Application of *Lactobacillus Plantarum* in Fermentation of *Moringa Oleifera* Leaves and How It Effects on Nutritional Quality

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ABSTRACT

Moringa oleifera leaves are highly nutritious but underutilized and have a very low shelf life. The major aim of this study is to examine the effect of nutritional quality of *Moringa oleifera* leaves by fermenting with *Lactobacillus plantarum*.

Sundried *Moringa oleifera* leaf powder followed with fermentation by *Lactobacillus plantarum* were investigated for % moisture, ash, crude protein, crude fat, crude fiber and total carbohydrates, based on AOAC methods. The antioxidant potential was analyzed via DPPH assay.

In different incubation periods, moisture content was significantly ($p<0.05$) increased from 10.24±0.04% (unfermented) to 11.46±0.07% (24h), 12.52±0.07% (48h), 27.48±0.20% (72h). Ash content was significantly ($p<0.05$) reduced from 8.25±0.05% (unfermented) to 7.33±0.03% (24h), 6.25±0.1% (48h), 5.33±0.13% (72h). Crude protein content was significantly ($p<0.05$) increased from 24.63±0.18% to (unfermented), 25.81±0.04% (24h), 28.43±0.11% (48h), 30.24±0.04% (72h). Crude fat content was significantly ($p<0.05$) reduced from 5.09±0.07% (unfermented), to 4.17±0.08% (24h), 4.17±0.08% (48h), 2.83±0.10% (72h). Crude fiber content was significantly ($p<0.05$) reduced from to 8.33±0.06% (unfermented) to 7.29±0.05% (24h), 6.12±0.03% (48h), 5.82±0.08% (72h). Total carbohydrate content was significantly ($p<0.05$) reduced from 41.45±0.19% (unfermented), to 39.63±0.17% (24h), 36.6±0.21% (48h), 30.49±0.17% (72h). The IC 50 values recorded for *Moringa oleifera* leaves fermented by *Lactobacillus plantarum* was 136.3 (unfermented), 115.6 (24h), 65.3 (48h), 50.2 (72h), indicating the highest antioxidant potential in 72h incubation period.

Therefore, 72 h fermented *Moringa oleifera* leaf powder is the best incubation period than unfermented, 24h and 48h.

Key words: *Moringa oleifera leaves*, *Lactobacillus plantarum*, Brine preservation, Proximate composition analysis, Antioxidant properties

INTRODUCTION

Moringa oleifera tree is a very versatile tree which is indigenous to tropical and sub-tropical countries. In Sri Lanka it is especially grown in dry zones. Fresh leaves, fruits, flowers and immature pods of *Moringa oleifera* are edible and are very delicious diets. *Moringa oleifera* leaves are highly nutritious and contain enormous health benefits. Due to the importance of *Moringa oleifera* tree it is known as “tree of life”, “miracle tree” and “God’s gift”(Martín Ortega & Segura Campos, 2018). The tree is also known by such regional names as “Drumstick tree” and “Horseradish tree” (Fahey, 2016).

The increasing population of many tropical countries led to awareness of the importance of *Moringa oleifera* leaves as a source of essential nutrients which may not be available in other food sources (Iheanacho & Udebuani, 2010). Recently, many researches have done to identify nutritional components of *Moringa oleifera* leaves and to develop it as a main food item or as a food supplement (Moyo et al., 2011). Since, *Moringa oleifera* leaves are rich in proteins and calcium it can be used as an alternative food source for children and even for pregnant mothers and feeding mothers (Gopalakrishnan, Doriya and Kumar, 2016). *Moringa oleifera* leaves can be used

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to fight against malnutrition and mineral deficiencies, because it contain significant amounts of phenolic compounds, dietary fiber, proteins and minerals.

Moringa oleifera leaves have brilliant antibacterial and fungicidal activities. Today many resistant bacterial strains have been developed against antibiotics. Due the low toxicity and low cost *Moringa oleifera* leaves extract can be used to fight against resistant bacterial strains and parasitic worms. Therefore, it can be used as alternatives or adjuvants for antibiotics. Methanol and ethyl acetate extracts of *Moringa oleifera* leaves prompt a great fungicidal activity. It can especially degrade the chitin in the cell walls of fungi. Since *Moringa oleifera* leaves have an extraordinary fungicidal activity it is used to cure skin diseases including skin lesions (DAAM, 2020). *Moringa oleifera* leaves can also be used as a cosmetic agent.

