THE INVESTIGATION OF ANTIMICROBIAL ACTIVITY OF SOME EXTRACTS FROM MOMORDICA CHARANTIA BY USING AS SOLVENT EXTRACTION AN IONIC LIQUID

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Keywords: pyrrolidinium hexanoate, Momordica charantia, antimicrobial efficiency, bioactivity, extraction

Abstract

The present study aimed to investigate the antimicrobial efficiency of an extract of Momordica charantia obtained by the sonication method, using an ionic liquid of pyrrolidinium hexanoate (PyrrHexa) as solvent extraction. The results were compared with those obtained by sonication for an extract based on ethanol (Et-OH 70%) and an extract based on ethanol: PyrrHexa (1:1) solvents mixture. The antimicrobial efficiency of ethanol and ionic liquid without vegetable mass was investigated. The results showed that PyrrHexa-based samples demonstrate a more significant antimicrobial potential than the common ethanol solvent. Also, the same variation of antimicrobial efficiency of the analysed extracts was observed for all the strains: alcoholic extract < ethanol: PyrrHexa (1:1) mixture extract < PyrrHexa extract. An efficiency 3.3 times higher than Nystatin 100 IU was demonstrated in the case of Candida albicans strain for both PyrrHexa based extract and PyrrHexa solvent without adding vegetal mass.

Rezumat

Scopul acestui studiu a fost investigarea eficacității antimicrobiene a unui extract de Momordica charantia obținut prin metoda sonicării, folosind ca solvent de extracție un lichid ionic:hexanoat de pirolidiniu (PyrrHexa). Rezultatele au fost comparate cu cele obținute prin sonicare pentru un extract bazat pe etanol și un extract pe bază de etanol și PyrrHexa (1:1). A fost investigată eficiența antimicrobiană a etanolului și a lichidului ionic, fără masă vegetală. Rezultatele obținute arată că eșantioanele pe bază de PyrrHexa prezintă un potențial antimicrobian mai ridicat decât cel a etanolului. De asemenea, aceeași ordine de variație a eficienței antimicrobiane a extractelor analizate a fost observată pentru toate tulpinile microbiene testate: extract alcoolic < extract mixt etanol:PyrrHexa 1:1 < extract pe baza de PyrrHexa. O eficiență de 3,3 ori mai mare decât Nystatin 100 UI a fost demonstrată în cazul tulpinii Candida albicans pentru extractul pe bază de PyrrHexa și pentru PyrrHexa pur.

Keywords: pyrrolidinium hexanoate, Momordica charantia, antimicrobial efficiency, bioactivity, extraction

Introduction

The uncontrolled and widespread use of antibiotics has led to an increase in antibiotic-resistant bacteria, which is a highly worrying fact and a major problem for public health in managing bacterial infections [1, 2]. A growing number of studies have focused on finding antimicrobial agents using plant sources [3, 4]. For some pathogens, the choice of available antibiotics is currently very limited [5]. The number of studies on the antimicrobial activity of certain classes of compounds has grown continuously over the years. The main objective of the research is to find new antimicrobial agents using plant-based resources to obtain extracts with a robust antimicrobial character [3, 4, 6].

In recent years, research has been focused on using ionic liquids as green solvents from the newest generation, in the extraction of the active principles from vegetal sources, that present besides the therapeutic importance and a rigorously demonstrated antimicrobial action, have been studied [7, 8]. They are salts composed exclusively of ionic species, primarily found in liquid aggregation at ambient temperature (RTILs) [9]. These solvents used in green chemistry combine physico-chemical properties due to their ionic structure and antimicrobial potential [10-13]. From 2011 to the present, the researchers continue to expand the area of biotechnological applications by using ionic liquids [14] as reaction mediums in the pharmaceutical industry. This field is in continuous development from the point of view of therapeutic and technological discoveries. At the basis of this advancement, there are particular properties of these
ionic compounds such as: potential antimicrobial and antibiofilm, antitumor properties, drug delivery vehicles like emulsions, microemulsions and ionogels [15]. Thus, beneficial and interesting ionic liquids have been discovered to be used as solvents for drug substances or as transport media for them (ionic liquids based microemulsion), ionic liquid salts forms as active pharmaceutical ingredients with pharmaceutically active cations and anions, ionic liquids as antimicrobial agents or antibiofilm agents etc. [16, 17].

