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Evaluation of antimicrobial potential of tetradecane extracted from *Pediococcus acidilactici* DSM: 20284 - CM isolated from curd milk

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Abstract

This study was oriented to production of bioactive tetradecane reported in extracts of *Pediococcus acidilactici* isolated from curd milk and 16S rRNA sequence analysis of isolate showed 98% correlation with *P. acidilactici* DSM: 20284-CM (accession no. NR042057.1). *P. acidilactici* activity has been tested by disc diffusion assay against *Staphylococcus aureus* MRSA ATCC 43300, *Listeria monocytogenes* ATCC 19116, *Pseudomonas aeruginosa* MTCC 1934, and *Escherichia coli* MTCC 1610, *Clostridium bifermentans* MTCC 11273 and *Candida albicans* MTCC 183 . The highest antibacterial and antifungal activities created by this strain were obtained. Tetradecane active metabolite which was extracted by *ethyl acetate* contained one observed bioactive spot on thin layer chromatography plate. Spectroscopic analyses confirm the presence of tetradecane as pure product. Minimal inhibitory concentrations (MIC) of tetradecane against indicators are noteworthy. In conclusion, this is the first work show tetradecane was obtained from a novel *P. acidilactici*-CM strain. Tetradecane act as antibacterial and antifungal.

Keywords: curd milk, *Pediococcus acidilactici*, ethyl acetate extraction, tetradecane, antimicrobial activity.

1. Introduction

1. Introduction

The increase in the risk of drug resistance to available commercial drugs by pathogenic microorganisms has become a global risk a worldwide issue [1]. In appropriate use of antibiotics, poor hygienic conditions and delay in diagnosis of the disease are among some of the important factors that favored these circumstances. This has led to the investigation, to look for an alternative source of antibiotic which having broad range of biological activities. [2].

Lactic Acid Bacteria (LAB) is a diverse group of microorganisms that are widely distributed in traditional fermented foods and milk products. The genera Pediococcus belong to LAB. Curd milk offers an excellent opportunity for isolating the potent P. acidilactici with solitary properties capable of producing useful bioactive natural compounds as antibacterial and antifungal agents [3].

Pediococcus are Gram positive cocci bacteria being able to survive at long range of temperature and pH; the most important criteria to be considered for the survival and potential benefits on the host are ability to overcome gastric pH and the toxic effects of bile salts, the advent in a viable physiological state at the site of action, should be capable of adhering effectively the intestinal mucosa and coaggregation ability to reduce the ill effects of pathogens[4, 5].So that, Pediococcus are frequently used on a large scale in the production and preservation of many foods or as probiotics for human and animals[6,7 &3].

The World Health Organization describes probiotics as "live microorganisms, which are when administered in adequate manner provide the host with a nutritional benefit'' [8] and can be used as various types of food product formulations which are incredibly useful to human beings. Gram-positive bacteria, including the Lactobacillus, Lactococcus, Leuconostoc, Pediococcus and Streptococcus genera, are primarily probiotic bacteria with commercial applications, especially in fermented food products. Consumption of food supplemented with live probiotic bacteria may impart many health benefits viz. intestinal microbial homeostasis, regulation of immune response/modulation, improvement of gastrointestinal health, improvement of immune and mucosal barrier functions [7, 9] and protective effects against colon cancer [10].

LAB secondary metabolites are endowed with a wide variety of chemical structures possessing

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strong biological activities and gain attention in view of their positive therapeutic effects to human health [11].

P. acidilacici is a source of secondary metabolites such as phenols, methyl tartronic acid and pyrrolo that are reported for various biological effects including antimicrobial and antioxidant activity [12].

Up to now, extracts from thousands of bacteria species have been screened for their activity against pathogenic microorganisms; but relatively few were found to be pure and
sufficiently active in addition to being non toxic to man [13].

Tetradecane is saturated aliphatic alkane hydrocarbon compound which has oil nature and have broad antibacterial and antifungal properties [14–17] and so become a subject of interest as alternatives to artificial compounds [18, 19].

