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Phytochemical Screening of Ethnomedicinal Herbal Extracts and their Effect on Microbial Quality of Sukuti

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Abstract

The effect of herbal extracts of four ethnomedicinal herbs (*Zanthoxylum armatum*, *Litsea cubeba*, *Heracleum nepalense*, and *Evodia fraxinifolia*) of culinary importance on the microbial quality of sukuti (a traditional dried meat product) was studied. Herbal extracts were prepared by grinding each herb to particle size < 250 µm, extracting in 50% (v/v) ethanol, and concentrating in a rotary vacuum evaporator at 50oC. Four of the spoilage and pathogenic microorganisms, viz., *Salmonella*, *Staphylococcus*, *E. coli*, and *Lactobacillus* were isolated from market sukuti samples and used as test-organisms for the study. Herbal extracts at the concentration of 40, 20, 10, and 2 mg/ml were tested against the test-organisms to determine the antimicrobial property of the extracts. The herbal extract showing the greatest antimicrobial activity was selected for use in optimized product (sukuti) development. The total phenolic content of the herbal extracts was also determined. The analyses were performed in triplicate. The data were checked for homogeneity before being analyzed with ANOVA in Genstat Release v12. The Fisher's Least Significant Difference (LSD) method was used to compare data means at a 5% level of significance.

Zanthoxylum armatum at 40 mg/ml concentration showed the largest zone of inhibition (ZOI) against the test-organisms and therefore selected for final product development. Meat strips (1 cm × 1 cm × 25 cm) were marinated with Zanthoxylum armatum (40 mg/ml) extract at the rate of 2%, aged (24 h at 4 ± 2oC), and dried in a cabinet dryer for 2 days at 55oC. The total plate counts (TPCs) of control (untreated)- and herbal (treated) sukuti were carried out for 20 days at an interval of 10 days to determine the microbial stability of the final product. The TPC for the treated sample was significantly lower ($p<0.05$) than that of the untreated sample.

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1. Introduction

The microbiological profile of meat products that are offered to consumers is a reflection of the overall health of the slain animal, the environment in which the animal was raised, the quality of the slaughtering, processing, packaging, and storage conditions. *Listeria monocytogenes*, *Clostridium perfringens*, *Staphylococcus aureus*, *Salmonella* spp., pathogenic strains of *Escherichia coli*, *Campylobacter* spp., *Yersinia enterocolitica*, and *Aeromonas hydrophila* are some of the most common pathogenic bacteria associated with meat. (Douglas and Farid, 2001).

Studies show that dried meat contains fairly high levels of many spoilage and pathogenic organisms (including Salmonella, E. coli, and toxin producing staphylococci and molds) capable of causing food poisoning (Garcia et al, 1995; Wolter et al, 2000; Hui et al, 2001; Mhlambi et al, 2010). Many documented outbreaks worldwide due to the consumption of dried meat have increased concern over growth and survival pathogen in dried meats (Nummer et al, 2004; Lake et al, 2012; Mindlin et al, 2013; Ha et al, 2019).

Meat and meat products are highly nutritious for human diet, but they also provide an ideal environment for microbial growth (Zhou et al; 2010). Synthetic

preservatives have been widely employed in the food industry because of their low cost and potent antibacterial activity to avoid the microbial contamination of meat products. However, use of synthetic chemical preservatives is recently being considered by customers due to concerns related to safety of synthetic food additives (Yu et al, 2021). Additionally, the use of artificial additives in foodstuffs is limited by food legislation (Devatkal et al, 2014). Therefore, the demand for the use of natural substances as food preservatives has increased lately (Yu et al, 2021).

Herbs, spices, aromatic plants, berries, and their extracts and essential oils have been studied as potential natural antimicrobials (Cowan, 1999; Burt, 2004; Tiwari et al, 2009; Ullah et al, 2012; Bajpai et al, 2012; Haghgoo et al, 2017; Koirala and Singh, 2017; Kamle et al, 2019; Dhakal et al, 2020) in meat and meat products (Hygreeva et al, 2014; Aminzare et al, 2016). Numerous studies on the antimicrobial effects of herbal essential oils in meat and meat products have been conducted, and it has been discovered that these essential oils exhibit good antimicrobial activity against a variety of pathogenic and spoilage microorganisms present in meat and meat products (Menon and Garg, 2001; Solomakos et al, 2008; Fratianni et al, 2010; Bajpai et al, 2012).

