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Bioactive Components, Nutritional Properties, and Antimicrobial Activities of Non-Fermented and Fermented Seeds of *Vitis vinifera*

Mansurat B. Falana[1]*, Muhammed R. Asinmi[2], Muhammad A. Dikwa[3] and Quadri O. Nurudeen[2]

[1]Department of Biological Sciences (Microbiology Unit), Al-Hikmah University, Ilorin, Nigeria.
[2]Department of Biological Sciences (Biochemistry Unit), Al-Hikmah University, Ilorin, Nigeria.
[3]Department of Microbiology and Biotechnology, Federal University, Dutse, Nigeria.

*Corresponding author Email: bolman4ever@yahoo.com, Tel: +234 (0) 806 041 1288

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ABSTRACT: The fermented and non-fermented seed powder of *Vitis vinifera*, a herbaceous plant with numerous biological activities was investigated in this study. The pH, *in vitro* antimicrobial assay against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*, phytochemical, proximate compositions were screened, while the active compounds of the fermented sample were further evaluated by High-performance liquid chromatography (HPLC). A decline in pH from 6.2 to 3.6 was recorded during fermentation for 14 days. Varying MIC/ MFC ranges of 12.5 – 25.0 mg/mL and 12.5-100.0 were noted for the fermented and unfermented samples, respectively. The fermented sample exhibited appreciable antimicrobial effects with varying inhibition zones at tested concentrations of 100 mg/mL (10.0-18.0 mm), 50 mg/mL (8.0-15.0 mm), and 25 mg/mL (6.0-12.0 mm) than the unfermented sample with inhibition zone at concentrations of 100 mg/mL, 50 mg/mL, and 25 mg/mL being 2.0-3.0 mm, 0.0-2.0 mm, and 0.0-2.0 mm, respectively. Ash, fat, and protein content increased with fermentation than moisture, fiber, and carbohydrate content. Alkaloids, anthraquinone, flavonoids, glycosides, saponins, tannins, and terpenoids were detected in the fermented sample while alkaloids, flavonoids, glycoside, saponins, and terpenoids were detected in the non-fermented sample. Gallic acid, quercetin, catechin, quercitrin, and epicatechin were detected by HPLC in the fermented samples. Conclusively, fermented, and non-fermented samples of *V. vinifera* have antimicrobial activities, however, the acidity of the fermented sample may be contributing to its better activity and higher components. Thus, their relevance as promising antimicrobial agents.

Keywords: *Escherichia coli*, Fermentation, HPLC, Protein, *Vitis vinifera*

Introduction

Plants are a great source of natural antimicrobial agents with numerous therapeutic values. Some scientific reports have stated that edible plants harbor compounds known to have antimicrobial potential against some pathogens (Oz and Kafkas, 2017). *Vitis vinifera*, raisins also known as dried grapes from the family Vitaceae, are edible fruits widely consumed as snacks for their traditional and natural medicinal values worldwide (Di Lorenzo *et al.*, 2016; Cordero-Bueso *et al.*, 2017).

Previous scientific reports have stated that fruits such as pomegranates, apples, grapes, and berries, possess anticancer, cardiovascular, antiallergic, anti-ageing, and antiviral properties (Schreiner and Huyskens-Keil, 2006; Karasaw *et al*., 2018). Sharafan *et al.* (2023) reported the antibacterial and anticancer properties of *V. vinifera* and its active agents. Furthermore, Yeung *et al.* (2006) attributed the antioxidant properties of raisins to their phenolic content. Akaberi and Hosseinzadeh (2016) mentioned the potential of various forms (dried,

unripe, juice) of grapes against many illnesses such as smallpox, eye infections, sore throat, cancer, cholera, liver, and kidney problems.

Different parts of *V. vinifera* such as the seeds, roots, leaves, and stems have been reported to contain various antioxidants such as bioflavonoids, proanthocyanidins, catechin monomers, procyanidin dimers, gallic acids, other polyphenolic compounds which have overall beneficial effects on humans (Monagas *et al.*, 2005; Vasavada *et al*., 2006; Siro *et al*., 2008).

