



# Characterization and *in vitro* Evolution of Antibacterial Efficacy of Novel Hesperidin Microemulsion

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## Abstract

Natural products used in the traditional systems of medicine exhibit a various and promising resource in human health. However, these products are often characterized for their chemical composition as well as mechanisms of action. Considering the bacterial infections play an important role in human diseases, it is necessary to develop new antibacterial herbal products. The aim of this study was to isolation and structural identification of hesperidin from *Citrus sinensis* L. and to formulate hesperidin loaded to water-in-oil (w/o) microemulsion as an inovative formulation for bactericidal treatment. This present study indicates that hesperidin microemulsion has antibacterial activity against various types of bacteria according to MIC method. All strains were showed logaritmic reduction. These findings indicated that the microemulsion as a drug carrier, especially hesperidin formulation, may be used as an effective antibacterial therapy.

**Keywords** —*Citrus sinensis*, Hesperidin, Antibacterial activity, Microemulsion, MIC

## 1. Introduction

In a worldwide, plant species are used as an alternative therapy and they are considered as potential source for development of new drugs [1]. According to World Health Organization (WHO) 80% of population in developing countries use plant origin pharmaceuticals [2]. Medicinal plants have been used for the treatment of various diseases such as infections, gastrointestinal ailments, reproductive and skeletal system problems [3-6]. Remarkable effects of medicinal plant materials indicated that they have bioactive substances mainly called as seconder metabolites [7]. They are primarily classified into alkaloids, terpenoids and phenolics whereas important and widely exploited groups of plants. Phenolic compounds are also common plant metabolites which are characterized bactericidal efficacy [8]. One of the largest and abundant class of phenolics is the flavonoids. Chemical complexity and biological activity of flavonoids enable wide range of their usage [9].

Hesperidin (Hsd) is a flavanon type glycoside which has been found mainly in *Citrus* fruits about 1000-5000 mg/kg [10]. Naturally multifunctional hesperidin has a great variety of biopharmaceutical activities, e.g. anti-cancer, anti-inflammatory, antioxidant and antimicrobial [11-13]. Consequently, hesperidin is widely used in the clinical treatment of many diseases, and it also serve as a raw ingredient for different drugs in pharmaceutical industry.

It is important to develop novel plant origin drugs to improve the outcome for patients. The water solubility of herbal drugs has been improved by many techniques. These techniques consist of physical modification of drug molecule, the use of excipients and development of dosage forms.

Hesperidin was isolated from the *Citrus sinensis* L. and its chemical structure was elucidated by using NMR as a beginning of this study. The one of the purpose of this study was to assess the hesperidin microemulsion development, thus microemulsions are homogeneous dispersions of water-in oil or oil-in-water droplets stabilized by surfactants. Additionally, results indicated that the novel herbal microemulsion was effective against *E. coli*, *S. epidermidis*, *B. cereus*, *E. faecalis*, *S. typhimurium*, *K. pnuemonie*, *P. aeruginosa*. This study is allowed to acquire data about this prototype of new drug's antimicrobial potential as a therapeutic material.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

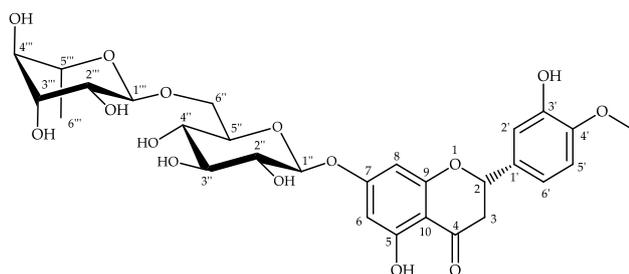
All chemicals used in extraction and isolation were supplied from Merck (Darmstadt, Germany) and used as received. Span 80 and Tween 80 were obtained from Merck (Darmstadt, Germany). Transcutol HP was received as a gift from Gattefosse (Nanterre, France). The following media were also used in MIC Test TSA (Tryptic Soy Agar, Merck, Germany), TSB-ST

(Tryptone soya broth, Biomerieux®, France), Diluent (MRTD, Biomerieux®, France), Mueller-Hinton broth (Oxoid, UK), Gentamycin (Sigma Aldrich Chemical Co. St Louis, USA) was also used..

