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Evaluation of Anticancer, Antioxidant, Antimicrobial and Antiviral Activities of Microbially Induced Novel Flavors and their Application in Carbonated Beverages

Abeer M. Abd-Alrahman [1], Rabab H. Salem [2], Manal M. Ramadan [2], and Fatma M. Saleh [1]

[1] *Food Science and Technology Dept., Faculty of Home Economics, Al-Azhar University, Tanta, Egypt*
[2] *Chemistry of Flavor and Aroma Dept., Food Industry and Nutrition, National Research Center, Giza, Egypt*

THE ABILITY of *Bacillus subtilis* to produce flavor from microbial fermentation of sugar beet pulp was evaluated and the effect of encapsulation on the quality of the flavor produced was investigated and add it to a flavored soft drink. The flavor was subjected to cytotoxicity testing and the antioxidant, anticancer, antiviral and antimicrobial properties of extract from sugar beet pulp supplemented with a mixture of L-Lysine and Yeast extract fermented by Bacillus subtilis NRCR22 using in vitro methods. Gas chromatography–mass spectrometry analysis of sugar beet pulp fermented by *B. subtilis* nrch123 and Bacillus subtilis NRCR22 identified 30 compounds with 2,6-ditert-butyl-4-methylphenol (97.88%) and 8 compounds with limonene (99.74%) as main constituents, respectively. The antioxidant activity of BLY2 extract was 66.02% at a concentration of 100µg/ml. The concentration of extract required to inhibit 50% cell viability when assessed against human liver cancer cell line HepG2 and the cytotoxicity were 0.20 µg/mL and 3.47 µg/ml respectively, indicating strong anti-cancer activity and at the same time safe on normal cells. The soft drink flavored with the natural, microbially produced flavor was more sensory palatable than the artificially flavored soft drink.

Keywords: De novo processes, agro- wastes, nanoliposomes, limonene, cytotoxicity, zeta potentials, soft drink.

1. Introduction

The chemical sensations produced by the molecules released during food consumption are known as flavor and they are the most significant aspect of food. Flavor is the complex sensation includes taste, smell, roughness or smoothness, hotness or coldness and pungency. Odor and taste are major components of flavor and mainly responsible for its discrimination (Fisher & Scott, 1997 and Shepherd, 2011). The flavor of food can be changed in using natural or artificial flavorings (Dubal et al. 2008). Artificial flavorings do not exist in nature and manufactured by chemical means (Ulloa, 2018). Natural flavors tend to come from natural raw materials and don't have any artificial constituents. Included in this group are flavoring ingredients produced from spices, fruit juices, vegetable juices, edible yeast, herbs, bud, bark, roots, leaves, or other similar plant materials, as well as meat, seafood, poultry, eggs, and dairy products (US Food and Drug Administration, 1990 and Rietjens et al. 2023).

Artificial flavors resulted in the production of environmentally unfriendly processes. It also resulted in the formation of unwanted products, reduced process productivity and increased final costs and health risks (Cheetham, 1997 and Bala et al. 2023). Natural flavor compounds have positive results in avoiding a variety of diseases, including cancer and neurological conditions like Alzheimer's. The presence of phenolic compounds, which have properties like free radical scavenging, anti-inflammatory, antiviral, and antimicrobial, is responsible for these effects. These compounds not only demonstrate a preventive mechanism against diseases but also stabilize the food product by having an antimicrobial effect (Kaur et al. 2019 and Akbari

*Corresponding author e-mail: beery.mahmoud22@gmail.com
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et al. 2022).

Research and creation of novel natural flavors plays a key role in modern society (Queiroz et al. 2022 and Sukumaran et al. 2023). Natural flavor agents (NFA) are produced through extraction from natural materials and biosynthetic pathway dependent on the use of microbial cells or enzymes in de novo microbial activities (fermentation) or bioconversions of natural precursors (Gupta et al. 2015). NFA produced by fermentation in two ways (a) submerged fermentation (SmF) and (b) solid state fermentation (SSF). SSF has attracted interest as a method of creating flavors and other substances important to the food industry. SSF has many benefits over submerged fermentation, including increased yields, decreased energy needs, efficient utilization, and the addition of value to wastes (El-Nahrawy et al. 2017, Cerda et al. 2019 and Elsakhawy et al. 2022). Agricultural residues are rich in bioactive compounds and used as the raw material in various industries (Sadh et al. 2018 and Bala et al. 2023). Agro-wastes have concentrated on biotechnological flavor synthesis through microbial fermentation since they contain a large number of reusable components for microorganisms (Laufenberg et al. 2003 and Astudillo et al. 2023).

Sugar beet pulp is a by-product from the sugar refining industry and use of that can be enhanced to produce bio and sustainable products (Kelly, 1983). Pulp has the ability to be converted into oligosaccharides made of pectin. It was oligosaccharides completely fermented by microorganism (Grahovac & Rončević, 2014). Besides that, nutrients such as amino acids added to the fermentation media to enhance the production of volatile compounds that produced during the enzymatic degradation of the fermentation media by microorganisms (Fadel et al. 2018). Sensitive food-grade bioactive materials can be protected and released using nanoliposomes in a safe manner. They are used to encapsulate various bioactive components for the enrichment and fortification of various foods as well as the production of functional products (Zarrabi et al. 2020). There are numerous uses for encapsulated flavoring particularly in drinks (Pegg 2012; Sobel et al. 2023).

Soft drinks are among the most popular drinks produced worldwide. There is no single definition available for soft drinks, but they are generally water-based sweetened beverages with a balanced acidity about 0.3 – 0.5% of acid. Soft drinks are dividing into carbonated and non-carbonated soft drink. Carbonated soft drinks are beverages with added carbon dioxide to give an effervescent taste (Jorge, 2003 and Troiano et al. 2000).

This study aims to 1) produce novel natural flavors to be used as an alternative to harmful industrial flavors by using available agricultural waste in Eco-friendly ways. 2) application of the novel natural flavors in most food products among adults and children such as soft drink.

2. Materials and methods

Raw materials

Sugar beet pulp (65.40% carbohydrate, 9.20% protein, 7.20% ash and 0.90% fat) were purchased from Food Technology Research Institute, Agric. Res. Center, Giza, Egypt. Sugar (sucrose) was purchased from local market at Tanta city, Gharbia, Egypt.

Microorganisms

Tow strains of *Bacilli* bacterial strains belonging to *Bacillus subtilis*, namely *B. subtilis NRCH123* (B_1), *B. subtilis NRCR22* (B_2) were purchased from Microbial Chemistry Lab., National Research Centre, Dokki, Giza, Egypt.

Chemicals and Reagents

Amino acid (L-Lysine), yeast extract, diethyl ether and sodium sulphate anhydrous were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Citric acid and food lemon flavor (E330) were obtained from Food Technology Research Institute, Agric. Res. Center, Giza, Egypt. All other solvents and chemicals were of analytical grade.

Technical methods

Inoculum preparation

B. subtilis (B_1 and B_2) were cultured on nutritional agar medium in petri dishes for 4 hours at 27 °C. By soaking each petri dish contents in 5 ml of sterile distilled water, cells were retrieved according to Besson et al. (1997).

Solid state fermentation with sugar beet pulp

Five grammes of sugar beet pulp moistened to 1:2 with distilled water, 1%w/v L-lysine+ 5% w/v yeast extract and autoclaved for 15 min at 121 °C. One ml of bacterial strain (B1) was added and incubated at 30 °C for 72 h. (BLY1). Five grammes of sugar beet pulp moistened to 1:2 with distilled water, 1%w/v L-lysine+ 5% w/v yeast extract and autoclaved for 15 min at 121 °C. One ml of bacterial strain (B2) was added and incubated at 30 °C for 72 h. (BLY2).

Volatiles compounds isolation

Using a dynamic headspace technique, the volatiles in the headspace of each sample under investigation were extracted. About 20 g of each sample added in conical flask containing 250 ml distilled water. The above mixture was stirred at 60 °C using Teflon-coated magnetic bar (B48.5). At a flow rate of 100 ml/min, nitrogen gas was used to purge the samples for 3 h. The headspace volatiles were drawn into diethyl ether-filled cold traps, maintained at -10°C and drying the volatile-containment solvents on anhydrous sodium sulphate. Solvents containing volatile matter were concentrated using a rotary evaporator (Heidolph, HEID31011, Germany) under normal pressure at 40°C to a final volume of 100 µl according to Abd El-Mageed (2007). The extracts were stored at -20°C until the samples were subjected to experiments. Before the samples were used in the studies, the extracts were kept at a temperature of -20°C.

Extracts encapsulation

Preparation of Nano liposome

To ensure total dissolution and homogeneity, lecithin powder (2%) was dissolved in 100 ml of acetate buffer (250 mM, pH 3.5 0.1) and stirred overnight. Different concentrations from the BLY2 extract (0.3, 0.6, and 0.9%) were added to the lecithin solution. A magnetic stirrer (Bibby Scientific Ltd, SERIAL No. 30794, U.K) was used to emulsify the mixture for 15 minutes. The entire mixture was then homogenised for 20 min at full sonicator power (200.0 W, 24 kHz) using a UP200S ultrasound homogenizer (IKA Hielscher, Number: HIEL_17007>, Germany). To inhibit any increase in emulsion temperature, the mixed vessel was thermally isolated by a freezing water bath during the ultrasound homogenization (Charve & Reineccius, 2009 and Kausadikar et al. 2015).

Novel flavored in flavored soft drink preparation

The basic formula of flavored soft drink (FSD) consists of drinking water, sugar, citric acid, flavor, and ascorbic acid (as antioxidant agent). Initially, simple syrup was prepared from dissolved of crystalline sugar in water at 85-90 °C until dissolves. After heating, the simple syrup was filtered. It was cooled to room temperature. The following components were added: citric acid, ascorbic acid and encapsulated flavor or artificial flavor with constant stirring.

Carbonization and packaging of flavored soft drink

The carbonation process was carried out using a manual carbon dioxide gas pump (iSoda, 410-000-3A, China) at 25 °C until a gas level of 7 mg / liter. Carbon dioxide was measured according to Rice & American Public Health Association (2012). Packaged in sealed plastic containers under cooling conditions.