Fermentation remains a successful technique used in industries for the production of biopharmaceuticals, food supplements, and compounds with inhibitory properties against pathogens (Kothari *et al*., 2020). This may be because fermented foods are associated with microorganisms that transform raw components of the food into bio-available nutrients with improved sensory properties, improved safety, and degraded toxic and antinutritive factors with better health-promoting compounds (Tamang *et al.*, 2016).

Less attention has been paid to the medicinal value and composition of fermented foods. Hence, in an attempt to improve the nutritional value and overall benefits of *V. vinifera*, a fermentation technique was adopted in this study to investigate the constituents and the biological activity mainly antimicrobial of fermented and non-fermented seeds of *Vitis vinifera*.

Materials and methods

Collection and processing of seeds of V. vinifera: *V. vinifera* seeds were purchased from a local market in Kano, Kano State, Nigeria. The seeds were identified and authenticated at the University of Ilorin Herbarium, Ilorin, Nigeria where a specimen was dropped (reference number UILH/004/1430/2021). The seeds were sorted, oven-dried at 30 ºC for 48 h, and milled to smooth, millipore-sized powder using Master Chef Blender (Mode MC-BL 1980, China).

Test organisms: Bacterial species (*Staphylococcus aureus* and *Escherichia coli*) and the yeast (*Candida albicans*) used in this study were obtained from the Microbiology Department, University of Ilorin Teaching Hospital, Ilorin, Nigeria. The isolates, obtained on Nutrient agar slants and Potato Dextrose Agar (PDA) slants, respectively, were confirmed by cultural, physiological, and biochemical identification techniques before being stored at 4 ºC until required for the study.

Standardization of inoculum: About five colonies grown within 18 to 24 h on a primary agar plate were picked with the end of a sterilized rod and suspended into the 5 mL sterile saline by agitation with a Vortex Genie mixer. The resulting suspension was checked for turbidity using a spectrophotometer at 530 nm that was adjusted to 1.5×10^8 CFU/mL and 1.5×10^4 conidia/mL for the bacteria and yeast isolate, respectively, equivalence of 0.5 McFarland standard (Chikezie, 2017).

Fermentation of V. vinifera seeds powder: *V. vinifera* seeds powder was fermented by employing a modified method of Kim *et al.* (2016). The raisins powder (100 g) was introduced into a 2-litre sterile mason jar (made of heat-tempered glass) containing 1000 mL of distilled water (1:10 w/v). The jar was immersed in a water bath at 72 ºC for 15 min to inhibit microbial growth and deactivate the enzymes, then cooled to room temperature (25 ºC). Ammonium dehydrogenase orthophosphate and yeast (Red Star® Quick-Rise, Lesaffre, Nigeria) were added at 0.2 % each to the mixture to serve as a nitrogen source for the yeast (starter agent) while the glucose and fructose content of the raisins powder served as a carbon source (Guarner and Schaafsma, 1998). The mixture was placed in a water bath at 43 ºC for 30 sec to fully activate the yeast. The cap of the mason jar was loosened to allow the carbon dioxide to escape. The jar mixture was kept for 14 days at 30 ºC. After 14 days, the sample was membrane filtered (0.4 mm) and refrigerated at -18 ºC for further analysis.

Sterility testing of the fermented V. vinifera seeds powder: Adopting the method recounted by Benkova (2020), serial dilution of 1 g of the fermented sample was done in sterile tubes containing 10 mL Mueller Hinton (Hi-Media) broth to reduce the concentration of the extract up to 10^{-1}. Subsequently, the tubes after incubation for 24 h at 37 ºC, were observed for clarity or turbidity of the broth. A clear broth is an indication that the extract was free of contaminants.