Today many researches have been undergone by using *Moringa oleifera* leaves. As a result researchers have been able to produce many productions such as *Moringa* tea, *Moringa* powder, *Moringa* capsules, *Moringa* soap, *Moringa* face wash, *Moringa* tablets, and *Moringa* beverages. Generally, a wide range of beneficial microorganisms are used for the preservation of *Moringa oleifera* leaves, in order to enhance the nutritional qualities and to provide numerous health benefits for the consumer.

Though there are many studies have been carried out in other countries such as India and China related to the preservation of *Moringa oleifera* leaves, there is a lack of studies on preservation of *Moringa oleifera* leaves by using microorganisms, in Sri Lanka. Therefore, the major objective of this study is to investigate the possibilities of using food grade microorganisms in fermentation of *Moringa oleifera* leaves.

METHODOLOGY

Collection of samples

Fresh *Moringa oleifera* leaves were plucked from a *Moringa oleifera* tree growing at Kalapaluwawa, Rajagiriya, Sri Lanka (I.F, Offor et al., 2014).

Preparation of samples

Drying of fresh *Moringa oleifera* leaves

Plucked *Moringa oleifera* leaves (200g) were weighed by using an electrical balance. Then leaves were dried under sunlight for 24 hours and were grounded into a fine powder.

Packaging and storage

Sundried *Moringa oleifera* leaf powder was immediately packed in air tight bottles which were sterilized by using alcohol (70%). Then bottles were stored in the room temperature (28℃ ± 2℃).

Preparation of starter culture of *Lactobacillus plantarum*

Stock cultures were grown in MRS broth (10.0 mL) at 37° C for 48 hours were inoculated into sterile MRS broth (10% w/v, 50.0 mL) with *Moringa oleifera* leaf powder (2.5 g), skim milk (1.0 g) and refined sugar (1.5 g). This was incubated at 37° C for 24 hours. Preparation of *Lactobacillus plantarum* starter cultures were done according to the method described in (Ouwehand et al., 2001) with slight modifications.

Preparation of fermented *Moringa oleifera* leaf powder

Sun dried *Moringa oleifera* leaf powder (50.0 g) were added separately into four conical flasks with sterile distilled water (500.0 mL). Flasks were sterilized in an autoclave at 121℃ for 5 minutes. Prepared starter cultures of *Lactobacillus plantarum* were transferred into each flask in order to carry out the fermentation process in different time durations such as 0h, 24 h, 48 h, and 72 h at 37° C. Then the fermented samples were pasteurized in order to stop the fermentation.

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Analysis of proximate composition unfermented and fermented *Moringa oleifera* leaf powder by *Lactobacillus plantarum*

Determination of moisture content

Moisture content was determined using AOAC, 2012 with slight modifications. Sample (2g) was weighed into a previously dried and weighed Petri dishes. Then it was dried in the oven (Philip Harris, UK) at 105 ±1°C for 4 hours. Finally, Petri dishes were cooled and weighed until a constant weight was obtained.

Determination of total Ash content

Total ash content was determined using AOAC, 2012 with slight modifications. Sample (2 g) was measured to a dry crucible and was incinerated in the muffle furnace (Wise Therm, South Korea) at 550°C for 6 hours. Finally, crucibles were cooled and weighed until a constant weight was obtained.

Determination of crude protein content. (Kjeldahl method)

In the digestion process the protein content was determined using the micro-Kjeldahl method (AOAC, 2012) with slight modifications. Sample (2 g) was weighed and was placed on digestion tubes. The catalyst [3.5 g K_2SO_4 + 0.4 g $CuSO_4$. 5 H_2O (Sigma Aldrich)] and conc. H_2SO_4 (95-98%, 5.00 mL) were added. Digestion was carried out in the digester for 60 minutes at 410°C. Tube rack was then taken out of the block digester and was placed on a rack holder, and was allowed to cool for 1 hour and distilled water (20 mL) was added to tubes.

In the distillation process H_3BO_3 solution (20 mL) and few drops of indicator were added to an Erlenmeyer flask. Flask was located under the tip of the condenser. Then a clean tube was placed in the distillation unit. Water (20 mL) and NaOH (20 mL) were added to it and the Distillation process was carried out.