Recent studies have shown that ionic liquids have a good antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and algae [7, 18, 19]. The use of ionic liquids as extraction solvents of bioactive compounds from numerous plant sources represents a novel trend of green chemistry. These green solvents present a much higher extraction efficiency of bioactive compounds from biomass than usual organic solvents, but the mixtures of the ionic liquid with organic solvents and water represent a successful alternative in solid-liquid extraction methods of bio-
molecules from different sources of biomass [20, 21]. Alkyl-3-methyl-imidazolium ionic liquids, associated with an anion of type Cl\(^{-}\), Br\(^{-}\) and BF\(_4\)\(^{-}\) are the most commonly used solvents in the separation of flavonoids, saponins, phenolic compounds, terpenoids etc. [22]. *Momordica charantia* is a hanging plant originating from Southeast Asia, which belongs to the *Cucurbitaceae* family and can be found in Romania in the Buzău Vegetable Research Station, where it was brought and cultivated due to therapeutic properties that generate pharmacological effects: hypoglycaemic, analgesic, anti-inflammatory, antitumor, antioxidant, antinutagenic, hypocholesterolemic, antimicrobial. These properties are due to the high content of polyphenols [23-26]. In addition to its polyphenol-rich content, this plant contains certain phytocompounds with a significant hypoglycaemic role, such as: charantin, momordicine, momordenol, zeatin, zeinoxanthin amino acids, P-polypeptides sometimes being referred to as insulin plant [27, 28].

![Figure 1.](image)

The ionic liquid structure realised with the ChemSketch program [31], ACD/2D (a) and ACD/3D (b)

The present study concerns the antimicrobial activity of an ionic liquid of pyrrolidinium-hexanoate (PyrHexa) (Figure 1), compared to that of a conventional organic solvent, ethanol 70° PyrrHexa, which belongs to the class of protic ionic liquids which has in their structure a pyrrolidinium cation (Pyrr\(^{+}\)) and an anion from hexanoic acid (CH\(_3\)-\((\text{CH}_2)_3\)-COO\(^{-}\), Hexa\(^{-}\)). Also, the efficiency of some extracts of *Momordica charantia*, obtained by sonication, using three types of solvents: ethanol 70°, PyrrHexa and mixture of ethanol:PyrrHexa (1:1), were tested.

### Materials and Methods

**Materials**

Liquid ionic: pyrrolidinium hexanoate (PyrHexa) synthesised in the laboratory according to the literature [29, 30] by the neutralisation reaction of pyrrolidine (Brönsted base) with hexanoic acid (Brönsted acid). *Physicochemical properties of synthesised PyrrHexa at ambient temperature*

The measured physicochemical parameters are presented in Table I.

| Physicochemical parameters | Value |
|----------------------------|-------|
| Density                    | 0.9673 (± 0.1%) g/cm\(^3\) at 18°C |
| Conductivity               | 1.41 (± 2%) mS/cm at 18°C          |
| Viscosity                  | 42.52 (± 0.1%) mPa/s at 20°C       |
| Refractive index           | 1.4615 at 20°C                     |
| pH                         | 7 - 8 at 20°C                      |

**Density measurement.** The PyrrHexa density was determined using an Anton Paar densimeter (DMA 4500M).

**Conductivity measurement.** The ionic conductivity was performed using a Consort (C862) digital multi-frequency conductimeter calibrated with KCl standard solutions (0.1 and 0.01 mol/L).

**Rheological measurement.** The dynamic viscosities were measured using a TA instrument rheometer (AR 1000) with conical geometry at various temperatures (from 15 to 50°C).
Refractive index. The refractive index was measured at a temperature of 20°C using an ABBE instrument, calibrated with deionised water. Coulometric Karl-Fischer titration. The water content was checked using coulorimetric Karl-Fischer titration before any crystallisation experiments. The water content of the final PyrrHexa product before crystallisation experiments was 3330 ppm, a result in agreement with the hydrophilic nature of pyrrolidinium ionic liquids.

Analysed samples
Cucumbers come from a national farmer from Ialomița County, Romania, with a certificate of compliance on microbiological quality and the absence of heavy metals. The plant sample was dried in a circulating-air oven at 45°C, for 24 hours and powdered in a blender, passed through a 2 mm Ø sieve and homogenised. The sample was stored at 4°C in the refrigerator until extraction.