Preparation of working concentration of the fermented sample: The testing concentrations (100 mg/mL, 50 mg/mL, and 25 mg/mL) were prepared by transferring 1mL of the sample into 9 mL of sterile distilled water (100 mg/mL), vortexed, another 1 mL was transferred from this mixture into a fresh test tube containing 9 mL of distilled water to have 50 mg/mL and 1 mL from this mixture was again transferred into another tube containing 9 mL of distilled water to have 25 mg/mL concentrations of the sample. The tubes were further centrifuged for 20 min at 25 ºC at 3000 rpm. The arising supernatant was collected by filtration using Whatman no. 1 filter paper and stored.

Determination of MIC of the sample: The microdilution techniques described by the Clinical and Laboratory Standards Institute (CLSI, 2008) were adopted to determine the minimum inhibitory concentration of each

extract that will prevent the growth of the test pathogens. The MIC was achieved by carrying out two-fold serial dilutions of the sample done in a microtiter plate of Mueller Hinton broth to obtain different concentrations (100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, and 3.25 mg/mL). Into each plate was introduced, 0.1 mL of the 18-h broth culture of the test inoculum that had been previously adjusted to 0.5 McFarland equivalence. Subsequently, each tube was covered with cotton wool and incubated at 37 ºC for 24 h. The tube with the lowest concentration of extract without growth (clear tube) after 24 h was recorded as the MIC.

Preparation and impregnation of sensitivity paper discs: Paper discs of 6 mm in diameter were prepared from Whatman No. 1 filter paper, arranged in a sterile glass petri dish, and sterilized in an oven at 160 ºC for about 1 h (Rashed *et al*., 2020) after which they were allowed to cool. The paper discs were soaked in the various concentrations of the sample. The discs were allowed to absorb 0.01 mL of the constituted extract (Bauer *et al*., 1966).

Antibiotic susceptibility test: The susceptibility testing of the sample was tested by the Kirby-Bauer disc diffusion technique following the recommendations of CLSI (2013). This was achieved by using a congealed Mueller-Hinton (Oxoid, UK) agar plate of 4.0 mm depth which was streaked with 0.5 mL broth culture of the standardized test organisms. The sample discs were loaded on the surface of the agar plate within 15 min of inoculation of the plates and gently pressed down to ensure contact of the disc with the agar surface. The plates were inverted and incubated at 35±1°C for 16–20 h. Clear zones around the disc were measured and recorded as the zones of inhibition. The experiment was done in triplicate and the average value of inhibition was calculated. DMSO (5%) was used as a control while standard antibiotics (erythromycin -30 µg and gentamicin -30 µg) discs were used as reference drugs for comparison (Shobowale *et al.*, 2017).

Proximate analysis: In this study, the modified method described by the Association of Official Analytical Chemists (AOAC, 2004) was adopted to determine the moisture, protein, fat, crude fiber, ash, and carbohydrate. Soxhlet extraction technique was adopted to determine the fat content while the crude protein content was determined by the Kjeldahl method. The weight difference was noted as the carbohydrate content:

Carbohydrate (%) = 100 – % (protein + fat + moisture + ash).

Qualitative phytochemical screening: The method given by Odebiyi and Sofowora (1978) was employed to determine the phytochemical composition of the sample (alkaloids, flavonoids, saponins, tannins, anthraquinones, glycosides, phlobatannin, steroids, terpenoids).

HPLC analysis: Evaluation of the components of the fermented seed powder of *V. vinifera* was done by HPLC analysis using the modular chromatographic system Shimadzu (Nexeramx). The system comprised an interface (CBM-10A), a column oven (CTO-10A), an LC-10AD pump, a UV-DAD detector (SPD-10A), and a Workstation (LC-10). The sample (1.0 g), suspended in acetonitrile/water (1:1 v/v) was centrifuged for 10 minutes at 3000 rpm and filtered. Then, analysis was performed on a 250 mm x 4.6 mm ID x 5 mm at 30 ºC. Using the mobile phase acetonitrile comprising of water (40:60 v/v) at a flow rate of 1 mL /min., separations of the constituents were done in the isocratic mode at a mobile phase of constant composition during the evaluation period (Springfield *et al.*, 2005).

Results

The pH value declined from 6.2 on the first day of fermentation to 3.6 on the last day (Figure 1).