In the present study, a number of in vitro tests were used to screen P. acidilacteci-CM of curd milk origin for its biological properties. This current work represents the first study to separate tetradecane in pure state.

2. Expermintal

2.1. Isolation of LAB

In the present study, antimicrobial agent-producing LAB were isolated and identified in fermented and unfermented dairy samples collected from various regions of Egypt and allowed to grow in De Man Rogosa Sharp medium (MRS) [20] under anaerobic condition (Mitsubishi AnaeroPak System, less than 0.1% of oxygen, more than 15% of CO2, Pack- Anaero, Mitsubishi Gas Chemicals, Tokyo, Japan) until biomass was obtained.

2.2. Antimicrobial activity of isolated LAB free supernatant

A 24 h loopful of indicators; Staphylococcus aureus MRSA ATCC43300, Listeria monocytogenes ATCC 19116, Clostridium bifermentans MTCC 11273, Escherichia coli MTCC 1610,Pseudomonas aeruginosa MTCC 1934 were purchased from Cairo MIRCEN, Faculty of Agriculture, Ain-Shams University and Candida albicans MTCC183 obtained kindly from the Regional Center for Mycology and Biotechnology, Al-Azhar University; was inoculated in 5 ml sterile saline solution and was set to 28×107 (CFU/ml), 10 µl was seeded in conical flask contains 100 ml Mueller-Hinton Medium (MH) for bacteria or Sabouraud dextrose agar (SDA) for fungus growth at 45˚C, then the inoculated medium was poured into sterile Petri-dishes or streaked the indicator strain on the surface of agar. The assay was performed using disc assay method [21].

2.3. Selection of the most potent LAB isolate

The most effective isolate showing the highest antimicrobial activity was selected for further investigations.

2.4. Identification of the potent LAB isolate

The most effective LAB isolate which exhibited the highest antimicrobial activity was identified using molecular technique based on 16S rRNA gene with the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) [22]. Sequencing was performed in Basic Local Alignment Search Tool (BLAST).

2.5. Extraction and purification of the antimicrobial substance

2.5.1. Solvent extraction method

Different trials of organic solvents systems have been applied on P. acidilactici extract viz.:

(1) Chloroform. (2) Ethyl Acetate. (3) n-Butanol. (4) Diethyl ether.

(5)Methanol+ethyl acetate (1:1, v/v). (6) Methanol+n-butanol (1:1, v/v).

(7) Ethyl acetate + n-butanol (1:3, 2:2 & 3:1, v/v).

Upon defatting the filtrate with n-hexane, 50 ml of each solvent was applied in a separating flask to 100 ml of filtrate (1:2), vortexes vigorously for 30 min, and repeated for three subsequent days. The upper or lower organic phase was extracted, evaporated under reduced pressure until viscous syrup was obtained. The residual syrup was dissolved in the least amount of separated solvent and filtered through Whatman No.1 filter paper. The separate agent's antimicrobial activity has been measured.

2.5.2. Solvent-solvent fractions

Solvents viz.: diethyl ether, n-hexan, chloroform, toluene, ethanol, methanol, n-butanol and ethyl acetate respectively were carried out for selection of the substances according to its polarity and then each fraction was tested for its activity after drying.

2.5.3. Thin layer chromatography (TLC)

The spotting of 10 µl of active fraction on thin layer chromatography (TLC) plates (aluminum sheets silica gel G-60, gf254 pre-coated 20x20 cm with layer thickness 0.2 mm, Merck, Darmstadt, Germany) has been permitted to separate the active fraction into its individual components.

2.5.4. Detection of separated zones on TLC plates

After processing the TLC plates in the best solvent, the plates were dried at room temperature. Separated zones were visualized in white normal light and UV at 254 nm and 365 nm [23]. The antimicrobial activity was checked by scrapping off the separated zones and studying their antimicrobial activities.