## 2.2. Isolation of Hesperidin

200 g of fresh peel of *Citrus sinensis* L. was dried in shade, then grinded and extracted with methanol (3×1.5 L) at room temperature. After filtration and evaporation under reduced pressure the methanolic extract (34.1 g) was suspended in water (250 mL) and partitioned successively with *n*-hexane (3×100 mL), chloroform (3×100 mL) and ethyl acetate (3×100 mL). The evaporation of organic solvents *in vacuo* resulted in 1.2 g of *n*-hexane, 1.7 g of chloroform, and 2.9 g of ethyl acetate extracts.

The ethyl acetate extract (2.9 g) was subjected to open column chromatography using silica gel (250 g) employing chloroform:ethyl acetate (9:0.5, 250 mL; 9:1, 200 mL; 8:2, 200 mL) and hesperidin was obtained in chloroform:ethyl acetate (9:1) solvent system as pale yellow powder (17 mg). <sup>1</sup>H- and <sup>13</sup>C-NMR (Spectrum 1 and 2) data were used to determine the structure of hesperidin [14] (Fig. 1).



**Figure 1.** Structure of Hesperidin

## 2.3. Preparation of Microemulsion

In order to develop the microemulsion of hesperidin, water titration method at ambient temperature was conducted as described in our previous study [15]. The existence of microemulsion fields that form microemulsions under dilution and gentle agitation were identified from ternary phase diagrams of systems containing oil–surfactant (S)–cosurfactant (coS). Oleique acide was selected as the oil phase. The effect of surfactants (the mixture of Span 80 and Tween 80 at 9.5:0.5 ratio) and cosurfactant (Transcutol HP) on the pseudo ternary phase diagram was systematically observed at room temperature. Distilled water was added dropwise to each clear oil and surfactants mixture with gentle stirring to allow equilibration. Following the addition of aliquot of water phase, the mixture was visually examined for transparency. Based on the results of pseudo ternary phase diagrams, one microemulsion was selected for further experiments. After that, hesperidine loaded microemulsion was prepared by containing 1 mg FEX per mL with vortex.

## 2.4. Determination of Antibacterial Activity

### Test Organisms

*In vitro* antibacterial activity of hesperidin microemulsion was tested against four gram negative (*Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumoniae* ATCC 13883) and four gram positive (*Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 14579) bacterial strains.

*E. coli*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis* were obtained from Biomerieux® (France) as a Bioball which contains a precise number of cells in a water soluble ball delivering unprecedented accuracy for quantitative microbiological quality control. *K. pneumoniae*, *S. epidermidis* and *B. cereus* were purchased from Microbiologics® (USA) as the lyophilized microorganism pellet after two passages from the reference strain in a KWIK-STIK format.

### Minimum Inhibitory Concentration (MIC) Test

Determination of MIC was carried out according to the microdilution method as described by NCCLS (2007) [16] with some modifications. Overnight bacterial suspension was adjusted to 0.5 McFarland standards (Biomerieux, France) and diluted 1:100 (v/v) was used to inoculate Mueller-Hinton broth and incubated for 24 hrs at 37 °C. The serial dilutions of hesperidin were prepared in test tubes and then were added to the broth previously dispensed in 96 well microtiter plates. The final concentration of hesperidin in the wells ranged between 256 and 0.5 µg/mL. The last well contained only 100 µL broth and 10 µL of bacterial suspension was used as negative control. All the plates were covered with a sterile plate sealer and incubated at 37 °C for 24 h. The MIC was showed no visible growth and no turbidity when viewed against a black back ground. Samples from clear wells were sub-cultured on Mueller Hinton agar. Wells containing Gentamycin dilutions ranging between 128 and 0.25 were used as positive control All of the assays were performed in triplicate.