Analytical methods

Analysis using Gas Chromatographic-Mass Spectrometry

The analysis was carried out by using a coupled gas chromatography Hewlett-Packard model (5890) / mass spectrometry Hewlett-Packard-MS (5970) (USA). A comparison was made between the spectra of the unknown component and the spectrum of the known components kept in the NIST library (Adams, 2001).

Anticancer activity

Cell viability is assessed using the sensitive, accurate, and trustworthy colorimetric MTT test. A spectrophotometer can be used to measure the absorbance of coloured solution at a wavelength typically between 500 and 600 nm. (Cory et al., 1991). Anticancer activity was measured by cytotoxic effect of extracts to human liver cancer cells line (HepG2) (Cell culture lab, Vacsera, Egypt). Using the MTT assay, the cytotoxic effect was investigated. This is how the viability percentage was determined: (OD of treated cells / OD of untreated cells) X 100 represents the cell viability percentage (Chen et al., 2009).

Cytotoxicity assay

Cytotoxic effect of BLY2 extract to African green monkey (Vero) cells lines (American Type Culture Collection ATCC-clone CCL-81). The 50% cytotoxic concentration (IC50), which is the concentration needed to reduce cell viability to 50% of the control, was used to express the cytotoxicity of extract.

Antiviral activity

a. Propagation of test virus on Vero cells

Hepatitis A virus (HAV) (Applied Research Sector VACSERA -Egypt) was propagated on Vero cells according to the method described by Afshar (1992) and Joseph & Thomas (1994).

b. Evaluation of antiviral activity against test virus

Antiviral activity of BLY2 extract safe concentrations against Hepatitis A (HAV) virus was determined according to Petricevich & Mendonça (2003), where, after a 24-hour treatment with and without extract, the virus was titrated on cell lines. The antiviral response is indicated by the difference between the virus titers in untreated and treated cells.

c. Evaluation of antiviral activity in cells pretreated with extracts

To assess the impact of the early stages of viral replication, Vero cells were primed with BLY2 extract for 24 h before being inoculated with the virus as Steven et al., (2000). The 50 % endpoint was calculated according to Reed & Muench (1938) as follows. 50 % endpoint (CCID50) = % CPE >50 – 50 Const X log dilution. /% CPE >50 % − % CPE < 50 %)

Antioxidant activity

The BLY2 extract was prepared at the following final concentrations: 25, 50 and 100 µg/ml. Determined was the DPPH-radical scavenging activity according to the method introduced by Kumaran (2006) and Rai et al. (2006). The following equation was used to estimate the antioxidant activity:

Antioxidan activity (%) = (control – sample)/(control) × 100

Antimicrobial activity

a. Microorganisms

Two different strains of bacteria that are food-borne pathogens were tested for the inhibitory impact of BLY_2 extract. Gram positive bacteria Staphylococcus aureus and gram-negative bacteria Escherichia coli. The stock cultures were grown on nutrient agar slant at 37 °C for 24 h before being stored in the fridge until use.

Two fungal species were used for antifungal assay Aspergillums Niger and Candida Albicans. The stock cultures were cultivated on potato dextrose agar slant for five days at 25 °C, and then stored in the refrigerator until use.

b. Disc diffusion technique

The disc diffusion method by Kirby-Bauer technique was used to determine the sensitivity test of BLY_2 extract with various bacterial cultures (Marrez et al., 2019). Measurements and expressions of inhibition zones were made as the diameter of the clear zone combined with the diameter of the paper disc (Bauer et al., 1966). By measuring the zone of inhibition (mm) against the studied fungus, antifungal activity was assessed (Medeiros et al., 2011).

c. Determination of minimum inhibitory concentration (MIC)

The micro broth dilution method was used to establish the minimal inhibitory concentration for BLY2 extract. MFC against fungi was created by utilising the Marrez & Sultan technique (2016).

Encapsulation efficiency tests

a. Dynamic Light Scattering (DLS) and Zeta potential Measurements

Nano BLY2 liposome was prepared via dilution of an adequate volume of sample about 0.1 ml, which added to about 3.5 ml of distilled water. A detection angle of 173° was chosen for the size measurement unless stated otherwise. Using a Zetasizer Nano ZS Nano DLS, the hydrodynamic diameters and zeta potentials were measured.

b. Transmission electron microscope (TEM)

The suspension of the Nano BLY2 liposome was sonicated for 10 min (Crest Ultrasonics Corp., USA). Next a few drops were loaded onto a copper grid that had been coated with carbon before drying. Few drops of phosphotungestic acid (1%) were loaded on the grid, left to dry. Then, HR-TEM analysis of the grid with the sample was performed (JEOL, JEM-2100, Japan).

Sensory evaluation procedure

a. Sensory evaluation of BLY2 extract

The evaluation was conducted by a well-trained panel consisting of 20 member (ten female and ten male) drawn from National Research Center, Giza, Egypt, according to Fadel et al. (2018) with some modifications. Sensory analysis had been carried out determine the odor sensory attributes of the sample to estimate the acceptability of odor. The odor acceptability was estimated on a 5 numbers scale (1: very unacceptable, 2: unacceptable, 3: average, 4: acceptable, 5: very acceptable).

b. Sensory evaluation of FSD

Sensory analysis was performed in National Research Center, Giza, Egypt, and included the following features: appearance, carbonation, odor, taste, color and overall acceptability according to Prado et al. (2015). A group of committee members comprising of 20 trained volunteers was selected for

admission test. The panelists recorded their scores on answer cards.

Statistical analysis

Using the Statistical Package for the Social Sciences, the statistical analysis employed the analysis of variance (ANOVA) and the Duncan test (SPSS 21). The findings were displayed as mean + SD. if differences were deemed significant at (p 0.05).

Table 1. Flavored soft drink formula with different ratios of flavor agents.

Ingredient%	Component ratios (%)				
	[1]BSD	[2]ASD	[3]NSD_1	[4]NSD_2	[5]NSD_3
Water	90.00	90.00	90.00	90.00	90.00
Sugar (w/v)	9.94	9.94	9.94	9.94	9.94
Citric acid (w/v)	0.05	0.05	0.05	0.05	0.05
Ascorbic acid (w/v)	0.01	0.01	0.01	0.01	0.01
Flavor (v/v)	0	0.10	0.50	0.50	0.50

[1]BSD: Soft drink without adding flavor. [2]ASD: Soft drink with adding artificial lemon flavor. [3]NSD_1: Soft drink with adding encapsulated flavor with 0.3 rates. [4]NSD_2: Soft drink with adding encapsulated flavor with 0.6 rates. [5]NSD_3: Soft drink with adding encapsulated flavor with 0.9 rates.

3. Results and discussion

3.1. Gas Chromatographic-mass Spectrometric analysis

3.1.1. GC/ MS analysis of the BLY1 extract

Gas chromatography and mass spectrometry are two well-known analytical methods combined to form GC-MS. To separate complicated mixtures, gas chromatography employs a well-chosen column with varying chemical retention time. A variety of detectors can be added at the end of the GC column to analyses the separated chemicals (Reber, 2020).

Volatile compounds in sugar beet pulp fermented by *Bacillus subtilis nrch123* with fortification of L-Lysine + Yeast extract (BLY1) as inducer for bacteria were extraction and analysed by GC–MS as seen in Table 2. The results indicated that nearly 30 different volatile compounds identified, representing seven groups of chemical classes including eleven aliphatic compounds, three phenols, seven alcohols, three esters, one acid, four ketones and one aldehyde.

As appears in Fig 1 and Table 2, all groups are present in very small percentages except for the phenols group (97.91%) which was represented by three compounds. The major compound was 2,6-ditert-butyl-4-methylphenol by 99.97% of the phenolic compounds, followed by 2,6-bis(1,1-dimethylethyl)-4-methyl-Phenol and 2,6-bis(1,1-dimethylethyl)-4-methyl-Phenol with very small proportion of about 0.03%.

In the same Table there were seven alcoholic compounds, the percentage was 0.4% the largest compound was 1-Ethynylcyclopentanol (55% of alcoholic compounds). They were followed in the order by three compounds from the group of esters with a rate of 0.27%, including Didodecyl phthalate, Octadecanoic acid, 2-oxo-, methyl ester and Undec-10-ynoic acid, dodecyl ester.

Volatile Compounds in fermented sugar beet pulp by Saccharomyces cerevisiae were ethanol with a percentage of more than 99% and percentage of less than 1%, namely Aldehydes, Esters, alcohols and Methanol (Gumienna et al., 2014).

It is concluded from this study that 2, 6-ditert-butyl-4-methylphenol is the main volatile compound of the media containing sugar beet pulp and fortified with L-Lysine + Yeast extract that was fermented by *Bacillus subtilis nrch123* and used solid-state fermentation method. This compound is an antioxidant known to be safe for use in foods (Yehye et al., 2015). Notably, it is a phenolic compound, so the predominant odor of the BLY1 extract was a light phenolic odor.

There have been very few studies of microbial production of 2,6-ditert-butyl-4-methylphenol, therefore the lack of information about the exact configurations of the production.

Lastly, 2, 6-ditert-butyl-4-methylphenol can be used in the preservation of fats and oils which used in cosmetics, pharmaceuticals as well as food preservation and as a defoaming agent, due to its antioxidant properties, so it is preferable to instead of synthetic antioxidants.

Table 2. GC-MS analysis of sugar beet pulp fortified with lysine and yeast extract fermented by *bacillus subtilis nrch123* using solid-state fermentation.