2.6. MIC determination of P.acidilactici extract against the most indicators

Micro broth dilution method has been used to determine the MIC value for an active antimicrobial fraction [24, 25]. Serial dilutions of the active fraction have been made with ether in a 96-well micro titer plate. The dilution factor was (100, 50, 25, 12.5, 6.3, 3.15, 1.575, 0.7, 0.3 and 0.19 mg /ml). To each dilution, 100 μl of the culture broths of the high sensitive indicators were added in their respective wells and the plate was incubated at 37˚C for 24 h. After incubation, the spectrophotometric analysis (Microplate spectrophotometer, Multiskan GO, Thermo Scientific, USA) was performed and the MIC value was detected.

2.7. Characterization of P.acidilactici active compound

The active compound was described by spectroscopic techniques to predict the proposed chemical composition and name using gas chromatogram analysis.

Statistical analysis

Data were statistically expressed in terms of means (n = 3) ± standard error (SE). Data variability was checked by one-way ANOVA at $P < 0.05$.

3. Results and Discussion

Increasing antibiotic resistance of microorganisms to traditional drugs involves the search for new, safe, and cost-effective ways to control infectious diseases. A huge number of antimicrobial agents produced from LAB have been found to be very active against certain diseases. There has been growing use of complementary and alternative medicines. This has contributed to a widening of the worldwide market for bacterial products [3, 26].

3.1. Screening of LAB isolates for their antimicrobial activity

Ten lactic acid bacteria (LAB) were isolated from Human breast milk (2), Curd (1), Yoghurt (1), Pickles (1), Cheese (2), Buffalo milk (2) and Goat milk (1) in Egypt (Table 1). LAB isolates were screened against the indicator strains, and the most promising isolate was selected for further investigations. Curd milk isolate (CM) was found exhibited the highest antimicrobial activity among all the tested LAB isolates, so it selected for further investigations.

Isolate CM showed the maximum inhibition zone against S. aureus (MTCC1430) and P. aeruginosa (MTCC 1934) with 23.2 and 22.4 mm respectively, followed by L. monocytogens (ATCC1614) and C. bifermentans (MTCC11273) with 21.5 and 19 mm respectively, while induced 20.9 and 15.1 mm for C. albicans (MTCC183) and E. coli (MTCC 1610) respectively, as shown in Table 1 and Fig. (1). P.acidilactici isolated strain showed prominent inhibitory spectrum with highlighted technical properties.

Broad spectrum antagonism by probiotic microorganism through various antimicrobial substances is currently well accepted phenomenon as compared to older concept where antimicrobial agent's inhibition was linked against closely related species only. There is much evidence reporting the secretory antibacterial components produced by LAB having broad range antagonism against Gram-positive and Gram-negative organisms [27].

Most of previous research indicated that, P. acidilactici Kp10 was exhibited high strong antimicrobial activity against several Gram-positive and Gram-negative food-spoilage and food-borne pathogens such as Listeria monocytgenes ATCC 15313, Salmonella enterica ATCC13311, Shigella sonnei ATCC 9290, Klebsiella oxytoca ATCC 13182, Enterobacter cloaca ATCC 35030 and Streptococcus pyogenes ATCC 12378 [28].

Also, the activity of non-neutralized cell free supernatant of *Pediococcus acidilactici* Ch-2 was studied against *Listeria monocytogenes* MTCC 839, *Clostridium perfringens* MTCC 1739, *Staphylococcus aureus* IGMC, *Bacillus cereus* CRI, *Enterococcus faecalis* MTCC 2729, *Escherichia coli* IGMC, *Pectobacterium carotovorum MTCC* 1428, *Leuconostoc mesenteroides* MTCC 107 and *Pseudomonas syringae* IGMC using well diffusion method; Their findings showed that, the antagonistic pattern of *P.acidilactici* Ch-2 against tested pathogens was assessed and was found to effectively inhibit them **[29]**.