Sukuti is a traditional dried meat product very popular in Nepal. Review of available literature (Thapa, 2017; Bhattarai and Lamichhane; 2022) reveals that researches on sukuti so far are limited to its preparation, process optimization, and general surveys. Microbial aspects of the sukuti, which are very important in the preservation of sukuti and prevention of health hazards, appear to have largely been ignored.

Among the rising meat safety concerns in recent years, microbial pathogens are traditionally associated with most serious meat safety issues in terms of foodborne illness. Also, the growing health concern and awareness has led public interest towards search of foods that are natural, less processed, and safe (Sofos, 2008; Teixeira and Rodrigues, 2019). As a result, the purpose of this study was to develop a novel sukuti (natural and microbiologically safe) by incorporating extracts from selected native herbs that have been hitherto-neglected or underutilized.

2. Materials and Method

2.1. Raw materials collection

Fresh male buffalo meat was purchased from the local market of Dharan. Meat (5 kg) from the round-cut was purchased and packed in low-density polyethylene (LDPE) bags to prevent contamination and moisture loss during transportation. The sample was transported to the laboratory and processed immediately (sukuti preparation). For the screening of microorganisms, sukuti sample (250 gm) was randomly collected from the 5 different local shops of Dharan. Similarly, the dried herbs (500 gm each) having culinary importance in the household of Kiranti community were also collected from the local market of Dharan, which were from Sankhuwasabha, a district in eastern region of Nepal. Four types of ethno medicinal herbs viz., *Zanthoxylum armatum* (Bokey Timur), *Heracleum nepalense* (Chimphing), *Litsea cubeba* (Sil timur) and *Evodia fraxinifolia* (Khanakpa) were purchased for the proposed study.

2.2. Preparation of herbal extracts:

Dried herbal materials (Bokey timur, Sil timur, Khanakpa and Chimphing) were ground separately in a grinder to particle size < 250 μm. 50 g of the powdered herb was added to 400 ml of 50% (v/v) ethanol in a stoppered Erlenmeyer flask and extraction done for 12 h with constant shaking (100 rpm) using a magnetic stirrer. The extract was then filtered through Whatman No. 2 filter paper, and the residue was extracted again for another 12 h with an additional 200 ml of 50% ethanol.The filtrates were pooled, concentrated in a rotary evaporator at 50oC and dried. Dried extracts were sealed in vials and kept at 4oC until analysis. (Zhang et al, 2016)

.2.3. Determination of total phenolic content in herbal extracts:

The total phenolic contents of the ethanolic extract of herbs were estimated using the Folin-Ciocalteau reagent as described by Singleton and Rossi (1965). In order to plot the calibration curve, 1 ml aliquots of 50–450 μg/ml gallic acid solutions were combined with 5.0 ml of Folin–Ciocalteau reagent (10-fold diluted) and 4.0 ml of Na2CO3 solution (75 g/L). . After 30 minutes, the absorbance at 765 nm was measured. For the ethanolic extracts (1 g/100 ml), 1 ml of extract was mixed separately with the same reagents as used for plotting the calibration curve. To calculate the total phenolic content of the extracts, the absorbance was measured after 1 hour.

2.4.Threshold study:

Novel attempts like incorporating herbs in traditional foods like sukuti can be daunting as these can mar the characteristic sensory quality of the product, resulting in a reduced acceptability, or even rejection of the novel product. Ultimately, it's the consumer acceptability that counts for any product to sustain in this competitive market. Threshold study was carried out by running trial experiments. Different batches of sukuti were prepared by marinating meat in different concentrations of herbal extracts. The maximum and minimum ranges at which herbs started to give off too strong a flavor and at which herb flavor just got detected (respectively) were noted. Threshold concentrations were ultimately selected from sensory analysis (ASTM, 2004).

2.5. Screening of microorganisms from sukuti:

Certain microorganisms such as *Salmonella*, *Shigella*, *E. coli*, *Staphylococcus*, *Lactobacillus* and were isolated from sukuti collected from the local market of Dharan.

2.5.1.*E. coli:*

E. coli was isolated on Eosine Methylene Blue Agar (EMBA) medium by pour-plate method as described by Harrigan and McCance (1976) using sterile water as diluent with slight modification. For identification, biochemical tests such as Gram staining, IMViC test, coagulase and catalase test were carried out using methods proposed by Gordon et al (1973).

2.5.2 Salmonella

Salmonella was isolated according to the Varadaraj (1993) with some modifications. Observation of colony characteristics and biochemical tests like Gram staining, IMViC test, coagulase test, H2S test and catalase test were done for confirmation.

