IPS Journal of Applied Microbiology and Biotechnology
IPS J Appl Microbiol Biotech, 4(1): 115-122 (2025)
DOI: https://doi.org/10.54117/ijamb.v4i1.48

Exploring the Phytochemical and Antimicrobial Properties of Fruit Vinegar: A Study on *Phoenix dactylifera* and *Malus sylvestris*

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Abstract

The increasing prevalence of antimicrobial resistance have sparked interest in exploring natural remedies. Vinegar is traditionally consumed for its potential health benefits, but the phytochemical composition and antimicrobial efficacy of vinegar produced from *Phoenix dactylifera* (date) and *Malus sylvestris* (apple) fruits require further investigation. This study investigated the phytochemical composition of *Phoenix dactylifera* (PD) and *Malus sylvestris* (MS) fruit vinegars, exploring their potential as natural antimicrobial agents. Yeast and acetic acid bacterium were isolated from spoilt fruits and characterized using appropriate microbiological techniques. Vinegar production was carried out using submerged fermentation of must extracted from PD and MS fruits. The physicochemical properties, nutrient and phytochemical constituents were determined using gravimetric and instrumentation techniques. *In vitro* and *in vivo* techniques were employed for the antimicrobial potency. The data generated from this study were analyzed at 95% confidence level using Analysis of Variance (ANOVA), and post-hoc analysis using Turkey's test. Alkaloids, saponins, flavonoids, tannins and glycosides were detected in the vinegar produced from MS and PD using *Saccharomyces cerevisiae* strain SR 128 (CS 128) and *Acetobacter aceti* strain WI (AAWI). There were significant ($P < 0.05$) inhibition of the vinegar samples against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica* serovar Typhi and *Candida albicans*, and the activity was most against *E. coli*. Therefore, the prepared vinegar samples from MS and PD possessed significant phytochemical and antimicrobial properties, and the sample produccd from PD was slightly better than the sample produced from MS.

Keywords: *Vinegar, Phytochemical, Antimicrobial, Saccharomyces, Acetobacter, Staphylococcus, Escherichia, Candida*

Article History
Received: 20 Apr 2025
Accepted: 08 May 2025
Published: 15 May 2025

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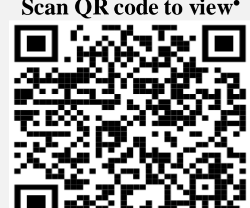
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How to cite this paper: Iheukwumere, I. H., Ajeh, J. C., Iheukwumere, C. M., Ike, V. E., Obianom, A. O., Ihenatuoha, U. A. ., Igboanugo, E. U., Onwuasoanya, U. F., Okereke, F. O., Nnadozie, C. H., Agbaugo, C. F., Nwike, M. I., Nwakoby, N. E., & Ilechukwu, C. C. (2025). Exploring the Phytochemical and Antimicrobial Properties of Fruit Vinegar: A Study on Phoenix Dactylifera and Malus Sylvestris. *IPS Journal of Applied Microbiology and Biotechnology*, *4*(1), 115–122. https://doi.org/10.54117/ijamb.v4i1.48

Introduction

Phoenix dactylifera (Date palm) belongs to the Arecaceae family, and traditionally, it is recognized as a valuable beneficial plant. The genus Phoenix is composed of 14 species, including *P. dactylifera*, and has been cultivated in the Middle East for 6000 years. Later, it was discovered in Bangladesh

and India, where it is also known as date sugar palm, and silver date palm. Fruits of *P. sylvestris* are considered beneficial, and they are used for their medicinal properties against hyperthermia, nervous debility, back pain, stomachache, toothache, headache, and arthritis (Hafzan *et al*., 2017).

Similarly, *Malus sylvestris (*Apple species) belonging to the genus *Malus* of the Rosaceae family, and thousands of cultivars are grown all around the world. This is in fact one of the most important economic fruit species according to the Food Agriculture Organization (FAO). The last available statistics from FAO are for the year 2018 and they reported that at worldwide level, the area for apple cultivation was 4,904 ha with a total production of 86,142 tones representing a trade value of around 8 billion US Dollars (Cusano *et al.,* 2020). Apples are extensively consumed in all countries around the world, being very popular because of their appreciated taste, juiciness, color, texture and nutritional contribution as reported by Matthew *et al*. (2019). Additionally, they have a good preservation capacity, they are available year-round in markets at relatively low prices, and they are seen as a healthy food (Hidalgo *et al*., 2010).

