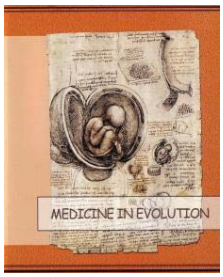
Preparation and characterization of ear drop solutions based on herbal extracts



Marin A., Marin A.M., Mot I.C., Horhat D., Trandafir C., Guran K., Chioreanu A., Balica N.C., Poenaru M.

"Victor Babes" University of Medicine and Pharmacy, Faculty of Medicine, 2nd Eftimie Murgu Sq., Timisoara-300041, Romania

Correspondence to:
Name: Balica N.
Address: Bd. Revolutiei 6., Timisoara
Phone: +40 746288006
E-mail address: balica@umft.ro

Abstract

Otitis is an infection in the ear caused by bacteria that causes pain and discomfort. If not treated in time, long-term complications can occur. An ear infection affects the middle ear; they are more common in children. Unlike the synthetic drugs, the herbal products have a complex composition, being complex mixtures of bioactive compounds. Aim and objectives: The main aims of this research were to develop a novel ear drop solution based on different phytocompounds and to characterize it by specific methods. Material and Methods: Mixed solutions based on Aloe vera and Tea tree extracts have been obtained and they have been characterized by pH measurements, UV-Vis spectroscopy, *in vitro* cytotoxicity and efficacy evaluations. Results and Conclusion: The present results indicate the obtaining of an ear drop solution that can be used in further clinical trials.

Keywords: Aloe vera, mesenchymal stem cells, MTT technique, otitis, pH, Tea tree, UV-Vis

INTRODUCTION

The ear has a very important role in terms of communication and socialization with other people, while also having a function in balance and spatial direction. The acoustic-vestibular apparatus has several segments, namely: a peripheral segment consisting of the outer ear, the middle ear and the inner ear; an intermediate segment represented by the acoustic and vestibular pathway; a central segment represented by the cortical and subcortical hearing centres and the balance centres. The peripheral auditory system is divided into three components, namely: the outer ear made up of the earlobe and the external auditory canal; the middle ear made up of the tympanic membrane, the ossicular chain, the muscles of the middle ear and the pneumatized portion of the mastoid; the inner ear located at the level of the temporal rock that is divided in the vestibule, the system of the vestibular semi-circular canals and the cochlea defined as the auditory organ. On the other hand, the middle ear is made up of the eardrum, the Eustachian tube, and the mastoid cells, while the inner ear is located at the level of the temporal rock and has two parts (one for the acoustic organ and one for the vestibular organ). It consists of multiple interconnected channels that are generically called labyrinths [1-3].

Aim and objectives

Ear pain is a painful sensation with auricular location; it can be felt as pressure, stabbing, stinging, throbbing, etc. and it may have variable intensity. Ear pain often scares the patient, who goes to the doctor. Of course, there may be mild, transient pain that resolves without treatment, but a persistent pain that does not yield to symptomatic treatment or that is accompanied by other manifestations such as fever, dizziness, balance disorders, ear discharge, should worry us and make us see a doctor [4]. There are three major groups of otitis, corresponding to the location of inflammation: external, medium and internal. Otitis externa is the inflammation of the earlobe and / or external auditory canal (the canal leading to the eardrum). Otitis media is the inflammation of the eardrum and of the tympanic cavity (a small chamber, located behind the eardrum, normally full of air, which in otitis media fills with fluids - serum, mucus or pus). Otitis media, less common, includes inflammation of the cochlear and vestibular labyrinth [5].

Man has always used plants for healing and almost as soon as he learned to write he recorded descriptions of their healing properties in different "handbooks on plants". The first known data was written almost 5,000 years ago during the Chinese Emperor Chi'en Nung; it was called Pen Tsao and it contains the descriptions of the medicinal uses of over 300 plants. By 2000 BC, the ancient Egyptians used plants in medicine, cosmetics and embalming; Greeks and Romans have perfected some of these techniques and developed new ones of their own [6]. They learned about their studies from the writings of Hippocrates in the fifth century BC and from the books "De Materia Medica" by Dioscorides and "Naturalis Historia" - 37 volumes by Pliny the Elder (both from the first century AD).

This paper describes the obtaining and the preliminary characterization of mixed solutions based on Aloe vera and Tea tree extracts, that can be used in the otitis treatment.