Finally, in titration process the solution in the Erlenmeyer flask was titrated with H_2SO_4 to the end point of the indicator. Crude protein content of the sample was calculated by multiplying the obtained Nitrogen content from the conversion factor of 6.25.

Determination of crude fat content

Fat content was determined using the method of AOAC, 2012 with slight modifications. Finely chopped sample (2 g) and anhydrous sodium sulphate (4.00g) were placed in an extraction thimble. Then the mouth of the thimble was plugged with a piece of cotton wool. Extraction thimble with the sample was kept in the Soxhlet apparatus. Petroleum ether (200.00 mL) and pumic chips were added into the cleaned and weighed round bottom flask. Flask was connected to the Soxhlet extractor and the condenser was fixed. Refluxing was carried out for 5 hours. After refluxing is over, the solvent was distilled off and flask and the contents were placed in an oven at 105°C for two hours. Finally, the flask and the contents were allowed to cool for 30 minutes and were reweighed.

Determination of crude fiber content

Crude fiber content was determined using the method described by AOAC, 2012 with slight modifications. 2g of defatted samples were transferred into a 400.0 mL beaker and H_2SO_4 (Sigma Aldrich) (5%, 50 mL) was added and the volume was made to 200 mL mark with distilled water. The content was brought to the boiling point and kept boiling for 30 minutes while stirring with a glass rod. Hot water was added time to time to keep the volume constant. It was then filtered through a 15.00 cm #4 Whatman filter paper on a Buchner funnel attached to filter pump. The residue was transferred to the funnel with a jet of hot water and was washed it with hot water until the filtrate was free from acid. This was checked by using a litmus paper. The residue was scraped off from the filter paper, and was placed in the same beaker and remaining last traces with a jet of hot water.

NaOH (5%, 50 mL) and distilled water (200 mL) was added. Then it was brought to the boiling point and was kept boiling for 30 min. It was filtered instantaneously through the same piece of filter paper and the residue was moved to the filter by means of a jet of hot water. Then the residue was washed with hot water and with HCl (Sigma Aldrich) (1%) and again with hot water until it was free from acid. After that it was washed twice with

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small amounts of alcohol (95%) and diethyl ether (Sigma Aldrich) and residue was transferred into a porcelain dish. Remaining liquid was evaporated in an oven (Philip Harris, UK) at 100° C until the residue comes to a constant weight. It was then allowed to cool and was weighed. Finally, it was kept in a muffle furnace (Wise Therm, South Korea) at 500° C and it was allowed to cool and the final weight was taken.

Determination of total carbohydrate content

Total carbohydrate content of fermented *Moringa oleifera* leaf powder by *Lactobacillus plantarum* was calculated from following equation. Total carbohydrate content = 100-(moisture content% + ash content% + crude fat content% + crude fiber content % + crude protein content).

Determination of antioxidant potential

The antioxidant potential of fermented *Moringa oleifera* leaf samples was determined by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay by using a 96 well microtiter plate according to the method described by (Wasana & Amarakoon, 2021) with slight modifications.

Determination of chemical quality (pH)

The chemical quality was determined by measuring the pH according to the method described by (Menaka et al., 2011) with slight modifications.

Statistical analysis

Minitab 16 statistical package was used. One-way ANOVA technique and Tukey's multiple comparison tests were used to determine the significant differences of mean values of fermented *Moringa oleifera* leaf powder by *Lactobacillus plantarum*. ($p<0.05$ was considered as significant.)

RESULTS AND DISCUSSION

Determination of Moisture content

Turkey's comparison in ANOVA shows that the means of moisture contents are significantly ($p<0.05$) different from each incubation periods suggesting that there is an effect of fermentation by *Lactobacillus plantarum*. The percentage of moisture content increases with increase of incubation period (unfermented, $10.24^{D}\pm0.04\%$ < 24h, $11.46^{C}\pm0.07\%$ < 48h, $12.52^{B}\pm0.07\%$ < 72h, $14.28^{A}\pm0.08$). The highest moisture content was observed in 72h fermented *Moringa oleifera* leaves by *Lactobacillus plantarum*. The percentage moisture content of fermented *Moringa oleifera* leaf powder shows a higher percentage of (>10%). Therefore, it confirms the fact that *Moringa oleifera* leaves are rich in moisture.