Type	Identified compounds	[1]Rt	Area %
Aliphatic	Hydroperoxide, hexyl	9.261	0.25
compounds	1-t-Butyl-4-(adamantyl-1) benzene	36.698	0.02
	(1,1-dimethylethyl)-Cyclohexane	40.080	0.04
	3-methyl-, (E)-1,5-Heptadiene	40.709	0.03
	3-oxiranyl-7-Oxabicyclo [4.1.0]heptane	44.377	0.03
	1-Undecyne	45.338	0.05
	Trans-cyclononene Oxide	45.601	0.04
	1-Nonylcycloheptane	47.633	0.06
	10-Undecanal	48.485	0.01
	(Z)-9-Tetradecenal	48.582	0.03
	1,2-15,16-Diepoxyhexadecane	49.744	0.12
Phenols	2,6-bis(1,1-dimethylethyl)-4-methyl-Phenol	27.829	0.01
	2,6-ditert-butyl-4-methylphenol	30.226	97.88
	2,6-bis(1,1-dimethylethyl)-4-methyl-Phenol	30.655	0.02
Alcohols	5-Methyl-1,5-hexadien-3-ol	24.069	0.03
	2-tert-Butyl-1-methyl-5-trifluoroacetylimidazole	31.954	0.03
	5-(methylenecyclopropyl)-1-Pentanol	33.253	0.06
	5,5-Dimethyl-cyclohex-3-en-1-ol	42.214	0.02
	2-Decyn-1-ol	43.364	0.01
	1-Ethynylcyclopentanol	48.794	0.22
	Levomenthol	50.076	0.03
Esters	Octadecanoic acid, 2-oxo-, methyl ester	37.928	0.03
	Didodecyl phthalate	46.797	0.21
	Undec-10-ynoic acid, dodecyl ester	49.498	0.03
Acids	17-Octadecynoic acid	48.411	0.06
Ketones	5,5-Dimethyl-3-(2'-methyl-1'-propen-1'-yl)-2-cyclohexen-1-one	25.277	0.02
	2,6-bis(1,1-dimethylethyl)-2,5-Cyclohexadiene-1,4-dione	28.555	0.09
	3,5-bis(1,1-dimethylethyl)-3,5-Cyclohexadiene-1,2-dione	28.698	0.21
	2-(1,1-dimethylethyl)-5-(2-methyl-2-propen-1-yl)-2,5-cyclohexadiene-1,4-dione	28.859	0.25
Aldehyeds	6-Methyl-3-cyclohexen-1-carboxaldehyde	12.928	0.11

[1]Rt: Retention time.

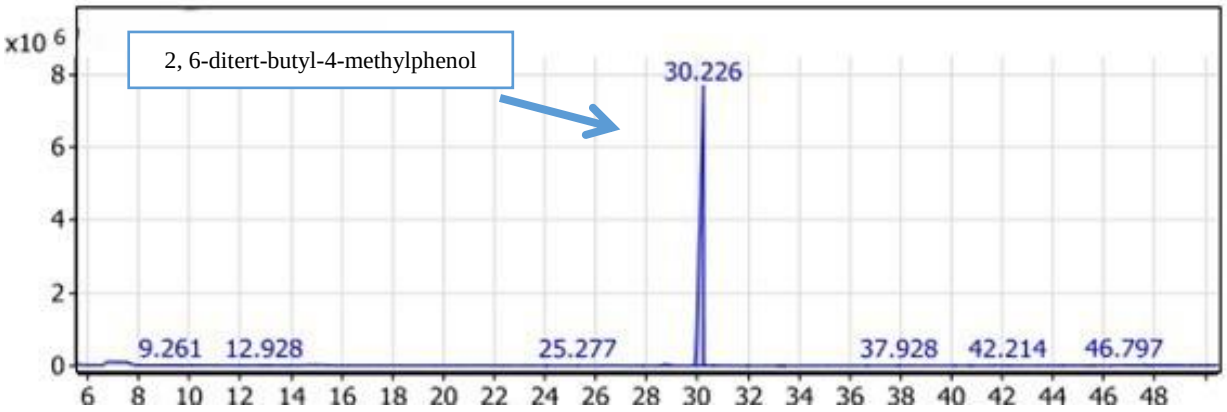


Fig. 1. GC-MS analysis of sugar beet pulp fortified with lysine and yeast extract fermented by *bacillus subtilis* nrch123 using solid-state fermentation.

3.1.2. GC-MS analysis of the BLY2 extract

Volatile compounds in sugar beet pulp fermented by *Bacillus subtilis NRCR22* with fortification of L-Lysine + Yeast extract were extraction and analysed by GC–MS mentioned in Table 3. The results showed that approximately eight different volatile compounds have been identified representing two groups of chemical classes including five aliphatic compounds and three alcohols.

As the same Table, aliphatic compounds were the largest groups with a percentage of 99.93 percent. Among the volatile aliphatic substances, limonene (C10H16) (Fig 2) was a major compound with almost 99.81% of the aliphatic compounds. Chemically limonene is 1-methyl-4-(1-methylethenyl) cyclohexane, it is a colorless liquid with characteristic odor of lemon (Tao et al., 2019).

It is concluded from this study that limonene is the main volatile compound of the media containing sugar beet pulp and fortified with L-Lysine + Yeast extract that was fermented by Bacillus subtilis NRCR22 by used the solid-state fermentation method. Therefore, the dominant odor in BLY2 extract was a lemon-like odor.

Limonene is a monocyclic monoterpene hydrocarbon and one of the most prevalent terpenes in nature, produced naturally by a monoterpene synthase in many plants from the complexation of geranyl pyrophosphate (Ibáñez et al., 2020). Limonene is utilized generally as a flavor and fragrance enhancer in everyday foods like soft drinks because of its pleasant lemon-like flavor. Also, it is regarded as secure for food preservation (Ruiz & Flotats, 2014 and Ravichandran et al., 2018).

Global limonene production is increasing at a rapid rate and is predicted to exceed 65 kilotons in 2023 (John et al., 2017). Limonene was produced microbially by biotechnological methods with strains E. coli DH1 (Alonso-Gutierrez et al., 2013), Synechocystis sp. PCC 6803 (Kiyota et al., 2014), S. cerevisiae AE9 (Jongedijk et al., 2015). Microbial enzymes (such as limonene synthase) used sugars (such as glucose) from media fermented by *Bacillus subtilis NRCR22* as a carbon source to produced terpenes (limonene) as shown in Fig 2.

From this the microbial strain *Bacillus subtilis NRCR22* can produce the flavor compound (limonene) from the fermentation of sugar beet pulp with L-Lysine and Yeast extract. Novel pathways should be sought for microbial production of limonene, by optimizing the microbial strain, selection of available and inexpensive substrates and addition of different growth promoters with solid stat fermentation method.

Since the second extract (BLY2) was the best in terms of its use as a food flavoring compound in addition to its technological and therapeutic properties, so other tests were conducted on it.

Table 3. GC-MS analysis of sugar beet pulp fortified with lysine and yeast extract fermented by *bacillus subtilis NRCR22* using solid-state fermentation.

Type	Identified compounds	[1]Rt	Area, %
Aliphatic compounds	3-ethyl-3-methyl-Pentane	4.775	0.07
	3-methyl-Heptane	6.538	0.06
	2,4-dimethyl-Heptane	6.921	0.01
	Limonene	7.888	99.74
	2,2,4,4-tetramethyl-Pentane	11.047	0.05
Alcohols	5-Methyl-1,5-hexadien-3-ol	5.857	0.04
	3,5-Dimethyl-3-hexanol	10.875	0.02
	3-(1,3-dimethylbutoxy)-2-Butanol	17.461	0.01

[1]Rt: Retention time.

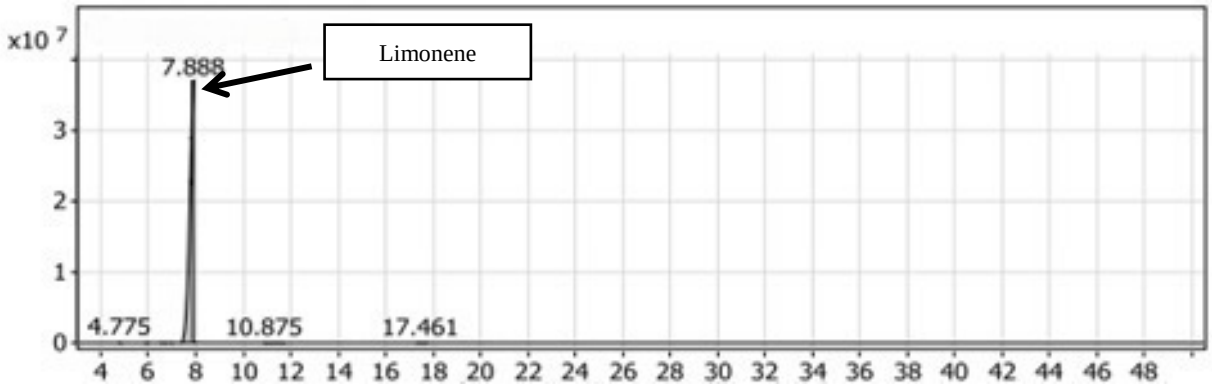


Fig. 2. GC-MS analysis of sugar beet pulp fortified with lysine and yeast extract fermented by *bacillus subtilis NRCR22* using solid-state fermentation.

3.2. Anticancer activity

An irregular growth of cells or tissues of body is called cancer (Baskar et al., 2012). Research on cancer prevention has received a lot of interest globally. It is claimed that adopting a healthy lifestyle and using natural agents can prevent certain types of cancer. Natural substances with chemopreventive action include dietary phytochemicals and polyphenols. Moreover, a number of natural substances have been the subject of current clinical trials to determine their safety and effectiveness in preventing or treating human cancer (Wang & Jiang, (2012).

Results in Fig. 3 showed the inhibition and viability effect of different concentrations of BLY2 extract and Cisplatin against human liver cancer cell line (HepG2) by MTT assay. Twenty concentrations (0.03: 60) µg/mL of BLY2 extract were prepared to study the inhibitory effect on cancer cell line (HepG2). By increasing the concentrations of BLY2 extract, the viability of the cells decreased and the cells did not exhibit any resistance even at the lowest concentration. Cisplatin did not show cytotoxicity at concentrations below 0.94µM as expected but with increasing concentrations of the positive control sample, cell viability decreased based on the results showed in Fig. 3.