## 3. Results

### 3.1. Chemical Characterization of Hesperidin

<sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>, 400 MHz) δ (ppm): 11.99 (1H, br s, 5-OH), 6.93 (1H, d, *J* = 2.0 Hz, H-2'), 6.91 (1H, *J* = 8.0 Hz, H-5'), 6.89 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 6.13 (1H, d, *J* = 2.0 Hz, H-8), 6.11 (1H, d, *J* = 2.0 Hz, H-6), 5.46 (1H, dd, *J* = 11.0, 5.0 Hz, H-2), 4.97 (1H, d, *J* = 7.2 Hz, H-1'''), 4.54 (1H, br s, H-1), 3.78 (3H, s, 4-OCH<sub>3</sub>), 3.16–3.64 (6H, m, H-2'' to H-6''), 3.16–3.64 (3H, m, H-2''' to H-6'''), 3.08 (1H, dd, *J* = 17.0, 11.0 Hz, H-3a), 2.82 (1H, dd, *J* = 17.0, 5.0 Hz, H-3b), 2.48 (1H, d, *J* = 6.0 Hz, H-5), 1.09 (3H, d, *J* = 6.0 Hz, H-6); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz) δ (ppm): 197.38 (C-4), 165.56 (C-7), 163.47 (C-5), 162.95 (C-9), 148.39 (C-4'), 146.88 (C-3'), 131.39 (C-1'), 118.38 (C-6'), 114.57 (C-2'), 112.55 (C-5'),

103.78 (C-10), 101.02 (C-1"), 99.92 (C-1"), 96.86 (C-6), 96.00 (C-8), 78.79 (C-2), 76.72 (C-5"), 75.98 (C-3"), 73.43 (C-4"), 72.54 (C-2"), 71.17 (C-4"), 70.72 (C-3"), 70.06 (C-2"), 68.75 (C-5"), 66.48 (C-6"), 56.13 (4-OCH<sub>3</sub>), 42.46 (C-3), 18.25 (C-6"). After comparing the results obtained to those of the literature, the structure of the isolated compound was identified as hesperidin [14,17,18].

### 3.2. Microemulsion Test Results

Phase behavior investigation of these systems demonstrated the suitable approach to determine the water phase, oil phase, surfactant concentration, and cosurfactant concentration with which the transparent phase microemulsion system was formed [19]. The construction of phase diagram makes it easy to find out the concentration range of components for the existence range of microemulsions. The exact composition of oil, surfactant, cosurfactant and aqueous phase were showed in Table 1.

**Table 1.** The Composition of Microemulsion (wt. %)

Hsd Formulation	Oil	S <sub>1</sub>	S <sub>2</sub>	CoS	Water
	34.0	1.4	28.3	29.7	6.36
	5	9	1	9	

### 3.3. MIC Test Result

It was demonstrated that hesperidin has inhibitory effects against the tested bacterial strains, with MICs ranging from 128 to 8 µg/mL indicating that hesperidin microemulsion formulation significantly inhibits bacterial growth (Table 2). Especially it showed strong activity on *E. coli* (8 µg/mL) and *E. faecalis* (16 µg/mL) with concentration equal to the standard antibiotic gentamicin.

**Table 2.** Antibacterial Activities of Hsd Microemulsion (µg/mL)

Strains	Hsd Microemulsion	Gentamicin
<i>E. coli</i> ATCC 10536	8	1.0
<i>P. aeruginosa</i> ATCC 15442	128	1.0
<i>S. typhimurium</i> ATCC 14028	128	1.0
<i>K. pneumoniae</i> ATCC 13883	128	4.0
<i>S. aureus</i> ATCC 6538	32	1.0
<i>S. epidermidis</i> ATCC 12228	32	1.0
<i>E. faecalis</i> ATCC 29212	16	16.0
<i>B. cereus</i> ATCC 14579	16	4.0

## 4. Discussion

The World Health Organization (WHO) reported that global market supports herbal medicinal products [20]. Recent studies within the last decades have shown that the use of complementary and alternative medicine has increased Worldwide [21]. Therefore, it is important to develop effective and non-toxic novel herbal-based products for human welfare.

Herbal medicine production requires processing of the raw material to obtain the desired compound. [22]. Therefore quality control is required to maintain the effectiveness of the processed raw materials. Manufacturers of herbal medicine should ensure the integrity and maintain the efficiency/activity of all plant based products [23].