Extraction procedures
Ultrasonic assisted extraction
Different solvents were tested for extractions: ethanol 70°, a mixture of ethanol and PyrrHexa (1:1) and PyrrHexa. Ethanol 96° and ultrapure water were purchased from Sigma Aldrich, USA. Ethanol 70° was prepared according to the alchoholometric tale [32] from ethanol 96° and ultrapure water. 2.5 g of dried plant samples were weighed into a 25 mL centrifuge tube and extracted with 25 mL extraction solvent by placing the tube in an ultrasonic bath for 60 min at 40 kHz. The samples were then centrifuged for 15 min at 3000 rpm; the supernatant was transferred to a 50 mL flask. Then the extracts were placed in the refrigerator and were analysed within 24 hours.

The analysed samples were the following:
Alcoholic extract based on cucumber, obtained by sonication in two types of solvents: ethanol 70° (Extract 1) and ethanol:PyrrHexa (1:1) (Extract 2); Cucumber extract in liquid extract 180° (Extract 3). Ethanol 70° and PyrrHexa solvents, separately, without cucumber.

The reference strains used were the following: Gram-negative bacteria: Enterobacter hormaechei ATCC 700323, Escherichia coli ATCC 25922; Pseudomonas aeruginosa ATCC 27853; Gram-positive bacteria: Staphylococcus aureus ATCC 25923; Streptococcus pyogenes ATCC 19615, Enterococcus casseliflavus ATCC 700327, Streptococcus pneumoniae ATCC 49619; Fungi: Candida albicans ATCC 90028.

Working method
Determination of the antibacterial activity – Disc diffusion method
Antibiotic susceptibility testing was determined using the Kirby-Bauer diffusion method, on the standardised culture medium (Müller Hinton, Müller Hinton with 5% sheep blood, Sabouraud), according to the Clinical and Laboratory Standard Institute (CLSI) [33]. An inoculum was performed in sterile 0.9% sodium chloride solution for each bacterial strain until the turbidity was equivalent to McFarland standard no. 0.5. Discs of gentamicin (10 μg), ampicillin (10 μg) and nystatin (100 U.I.) were considered as positive controls, and the rounds of filter paper of 6mm soaked in 25 μL from the analysed extracts, but also from solvents. Also, the test was performed by the procedure using a 50 μL sample. After the disks were applied to the culture media, the plates were incubated at 37°C for 18 - 24 hours. The inhibition zones were read in mm, using a graded ruler, including the disk size. The experiment was conducted in duplicate.

Results and Discussion
Tests on cultures of Gram-negative bacteria
Table II presents the results of tests carried out to investigate the antimicrobial activity on Gram-negative bacteria of 3 extracts: alcoholic extract (Extract 1), extract with a mixture of solvents, Et-OH:PyrrHexa = 1:1 (Extract 2), extract with PyrrHexa (Extract 3). The results obtained were compared with those obtained with the two solvents, Et-OH and PyrrHexa, without vegetable mass.

The analysis of the results for the investigated culture media led to the following conclusions:
For all bacterial strains, Enterobacter hormaechei, Escherichia coli, Pseudomonas aeruginosa, the samples show the following variation of the inhibition zone: Extract 1 < Extract 2 < Extract 3. It can be concluded that the extracts based on PyrrHexa show the highest antimicrobial efficiency, superior to the alcoholic extract. The solvent mixture, Et-OH: PyrrHexa 1:1, is also more effective than the alcoholic extract. The extract based on PyrrHexa has an efficiency of 2.3 to 2.7 times higher than that of the conventional organic solvent.

The values of the diameters of inhibition zones for Escherichia coli and Pseudomonas aeruginosa are identical in both cases of the use of pure PyrrHexa and of the cucumber extract based on PyrrHexa. This result indicates that the antimicrobial activity is independent of certain compounds of Momordica charantia. Antimicrobial activity is an intrinsic property of the ionic...
For Enterobacter hormaechei, pure PyrHexa determines a higher value of the inhibition zone diameter than that obtained by using the extract based on PyrHexa (28 mm vs. 22 mm). This result indicates a better antimicrobial efficiency in the case of pure ionic liquid. The obtained value is slightly higher than that obtained with the reference antibiotic, gentamicin (24 mm for the reference antibiotic and 28 mm for pure PyrHexa).

For all the analysed bacterial strains, samples based on the ionic liquid determine for the inhibition zone the values which are very close to those corresponding to gentamicin 10 μL. This highlights the antimicrobial nature of PyrHexa solvent.