Table 1: Antimicrobial activity of different LAB against indicator strains

LAB Isolate Code	LAB Source	Inhibition zone (mm)					
		C.bifermentans (MTCC11273)	*E.coli* (MTCC 1610)	*L. monocytogens* (ATCC1614)	*P. aeruginosa* (MTCC 1934)	*S. aureus* (MTCC1430)	*C. albicans* (MTCC183)
hBM1	Human breast milk1	15.21 ± 0.34	14.75 ±0.24	13.17± .42	13.25± .51	14.51±0.12	11.3± 0.32
hBM2	Human breast milk2	10.41± 0.51	11.0±0.22	9.5±0.41	8.4±0.12	6.21±0.29	9.91±0.52
CM	**Curd milk**	**19.01± 0.21**	**15.14± 0.11**	**21.51± 0.41**	**22.40± 0.51**	**23.2± 0.91**	**20.91±0.13**
Y	Yoghurt	11.57± 0.19	14.07± 0.11	12.61± 0.21	13.25± 0.25	9.26± 0.28	10.4± 0.43
P	Pickles	6.25± 0.25	8.80± 0.32	5.16± 0.22	10.00± 0.25	11.21± 0.14	00.0
Ch1	Cheese 1	12.75± 0.21	14.17± 0.12	13.00± 0.25	10.41± 0.51	12.50± 0.51	00.0
Ch2	Cheese 2	13.50± 0.12	15.50± 0.31	12.0± 0.24	13.06± 0.34	13.21± 0.14	00.0
BM1	Buffalo milk 1	14.30± 0.41	13.50± 0.51	8.00± 0.42	10.40± 0.10	12.7± 0.14	5.8± 0.29
BM2	Buffalo milk 2	13.40± 0.52	14.12± 0.14	14.17± 0.91	12.00± 0.22	13.10± 0.23	00.0
GM	Goat milk	14.17± 0.29	17.60± 0.51	12.80± 0.15	12.50± 0.12	13.92± 0.34	8.94± 0.41

Where the diameter of disk = 5mm

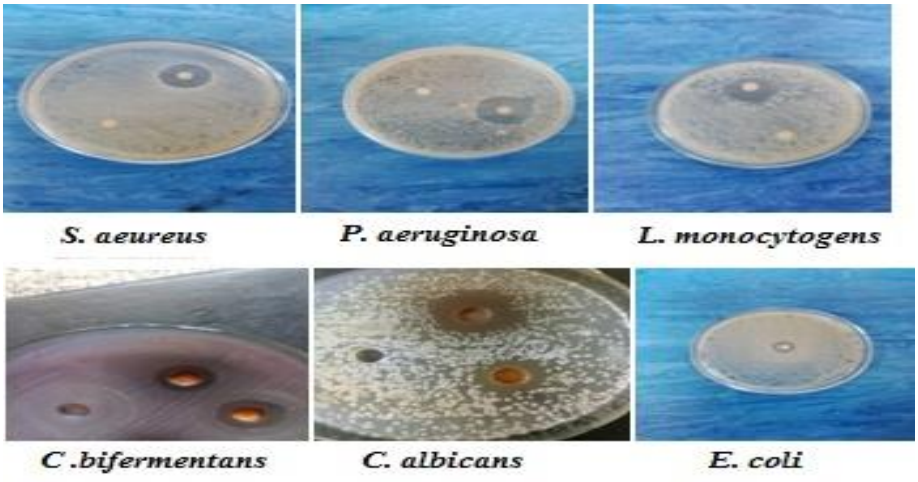


Fig. (1): Antimicrobial activity of curd milk isolate against indicators

In the present study, the most effective LAB isolate (CM) was subjected to identification by 16SrRNA and was found belongs to *Pediococcus. acidilactici* DSM*:* 20284 with 98.8% similarity. A dendogram showed the sequence relationships between *P. acidilactici*-CM, the most effective LAB, in respect to other *Pediococcus* spp. based on 16S rRNA gene sequence **(Fig. 2)**