MATERIAL AND METHODS

The obtaining of extracts: Vegetal material (leaves of Aloe vera and Tea tree - *Melaleuca alternifolia*) was kindly donated by our colleagues from the Biology Dept. of West University Timisoara; they have previously analysed and labelled the samples. The material was rapidly dried at 90 ^{o}C for 48 hours and the dried material was deposed in paper boxes, in darkness, at room temperature.

The phytocompounds from this vegetal material were extracted using 70 % ethanol (dried material/ solvent ratio = 1:5, w/v) for 12 hours. Then, the mixture was filtered and centrifuged at 800 rpm for 15 minutes. The two extracts were concentrated using a rotavapor at 85 ^{o}C until constant weight. This procedure was repeated two times in order to obtain different extracts - samples Ex_1 and Ex_2 (Table 1).

Table 1. The ratios between the raw materials

Sample code	Dried vegetal material, mg		Solvent
	Aloe vera	Tea Tree	70 % ethanol, mL
Ex_1	5	10	75
Ex_2	10	5	75

Preparation of the ear drop solution: Aqueous solutions (1:100, w/v) of vegetal extracts were prepared to evaluate their properties, their toxicity, and their efficacy; a sterile saline solution (9 mg/mL NaCl) was used as the solvent. Polypropylene sterile vials containing these solutions were kept at room temperature before any evaluation [7].

Characterization of the extracts: The pH values of the samples based on the ear drops solutions were determined using a HI 2221 (Hanna Instruments, USA) with a combined electrode (a glass electrode and a calomel reference electrode) at 25 oC. Three standard buffer solutions (pH=4.50, 7.00, and respectively 9.50, at 25°C) were previously used to calibrate the instrument. The electrode was rinsed repeatedly with distilled water and dried prior to pH measurement.

The presence of the main phytochemicals was controlled using a UVi Line 9400 (SI Analytics, Germany); the main components of Aloe vera extract (phenols, around 70 %) have been determined at 765 nm according to O.A. Wintola et al. [8], while myrtenal from Tea tree extract have been determined at 255 nm according to G. V. Buxton et al. [9].

In vitro evaluations: Bone marrow was obtained from patients admitted to The County Clinical Hospital Timisoara (Romania), who have been submitted for bones' surgery; the protocol was previously reviewed and approved by our Ethical Committee. The volunteers were informed about the goal of this study and they signed an Informed Consent according to the Helsinki Declaration. 10.0 mL bone marrow, as source for mesenchymal stem cells (MSCs), was diluted with phosphate-buffered saline (PBS), centrifuged, and placed in a proliferation medium (Dulbecco modified Eagle's medium - high glucose, with 4.5 mg/mL glucose, L-glutamine, and sodium bicarbonate) supplemented with 10 % Fetal calf serum (FCS), 10 ng/mL fibroblast growth factor, and 2% mixture of penicillin/streptomycin in plastic culture plate specific for adherent cell culture. These were incubated at 37 oC and the medium was replaced after 48 hours with a fresh one; the plates were washed after 7 days using PBS and the medium was replaced every 4 days; the cells were passed until they reached 90 % confluence. The culture plate was washed again with PBS and preheated Trypsin-EDTA was added to act on the incubated MSCs after that the microscope was used to observe their separation. The cells were counted using Trypan Blue as vital dye and depending on their number, there were distributed and reinoculated in other culture to ensure optimal proliferation according to M.F. Munteanu et al. [7].

Efficacy evaluation: The murine macrophage cell line RAW 264.7 was achieved from the American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 5.5 % heat-inactivated FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a 5 % CO2 incubator at 37 °C. The viability of cells treated with the samples was evaluated by the MTT technique (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay). This method assesses the activity of mitochondrial dehydrogenase from metabolically viable cells. The effect can be quantified through a colorimetric reaction in which the MTT (yellow compound) is reduced by viable cells to formazan (dark blue compound) according to a previous study [10].

Statistics: All the measurements from this research were done in triplicate for each sample; the results were expressed as mean ± standard error. Paired Student's t tests or One-way Anova followed by Bonferroni's post-tests were used to determine the statistical difference between different experimental and blank groups. $p < 0.05$ was considered statistically significant; *, ** and *** indicate $p<0.05$, $p<0.01$ and <0.001.