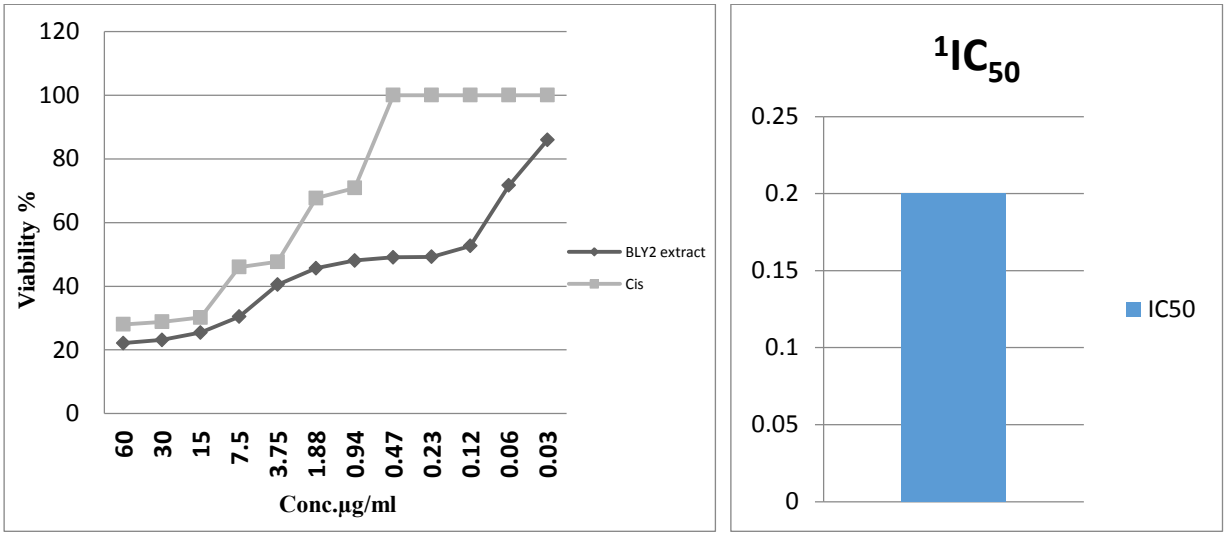
The same Figure offered that the cell death resistance at the maximum extract concentration of 60µg/ml was low resulting in inhibition of 77.67 and 72% of the extract and Cisplatin cell line. But the concentration that inhibited the lowest percentage of the extract was 0.03µg/mL which affected 14.07% of the cell line. While the lowest inhibitory concentration of the positive control sample on cell lines was 0.94µg/ml.

The inhibitory concentration causing toxic effects of BLY2 extract was mentioned in 50% cell which expressed as IC50, where the IC50 values of the extract equal 0.20 µg/mL.

According to the results obtained in this study, limonene, which is the main compound in BLY2 extract (Table 3), has a remarkable effect on liver cancer (HepG2) cells even at very low concentrations, thus this result proved that limonene can enhance anti-cancer activity. The possible mechanism of the cytotoxic effect could be due to the induction on cell death by apoptosis, which leads to increased cell permeability and thus loss of many cytoplasmic organelles.

Limonene is shown to have wide clinical applications in cancer as well as in other disease conditions (Igimi et al., 1976). It has been shown to prevent or delay the growth of several cancer types including liver cancer (Kaji et al., 2001). The toxic effect limonene on HepG2 liver cancer was studied, as the results showed that the survival percentage of HepG2 cells treated decreased significantly and reported a decrease in viability in the range from 0.06 to 0.90µg/µl (Elshafie et al., 2017).

Finally, limonene is recommended as a therapeutic anti-cancer agent and for overcoming the development of multidrug resistance and its negative side effects. Since it is toxic to HepG2 cells, it is recommended that it be tested on several other types of cancer cells.



1IC50: The half maximal inhibitory concentration

Fig. 3. Anticancer activity of BLY_2 extract concentrations compared with Cisplatin and IC_{50} against human hepatocellular carcinoma cell line ($HepG_2$).

3.3. Cytotoxicity assay

Cytotoxicity is the toxicity brought on by chemotherapeutic drugs acting on live cells (Orlowski et al., 1988). The cytotoxicity of the BLY2 extract was assayed on the African green monkey (Vero) cell lines, by MTT assay compared with DMSO. The concentrations of BLY2 extract and DMSO examined ranged from 60µg/mL to 0.03 g/mL as reported in Fig. 4.

This Fig. presents the toxic effect data on normal cells to know the extent of cytotoxicity of BLY2 extract In comparison with positive control sample. The study of the cytotoxicity based on MTT assay revealed that extract expressed low cytotoxicity toward Vero cells lines, with an IC50 approaching a concentration of 3.47 µg/ml.

It is appeared from the obtained data (Fig. 4) that at the highest tested concentration of the extract (60 µg / ml) there was a decrease in cell activity less than that of the positive control sample, as the percentage of live cells was (38.97%, 11.57%) for both the extract and the positive control sample respectively. Also, for the following three concentrations (30, 15, and 7.50 µg/ml), the percentage of live cells was less than 50%, but the viability was higher than that of the positive control. These results indicate greater viability of Vero cells upon treatment with BLY2 extract than DMSO at higher concentrations.

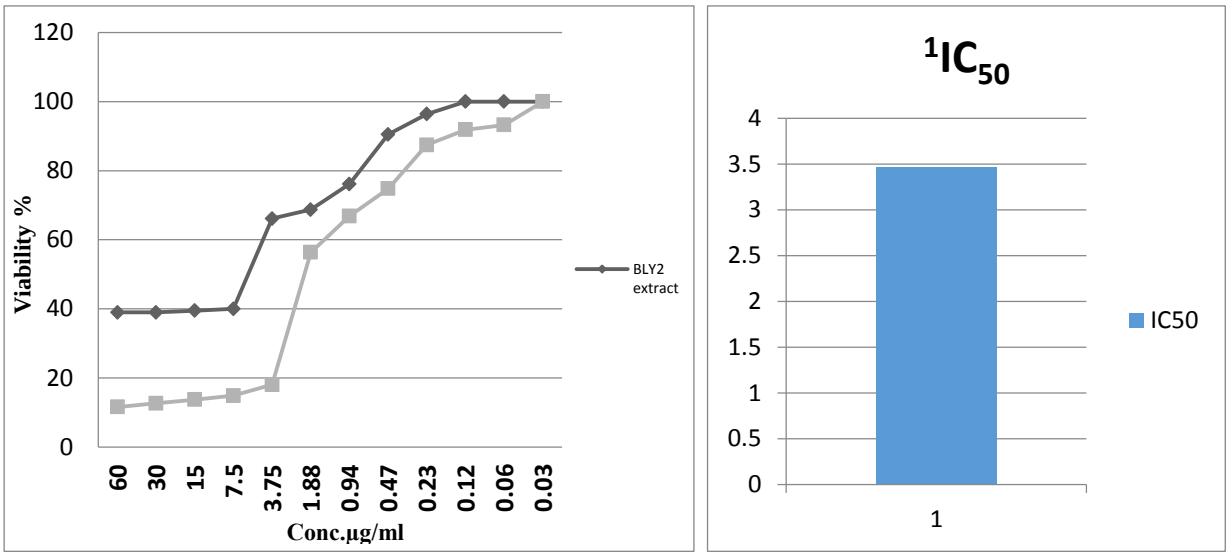
In addition, at the following concentrations up to (0.94 µg/ml), the cell viability percentage was higher than 50%, where was the inhibitory effect of the extract 23.58% at this concentration. While at the concentration (0.47 µg/ml), which is highly safe for cells, the percentage was (90.51%).

It was concluded from these results that limonene

compound, which is the main component of BLY2 extract (Table 3), is considered safe on normal cells whenever the concentration is low. At high concentrations by the same extract, it shows some low toxicity. Hence the low toxicity of limonene provides good safety to healthy cells.

D-limonene is fairly low toxic and showed no risk of evidence in cells studies (Sun, 2007). Subsequently D-limonene is reported to be non-mutagenic and noncarcinogenic in humans and shows low toxicity even after continues dose for up to several years (Rolseth et al., 2002).

Ultimately, microbially produced limonene is a safe compound on normal cells in concentrations higher than those added to foods and beverages, when added as a flavoring agent. However, it should be later tested in vivo and particularly in animal models.



1IC50: The half maximal inhibitory concentration

Fig. 4. Cytotoxicity assay of BLY_2 extract concentrations compared to DMSO and IC_{50} against Vero cell lines.

3.4. Antiviral activity

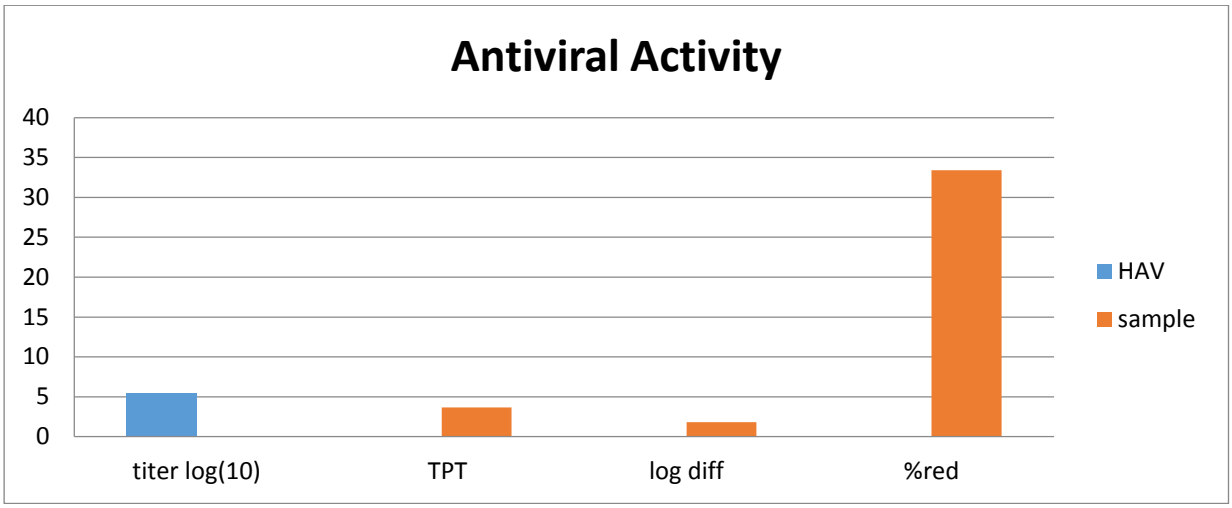
Foodborne viral infections are a serious threat to public health and have a significant negative impact on both industrialised and developing nations' economy. The majority of outbreaks and illnesses caused by foodborne pathogens are caused by enteric viruses. The WHO lists Egypt as having intermediate to high endemicity for several enteric viruses. This is demonstrated by the high prevalence rates of various enteric viral diseases, including Hepatitis A, among the Egyptian population (Aboubakr and Goyal, 2019).