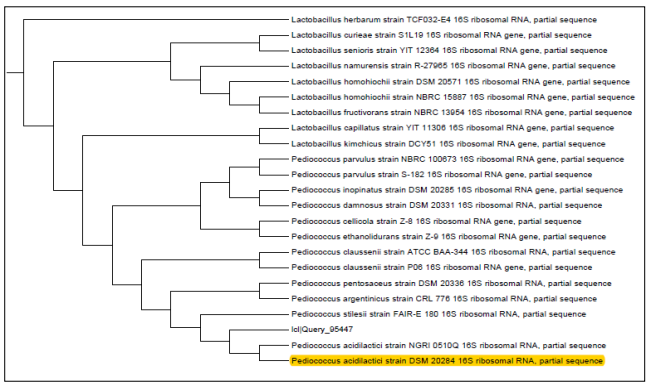


Fig. (2): A dendogram showing the sequence relationships between *P.acidilactici*- CM in respect to other *Pediococcus* spp. based on 16S rRNA gene sequence.

Pediococcus acidilactici was grown over night on MRS growth medium at 37 ˚C until mid-log phase as shown in **Fig. (3)**. The exponential phase began after 4 h in all tested conditions and the maximum biomass was reached after about 16 h of incubation, which remained constant until the end of the stationary phase.

3.2. Extraction & purification of the active

antimicrobial substance from P. acidilactici

Extraction is the key stage for the recovery and isolation of bioactive components from bacteria. The focus of the present study was to provide a more systematic method of extracting the antimicrobial agent from bacteria. Organic solvent approach was used for this purpose.

Ethyl acetate was the best extraction solvent amongst all the solvents tested. *Ethyl acetate* extract's inhibition zone against *S. aureus* was 34 mm as in **Table 2 and Fig. (4**).

Table 2: Extraction of antimicrobial substances from *P. acidilactici* by different solvent

Solvent used	**Antimicrobial activity in terms of mean diameter of inhibition zones (mm)against *Staphylococcus aureus* ATCC 43300**	
	Inhibition zoneof crud	**Inhibition zoneof solvent**
1. Chloroform	0	0
2. Ethanol	0	0
3. N-hexane	0	0
4. propanol	0	0
5. Methanol	0	0
6. Toluene	0	0
7. Ethanol	0	0
8. Diethyl ether	0	0
9. Acetone	0	0
10. Ethyl acetate	**34.0 ± 0.0**	**0**
11. N-butanol	5	5

Bold indicates the more suitable ratio to give the best extraction compound

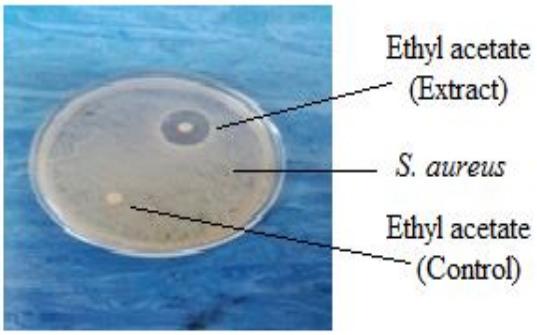


Fig. (4): Activity of *P. acidilactici*-CM *ethyl acetate* extract against *Staphylococcus aureus* ATCC 43300

In the present investigation, the antimicrobial substance was fractionated by solvent-solvent fraction method.

In a trial to isolate the active compounds; the active fraction was applied on TLC plates; *Hexane*: *ethyl acetate* (4:1, v/v) was found as the best active fraction separating solvent system.

The active fraction gave only one pale yellow color spot under normal lab light and pale turquoise fluorescent color under long wavelength as in **Fig. (5)**; the active fraction displayed antimicrobial activity against the most indicators under study.