Determination of Ash content

Turkey's comparison in ANOVA shows that there is a significant ($p<0.05$) reduction in the ash content between unfermented ($8.25^{A}\pm0.05$) and 72h fermented *Moringa oleifera* leaf powder ($5.33^{D}\pm0.13\%$) by *Lactobacillus plantarum*. This may be because minerals present in *Moringa oleifera* leaf powder has been used by *Lactobacillus plantarum* for its metabolic purposes. (Scheuer et al., 2021). As a result percentage of ash content has been decreased with the increase of incubation period (unfermented, $8.25^{A}\pm0.05\%$ < 24h, $7.33^{B}\pm0.03$ < 48h, $6.25^{C}\pm0.1$ < 72h, $5.33^{D}\pm0.13$).

Determination of Crude protein content

Turkey's comparison in ANOVA shows that there is a significant ($p<0.05$) increment in the crude protein content between unfermented ($24.63^{D}\pm0.18$) and 72h fermented *Moringa oleifera* leaf powder (30.24 ± 0.04^{A}) by *Lactobacillus plantarum* indicating the highest protein content. This increment may be due to the extracellular enzymes secreted by *Lactobacillus plantarum* in order to degrade cellulolytic materials during the time of fermentation (Munishamanna et al., 2017).

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Determination of crude fat content

Turkey's comparison in ANOVA shows that the means of crude fat contents are significantly ($p<0.05$) different from each other suggesting that there is an effect of fermentation by *Lactobacillus plantarum*. A significant ($p<0.05$) reduction in crude fat content can be observed at 72h incubation period ($2.83^{D}\pm0.10\%$). There is no significant ($p<0.05$) difference between the crude fat contents of 24h ($4.17^{B}\pm0.08\%$) and 48h fermented *Moringa oleifera* leaf powder ($4.17^{B}\pm0.08\%$) by *Lactobacillus plantarum*.

According to Scheuer et al. (2021) extensive breakdown of large molecules of fat into simply fatty acids causes the enhancement of crude fat during fermentation. However, the results obtained in this study does not agree with that. This is due to the activity of *Lactobacillus plantarum* in *Moringa oleifera* leaf powder.

Determination of crude fiber content

A significant ($p<0.05$) reduction in crude fiber content can be observed at 72 h incubation period ($5.82^{D}\pm0.08\%$). This is a result of unstiffening of fibrous tissues at the time of fermentation. Bioconversion of dietary fiber and lignocelluloses in to protein also reduces the fiber content (Scheuer et al., 2021).

Fiber is an essential component in food which proliferates the digestibility and reduces constipation. This study shows a higher fiber content in fermented *Moringa oleifera* leaf powder Therefore, it could function as a good source of dietary fiber providing more health benefits.

Determination of total carbohydrate content

Based on Turkey's comparison in ANOVA, it can be concluded that there is a significant ($p<0.05$) difference between the means obtained for the total carbohydrate content in different incubation periods indicating an effect of fermentation by *Lactobacillus plantarum*. The amount decreases in the order of unfermented ($41.45^{A}\pm0.19\%$> 24h ($39.63^{B}\pm0.17\%$) > 48h ($36.6^{C}\pm0.21\%$) > 72h ($30.49^{D}\pm0.17\%$). Statistical analysis revealed that there is a significant ($p<0.05$) reduction in the total carbohydrate content in 72h incubation period. Reduction arises as a result of utilizing carbohydrates by *Lactobacillus plantarum* as a source of energy for metabolic functions and growth. During the period of fermentation oligosaccharides are converted into simple sugars. This reaction also reduces the total carbohydrate content (Scheuer et al., 2021).

Determination of Antioxidant activity

Moringa oleifera leaves are rich in phenols, flavonoids, and para-anthocyanin making it a good source of antioxidants. (Iqbal & Bhanger, 2006).

IC50 value is the concentration of antioxidant required to give 50% inhibition of the probe in the antioxidant assay. Hence, lower IC50 value denotes high antioxidant activity of a given food source. According to the results, IC50 values decrease in the order of unfermented (136.3) > 24h (115.6)> 48h (65.3)> 72h (50.2).