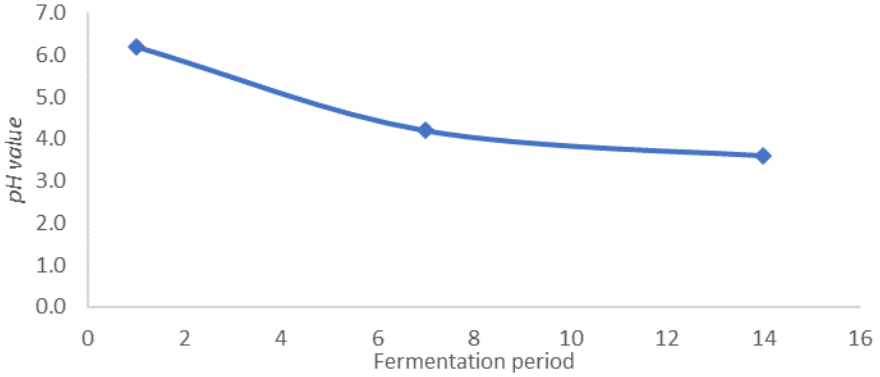

Figure 1: pH value of *V. vinifera* powder during the period of fermentation

A lower concentration of the fermented sample (12.5 – 25.0 µg/mL) is required to inhibit the growth of the test organisms while the MIC range (50.0 – 100.0 µg/mL) of the non-fermented sample is required to inhibit the growth of the test organisms (Table 1).

Table 1: Comparative Minimum Inhibitory Concentration (µg/mL) of non-fermented and fermented samples of *V. vinifera* powder against selected pathogens

	Minimum Inhibitory Concentration (µg/mL)		
Sample type	***S. aureus***	***E. coli***	***C. albicans***
Non-fermented	100	50	50
Fermented	25	25	12.5

The diameter of the inhibition zone exhibited by the non-fermented sample at concentrations of 100 mg/mL, 50 mg/mL, and 25 mg/mL are 2.0-3.0 mm, 0.0-2.0 mm, and 0.0-2.0 mm, respectively while the fermented sample exhibited varying diameters of inhibition zones at tested concentrations of 100 mg/mL (10.0-18.0 mm), 50 mg/mL (8.0-15.0 mm), and 25 mg/mL (6.0-12.0 mm) (Figures 2 and 3)

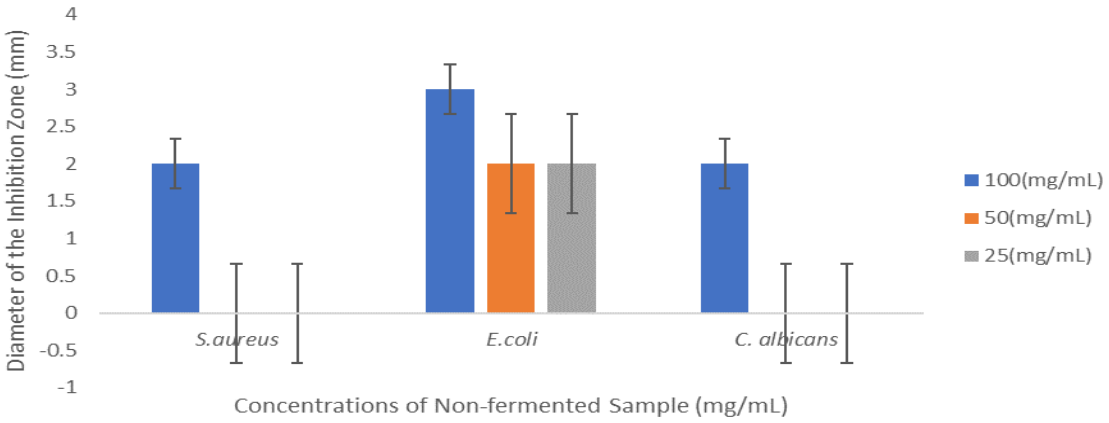

Figure 2: Antimicrobial effects of the non-fermented sample of *V. vinifera* powder against selected pathogens