BLY2 extract has been reported to reduce viral infectivity titers after treatment of hepatitis A virus (HAV)with the safe concentration of extract previously calculated on Vero cells as shown in Fig 5.It was observed from the results that after treatment, the BLY2 extract showed a decrease in the viral infection titer by 1.84 log (10) / 0.1 ml, where the virus titer before treatment with the extract was 5.5 log (10) / 0.1 ml, which decreased to 3.66 log (10) / 0.1 ml after treatment of cells with BLY2 extract. The recorded data also revealed that BLY2 extract has a high potential against HAV with a reduction percentage of 33.4%.

It is clear from these results that limonene, which is the dominant compound in BLY2 extract (Table 3), has a remarkable ability to reduce the activity of hepatitis A virus, which is a highly circulated virus and considered a threat to public health. Limonene reduced the activity of the virus by 33.4% at a low concentration, which is a high value, therefore limonene is considered a powerful antiviral.

In viral suspension assays, limonene had strong antiviral efficacy against HSV1. Plaque formation for limonene was inhibited by 100% at maximum noncytotoxic doses. Either cells were pretreated before to viral infection or viruses were incubated with noncytotoxic drug doses prior to infection in order to determine the method of antiviral action (Astani & Schnitzler, 2014).

In conclusion, it is recommended to use limonene as a natural antiviral and as an alternative to drugs that may cause side effects. It also suggests evaluating the antiviral activity against other circulating viruses that pose a threat to health.



TPT: titer post treatment. %red: reduction percentage

Fig. 5. Antiviral activity of BLY_2 extract against Hepatitis A virus (HAV) virus using cell culture.

3.5. Antioxidant activity

When the body is exposed to various environmental stresses, free radicals, which are extremely unstable and reactive molecules, are created. Cells experience oxidative stress as a result of the damaging impact that these free radicals have on them. An essential means of defence against these free radicals-mediated stresses is provided by the cellular antioxidants (Berger et al., 2012). The scavenging activity by 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) method is an electron transfer-based antioxidant method (Pyrzynska & Pękal, 2013).

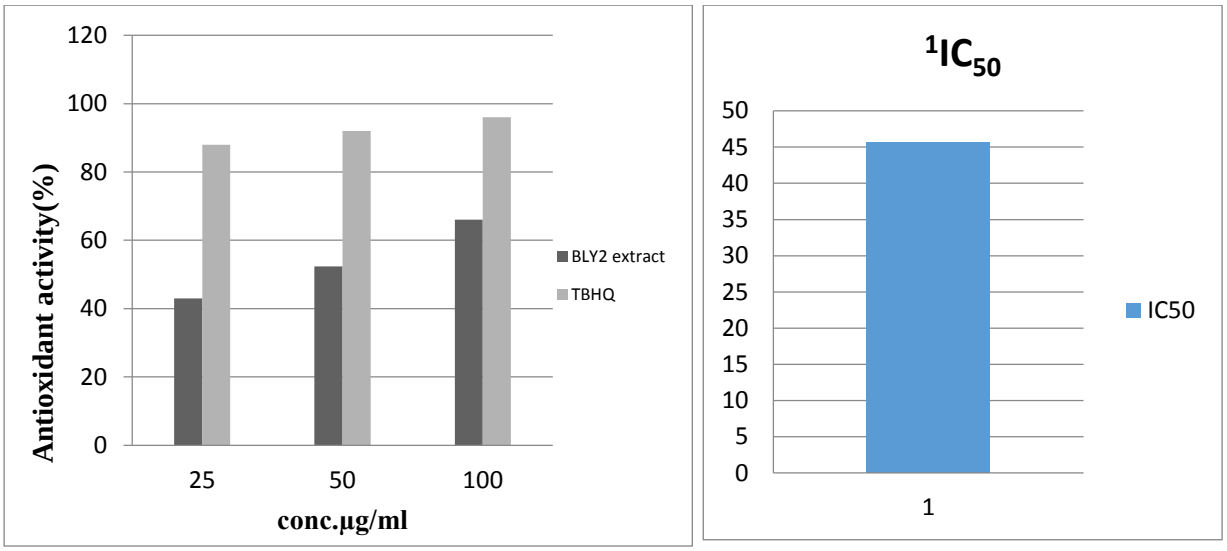
Data given in Fig. 6 noticed when extract concentration enhanced, the sample extract's ability to scavenge free radicals on DPPH increased. When compared to TBHQ (96%) at the same concentration, the 100 ug/ml sample had the strongest efficacy for reducing the DPPH radical by 66.02%. The effect was less for BLY2 extract with a concentration of 25 µg/mL where the percentage of antioxidant activity was (43.02%), on the other hand TBHQ recorded by (88%) with the same concentration. At the level of 50µg/ml the BLY2 extract recorded (52.40%), while the TBHQ recorded (92%).

As showed in Fig. 6, the parameter IC50 (effective concentration value), it is used to understand the data from the DPPH method and is described as the substrate concentration that results in a 50% reduction of DPPH activity (Mishra et al., 2012). For the extract, the scavenging activity value of 50% (IC50) was found to be 45.69µg/mL.

Since limonene is the predominant compound in the extract as listed in Table 3, then it is the main compound responsible for the antioxidant activity. Several researches showed that D-limonene has antioxidant properties, inhibits lipid peroxidation, and combats cell damage brought on by free radicals (Yu et al., 2017).

In this study, limonene was appreciably reduced free radical formation. By giving an atom of hydrogen, the DPPH radical in the limonene DPPH assay was transformed into a stable state. The antioxidant impact of limonene was measured by the removal of the DPPH radical in test samples, which was directly proportional to limonene's antioxidant activity.

Finally, limonene can be used as a natural antioxidant alternative to industrial antioxidants. Thus, it is preferable to test the antioxidant activity of BLY2 extract and calculate the IC50 value by other methods than DPPH such as ABTS and beta-carotene.



TBHQ: Tert-butyl hydroquinone, standard synthetic antioxidant

[1]IC50: The half maximal inhibitory concentration

Fig. 6. Antioxidant activity of BLY_2 extract compared with Tert-butyl hydroquinone (TBHQ) and IC_{50} against DPPH assay.

3.6. Antimicrobial activity

3.6.1. Antibacterial activity

Inhibition zone diameter

The inhibitory zone diameters recorded with the agar diffusion are established in Fig. 7. The results reported that, the BLY2 extract was effective against two tested microbial strains. The size of the inhibition zones was 30 mm for Gram-positive bacteria represented by *S. aureus* and 32 mm for Gram-negative bacteria stated by E. coli. The data obtained in the Table exhibited that the antibacterial activity of the extract was more effective on *E. coli*, followed by S. aureus. Compared to standard commercial antibacterial Ceftriaxone (positive control sample) which inhibition zone (27.2mm and 18.7mm), respectively.

It is evident from the previous results, limonene which represented the major component in BLY2 extract (Fig. 2) has significant antimicrobial activity against Gram-positive and Gram-negative bacteria higher than the positive control sample.

Modern antimicrobial chemicals especially those that are effective against pathogenic microorganisms, may come from essential oils. Typically, the antibacterial activity of essential oils had many sites of action at the cellular level. The main process is irreversibly damaging the bacterial membrane, which causes cytoplasmic losses, energy substrate losses (such as glucose and ATP), bacterial lysis, ions leakage and death. Protease inhibition, which leads to cell content coagulation, is yet another potential action method (Di Pasqua et al., 2007 and Bakkali et al., 2008).

The antibacterial activity of oil extracted from dried lemon leaf powder containing limonene was evaluated. The volatile leaf oil showed remarkable inhibition against *S. aureus* bacteria (32 mm), but in the same study it did not affect *E. coli* (Asker et al., 2020).

An antimicrobial agar diffusion technique was used to evaluate the potency of (lemongrass) against selected microbial pathogens. The inhibition zone for the test organisms *S. aureus* was located between (11.33, 11.66), *E. coli* (16.33, 15.66) (Ewansiha et al., 2012).

Finally, in the prospective study it is suggested to evaluate the antibacterial activity of the BLY2 extract on other Gram-positive and Gram-negative strains.