Hesperidin is a flavanone that is known for its antioxidant, anti-inflammatory and antibacterial properties [24]. Additionally, it is known to possess remarkable medicinal value and have been pharmacologically proved to be an antimicrobial, analgesic and immunomodulatory [25]. At the beginning of the isolation of hesperidin from *Citrus sinensis* and structural elucidation was carried out.

During the study, hesperidin microemulsion was conducted on phase diagram system according to water titration method. It is characterized as a dispersion consisting of oil, surfactant, cosurfactant and aqueous phase. This formulation has been used for various type of drug administration such as oral, dermal, intramuscular and intravenous. Moreover, particle size, transparency, low viscosity, thermal and thermodynamic stability over a wide range of pH's and ionic environments and ease of preparation are considerable and important physicochemical advantages of microemulsions [26,27]. It was essential to find an appropriate method to exert the efficacy for acquiring ideal microemulsion formulation. The minimum inhibitor concentration test was used to analyse the antibacterial activity of hesperidin microemulsion against the bacteria *E. coli*, *P. aeruginosa*, *S. aureus*, *S. typhimurium*, *K. pneumoniae*, *S. epidermidis*, *E. faecalis*, *B. cereus* which are one of the main causes of infections and diseases.

The results of this study indicate that the hesperidin microemulsion possess a relatively high antibacterial response while displaying novel plant origin drug capacity. Previously it has been shown that hesperidin has antibacterial efficacy against the gram-positive and gram-negative bacteria [28]. Besides it has been indicated that pretreatment with hesperidin could suppress infection-induced endotoxic shock in mice and reduce bacterial colonies during infection [29]. Regarding the evaluation of the humoral response, the present study showed that hesperidin microemulsion significantly decreased bacterial growth. The observed



outcomes however confirm the anti-bacterial mechanisms of hesperidin.

This study provides evidence of innovative formation as an alternative antibacterial therapy of novel hesperidin microemulsion for drug delivery.

### Conflict of interest

The authors have no conflicts of interest to declare.