2.5.3 *Staphylococcus:*

Staphylococcus was isolated on Mannitol Salt Agar by the pour-plate method according to Harrigan and McCance (1976). The bacteria were characterized by microscopic morphological examination and conventional biochemical tests.

2.5.4 Lactobacillus:

Lactobacillus was isolated by the pour plate method on De Man Rogosa and Sharpe agar (MRS) agar according to Harrigan and McCance (1976). The bacteria were characterized by microscopic morphological examination and conventional biochemical test as described by Buchanan and Gibbons (1974).

2.6. Preparation of bacterial inoculum/suspension:

Required colonies of freshly cultured test-organisms were inoculated aseptically to glass vials containing sterile nutrient broth and incubated at 32oC for 24 h (Phuyal et al, 2020).

2.7. Determination of antimicrobial activity of herbal extracts:

Using the agar well diffusion method, the antibacterial activity of the herbal extracts was tested in triplicate to detect growth inhibition of *Salmonella*, *Lactobacillus*, *E. coli*, and *Staphylococcus* (Balouiri et al, 2016). At the following diluted herbal extract concentrations in 50% ethanol: 40, 20, 10 and 2 mg/ml (concentration range selected from trial experiment), tests for individual antimicrobials were conducted. A sterile cotton swab was used to prepare carpet cultures by swabbing microbial suspension over the dry surface of sterile nutrient agar plates. The agar plates were aseptically punctured with a sterile cork-borer (5.2 mm in diameter), and 0.1 ml of herb extracts were then aseptically placed into the well at each dilution whereas 0.1 ml of 50% ethanol is taken as control. The plates were incubated with the lid on for 24 h at 35 ± 2°C.With the aid of a ruler, the diameter of the zone of inhibition (ZOI, clear zone) around the extract was measured to determine the inhibitory effect (Phuyal et al, 2020).

2.8. Preparation of untreated sukuti (control) and herbal extract incorporated sukuti (optimized):

Sukuti was prepared using a cabinet dryer as described by Thapa (2017) with slight modification. Buffalo meat was cut into strips of 1 cm thick and 25 cm long. Herbal extract having the best antimicrobial activity was selected and dissolved in distilled water for application on meat strips. Herbal extract was applied at the rate of 2% on meat strips. The marinated meat strips were kept in the refrigerator for 24 h at 4 ± 2oC. Then they were placed on trays and dried in a cabinet dryer at 55oC for 2 days. Similarly, control was prepared without applying herbal extract.

2.9. Microbiological analysis of prepared sukuti (control and optimized):

Total plate counts (TPCs) of control and optimized sukuti were carried out by the pour-plate method as described by Harrigan and McCane (1976). The TPC of the sample was expressed in terms of log colony forming units (cfu) per gram and the results obtained were statistically processed.

Statistical analysis: The analyses were performed in triplicate. The data were examined for homogeneity before being analyzed with ANOVA using Genstat Release v12. The Fisher's Least Significant Difference (LSD) method was used to compare means of the data at a 5% level of significance.

3. Results and discussion

3.1. Total phenolic content of herbal extract:

Table 1 shows that the ethanolic extract of *Zanthoxylum armatum* had the highest total phenolic content, followed by *Litsea cubeba*, H*eracleum nepalense* and *Evodia fraxinifolia*. The result is in agreement with those of Ullah et al (2012) and Koirala and Singh (2017).

Table 1 Total phenol content of herbal extract

Ethanolic extract	Total phenolic content (mg GAE/g)
Zanthoxylum armatum	21.414 ± 0.204^{a}
Litsea cubeba	16.9220 ± 0.1508^{b}
Heracleum nepalense	8.860 ± 0.264^{c}
Evodia fraxinifolia	5.851 ± 0.180^{d}

*Results are the average values of the three replicates. Values are presented in the form of Mean ± Standard deviation. Means in the different row bearing different alphabets in superscript are significantly different at $p<0.05$.

3.2. Antimicrobial activity of herbal extracts:

Data on antimicrobial activity of the herbal extracts as determined by well diffusion method and ZOI against the four test organisms (*Salmonella*, *E. coli*, LAB and *Staphylococcus*) at four different levels of concentration (2 mg/ml, 20 mg/ml, 30 mg/ml and 40 mg/ml) are shown in Fig. 1 (Panel 1 & Panel 2).