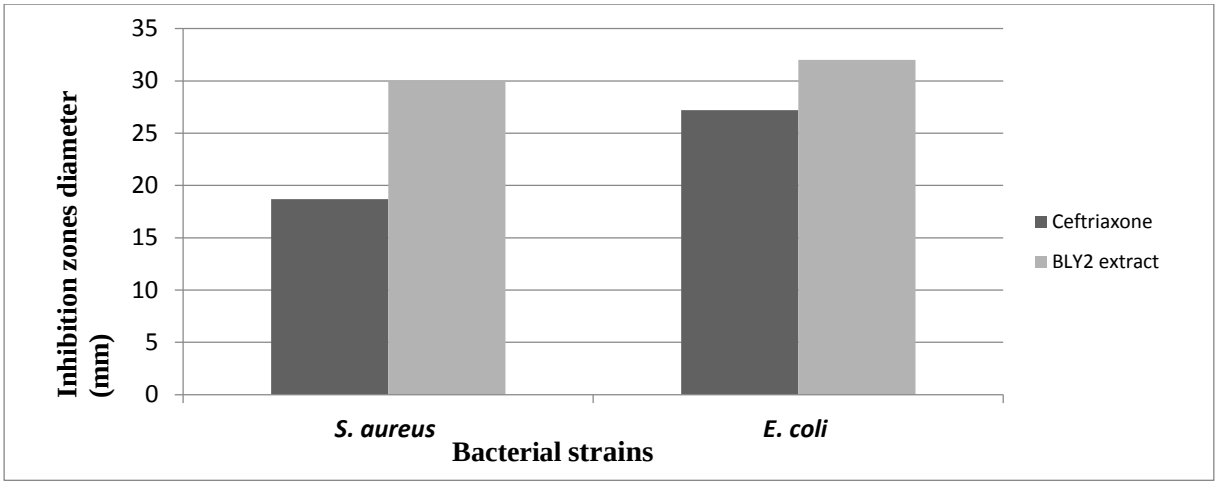


Fig. 7. Inhibition zone diameter of BLY_2 extract compared to Ceftriaxone against bacterial species.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was checked to examine the dosage required to inhibit the growth of bacterial organism. The results of MIC obtained against tested bacteria strains have been given in Fig. 8. Before the interaction study, extract was subjected to the micro broth assay to determine the MIC values for *S. aureus* and *E. coli*. Fig. 8 showed the lowest MIC value which gave very high antimicrobial susceptibility of extract were (0.78 µg/ml) for *S. aureus* and *E. coli*.

Eventually, microbial limonene shows strong antibacterial activity at very low concentrations compared to plant extracts containing the same compound, since its concentration is higher and its effect is stronger.

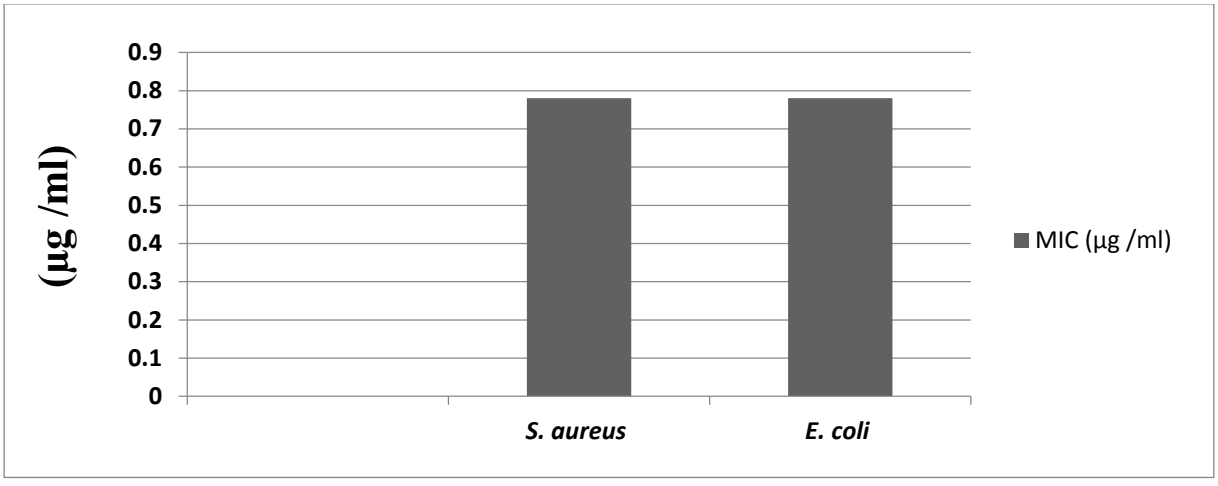


Fig. 8. Minimum inhibitory concentration (MIC) of BLY_2 extract against bacterial species.

3.6.2. Antifungal activity

Inhibition zone diameter

Recorded data in Fig. 9 illustrate that BLY2 extract showed strong antifungal activity against *C. albicans* and *A. niger* which the size of inhabitation zones was (28, 30 mm), respectively. It is evident from results that the antifungal activity of extract was more effective on *C. albicans* and *A. niger* compared to standard commercial antifungal Miconazol (positive control sample), which showed antifungal activity (15.2, 15.8 mm), respectively.

The limonene presented strong antifungal activities at Minimum inhibitory concentration (0.75µL/mL). Scanning electron microscopy showed that limonene compound had destructive effects on the yeast cell surface the damaging effects of the limonene compound on the yeast cell surface were revealed by scanning electron microscopy. After treatment with limonene, the relative electrical conductivity and DNA leakage were significantly

increased (Cai et al., 2019).

Disk diffusion was used to measure the antifungal impact of limonene on yeasts as well as the lowest inhibitory concentration (MIC). All the microorganisms that were tested developed an inhibitory zone. The inhibition zones were wider than those seen when the antibiotic Fungizone was administered as a control. As limonene concentrations rose, the widths of the inhibition zones increased (Ünal et al., 2012).

Finally, limonene is the predominant compound in BLY2 (Table 3) extract plays an effective role as an antifungal. As a result, there is potential for limonene to be used as a natural preservative to control fungal spoilage and protect against mycotoxins.

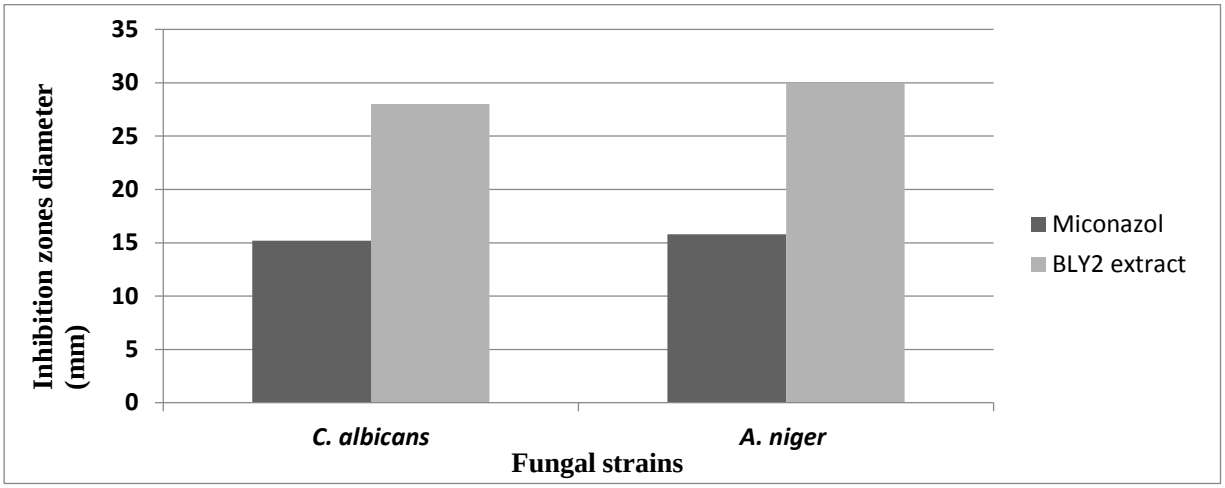


Fig. 9. Inhibition zone diameter of BLY_2 extract compared to Miconazol against fungal species.

Minimum fungicidal concentration (MFC)

Results of the MFC values of extract against potentially pathogenic fungi were illustrated in Fig. 10. Data presented the lowest MFC value which gave very high antifungal susceptibility of extract were (1.65 µg/ml) for *C. albicans* and *A. niger*.

From tabulated data in the same Fig displayed the lowest MFC values that gave high sensitivity against *C. albicans* and *A. niger* were very low (1.65 µg/ml). Which indicates that microbial limonene (Table 3) is a natural antifungal that can be used as an alternative to synthetic antifungals.

Due to its excellent antibacterial action, safety, and low toxicity, limonene has a very promising future as a chemical for antimicrobial and food preservation applications (Han et al., 2019).

In potato dextrose broth (PDB) medium, the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of d-limonene against *Candida* were 20 mL/mL and 40 mL/mL, respectively. The failure of ion trans-shipment and ATP generation in the membrane, the disruption of intracellular ion homeostasis, the leakage of intracellular proteins of *Candida*, and the increase in permeability and integrity damage of the cell membrane after treatment with d-limonene indicated these events. These changes also affected the cell morphology. These findings imply that d-limonene may be able to stop *Candida* contamination in the food sector (Yu et al., 2022)

Eventually, in a prospective study, it is proposed to evaluate the antifungal activity of BLY2 extract on several other fungal strains that cause food spoilage and produce mycotoxins.

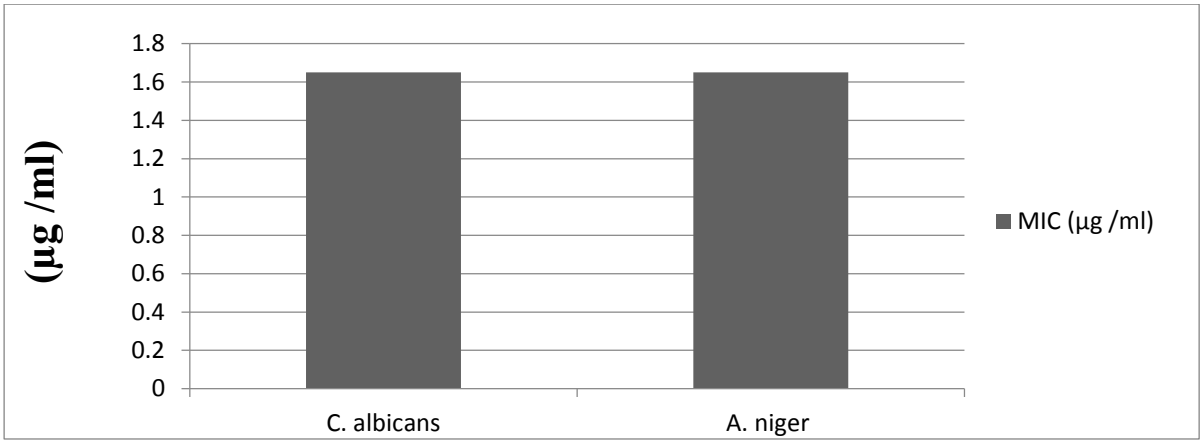


Fig. 10. Minimum fungicidal concentration (MFC) of BLY_2 extract against fungal species.