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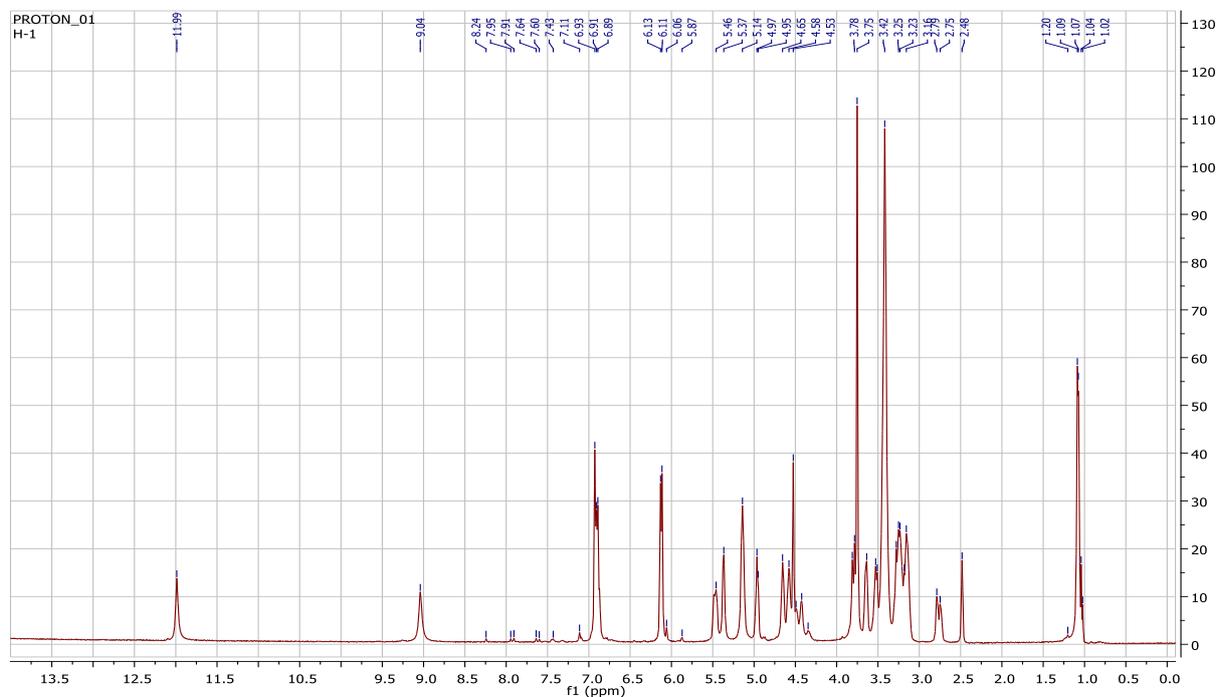
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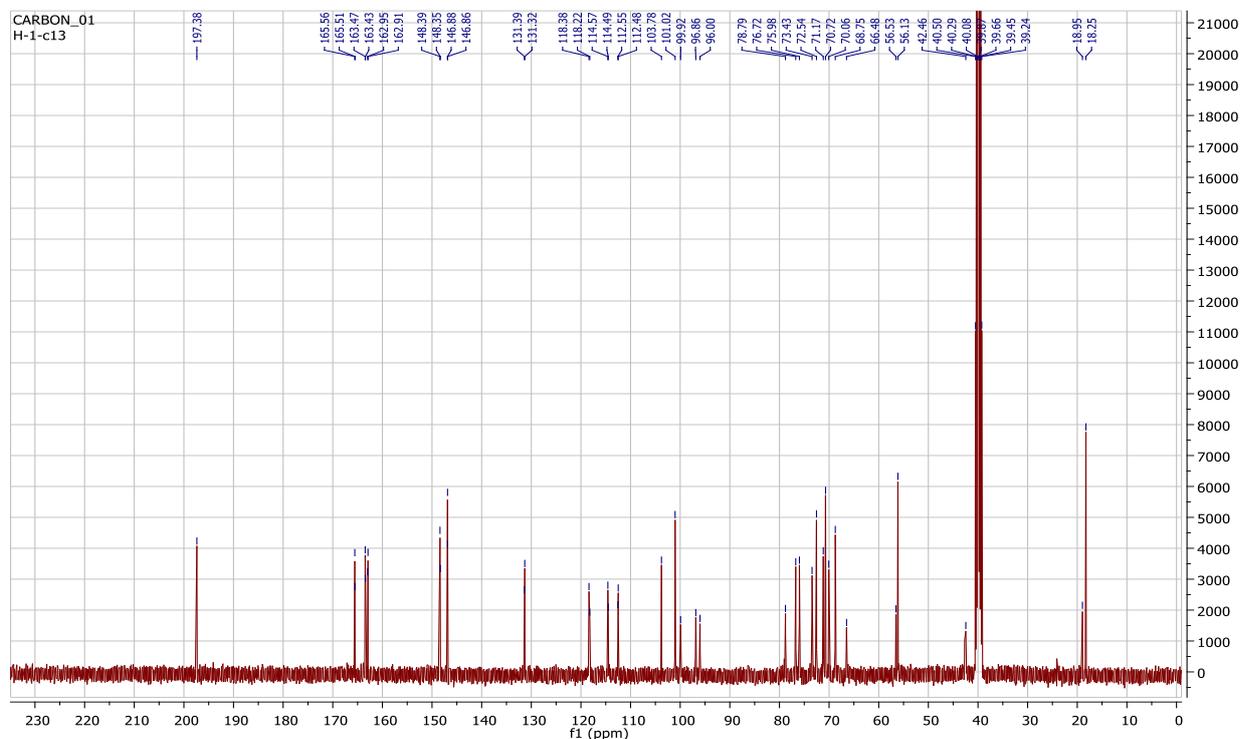
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**Spectrum 1.** <sup>1</sup>H-NMR Spectrum of Hesperidin (400 MHz, DMSO-*d*<sub>6</sub>)



**Spectrum 2.** <sup>13</sup>C-NMR Spectrum of Hesperidin (100 MHz, DMSO-*d*<sub>6</sub>)