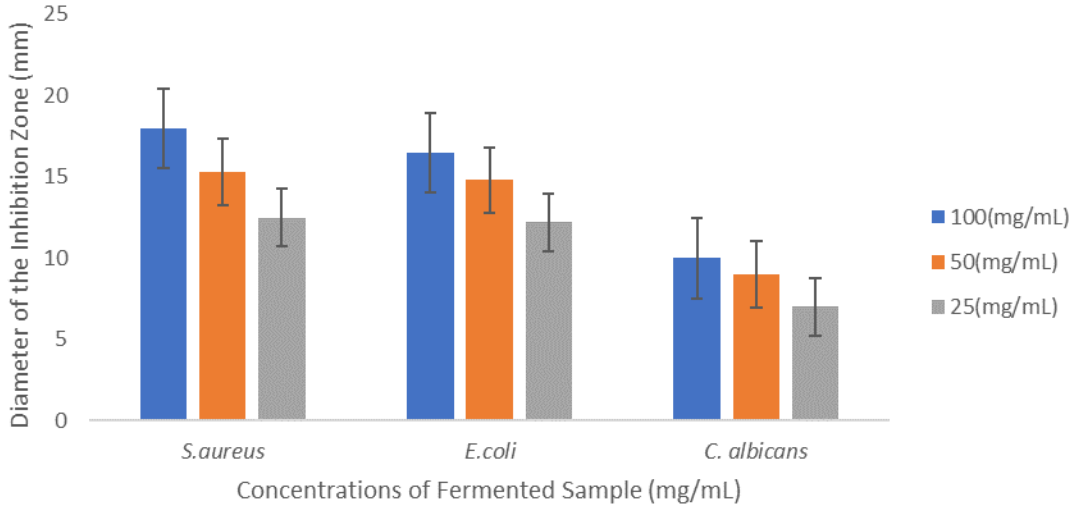

Figure 3: Antimicrobial effects of the fermented sample of *V. vinifera* powder against selected pathogens

Varying diameters of the inhibition zones were exhibited on the test organisms by the commercial drugs-erythromycin (22 mm against *E. coli* and 17 mm against *S. aureus*), gentamicin (18 mm against *E. coli* and 15 mm against *S. aureus*), and griseofulvin (14 mm against *C. albicans*) (Figures 4)

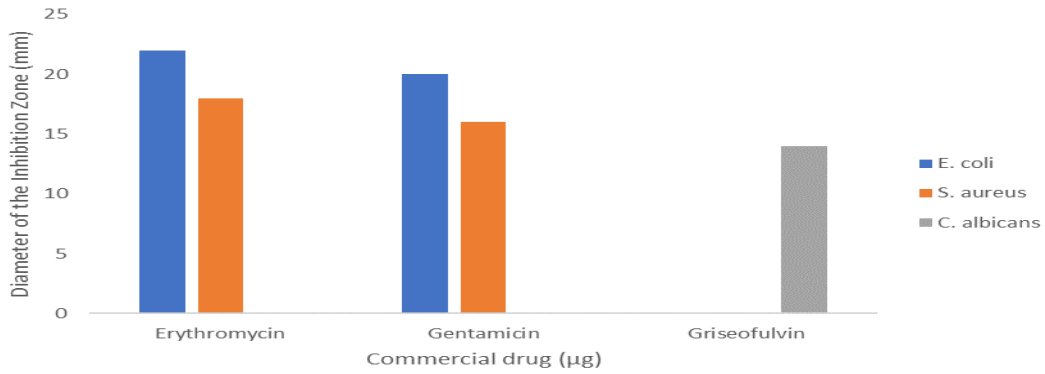

Figure 4: Antimicrobial effects of commercial drugs against the selected pathogens

The proximate composition values ranged between the non-fermented and fermented samples. While the ash, fat, and protein levels increased with fermentation, the levels of moisture, carbohydrate, and fiber decreased (Table 2).