Research had revealed that *Phoenix dactylifera* and *Malus sylvestris* could be optimized in biological production of juices as well as vinegar (Matthew *et al*., 2019). Biological production of vinegar involves agricultural-based fruits as source of fermentable sugar and microorganisms such as *Saccharomyces cerevisiae*, acetic acid bacteria such as *Acetobacter aceti* (Matthew *et al.,* 2019). Some of the substrates that are mostly optimized in vinegar production are orange, date, apple, pineapple, mango etc. The sugar molecules in the substrates undergo alcoholic fermentation to produce ethanol (Matthew *et al*., 2019).

It is worthy to note that vinegar produced using natural sources such as *Phoenix dactylifera and Malus sylvestris* exhibit high profile characteristics, due to the presence of bioactive compounds (Guedes *et al.,* 2014). Apples and dates are recognized as providing a high amount of bioactive compounds with health promoting benefits. They are major dietary sources of flavonoids being particularly rich in the flavonol quercetin and its derivatives, which are bioactive compounds object of several studies that confirm their antioxidant (Duarte *et al.,* 2010; Guedes *et al.,* 2014), anti-inflammatory and antimicrobial properties, as well as antidepressive and anticarcinogenic effects.

Research had shown that vinegars produced using natural substrates are capable of exhibiting antimicrobial properties (Jia *et al*., 2012). These potentials have been attributed to the presence of phytochemical compounds such as flavonoids, tannins, saponins etc. and organic acids such as acetic acid, lactic acid etc. These bioactive compounds interfere with the metabolic processes in pathogens, thereby inhibiting their proliferations (Jia *et al*., 2012).

Several studies are available on vinegar production using natural substrates such as Duarte *et al.* (2010), Jia *et al*. (2012), Guedes *et al.* (2014), and Siddeeg *et al*. (2016) but few studies are available on the phytochemical and antimicrobial properties of vinegar produced using *Phoenix dactylifera and Malus sylvestris.* Hence, this study aims to explore the phytochemical and antimicrobial properties of vinegar produced using *Phoenix dactylifera and Malus sylvestris*

MATERIALS AND METHODS

Isolation and Characterization of Saccharomyces Species from Spoilt Fruit Samples

Sample collection

This was done following the method described in a study published by Iheukwumere *et al*. (2025a). Spoilt *Musa paradisicum* (Banana) and *Citrus aurantium* (orange) fruits were collected from different points in Nkwo Oba market, Idemili South LGA, Anambra State. The fruits were detected through sight and nasal perception, followed by carefully and selectively picking the detected fruits into polyethene bags. The polythene bags were appropriately labelled and transported immediately to the laboratory for further analysis.

Sample preparation

The fruit samples were thoroughly washed using distilled water and their ectocarps were appropriately peeled using stainless chicken knife. The peeled fruits were pulverized using electric blender (SMX425/Japan). This was serially diluted (1:10) using 250 mL conical flask (Pyrex) in the capacity of 10 g of the fruit sample to make up 200 mL of the sample solution. The solution was thorough shaked, stoppered and kept for further analysis.

Isolation of yeast

This was done following the method described in Iheukwumere *et al*. (2022) and Iheukwumere *et al*. (2020). The Sabouraud Dextrose Agar (SDA) and Yeast Extract Agar (YEA) were prepared according to the manufacturer's direction. The prepared media were autoclaved at standard conditions (121◦C 15PSI at 15 min). The media were aseptically poured in Petri dishes and allowed to solidify. An aliquot of 0.1 mL of the prepared sample was aseptically spread on the surfaces of the agar poured plates and incubated at an inverted position at 35±2◦C for 24 hours.

Characterization of the yeast

The yeast isolate was characterized morphologically, biochemically, and molecularly using the method described in Cheesbrough (2010) and Iheukwumere *et al*. (2020).The yeast isolate was physically examined; the colour, the shape, texture, elevation and the consistency were examined and recorded.

Isolation of Acetic Acid Bacterium from Spoilt Fruit Samples

This was carried out using a method described in Okpalla *et al*. (2012). Glucose-Yeast Extract Calcium Carbonate (GYC) agar prepared from glucose (10%), CaCO3 (2%) and agar (1.5%). The re-constituted medium was autoclaved at standard conditions (121◦C, 15 PSI at 115 min). The medium was aseptically distributed into different Petri dishes and allowed to solidify. An aliquot of 0.1 mL of the prepared sample from the spoilt fruits was aseptically spread on the surfaces of the prepared agar medium and these were incubated on the

inverted position at room temperature (30±2◦C) for 48 h. Colonies with large clear zones around them were subcultured.