To check if the antimicrobial efficiency is only due to ionic liquid and not to the effect of the compounds contained in Momordica charantia, the study was repeated on new extracts obtained by sonication, using the same volume of solvent and a double amount of the vegetal mass compared to the one used initially (5 g vegetal mass for 25 mL extraction solvent). The droplets volume was 50 μL. Our study focused on the following samples: an alcoholic extract (Extract 1), a mixture of EtOH:PyrHexa 1:1 extract (Extract 2), a PyrHexa extract with 5 g vegetal mass (Extract 3), PyrHexa extract with 2.5 g of vegetal mass with a double drop (Extract 4), and ionic liquid without vegetable mass (called ionic liquid). The diameter of the inhibition zones obtained on stems of Escherichia coli ATCC 25922 is presented in Figure 2. As can be observed from the results, the diameters of inhibition zones vary as follows: Extract 1 < Extract 2 < Extract 3 = Extract 4 < PyrrHexa. This tendency of variation shows, on the one hand, that the extracts based on PyrrHexa present the highest antimicrobial efficiency. On the other hand, PyrrHexa without vegetal mass determined the highest antimicrobial efficiency. The close values of the two samples, 36 mm for the extract based on PyrrHexa and 40 mm for PyrrHexa without vegetal mass, confirm the antimicrobial character of the protic ionic liquid taken in the study.

Inhibition zone (mm) for Escherichia coli ATCC 25922
Tests on cultures of Gram-positive bacteria
The results of tests carried out on stems of Gram-positive bacteria to investigate the antimicrobial efficiency of the 3 types of extracts are made on the following samples: an alcoholic extract (Extract 1), an extract-based in EtOH:PyrHexa 1:1 solvents mixture (Extract 2) and an extract based on PyrrHexa (Extract 3). The results obtained for these extracts have been compared to those obtained on the same strains with ethanol and ionic liquid without vegetal mass. The volume of drops was 25 μL. The diameters of the inhibition zones obtained on stems of Gram-positive bacteria are presented in Table III.

For Enterococcus casseliflavus cultures, all the values are lower than those obtained using the antibiotic taken as a reference (ampicillin 10 μg). The results obtained for the extracts based on PyrrHexa are identical to those obtained with alcohol 70°, except for the extract based on EtOH:PyrHexa 1:1 mixture solvent, which presents a slightly increased value.

Table III

| Bacterial strain                  | Gentamicin | Ampicillin | Ethanol | PyrHexa | Extract 1 | Extract 2 | Extract 3 |
|-----------------------------------|------------|------------|---------|---------|-----------|-----------|-----------|
| Enterococcus casseliflavus        |            | 25 ± 3.5   | 8 ± 1.4 | 8 ± 0.7 | 6 ± 1.4   | 10 ± 2.1  | 8 ± 0.7   |
| ATCC 700327                       |            |            |         |         |           |           |           |
| Staphylococcus aureus             |            |            | 6 ± 0.7 | 27 ± 2.1| 7 ± 2.2   | 8 ± 1.4   | 12 ± 2.8  |
| ATCC 25923                        | 33 ± 1.7   |            |         |         |           |           |           |
| Streptococcus pyogenes            |            |            | 11 ± 2.2| 39 ± 2.8| 38 ± 3.5  | 32 ± 2.8  | 28 ± 3.5  |
| ATCC 19615                        |            | 50 ± 4.2   |         |         |           |           |           |
| Streptococcus pneumonia           |            |            | 9 ± 2.2 | 32 ± 3.8| 14 ± 0.7  | 28 ± 3.5  | 40 ± 3.8  |
| ATCC 49619                        |            | 40 ± 2.1   |         |         |           |           |           |

For Enterococcus casseliflavus cultures, the obtained values are slightly higher than those corresponding to gentamicin values which are very close to those corresponding to gentamicin 10 μL. This highlights the antimicrobial character of the protic ionic liquid.
For *Staphylococcus aureus*, the presence of the ionic liquid slightly increases the antimicrobial efficiency of the extracts. The diameters of the inhibition zone range in the following order: Extract 1 ≤ Extract 2 < Extract 3. However, the extract based on PyrrHexa shows the highest value, being 1.7 times more effective than the alcoholic extract.

The alcoholic extract, the extract based on mixture solvents, and the sample based on alcohol without vegetable mass show similar small values, which remain low compared to the reference antibiotic, demonstrating their ineffectiveness.