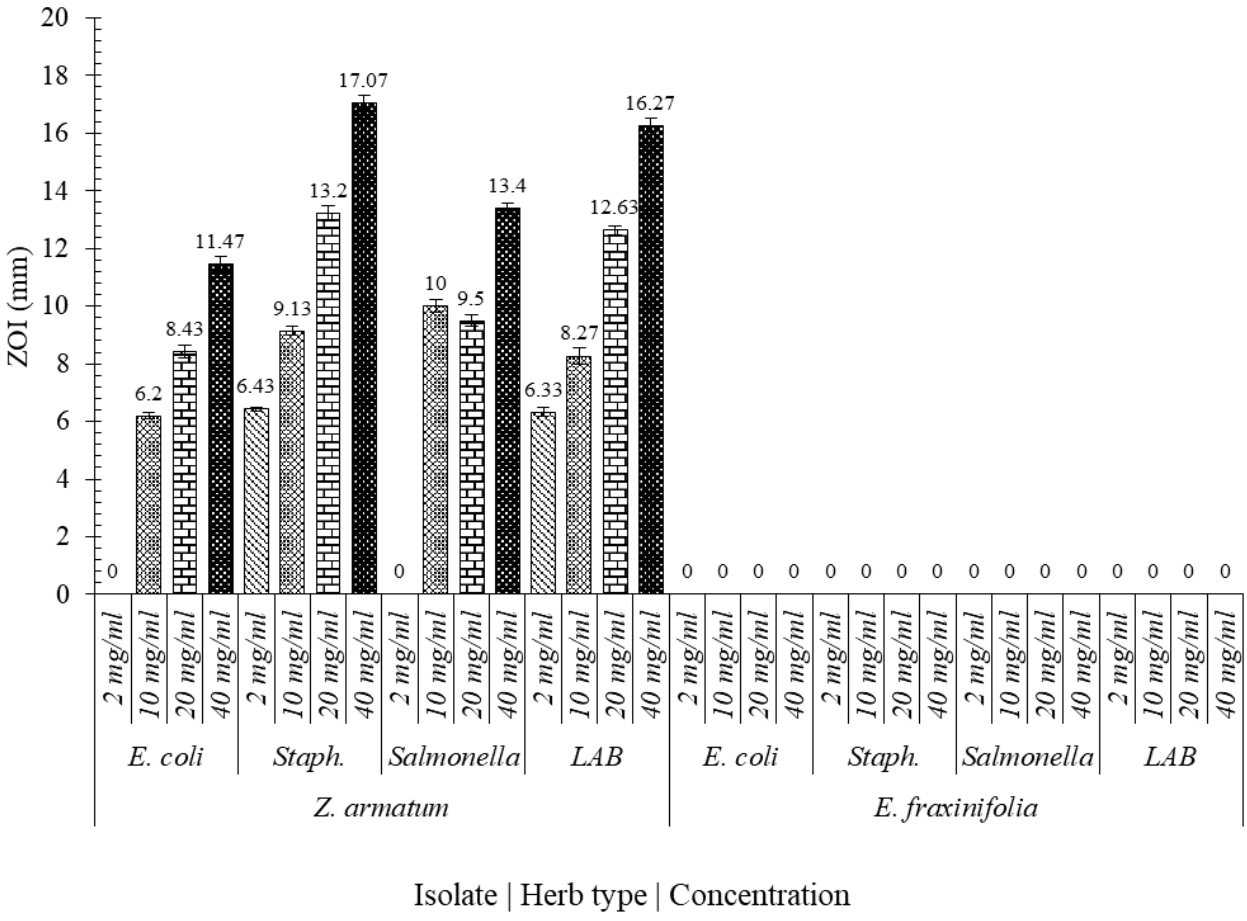

Figure 1(Panel 1): Graphical representation of ZOI showed by herbal extracts for the test organisms at different level of concentration (continued)