RESULTS AND DISCUSSIONS

The pH of samples that are biomedical applications is a very important parameter because it may modify the therapeutic activity, solubility, stability and comfort to the patient [11]. The following pH values of the solutions have been found: 6.83±0.09 (Ex_1), and 6.88±0.22 (Ex_2). The present values are proper for solutions with a possible application as otitis treatment.

Table 2 describes the results of the UV-Vis characterization. Different absorption levels were found for the investigated compounds (phenols from Aloe vera and respectively myrtenal from Tea tree extract).

Table 2. The absorption of investigated phytocompounds

Sample code	Absorption values	
	Phenols	myrtenal
Ex_1	0.82 ± 0.06	1.37 ± 0.11
Ex_2	1.21 ± 0.14	0.54 ± 0.09

The molar extinction coefficient for the oxidized Alamar Blue at 570 and 600 nm, respectively the molar extinction coefficient for the reduced Alamar Blue at the same wavelengths and the absorbance of tested cells were used based on a formula that was described in the literature [12]. Figure 1 presents the cytotoxicity evaluations of the samples tested on MSCs at 24- and 48-h.

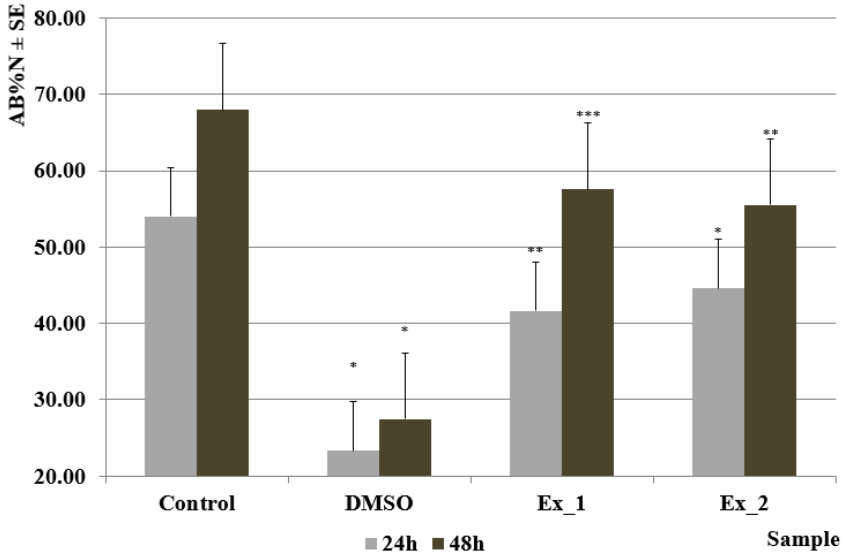


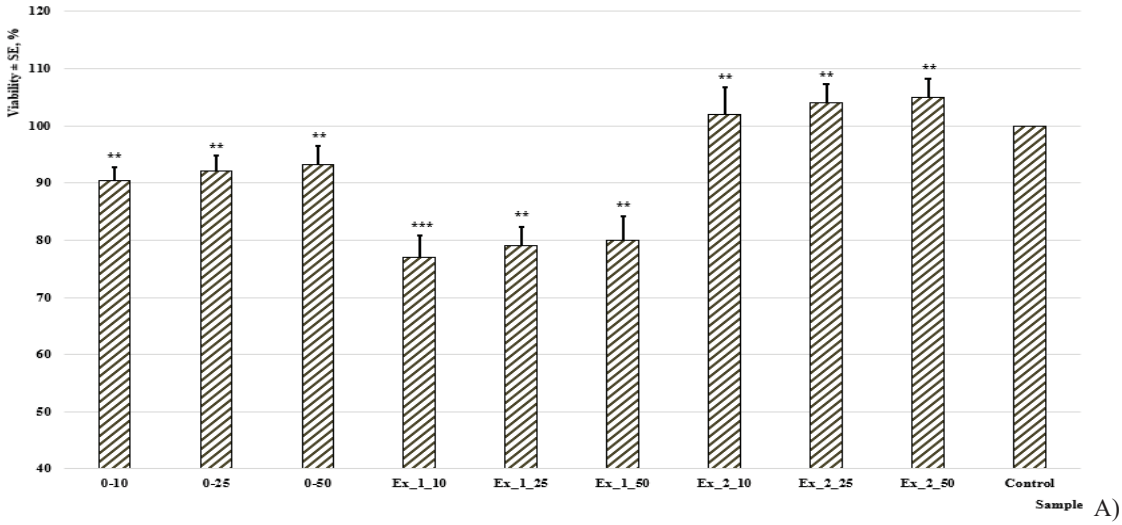
Figure 1. The cytotoxicity potential of the tested samples compared to control and DMSO