However, it should be noted that PyrrHexa, without vegetable mass, has the highest inhibition diameter zone (27 mm) of all the tested samples. This value is close to that obtained for gentamicin (3 mm), demonstrating their efficiency against this type of bacteria. Therefore, PyrrHexa ionic liquid presents an intrinsic antibacterial potential.

As observed from the results presented in Table III, the diameters of inhibition zones for *Streptococcus pneumoniae* vary as follows: Extract 1 ≤ Extract 2 < Extract 3. We can conclude that the presence of PyrrHexa increases the antimicrobial efficiency of the analysed extracts. The extract based on ethanol is 1.5 times more effective than pure EtOH 70° without vegetable mass. The extract based on ionic liquid is 1.25 times more effective than pure ionic liquid without cucumber. The extract based on PyrrHexa is 2.85 times more effective than the alcoholic extract. The extract based on ionic liquid showed the highest value, which is similar to that of ampicillin (40 mm). Therefore, the bioactive compounds of *Momordica charantia* increase the efficiency of the extracts.

For *Streptococcus pyogenes*, the inhibition zones diameters vary in the opposite direction from the order observed for the strains of Gram-positive bacteria studied before: Extract 1 > Extract 2 > Extract 3, and the alcoholic extract seems to be the most effective. However, the pure ionic liquid solvent also shows a value approximately equal to that obtained for the alcoholic extract (39 mm vs. 38 mm), which demonstrates the antimicrobial potential of PyrrHexa. However, these values remain lower than those obtained with the reference antibiotic (50 mm for ampicillin 10 μg).

Inhibition zone diameter. This result shows that the antimicrobial potential is an intrinsic property of ionic liquid solvent, which does not depend on the bioactive constituents of the vegetal source. The solvents based on PyrrHexa ionic liquid are 3.3 times more effective than the reference, Nystatin 100 UI.

Tests on fungi cultures

As can be observed from the results presented in Figure 3, the diameters of inhibition zones for the fungal strains increase significantly with the presence of PyrrHexa in the samples. The values ranging in the following order: Extract 1 < Extract 2 < Extract 3. The extract based on Et-OH: PyrrHexa mixture solvents is 4 times more effective than the alcoholic extract, and the extract based on ionic liquid is 6 times more efficient than the alcoholic extract.

All samples based on the ionic liquid, with or without the vegetable mass of cucumber, present an identical inhibition zone diameter. This result shows that the antimicrobial potential is an intrinsic property of ionic liquid solvent, which does not depend on the bioactive constituents of the vegetal source. The solvents based on PyrrHexa ionic liquid are 3.3 times more effective than the reference, Nystatin 100 UI.

The association of the pyrrolidinium cation with the carboxylate anion leads to a water-miscible compound, which belongs to the class of protonic ionic liquids. According to our results, this ionic liquid presents encouraging effectiveness of an antimicrobial activity against *Escherichia coli*, *Enterobacter hormaechei*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Candida albicans*. The obtained results are confirmed by other studies based on ionic liquids with pyrrolidinium and imidazolium cations [33], showing high efficiency, primarily when associated with anions such as Cl⁻, Br⁻ [7, 34].

Ionic liquids are significant candidates for the category of modern antiseptics and disinfectants. This makes it possible for their use in mainly environmental disinfection, a surgical application like disinfection of surface and medical equipment, or as a disinfectant in the food industry.

Conclusions

The solvents based on ionic liquids demonstrate a higher antimicrobial potential than the classical EtOH 70°, with only one exception (*Enterococcus casseliflavus*). The same variation of the antimicrobial efficiency is observed for all the extracts: alcoholic extract < alcohol:PyrrHexa (1:1) extract < PyrrHexa extract. The samples based on PyrrHexa ionic liquid present an antimicrobial activity, approximately identical to some antibiotics taken as reference (*Escherichia coli* and *Pseudomonas aeruginosa*).

In terms of *Enterobacter hormaechei*, PyrrHexa presents a slightly higher antimicrobial efficiency than that observed for gentamicin 10 μg.

The same antibacterial efficiency as the one determined for the reference antibiotic ampicillin 10μg has been observed in the case of *Streptococcus pneumoniae*.
for the extract based on PyrrHexa (40 mm for both the inhibition zones diameters).
An efficiency 3.3 times higher than Nystatin 100 IU
was obtained in the case of fungi.
Lower values, but close to the reference antibiotics,
were found in the case of Streptococcus pyogenes
and Staphylococcus aureus).

Conflict of interest
The authors declare no conflict of interest.

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