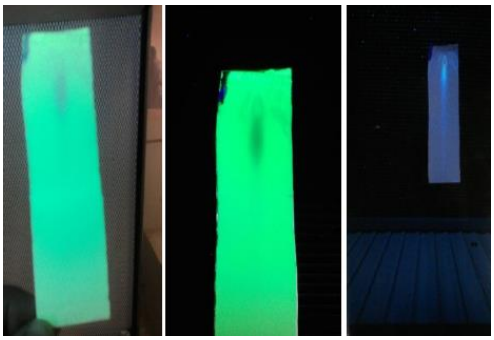


Fig. (5): identification of spot fraction under UV.

In addition, **[30]** reviewed that, *P.acidilactici* ethyl acetate: n- hexane extract scoring strong antimicrobial activity over TLC silica gel after column fractionation.

3.3. Determination of MIC value of the P. acidilactici-CM extract against S. aureus ATCC4330 *and C. albicans* MTCC183.

Data announced in **Table 3** showed that, the MIC of *P. acidilactici* extract against *S. aureus* and *C. albicans* was recorded 3.15 and 0.095 mg/ml, respectively.

Table 3: Minimum inhibitory concentration (MIC) of *P.acidilactici*-CM extract against *S. aureus* and *C. albicans.*

***P.acidilactici*-CM extract (mg/ml)**	**Concentration (mg/ml)**	**Inhibition zone (mm)**	
		S. aeureus	***C. albicans***
Compound	100	20	19
	50	18	16
	25	18	15
	12.5	16	14
	6.3	14	13
	3.15	***11***	12
	1.575	0	12
	0.7	0	11
	0.3	0	11
	0.19	0	10
	0.095	0	***9***
	0.047	0	0
	0.023	0	0

Bold and Italics indicates the minimum concentration from the extracted compound which inhibit the growth bacteria or fungi.

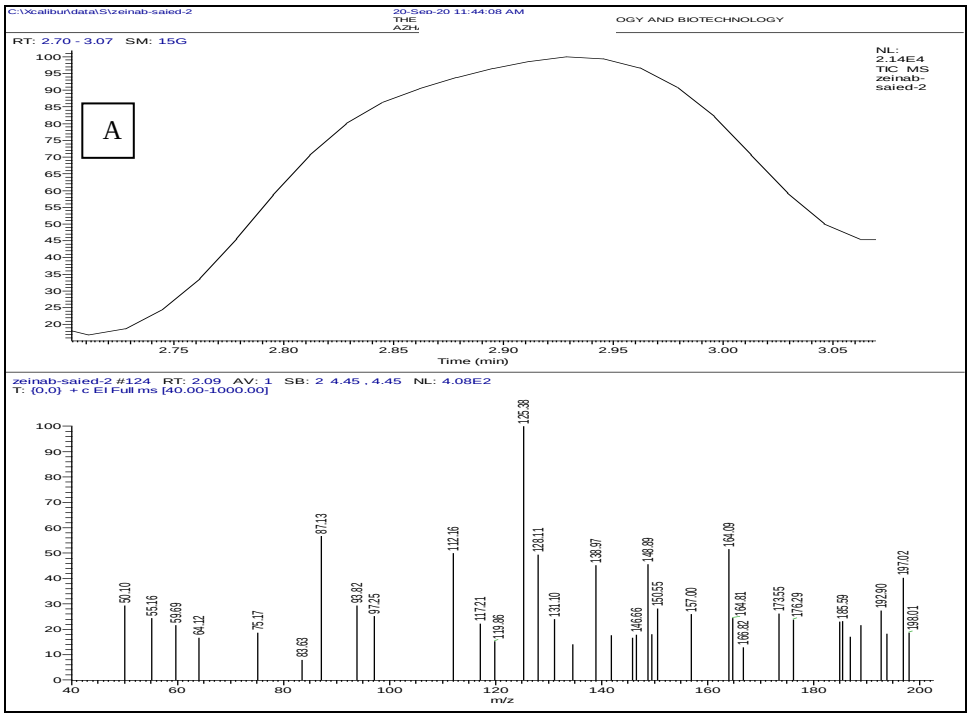
3.4. Characterization of P.acidilactici active extract