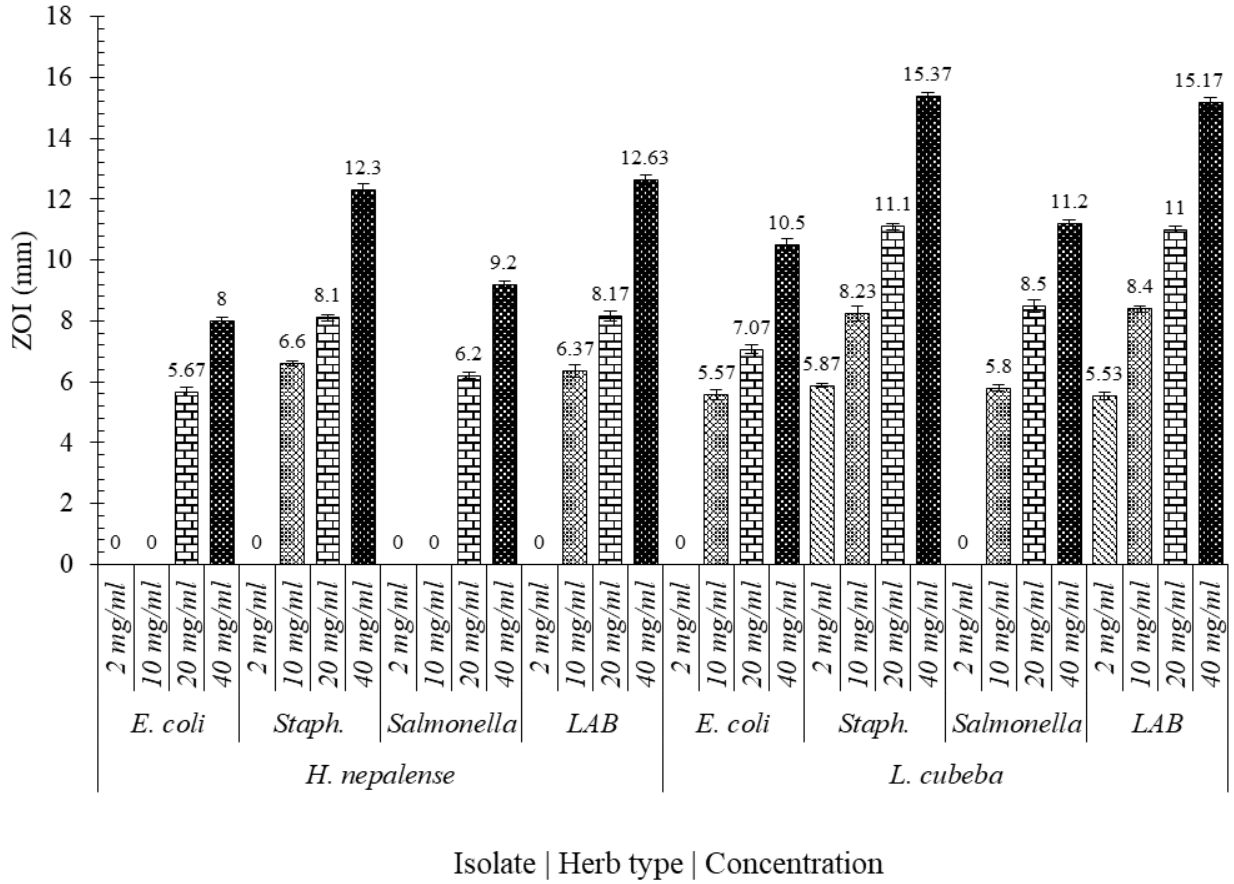

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Figure 1 (Panel 2) Graphical representation of ZOI showed by herbal extracts for the test organisms at different level

3.3. Effect of extract concentration on ZOI

Table 2 reveals that 40 mg/ml concentration of extract showed highest ZOI (9.54 □ 6.05 mm) whereas 2 mg/ml concentration showed the least ZOI. The result showed positive correlation between extract concentration and ZOI whereas the correlation matrix table for the same is shown in Table 3. It has been reported that the antibacterial effect of the plant extract increases with increase in its concentration (Haghgoo et al, 2017). This may be due to the higher amount of active antimicrobial principles that naturally occur at higher extract concentration.

Table 2: Summary of effect of concentration on ZOI

Concentration	2 mg/ml	10 mg/ml	20 mg/ml	40 mg/ml	LSD (5%)
ZOI (mm)	1.51 ± 2.65[a]	4.43 ± 3.6[b]	6.85 ± 4.48[c]	9.54 ± 6.05[d]	1.764

*Values represent means ± standard deviation. Means in different columns with different alphabets in superscript differ significantly ($p<0.05$).

Table 3 Correlation matrix of different variables (concentration and ZOI)

	Concentration	ZOI (mm)
Concentration	1	
ZOI (mm)	0.972581	1

3.4. Effect of herb type on ZOI

Table 4 shows that *Z. armatum* and *L. cubeba* were most potent ($p<0.05$) against the test organisms (*Salmonella*, LAB, *Staphylococcus* and *E. coli*), followed by *H. nepalense*. Unlike the rest of the herbs, E. fraxinifolia showed no ZOI against any test organism and thus can be considered ineffective. The reason behind inferiority of *E. fraxinifolia* and high efficacy of Z. armatum against test organisms may be due to their phenolic content (Table 1): the higher the amount of phenolic compounds in the herb extract, the higher will be the antibacterial properties against food pathogens (Burt, 2004).