Characterization of the Bacterial Isolate

The pure isolates will be characterized using the morphological, biochemical and molecular characteristics as described by Iheukwumere *et al.* (2018). The cultural descriptions (size, appearance, edge, elevation, colour) of the isolates will be carried out as described in Iheukwumere *et al*. (2018a). The Gram staining technique which revealed the Gram reaction, cell morphology and cell arrangement will also be carried out using the procedure described by Cheesbrough (2010), and Iheukwumere *et al*. (2018b). The presence or absence of a capsule will also be carried out as described by Iheukwumere *et al*. (2018c). The presence or absence of flagellum will be determined by carrying out a motility test as described by Cheesbrough (2010). The capability of the isolates to produce catalase, indole, oxidase, acetoin, grow in 6.55 % NaCl and utilize sugars, sugar alcohols and other substances (ribose, sorbitol, arabinose, saccharose, glucose trehalose, lactose, starch, inulin, salicin, hiparate) and also the haemolytic activity of the isolates were done using the methods described by Cheesbrough (2010) and Iheukwumere *et al*. (2018d). The molecular characterization involved DNA extraction, authentication, amplification and sequencing of the amplicons (Iheukwumere *et al*., 2018a)

Vinegar Production

Collection and preparation of fruit samples for production of vinegar

Phoenix dactylifera (commonly known as Date) and *Malus sylvestris* (commonly known as English Apple) fruits were bought from Eke Awka Market, Anambra State. The fruit samples were thoroughly washed using distilled water and their ectocarps were thoroughly peeled. These were separately pulverized using electric blender (SMX 425/Japan). The pulverized fruits were extracted using distilled water. The solutions were then filtered using muslin cloth.

Production of alcohol

Here, 400 mL of the fruit extract was dispensed each into 500 mL conical flask (Pyrex). The extracts were sterilized using an Autoclave at standard conditions (121◦C, 15 PSI at 115 min). The sterilized extracts were allowed to cool. The extracts were each inoculated *Saccharomyces cerevisiae* strain and allowed for 28 days with manually daily shaking at 30±2◦C. After the fermentation, the alcohol was decanted and poured into sterile 2000 mL bottle and allowed open for 2 days.

Alcohol tolerance test

The ability of the acetic acid bacterium to grow in the presence of alcohol was carried out using the method described in the study published by Tharinee *et al*. (2015). The tested isolate was grown in yeast extract agar (0.50% yeast extract, 2% agar) supplemented with 2%, 4%, 6%, 8%, and 10% (v/v) absolute ethanol. The above procedure was then modified by growing the isolate in Glucose-Yeast Extract Calcium Carbonate (GYC) broth/agar supplemented with 2%, 4%, 6%, 8%, and 10% (v/v) absolute ethanol.

Vinegar production

The colonies of *Acetobacter aceti* strain was aseptically transferred into the container containing the alcohol. The bottles were thereafter covered with sack cloth to prevent the entry of insect. The set-up was allowed for 28 days at room temperature (30±2◦C.). At the end of the fermentation period, a thick film known as mother of vinegar had covered the surface of the vinegar and was carefully scooped out to avoid contamination. The vinegar was thereafter filtered.

Phytochemical Analysis of the Vinegar Samples

The phytochemical components (alkaloids, glycosides, flavonoids, phenolics, tannins, steroids and saponins) of the samples were determined quantitatively using the methods described in the study published by Iheukwumere *et al.* (2017a)

Alkaloids: Five milliliters of the sample was mixed with 96% ethanol and 20% tetraoxosulphate (VI) acid (1:1). One milliliter of the filtrate from the mixture was added to 5 ml of 60% tertraoxosulphate (VI) acid and allowed to stand for 5 minutes. Then 5 ml of 0.5% formaldehyde was added and allowed to stand for 3 h. The reading was taken at absorbance of 550nm.

Glycosides: This was carried out using Buljet's reagent. One millimeter of the sample was added in 9 ml of 70% alcohol for 2 h and then filtered with Whatman No. 1 filter paper. The extract was then purified using lead acetate solution and disodium hydrogen tetraoxosulphate (VI) solution before the addition of freshly prepared Buljet's reagent. The absorbance was taken at of 550nm.