The results from the *in vitro* evaluation (Figure 1) show that the tested extracts had no major toxic activity on MSCs. After 24 and 48 h, the first extract (with an increased amount of tea tree leaves) reduced the viability of cells with around 15 % compared to control, but the best results of cells' viability were obtained in the case of the second extract.

The popular murine macrophage cell line, RAW 264.7, is often used to initially screen natural products for bioactivity and to predict their anti-inflammatory effects [13]. The cells were stimulated with different volumes of tested compounds (10, 25 and respectively 50 µl). Figure 2 shows that the second extract (with an increased quantity of Aloe vera) presents the best anti-inflammatory effect compared to control sample and to the other sample.

The viability of the RAW 264.7 cells was greater after the treatment with the second extract, versus control. By contrast, in the case of RAW cells treated with the first extract, the numbers were reduced (Figure 2A and B). These results indicate that the second extract induced RAW 264.7 cell proliferation, whereas the first extract probably had the opposite effect, suggesting that these increases may indicate increases in antigen presenting cells and enhanced cellular immunity.

On the other hand, it is important to mention that both samples based on these extracts indicate that the obtained effects are directly proportional to the concentration of samples.



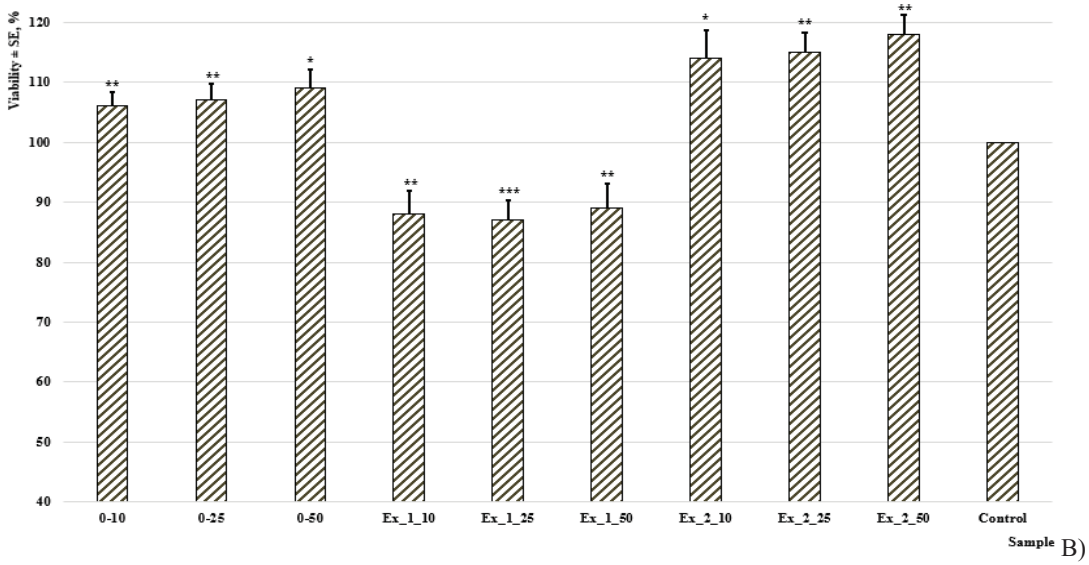


Figure 2. The efficacy of the tested samples as anti-inflammatory agents at (A) 48-h and (B) 72-h.

CONCLUSIONS

Otitis media can affect people of any age. Its typical symptoms include pressure sensation, ear pain and hearing loss; the existing treatments are effective with a good chance of cure, but also with the help of some natural methods. In this study, two different samples based on herbal extracts (Aloe vera and Tea tree) have been obtained and characterized. The comparative analysis revealed that the best effects were obtained in the case of the sample with an increased amount of Aloe vera. The present results indicate that an ear solution based on a mixture of Aloe vera and Tea tree extract can be used in the management of otitis, that continues to affect millions of people around the world annually.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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