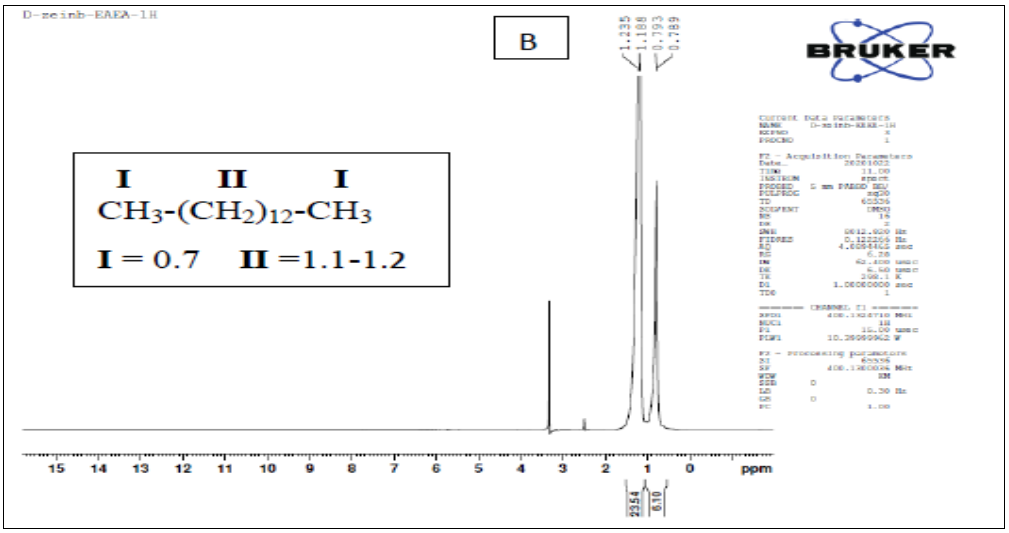
By studying the physical properties of the purified compound, the results showed that, this compound has boiling point; 253˚C, in soluble in water but dissolved in CCL_4 or ether; had a structure ($C_{14}H_{30}$) and named tetradecane.

More spectroscopic studies are needed to elucidate the compound's structure and can serve as an important tool for recognizing active bacterial metabolites.

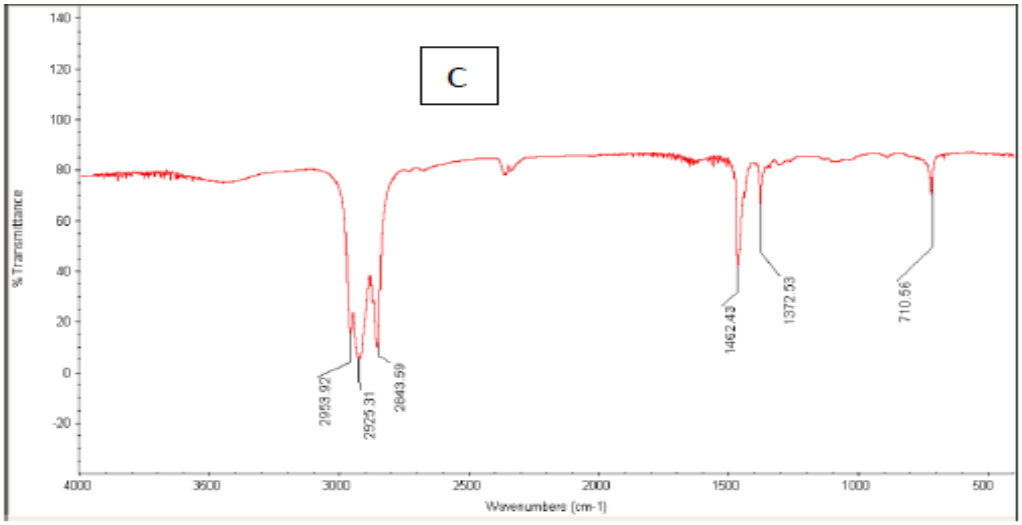
After the purity of the compound was checked by chromatographic separation on silica gel TLC plate that showed one band; total ion chromatogram resulted from an analysis of this compound by MS,^{1}H NMR spectroscopy and IR was confirmed this purity and chemical structure of the active compound as shown in **Fig. (6 A, B & C)**.