Flavonoids: Five milliliters of the sample was mixed with 5 ml of dilute hydrochloric acid and boiled for 30 minutes. The boiled mixture was allowed to cool and then filtered with Whatman No. 1 filter paper. One milliliter of the filtrate was added to 5 ml of ethyl acetate and 5 ml of 1% ammonia solution. The absorbance was taken at 420nm.

Phenolics: Ten milliliters of the sample was boiled with 50 ml acetone for 15 minutes. Five milliliters of the solution was pipetted into 50 ml flask. The 10 ml of distilled water was added. This was followed by addition of 2 M ammonium hydroxide solution and 5 ml of concentrated amyl alcohol solution. The mixture was left for 30 minutes and absorbance was taken at 550nm.

Tannins: Ten milliliters was pipetted into 50 ml plastic containing 50 ml of distilled water. This was mixed for 1 h on a sterile mechanical shaker. The solution was filtered with Whatman No. 1 filter paper, and 5 ml of the filtrate was mixed with 2 ml of iron (III) chloride solution in 0.1 N hydrochloric acid. The absorbance was taken at 550nm.

Steroids: The sample was mixed with normal ammonium hydroxide solution. Two milliliters of eluate was mixed with 2 ml of chloroform in a test tube. Three milliliters of ice cold acetic anhydride was added to the mixture and allowed to cool. The absorbance was taken at 420nm.

Saponins: Five milliliters of the sample was dissolved in aqueous methanol. The 0.25 ml of aliquot was taken for spectrophotometric determination for total saponins at 544nm.

Antimicrobial Activity of the Vinegar Samples

Collection and preparation of test organisms: The test organisms used for this study were *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica* serovar Typhi and *Candida albican*. The test organisms were collected from ZAHARM Analytical and Research Laboratory, Awka, Anambra State. Catalase test, indole test, citrate/hydrogen sulphide production test and germ tube test were respectively carried out to confirm the identities of the test organisms. The pure isolates were scrapped in normal saline solution, subsequently diluted to match the turbidity of 0.5 MacFarland matching standard that was prepared by mixing 0.5 mL of 1.175 % $BaCl.2H_2O$ and 9.95 mL of 1 % of concentrated H_2SO4 to form 1.50×10^8 cells/mL

Agar-welled diffusion technique: This was carried out using the method described by Iheukwumere *et al.* (2017b). Each labeled plate was uniformly inoculated with the test organism using pour plate method. A sterile cork borer of 5 mm diameter was used to make the wells on the medium. One tenth milliliter of test sample was dropped into each labeled wells and then incubated at 37±2˚C for 24 h. Antimicrobial activity was determined by measuring the diameter of the zones of inhibition (mm) produced after incubation.

Statistical Analysis

The data generated from this study were analyzed at 95% confidence level using Analysis of Variance (ANOVA), and post-hoc analysis using Turkey's test (Iheukwumere *et al*., 2022a).

RESULTS

Characterization of the Yeast Isolate and Acetic Acid Bacteria Strains

The yeast isolate (XI) showed characteristic features of yeast such as cream white colonies on Sabouraud Dextrose Agar (SDA) plate, smooth surface, spherical morphology and utilization of glucose and sucrose. The yeast was also resistant to cycloheximides as shown in Table 1. The acetic acid bacterium (AI) showed cream to yellow colonies on glucose yeast extract calcium carbonate agar (GYA). The isolate was also Gram-negative rod, motile, catalase, methyl red and Voges Prokauer positive, but indole, oxidase and citrate negative as shown in Table 2. The quality and nature of the extracted nucleic acid revealed 260/280. Hence, Deoxyribonucleic acid (DNA) as shown in Table 3. The molecular identities of the isolates revealed 100% query cover and 100% identities. This revealed that sample 1D AI was *Acetobacter aceti* strain WI (AAWI) whereas sample ID XI was *Saccharomyces cerevisae* strain Ysr128 (SC 128) as shown in Table 4

Alcohol Tolerance Potential of the Test Isolate

The study revealed that the test isolate was able to grow in the presence of 10% absolute alcohol. There was significant ($P < 0.05$) number of colonies of acetic acid bacteria in 10% absolute alcohol level in both yeast extract agar (YEA) and glucose-Yeast extract calcium carbonate agar (GYA). The number of colonies slightly decreased as the concentration of alcohol increased as shown in Table 5 but the decrease was statistically non-significant ($P > 0.05$).

Phytochemical Constituents of the Vinegars

The study revealed the presence of alkaloids, saponins, flavonoids, tannins, and glycosides as shown in Table 6. Alkaloids and saponins were non-significantly ($P> 0.05$) detected most in sample VA. Tannin was significantly ($P > 0.0$) detected most in VA. Flavonoids was non-significantly (P. 0.05) detected most in VD whereas glycosides was detected most in VD.