Table 4 Summary of effect of herb type on ZOI

Herb	*E. fraxinifolia*	*H. nepalense*	*L. cubeba*	*Z. armatum*	LSD (5%)
ZOI (mm)	0^{a}	5.21 ± 4.48^{b}	8.08 ± 4.31^{c}	9.04 ± 4.84^{c}	1.586

*Values represent mean ± standard deviation. Means in the different columns with different alphabets in superscript are significantly different ($p<0.05$).

3.5. Effect of herbal extracts on ZOI for different isolate types:

Table 5 shows that *Staphylococcus* was more susceptible to be herbal extract among the test organisms. Similarly, LAB was second most susceptible after *Staphylococcus*. However, E. coli showed the least ZOI followed by *Salmonella*. This may be due to the difference in the composition of their cell wall, which affects the antimicrobial properties of herbal extracts. Herbal extracts have reportedly been shown to affect Gram positive bacteria slightly more than Gram negative bacteria (Burt, 2004). This might be explained by Gram positive bacteria having a single layer of cell wall construction as opposed to Gram-negative bacteria, which limits the permeability of extracts into the bacterial cell.

Table 5 Summary of effect of herbal extracts on ZOI for different isolate type

Isolate	*E. coli*	*Salmonella*	LAB	*Staphylococcus*	LSD (5%)
ZOI (mm)	3.93 ± 4.24^{a}	4.38 ± 4.77^{a}	6.92 ± 5.56^{b}	7.1 ± 5.7^{b}	2.055

*Values represent mean ± standard deviation. Means in different columns with the same alphabets in superscript are not significantly different ($p<0.05$).

3.6. Microbiological analysis of the final (optimized) herbal sukuti:

Having obtained the highest ZOI against *Staphylococcus* for *Z. armatum* extract at 40 mg/ml concentration, a final (optimized) herbal sukuti was prepared and the effect of the said herbal extract was compared against a control (lab prepared sukuti) by analyzing the change in TPC during the storage of the samples (optimized and control) for 20 days (Table 6).

Table 6 Comparison between total plate count of control and optimized samples

Treatment	Total Plate Count (log cfu/g)		
	Day 0	Day 10	Day 20
Untreated *sukuti* (Control)	3.46 ± 0.151^{a}	3.86 ± 0.035^{a}	4.378 ± 0.068^{a}
Herbal *sukuti* (Optimized)	2.36 ± 0.104^{b}	2.507 ± 0.18^{b}	2.66 ± 0.794^{b}

*Values represent mean ± standard deviation. Means in the same column with different alphabets in superscript are significantly different ($p<0.05$).

.Table 6 shows a significantly lower ($p<0.05$) TPC for optimized samples than that for control samples. Overall, the results demonstrate the effectiveness of the tested herb for inhibiting microbial growth, which agrees with what Hygreeva et al (2014) have mentioned. This may be due to the active principles present in the herbal extract that had an antibacterial impact on sukuti. Herbs' phenol components, in particular, are the primary cause of their antibacterial properties (Cowan, 1999). Altering microbial cell permeability, interfering with membrane function (nutrient uptake, electron transport, protein, nucleic acid production, and enzyme activity), and interacting with membrane proteins leading to deformation in structure and functionality can all be potential mechanisms for the antimicrobial effects of phenol compounds (Tiwari et al, 2009).

4. Conclusions

Based on the present findings, however, not all herbal extracts are equally effective as antimicrobials. Moreover, the effect is contingent upon several factors

like the herb type, concentration of the antimicrobials, and the type of target organism. Dried meat (sukuti) can harbor some of the important foodborne pathogens. Therefore, it is reasonable to apply selected ethnomedicinal herbs on sukuti given their antimicrobial efficacy (because the study found that market sukuti harbored *Salmonella*, a zero-tolerance pathogen) and feasibility (because the herbs are readily available). However, it is recommended that a threshold study be first carried out by sensory trials to optimize the quantity of herbs (or their derivatives) to be used. *Zanthoxylum armatum* extract at the concentration of 40mg/ml can be incorporated in sukuti to improve microbial stability of the product without affecting the product's palatability.

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Competing interests

The authors declare no competing interests.

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