Table 2: Nutritional components of the non-fermented and fermented samples of *V. vinifera* powder

Component	Non-fermented	Fermented
Moisture content (%)	82.41 ± 0.21	70.21±0.23
Ash (%)	3.86 ± 1.03	6.26 ± 0.83
Fat (%)	9.24 ± 0.01	12.21 ± 0.32
Carbohydrate (%)	78.59 ± 0.42	71.32 ± 1.02
Fiber (%)	1.33 ± 0.28	0.43 ± 0.22
Protein (%)	6.98 ± 0.59	9.78 ± 1.03

All values are presented in % and represent mean ± standard deviation of triplicate

In addition to the five (5) components (alkaloids, anthraquinones, phlobatannins, saponins, steroids, and terpenoids) present in the non-fermented sample, flavonoids, and tannins were present in the fermented sample (Table 3)

Table 3: Phytochemical constituents in the non-fermented and fermented samples of *V. vinifera* powder

Phytochemical components	Non-fermented	Fermented
Alkaloids	+	+
Anthraquinones	+	+
Flavonoids	-	+
Glycosides	-	-
Phlobatannins	+	+
Saponins	+	+
Steroids	-	-
Tannins	-	+
Terpenoids	+	+

+ = Present - = Absent

The HPLC components identified in the fermented sample of *V. vinifera* in order of peak heights are gallic acid, quercetin, catechin, quercitrin and epicatechin (Figure 5).