More importantly, a significant ($p<0.05$) increase in the antioxidant potential was observed in 72 h fermented *Moringa oleifera* leaf powder by *Lactobacillus plantarum*. Therefore, 72 h fermented *Moringa oleifera* leaf powder has the highest antioxidant potential. Antioxidant properties are mostly due to the phytochemicals present in plants. These phytochemicals are important for the human health making them functionally vital for consumption. As a result of bioactive peptide synthesis by *Lactobacillus plantarum* via hydrolysis of proteins, antioxidant properties are enhanced during the fermentation (Nkhata et al., 2018).

Analysis of chemical quality (pH)

According to the statistical analysis there is a significant ($p<0.05$) difference between the means obtained for pH in fermented *Moringa oleifera* leaf powder by *Lactobacillus plantarum*. There is a gradual reduction of pH with increasing incubation time in the order of unfermented ($6.10^{A}\pm0.04$) >24h ($5.50^{B}\pm0.06$) >48h ($4.80^{C}\pm0.05$> 72h ($4.20^{D}\pm0.03$). This result agrees with almost all the lactic acid bacteria mediated fermentation and natural fermentation reported by (Obadina et al., 2013). This may be due to the production of lactic acid by *Lactobacillus*

plantarum during fermentation period in the process of energy synthesis for their metabolism (Obadina et al., 2013).

CONCLUSION

Proximate composition analysis of sundried *Moringa oleifera* leaf powder followed with fermentation by *Lactobacillus plantarum* in different incubation periods, moisture content 10.24±0.04% (unfermented) 11.46±0.07% (24h), 12.52±0.07% (48h), 27.48±0.20% (72h). Ash content 8.25±0.05% (unfermented) 7.33±0.03% (24h), 6.25±0.1% (48h), 5.33±0.13% (72h). Crude protein content 24.63±0.18% (unfermented), 25.81±0.04% (24h), 28.43±0.11% (48h), 30.24±0.04% (72h). Crude fat content 5.09±0.07% (unfermented), 4.17±0.08% (24h), 4.17±0.08% (48h), 2.83±0.10% (72h). Crude fiber content 8.33±0.06% (unfermented), 7.29±0.05% (24h), 6.12±0.03% (48h), 5.82±0.08% (72h). Total carbohydrate content 41.45±0.19% (unfermented), 39.63±0.17% (24h), 36.6±0.21% (48h), 30.49±0.17% (72h) were recorded respectively. The IC 50 values recorded for *Moringa oleifera* leaves fermented by *Lactobacillus plantarum* were 136.3 (unfermented), 115.6 (24h), 65.3 (48h), 50.2 (72h). The highest antioxidant potential was recorded in 72h fermented *Moringa oleifera* leaf powder by *Lactobacillus plantarum*.

Fermentation by using *Lactobacillus plantarum* had significantly ($p<0.05$) affected on ash content, crude protein content, crude fiber content, crude fat and total carbohydrate content at the maximum incubation period. Based on the above results, it can be concluded that the 72 h fermented *Moringa oleifera* leaf powder by *Lactobacillus plantarum* is the best preservation method in terms of nutritional quality to increase its' shelf life.

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Table 1: Nutrient content of unfermented and fermented *Moringa oleifera* leaf powder by *Lactobacillus plantarum*

Treatment	Moisture *	Ash *	Crude protein *	Crude fat *	Crude fiber *	Total carbohydrate *
0h (unfermented)	10.24^{D}±0.04	8.25^{A}±0.05	24.63^{D}±0.18	5.09^{A}±0.07	8.33^{A}±0.06	41.45^{A}±0.19
24 h	11.46^{C}±0.07	7.33^{B}±0.03	25.81^{C}±0.04	4.17^{B}±0.08	7.29^{B}±0.05	39.63^{B}±0.17
48 h	12.52^{B}±0.07	6.25^{C}±0.1	28.43^{B}±0.11	4.17^{B}±0.08	6.12^{C}±0.03	36.6^{C}±0.21
72 h	14.28^{A}±0.08	5.33^{D}±0.13	30.24^{A}±0.04	2.83^{C}±0.10	5.82^{D}±0.08	30.49^{D}±0.17

*The values are mean ±standard deviation of the replicates. The values with common superscript letters in each column are not significantly different ($p<0.05$).