3.7. Encapsulation efficiency

3.7.1. Particle size distribution and Polydispersity Index (PDI)

Some factors that can affect the encapsulation efficiency of food flavors and oils are the properties of the wall and core materials (Jafari et al., 2008). Particle size droplets means diameter of the treatments produced with different ratios of the wall material as seen in Table 4 and Fig 11, the diameter decreased from 196.6 nm in the nanoliposomes that contained 0.3% (L1) of BLY2 extract to 160.6 nm in the nanoliposomes that contained 0.9% (L2) of the extract in the lecithin solution.

In addition, the diameter was 188.2 nm in nanoliposomes that contained 0.6 % (L3) from the extract. Lecithin is known to have stabilizing and emulsifying effects on encapsulation as reported by (Ding et al., 2021). From these results, notice that by increasing the percentage of the extract, the size of the microcapsules decreases by a slight difference.

On the other hand, Polydispersity index is a dimensionless measure of the heterogeneity of particles size in a mixture and is used to show distribution pattern of particles (Degobert & Aydin, 2021), where the Polydispersity (PDI) indicates the level of dispersion homogeneity, which ranged from 0 to 1. If this number is near to 0, it suggests that the size distribution of the dispersion particles is homogeneous. If the PDI value is more than 0, there are non-uniform sized particles present (Yadav & Sawant, 2010).

Illustrated in the same Table, formula (L3) prepared at (0.9%) presented the lowest PDI value (0.253). But (L2) prepared at (0.6%) recorded the highest PDI value (0.958), attributed to the accumulation of particles during the homogenization process. While (L1) prepared at (0.3%) recorded a value for PDI (0.400).

Using soy-lecithin, thin-film hydration and sonication were used to create nanoliposomes with an average size of 107-136 nm and a 70% encapsulation efficiency. The addition of nanoliposomes increased the mechanical rigidity and thermal stability while reducing water vapour permeability, moisture absorption, and water solubility (Haghju et al., 2016).

3.7.2. Zeta potentials

Zeta potential significantly contributes to the emulsions' physical stability (Wu et al., 2018). The emulsions are more stable when the zeta potential is higher, whether it is positive or negative (Wiącek & Chibowski, 2002). There are various factors affecting zeta potential including the chemical nature of the polymer, the chemical nature of the stabilizing agent and pH of the medium (Mora-Huertas et al., 2010).

Table 4 and Fig 12 showed that the zeta potential of L1, L2 and L4 capsules that has negative zeta potential within -15.0, -12.9 and -12.6 mV, respectively. The greatest zeta potentials were obtained in L1 sample (-15.0), in which small particle size (196.6 nm) was also achieved, demonstrating more stability than other samples.

Using the use of the thin layer hydration approach in conjunction with homogenization and sonication, essential oil-loaded nanoliposomes were created. Nanoliposomes were determined to have a size of 150 nm and a zeta potential of 10.9 to 17.4 mV using dynamic light scattering (DLS). The size of the nanoparticles obtained by DLS was validated by scanning electron microscopy. Antimicrobial and antioxidant activity could be safeguarded by encapsulation (Nahr et al., 2019).

In general, nanomaterials enhance stability,

solubility, cellular infusibility (Chen et al., 2015). Particle size and z-potential are the most important properties that determine fate and stability of liposomes.

Table 4. Particle size, Polydispersity index and zeta potential of encapsulated novel flavor by nanoliposomes.

Formulation Code	Particle size (nm)	Polydispersity index	Zeta potential (mV)
[1]**L_1**	196.6	0.400	-15.0
[2]**L_2**	188.2	0.958	-12.9
[3]**L_3**	160.6	0.253	-12.6

[1]L_1: Capsules loaded with 300 µg/ml flavor
[2]L_2: Capsules loaded with 600 µg/ml flavor
[3]L_3: Capsules loaded with 900 µg/ml flavor

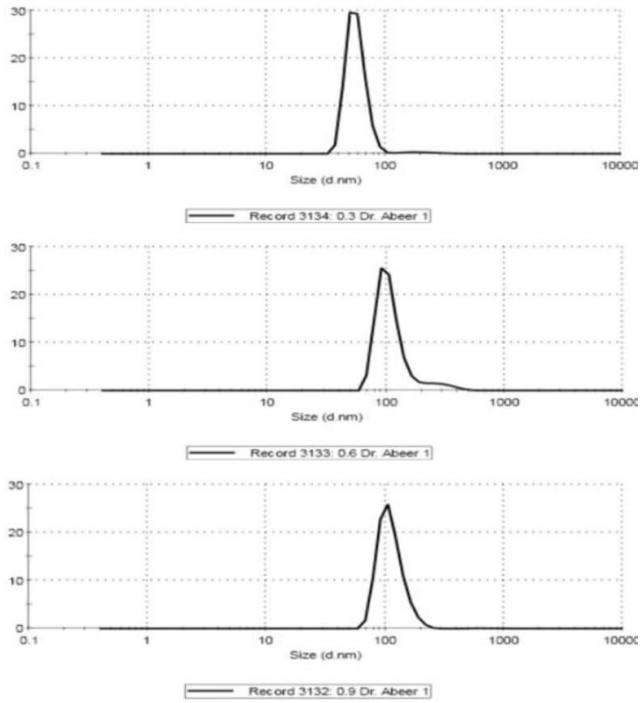


Fig. 11. Particle size distribution of encapsulated novel flavor by nanoliposomes.

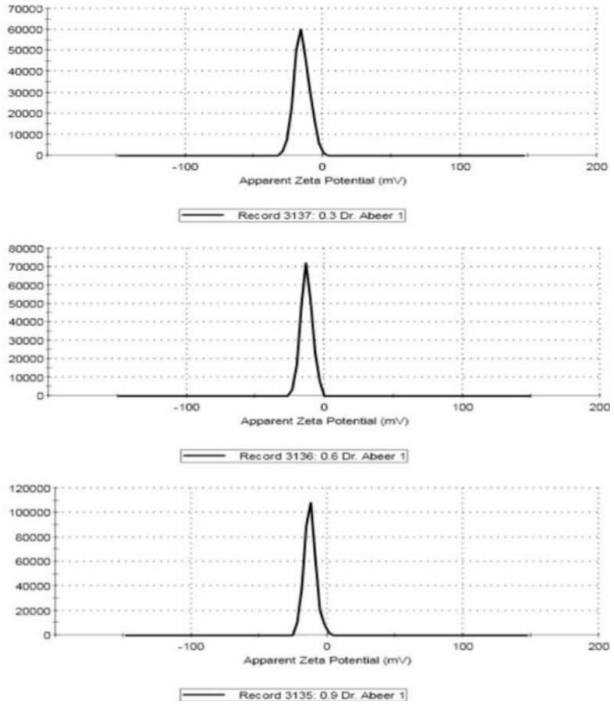


Fig. 12. Zeta potential of encapsulated novel flavor by nanoliposomes.

3.7.3. Transmission electron microscopy

Using a combination of high-resolution electron microscopy imaging, selected area electron diffraction, and electron energy loss spectroscopy, transmission electron microscopy (TEM) is used to examine the evolution of the surface structure at room temperature (Hwang et al., 2014).

TEM was used to investigate the morphology of liposomes and the complex formation between the extract and lecithin. It may display phospholipid form, size, and packing arrangement. Liposomes should be tiny and spherical in form, with roughness or no rupture. Fig. 13 showed TEM pictures of essential the extract loaded liposomes which revealed spherical vesicles in all pictures.

As detailed, TEM imaging indicates that there were no cracks appeared and capsules have a regular shape. Furthermore, the size distribution was homogeneous, as the wall material showed small particles diameter. The results indicated that utilization of lecithin as wall material is capable for microencapsulation of the extract loaded with ultrasoundas.

The formation of the liposomes in Fig. 13 was verified by microscopic analysis of the mixture. With the use of transmission electron microscopy, morphological characterization of the encapsulated extract nanoliposomes indicated the presence of presence of uniformly sized (100: 200 nm) spherical symmetry vesicles, along with the presence of very few large size aggregates (500 µg).

According to Fig. 2, the main ingredient in the extract was determined to be limonene. Despite being employed as an antibacterial agent, the literature described nanoliposomes containing limonene with a particle size of 100 nm (Chan & Tian, 2006). In a different study, 114 nm-sized liposomes containing limon were also found (Palmas et al., 2020).

Where the size of the capsule was small, its stability high and it has a homogeneous spherical shape. Therefore, limonene is generally recognized as safe and therefore widely used as a flavor and fragrance additive in the food industry (Sun, 2007).