As shown in **Fig. (6A)**; the purified active compound has MW= 198.01 Dalton. Also, in **Fig. (6B)**; the integration gives the relative number of hydrogens; Tetradecane only have 2 types of hydrogen atom, CH_3 (group I) and CH_2 (group II); so, we only saw 2 peaks at about 0.7 and 1.1ppm.





The downfield peak at about 0.7 ppm is the CH_3, while the upfield peak at about 1.1&1.2 ppm is the CH_2.



The important characteristic peaks of IR are: 2953 cm^{-1} = C-H stretch of – CH_3 group, 2925 cm^{-1};= C-H stretch of methylene group, 2843,&1462 cm^{-1}= C-H bending of methylene group, 1372 cm^{-1}= C-H binding of – CH3 group; 721 cm^{-1} = C-H binding of methylene group (methylene rocking) which seen only in long chain alkanes which contain at least four methylene groups, e.g. $[-CH_2]_n$, where $n \geq 4$.

Fig. (6): Structure elucidation of tetradecane. MS spectrum (A), 1H NMR spectrum (B), IR spectrum (C).

As shown in **Fig. (6C)**, bands lower than 3000 cm^{-1} for – C-H stretch (alkans) **[31]**.

Most of previous research extracted tetradecane from microorganisms by GC-MS; but mixed with other compounds **[29, 32 & 33];** but in present study; tetradecane was extracted from *P. acidilactici*-CM in a pure state.

Tetradecane content has been found to be active against Gram-positive and Gram-negative bacteria **[34]**.

Moreover, the extracts of *Spirulina* sp. have showed antibacterial activity of octadecane and tetradecane **[35].** The antibacterial activities of pentadecane and heptadecane compounds extracted from Sea Urchin have also been reported to possess potent activity against Gram-positive and Gram-negative bacteria **[36].** The antifungal activity of tetradecane and octadecane has been reported against *C. albicans* **[37].**

In addition, further reviewed that, tetradecane *Streptomyces cheonanensis* VUK-A extracted with ethyl acetate possesses both significant antibacterial and antifungal activity **[38]**.

The activity may be due to tetradecane oil nature components or due to the synergistic effect of its major and minor components **[32&33]**.

Conclusions and prospects

In the present study, tetradecane produced by *P. acidilactici* exhibited significant bioactivity against the tested bacteria and fungi and reveals that that strain is promising producer of antimicrobial compounds. Tetradecane identified by using MS, NMR and IR analysis are not yet reported from the genus *Pediococcus* and also as natural products from lactic acid bacteria; so that *P. acidilactici* can be a good source of bio active components like alkanes.

Despite their omnipresence and a large body of obtainable information on *P. acidilactici* metabolites and their biological properties, there is a paucity of application-oriented research regarding their roles in drug development and production of new industrial compounds. Collective efforts are needed to scout the chemical, biological and genetic diversity of *P. acidilactici* for controlling plant, human and animal diseases that hinder advances in agriculture, drug industry or proliferation of human life. Future research must also contribute to bio prospecting of *P. acidilactici* diversity in symbiotic interactions for novel chemicals. Also, efforts need to be taken to identify novel genes/molecules or drug discovery from isolates belonging to new habitats.

A perusal of literature revealed that, there was no report on the occurrence of tetradecene as natural products from *P. acidilactici*-CM, and this is the first report of their isolation from *P. acidilactici*-CM.

Conflicts of interest

There are no conflicts to declare.

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