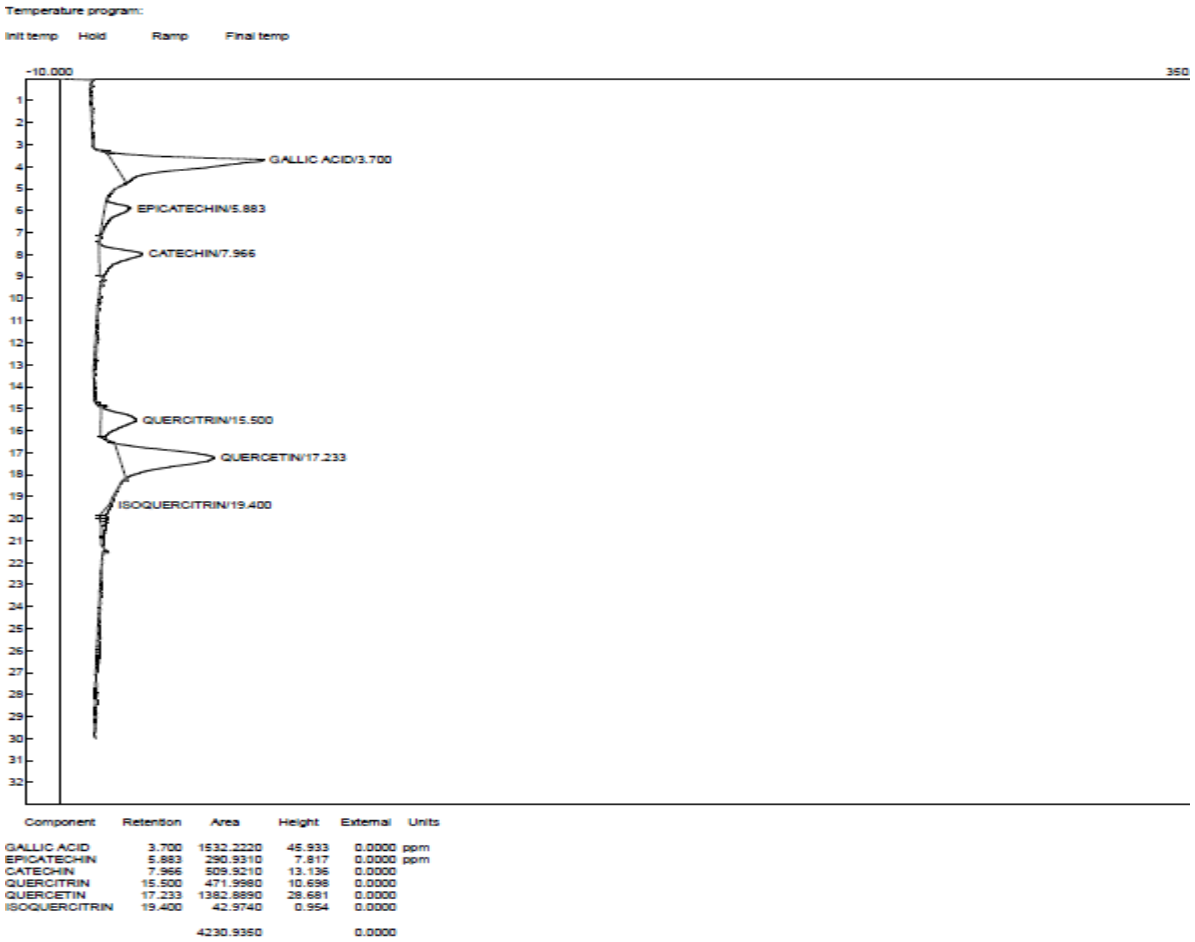

Figure 5: HPLC chromatogram of the components of the fermented sample of *V. vinifera* powder

Discussion

Interest in research on medicinal plants has continued to grow rapidly due to the successful use of active components of the plants in alternative medicines for the treatment of numerous ailments (Abdulrahman and Hamad, 2021). However, despite the acceptance of the use of medicinal plants in medicine, microbial resistance due to the emerging and reemerging of new diseases has compromised the successful application of medicinal plants in the treatment of ailments (Kayfi and Abdulrahman, 2021).

Fermentation of foods, vegetables, and other products is a microbial and enzyme-aided process that goes beyond food preservation and nutritional improvement (Rolle and Satin, 2002; Di Cagno *et al*., 2013), it also confers health-promoting properties such as better immunity, reduced risk of various diseases, and better gastrointestinal functions (Swain *et al*., 2014).

In this study, the better activity recorded for the fermented sample than for the non-fermented sample may be attributed to the reduced pH of the fermented sample by the organic acids and alcohol imparted by the associated fermenting organisms. Organic acids and alcohol are linked to the development of flavor, reduced toxicity, and enhanced biological activity of fermented foods (FAO and Azam-Ali, 1998; Rolle and Satin, 2002). A pH below 4.7, as observed in this study, does not support the growth of spoilage organisms (Nigatu and Abegaz, 1994) thus, it may mean that the fermented sample of *V. vinifera* seed powder has antimicrobial activities through the induction of damage to the cell membrane of the tested pathogen (Sánchez *et al*., 2010).

Several biological activities have been attributed to different parts of *V. vinifera* (Jeong *et al.,* 2010; Shrestha *et al*., 2012). The anti-bacterial and other medicinal values of *V. vinifera* have been reported by Kim *et al*. (2012). This is corroborated by our findings in the MIC of this study where the lowest concentration of the samples inhibited the growth of the test pathogen. The susceptibility of *S. aureus, E. coli*, and *C. albicans* to fermented and non-fermented fruits of *V. vinifera* agrees with the activity of wine extract against the same set of organisms (Papadopoulou *et al*., 2005). In comparison with the non-fermented sample, the higher activity of the fermented

V. vinifera may be attributed to the antimicrobial contents such as bacteriocin produced by the associated fermenting organisms and the polyphenolic contents of *V. vinifera* as reported by Iacopini *et al.* (2008). However, the higher activity observed for non-fermented samples against *E. coli* at all tested concentrations corroborates the report of Vaquero *et al*. (2007) which mentioned the inhibitory activity of grape wine against *E. coli*.