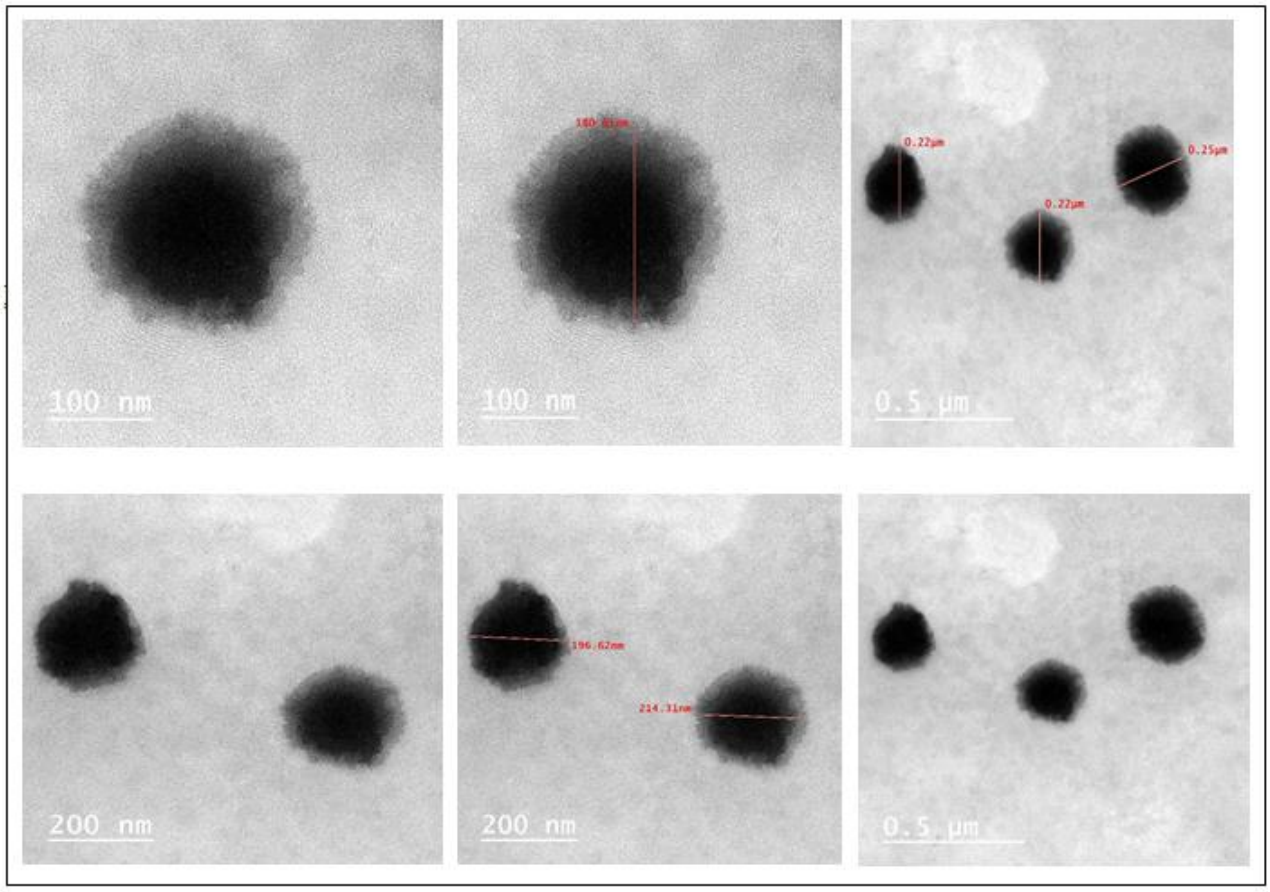


Fig. 13. Transmission electron microscopy (TEM) of encapsulated novel flavor by nanoliposomes.

3.8. Sensory evaluation of encapsulated novel flavor

Sensory evaluation measures individuals' response to stimuli (Booth, 2015). Sensory evaluation of the flavor resulting from the fermentation of sugar beet pulp by Bacillus subtilis NRCR22. The odor was detected after three days on the farm supplemented with the amino acids L-Lysine and Yeast extract. In general, the perceived odor has been described as a lemon-like flavor. As results of GC–MS analysis confirmed those of odor sensory evaluation of the lemon-like flavor.

The data presented in Fig. 14 showed the degree of acceptance of limonene extracted from the fermentation environment as a percentage of arbitration by the arbitrators. The data indicated that 50% of the arbitrators gave the extract a very acceptable degree, while 40% were acceptable and 10% gave the sample a medium degree. While it did not record any value in unacceptable or very unacceptable.

According to the obtained results, the strain Bacillus subtilis NRCR2 is capable of fermenting sugar beet pulp with the addition of the amino acid L-Lysine and yeast extract and forming the limonene flavor. Limonene compound, which is the main responsible for the lemon flavor and the microbial product, found sensory acceptance by the arbitrators, thus its application in food products.

Finally, it is recommended to use the limonene compound, which is responsible for the lemon flavor, as a natural flavoring in food and beverages, as an alternative to the artificial lemon flavor that is widely used in the food field.

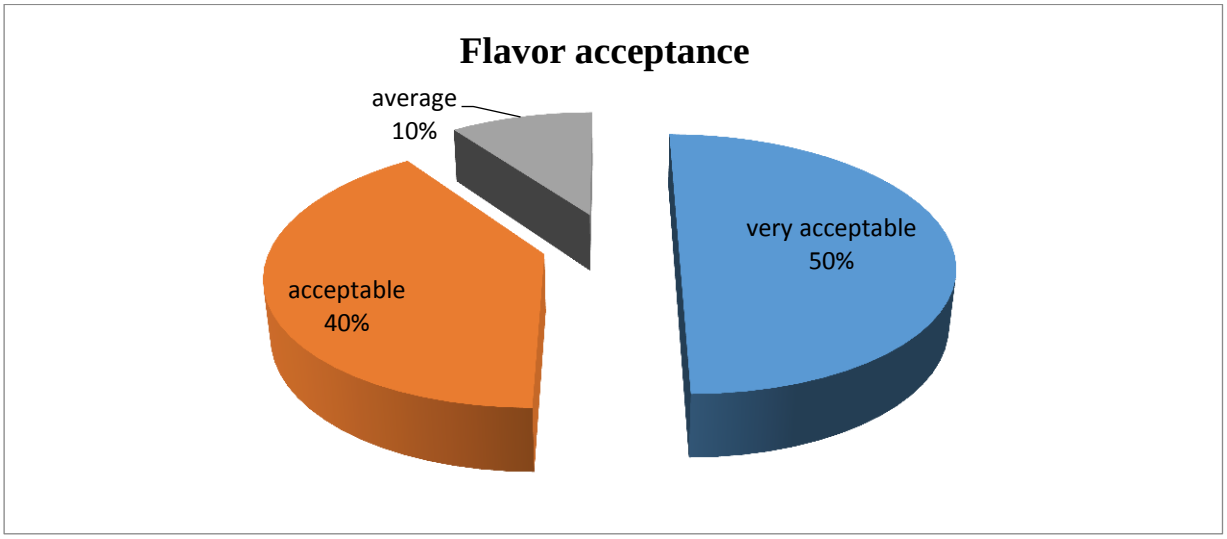


Fig. 14. Flavor acceptance of encapsulated novel flavor by nanoliposomes.

3.9. Sensory evaluation of flavored soft drink

Sensory evaluation of food to which new flavors have been added is carried out to know the degree of their acceptance by the consumer before they are put on the market (Lawless et al., 1999).

Sensory evaluation of soft drink samples prepared by addition of the encapsulated flavor was presented in Table 5. Each the samples were evaluated, the first control sample (BSD), the second control sample (ASD) and the encapsulated samples supplemented with natural flavor of 0.3% (NSD1), 0.6% (NSD2) and 0.9% (NSD3) from appearance, carbonation, odor, taste, color and overall acceptability.

The data in the same Table showed that there were no significant differences ($p > 0.05$) in the sensory evaluation of the flavored soft drink for each of appearance and carbonation between the two control samples and the samples which the natural flavor was added. As for Odor and Color, there was a significant difference ($p \leq 0.05$) between the first control sample (BSD) and the rest of the samples, as the percentages were lower than the other samples (6.00, 7.50) for each of the odor and color, respectively.

On the other hand, the same Table determines the presence of statistically significant differences ($p \leq 0.05$) between the samples in terms of taste, as the percentage decreased for the first control sample, followed by the second control sample, while the percentage was high for the samples containing encapsulated natural flavor, where NSD2 recorded the highest score for taste (8.80), followed by NSD3 (8.70), then NSD1 (8.40) without statistically significant differences. For Overall acceptability, there were significant differences ($p \leq 0.05$) between all samples, as the NSD2 sample recorded the highest value (42.50) and the BSD sample recorded the lowest value (36.60).

From these results, conclude that the sample NSD2 recorded the highest value in terms of taste, smell, and overall acceptability, followed by NSD3, then NSD1, followed by ASD, and the least acceptable was the BSD sample. From this, soft drink produced from the microbial flavor that was encapsulated has good sensory characteristics and better acceptance when compared to the sample containing artificial flavor and the sample to which no flavor was added.

Lastly, microbially produced flavors have wide sensory acceptance when applied in the food field. Therefore, it is recommended to replace artificial flavoring, which has many harms to health, especially children, with natural microbial flavoring, which has many health benefits in addition to its high acceptance.

Table 5. Sensory evaluation of flavored soft drinks prepared with encapsulated novel flavor by nanoliposomes at different loading percentages.

Samples	Appearance (10)	Carbonation (10)	Odor (10)	Taste (10)	Color (10)	Overall acceptability (50)
[1]**BSD**	8.10a ± 0.99	8.50a ± 1.18	6.00b ± 0.94	6.50c ± 1.08	7.50b ± 1.08	36.60e ± 3.53
[2]**ASD**	8.30a ± 0.67	8.70a ± 0.28	8.20a ± 0.79	7.60b ± 0.52	8.00a ± 0.94	40.80d ± 2.25
[3]**NSD_1**	8.50a ± 0.71	8.50a ± 0.85	8.30a ± 0.95	8.40a ± 0.84	7.80a ± 0.42	41.50c ± 2.76
[4]**NSD_2**	8.40a ± 0.84	8.50a ± 1.08	8.70a ± 0.83	8.80a ± 0.63	8.10a ± 0.74	42.50a ± 2.68
[5]**NSD_3**	8.30a ± 0.82	8.20a ± 1.03	8.50a ± 0.85	8.70a ± 0.48	8.10a ± 0.74	41.80b ± 2.15

All the values are Mean ± *Sd. abcdMean values in a column with at least one similar superscript do not differ significantly ($P < 0.05$). 1BSD: Soft drink without adding flavor. 2ASD: Soft drink with artificial lemon flavor. 3NSD1: Soft drink with encapsulated flavor (0.3 rates). 4NSD2: Soft drink with encapsulated flavor (0.6 rates). 5NSD3: Soft drink with encapsulated flavor (0.9 rates).

Conclusions

From these results, it was concluded that *Bacillus subtilis NRCR22* strain is capable of fermenting sugar beet pulp fortified with L-Lysine and yeast extract and producing acceptable sensory and non-toxic flavor compounds, and has anticancer, antioxidant, antiviral and antifungal properties. It was also concluded that the microbially produced flavors have wide sensory acceptance when applied in the field of food, as the soft drink produced from the microbial flavor that was encapsulated had good sensory characteristics and better acceptance when compared to the sample that contained an artificial flavor and the sample to which no flavor was added.

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