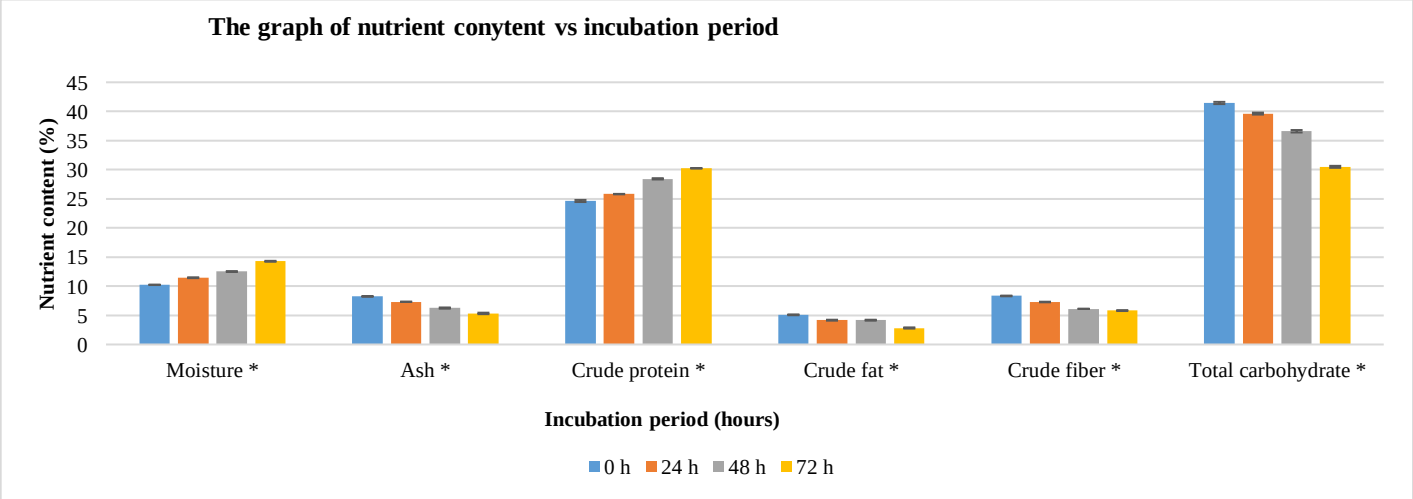

Figure 1: Bar chart representing the nutrient content of unfermented and fermented *Moringa oleifera* leaf powder by *Lactobacillus plantarum*

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Table 2: Representing % inhibition of unfermented and fermented *Moringa oleifera* leaf powder by *Lactobacillus plantarum*

Sample concentration	0 h (unfermented)	24 h	48 h	72 h
1500 *	66.7	48.0	50.8	60.6
250 *	56.4	40.1	39.2	45.2
125 *	42.3	31.9	33.4	36.2
62.5 *	35.4	30.8	19.8	25.9
125 *	32.1	28.7	16.1	15.3

*The values are mean ±standard deviation of the replicates. The values with common superscript letters in each column are not significantly different ($p<0.05$).