Antimicrobial Activities of the Vinegar Samples

The study revealed significant activities of the vinegar samples against *S. aureus, E. coli, S. enterica* ser. Typhi and *C. albicans* as shown in Table 7. The study showed that sample VD showed the highest zones of inhibition when compared to samples VA and VS but the variations in their zones of inhibition were statically non-significant ($P > 0.05$). The zones of inhibition of the vinegar samples against *S. aureus*, *E. coli*, and *S.* ser. Typhi were significantly ($P < 0.05$) lower than that of ciprofloxacin (standard antibiotic) as shown in Table 7. Ketoconazole (standard antifungal agent) was able to inhibit only *C. albicans*, and the zone of inhibition is significantly (P < 0.05) higher than the zones of inhibitions recorded by the vinegar samples against *C. albicans*.

Table 1: Morphological and biochemical characteristics of the yeast isolates

Parameter	X1	X2
Appearance on GYA	Cream white colonies	Cream white colonies
Surface	Smooth	Smooth
Margin	Circular	Circular
Elevation	Convex	Convex
Shape	Spherical	Spherical
Bud	Present	Present
Ascospore	Present	Present
Glucose	+	+
Sucrose	+	+
Maltose	+	+
Gelactose	+	+
Raffinose	+	+
Mannitol	_	_
Lactose	_	_
Xylose	_	_
Cyclohexide	Resistance	Resistance
Suspected yeast	Saccharomyces cerevisae	Saccharomyces cerevisae

Table 2: Morphological and biochemical characteristics of the acetic acid bacterium

Parameter	A1	A2
Appearance on GYA	Cream to yellow colour	Cream to yellow colour
Surface	Smooth	Smooth
Elevation	Convex	Convex
Opacity	Opaque	Opaque
Shape	Rod	Rod
Arrangement	Clustered	Clustered
Gram Reaction	_	_
Motility	+	+
Indole	_	_
Citrate	_	_
Catalase	+	+
Methyl red	+	+
Voges Proskauer	+	+
Oxidase	—	—
Glucose	+	+
Sucrose	+	+
Mannitol	+	+
Bacterium	*Acetobacter* species	*Acetobacter* species

Table 3: Quality and nature of the extracted nucleic acid

Sample ID	Nucleic acid(µg/mL)	260 nm	280 nm	260/280
A1	120.20	3.412	1.875	1.82
X1	102.10	3.104	1.687	1.84

Table 4: Molecular identities of the isolates

Parameter	A1	X1
Max Score	2676	6205
Total Score	2676	6604
Query Cover (%)	100	100
E-Value	0.0	0.0
Identity (%)	100	100
Accession Length	1449	224595
Accession Number	1ICC662508.1	CP036471.1
Description	*Acetobacter aceti* strain W2 (AAW1) 16S rRNA gene partial sequence	*Saccharomyces cerevisiae* strain Ysr128 (SC128) chromosome 1, complement sequence

Table 5: Alcohol tolerance of the test isolate

Alcoholic Content (%)	Yeast Extract Agar		Glucose-Yeast Extract Calcium Carbonate	
	Count (CFU/mL)	Log CFU/mL	Count (CFU/m)L	Log CFU/mL
2.0	$5.10X10^2$	2.71	$6.40X10^2$	2.81
4.0	$4.70X10^2$	2.67	$6.10X10^2$	2.79
6.0	$4.30X10^2$	2.63	$5.70X10^2$	2.76
8.0	$4.10X10^2$	2.61	$5.40X10^2$	2.73
10.0	$3.80X10^2$	2.58	$5.10X10^2$	2.71

Table 6: Phytochemical constituents of the vinegars

Parameter	VA (%)	VD (%)	VS (%)
Alkaloids	0.29±0.01	0.17±0.01	0.11±0.00
Saponins	2.11±0.03	1.93±0.04	1.82±0.13
Flavonoids	1.74±0.11	2.19±0.31	2.04±0.07
Tannins	3.05±0.17	1.86±0.11	2.23±0.13
Glycosides	1.04±0.01	2.14±0.14	2.04±0.01
Steroids	0.00±0.00	0.00±0.00	0.00±0.00