Compared to our findings, Santos *et al*. (2011) previously reported the proximate composition values of different varieties of *V. vinifera*. The increase in the proximate composition (ash, fat, protein, and carbohydrate) due to fermentation during our study may mean that the fermenting organisms promote polymer buildup and have improved the quality of protein by synthesizing more amino acids. Fermentation improves the nutritional properties of cereals (Mattila-Sandholm, 1998) and improves the synthesis of nutrients (Blandino *et al.,* 2003). Obadina *et al*. (2013) linked the increase of crude protein during fermentation to anabolic processes leading to polymer build-up or due to microbial cell proliferation. In this study, the decrease in moisture value with fermentation may signify a positive fermentation and a better shelf life of the fermented *V. vinifera*, since moisture is a factor that enhances the growth of spoilage organisms (Ashenafi, 2006; Zewdie *et al*., 2008). Andeta *et al*. (2018) has also reported a decrease in moisture content with increased fermentation time. The reduction in the level of fiber after fermentation may be linked to its use by the fermenting organisms (Xu *et al.,* 2020). The increase in protein level in our study also concurs with the report of Asensio-Grau *et al*. (2020) who reported an increase in the protein level of fermented lentils. Fagbohun *et al*. (2012) reported that ash-content plants are indicators of the presence of mineral elements in them. Hence, the higher content of ash recorded in the fermented sample may be pointing to a higher mineral content than in the non-fermented sample. The higher carbohydrate content of the non-fermented sample may mean its potential to confer better activity to the body tissue and promote the optimum function of the cardiovascular and immune systems (Offor *et al*., 2014) than the fermented sample.

Numerous phytochemical compounds with beneficial effects on humans have previously been reported in *V. vinifera* including phenolic compounds, flavonoids, and proanthocyanidins (Filocamo *et al*., 2015). In the present study, alkaloids, anthraquinones, saponins, phlobatannins, and terpenoids were detected in both fermented and non-fermented samples of *V. vinifera* seed powder. Alkaloids are reported to exert numerous antibacterial effects such as through depolarization of the microbial cell wall (Doncheva *et al*., 2020; Gaber *et al*., 2020). Several biological activities such as antibacterial, antifungal, antiviral, anti-inflammatory, and anti-ulcer have been attributed to anthraquinones, terpenoids, phlobatanins, and saponins (Chwalek *et al*., 2006). Flavonoids and tannins, which were only present in the fermented sample of this study may contribute to its better antimicrobial effects as previously reported that flavonoids and tannins possess numerous biological benefits such as antioxidant, antimicrobial, and antimicrobial properties (Nagy *et al*., 2017).

The results of compounds separated by HPLC in this study (gallic acid, quercetin, catechin, and epicatechin) agree with the report of a few researchers. Grape seed extract contained procyanidin, gallic acid, epicatechin, catechin, and quercetin (Cádiz-Gurrea *et al*., 2017). Abouzeed *et al*. (2018) reported the composition of raisins by HPLC determination to include catechin, quercetin, and rutin. A previous study by Sochorova *et al.* (2020) also reported quercetin, catechin, and epicatechin as part of the HPLC-evaluated components of grapeseed extracts. Quercetin has been reported to have antioxidant properties and, hence, has the potential to reduce the risks of various diseases (Russo *et al*., 2014). Catechin has been shown to possess antioxidant properties (Pietta, 2000) and has been implicated in a reduction in the complications of ischaemic heart disease (Arts *et al*., 2001). Being a natural phenolic compound occurring in most fruits, gallic acid has been reported to have antioxidant, antimicrobial, anti-inflammatory, and other beneficial properties. Nohynek *et al.* (2006) ascribed the potential of outer-membrane disintegration of Gram-negative bacteria to gallic acid.

Conclusion

In our study, the decrease in pH during fermentation indicated a rise in acidity which deters spoilage organisms from surviving in such an environment, hence, better safety of the fermented sample of *Vitis vinifera.* This study also revealed that *V. vinifera* possesses antimicrobial properties which may be attributed to the associated phytochemical constituents that were detected during screening. Better antimicrobial activities exerted by the fermented sample may be attributed to its higher number of phytoconstituents and other active compounds detected by HPLC with proven antimicrobial properties. Thus, our study proves that fermentation improves the nutritional, antimicrobial, and composition of *V. vinifera*, making it a promising antimicrobial agent.

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