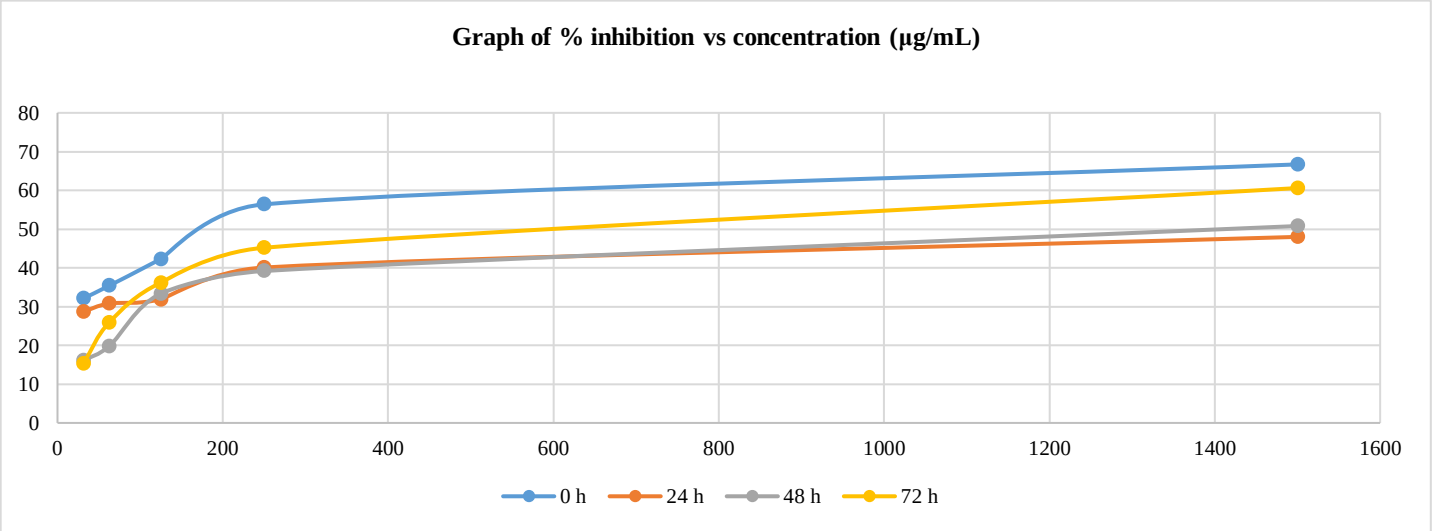

Figure 2: Variation of antioxidant potential in unfermented and fermented *Moringa oleifera* leaf powder by *Lactobacillus plantarum*

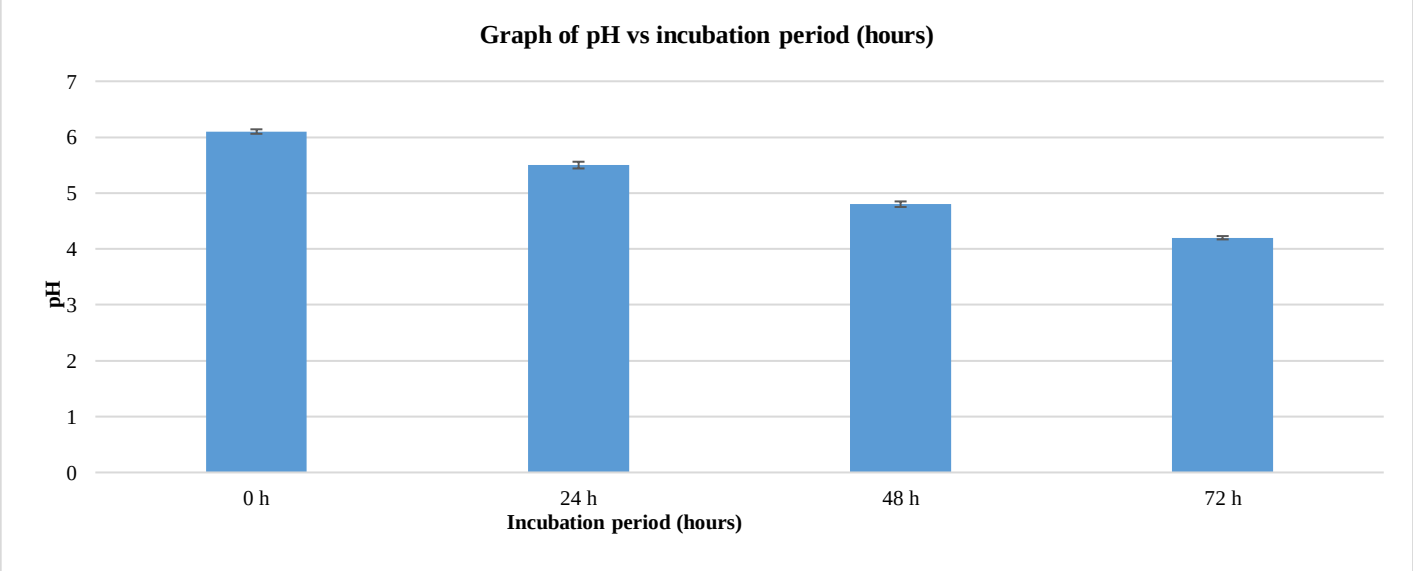

Figure 3: Bar chart representing the pH of unfermented and fermented *Moringa oleifera* leaf powder by *Lactobacillus plantarum*