Table 7: Antimicrobial activities of the samples

Test organism	Diameter Zones of INHIBITION (ẋ±SD) mm				
	VA	VD	VS	CPX	KET
S. aureus	6.70±0.14	7.40±0.11	6.90±0.21	14.10±0.33	0.00±0.00
E. coli	7.80±0.42	8.50±0.00	8.40±0.21	28.50±0.11	0.00±0.00
S. ser. Typhi	7.10±0.81	7.90±0.14	7.90±0.11	19.50±0.14	0.00±0.00
C. albicans	6.80±0.11	7.30±0.58	7.10±0.22	0.00±0.00	17.10±0.00

CPX= Ciprofloxacin; KET= Ketoconazole

DISCUSSION

The presence of *Saccharomyces cerevisiae* strain Ysr128 (SC 128) from the spoilt banana samples corroborated with the findings of Jayamma *et al*. (2020). The characteristic features of the yeast isolate such as cream white appearance on Sabouraud Dextrose Agar (SDA), resistant to cycloheximide, utilization of sugars were also reported by many researchers (Amanul *et al.,* 2017; Kechkar *et al*., 2019; Kumari *et al*., 2019; Jayamma *et al.,* 2022; Petruzziello *et al*., 2023). The presence of *Acetobacter aceti* strain w1 (AAWI) in banana juice supported the findings of Srivastava and Rani (2019) and Wang *et al*. (2022). The characteristic features of *Acetobacter* revealed in this study corroborated with the findings of Afrifuzzaman *et al.* (2018), Quattara *et al*. (2018), Srivastara and Rani (2019) and Wang *et al*. (2022).

Vinegar is a liquid fermented product that contains fruits juice as the main ingredients which contain many functional compounds such as organic acids, vitamins, minerals, amino acids and phytochemicals such as phenolics, flavonoids, tannins and other phytochemicals. A similar report was made by Hamidatun (2014). In the present study, the production of vinegar from Malus sylvestris (green apple) and Phoenix dactylifera (date) agrees with the findings of Kechka *et al.* (2019). Other researchers (Quattera *et al*., 2018; Srivastava and Rani, 2019; Wang *et al.,* 2022; Safrida *et al*., 2023) produce vinegar from various plants. The maximum acetic acid using green apples agrees with water Klawplyapamornkun *et al*. (2015) and Quattara *et al*. (2018) produced from fruits and mango juice, respectively. Vinegar produced from date fruits gave 5.2% of acetic acid and this was more than acetic acid produced from many other fruits.

The presence of alkaloids, saponins, saponins, flavonoids, tannins and glycosides in the vinegar samples supported the findings of Hamidatun (2014), Zubaidah (2015), Yuliant *et al.* (2019) reported that the combination of acetic acid in the vinegar, flavonoids and other antioxidants prevent oxidative caused by free radicals. Yuliant *et al*. (2019) also reported that the antioxidant potentials of flavonoids, tannins and phenolics prevent cell and organ damage due to oxidative stress. The antioxidant activities of flavonoids were also reported by Han *et al*. (2016). Safrida *et al*. (2023) stated that saponin has many biological activities such as medicinal properties and antimicrobial activities.

The inhibitory activities of the vinegar samples against *S. aureus, E. coli, Salmonella enterica* serovar Typhi and *Candida albicans* could be attributed to the activities of the phytochemical constituents/bioactive substances in the vinegar samples. Similar conclusion was drawn by many researchers (Senguen *et al*., 2020; Singh and Garg, 2022). The slight increase in the inhibitory activities from *P. dactylifera* fruits could be attributed to the potency of the phytochemicals and the high acetic acid content of the vinegar samples. The highest activity of the vinegar samples against *E. coli* could be attributed to the nature of the organism, majorly the nature of the bacterial cell wall.

CONCLUSION

The study has shown that vinegar samples produced from *Malu sylvestris* (MS/Apple) and *Phoenix dactylifera* (PD/Date) fruits possessed significant phytochemical and antimicrobial properties, and the sample produccd from PD was slightly better than the sample produced from MS, mostly against *Escherichia coli*

Acknowledgments

We are grateful to all our study participants who join the study voluntarily. We are grateful to ZAHARM Analytical and Research Laboratory, Amawbia, Awka Anambra State, Nigeria for providing enabling environment, resources and techniques for this study. We really salute their wonderful efforts.

Conflict of interests: The authors declare that they have no conflict of interests.

Funding: This research did not receive specific grant from any funding agencies.

Ethical approval: Not applicable

Authors Contributions: All contributed towards the study design, experiment execution, data analysis, and manuscript drafting.

Availability of Data and Materials: All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request.

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