPH scavenging activity with time from the first date of production to three months of storage. According to Lugasi (2003), metal sulphites had relatively high antioxidant potential which has been used by modern breweries for decades. But due to legislation on the health of consumers, many developed countries avoid using this metallic salt.

DPPH scavenging activity for beer samples treated with the extracts revealed linear increment with a concentration of 60.14, 71.60 and 86.44% for 400, 600 and 800 ppm respectively. Intensification of DPPH scavenging potential with the extract concentration is due to the abundantly found bioactive compounds and antioxidants (Tesfaye and Solomo, 2014) in the leaf of *M. stenopetala*. This finding also showed a reduction of DPPH scavenging potential with storage time for beer samples enriched with the extracts. The reduction was from 60.14-34.29% (400 ppm sample), 71.60-41.61% (600 ppm sample) and 86.44-50.40% (800 ppm sample) within three months of storage time (Figure 1).

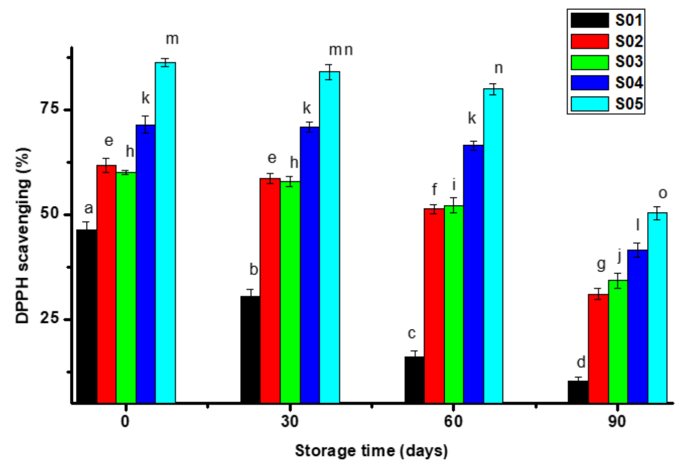

Figure 1. Effect of *M. stenopetala* leaf extract on DPPH scavenging potential of beer. Beer treated with different concentrations of 80% ethanol leaf extracts of *M. stenopetala*, S01: untreated, S02: treated with 12 ppm potassium metabisulphite, S03: treated with 400 ppm extract, S04: treated with 600 ppm extract S05: treated with 800 ppm extract. Values are average of duplicated experiment (mean± SD). Values of the same concentration with different letter in the histogram bar are significantly different at p<0.05.

3.4 Effect of leaf extract of Moringa stenopetala on total antioxidant potential of beer

The total antioxidant potential of beer enriched with leaf extract of *M. stenopetala* with storage time is illustrated in Figure 2. The total antioxidant potential for untreated beer was found 139.12 mg BHTE/L which is almost similar to the study of light laager beers by Zhao *et al*. (2013) which was 140.23 mg BHTE/L.

The total antioxidant potential of the beer sample enriched with ethanol 80% leaf extract of *M. stenopetala* increased with increasing concentration of the extract. The total antioxidant potential increased by 30% with the addition of 400 ppm of the extract and increasing the concentration of extract by 200 ppm raised the beer antioxidant potential by 5%. The increment was linear for all extract-treated beer samples. Accordingly, the total antioxidant potential of beer treated with 400, 600 and 800 ppm was 190.16, 213.00 and 216.67 mg BHTE/L respectively. The beer enriched with potassium metabisulfite also showed similar trained with beer treated with 400 ppm extract. The total antioxidant potential of

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untreated beer showed a linear reduction with increasing storage time by 27% (139.12-101.72 mg BHTE/L) within three months of storage time. The reduction was drastic when compared with extract treated samples. A similar study conducted by Piazzon *et al*. (2010) who analysed the antioxidant potential of different beer types explained that light beer storage without antioxidants would reduce its total antioxidant by 20% to 30% within three months of storage. Storage time had a slight reduction of total antioxidant potential for treated beer when compared with untreated beer. The slight reduction of total antioxidant for potassium metabisulphite and extract-treated beer showed that these additives had strong antiradical potential when compared with untreated beer.

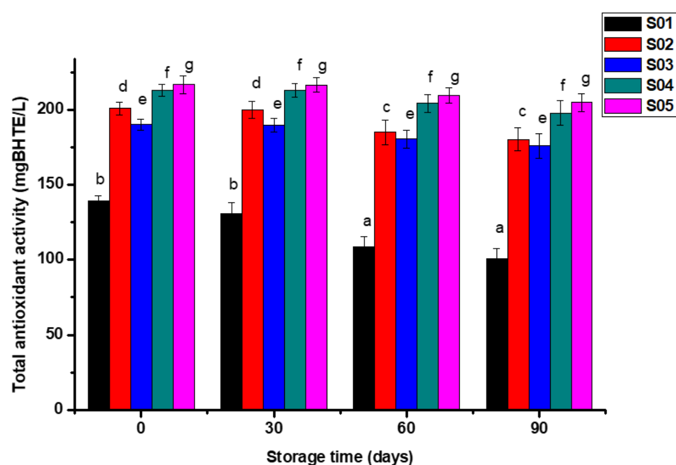

Figure 2. Effect of leaf extract of *M. stenopetala* on total antioxidant potential of beer. Beer treated with different concentrations of 80% ethanol leaf extracts of *M. stenopetala*, S01: untreated, S02: treated with 12 ppm potassium metabisulphite, S03: treated with 400 ppm extract, S04: treated with 600 ppm extract and S05: treated with 800 ppm extract. Values are average of duplicated experiment (mean± SD). Values of the same concentration with different letter in the histogram bar are significantly different at $p < 0.05$.

According to the study dictated by Ulloa *et al*. (2017), the total antioxidant potential of propolis treated beer sample linearly increased with increasing the concentration of propolis which is related to our findings. A similar study by Florence *et al*. (2016) proved that 600 ppm *M. stenopetala* treated pito improved the total antioxidant potential of beer by 32% and showed a slight reduction in storage time. These two studies proved our finding to use 600 ppm of ethanolic extract of *M. stenopetala* in modern beer production for shelf life improvement.

4. Conclusion

This study highlighted the promising potential for the development and use of leaf extract of *M. stenopetala* as an additional source of bioactive compounds and increase antioxidant activity in beer. The incorporation of the leaf extract at a moderate level in beer reduces oxidation and increase the phenolic content that is typically reduced during the boiling, filtration, bottling, and storage stages of the brewing process. The finding has a potential promise to improve the stability and shelf-life of commercial beers without the incorporation of artificial or chemical preservatives (for example, potassium metabisulfite). Further study is also required on physicochemical characteristics and sensory acceptability of *M. stenopetala* leaf extract treated beer.

Conflict of interest

The authors declare no conflict of interest.

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