

## Article

## Novel Formula of Antiprotozoal Mixtures

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**Abstract:** Antimicrobial resistance (AMR) is becoming more common in both bacteria and pathogenic protozoa. Therefore, new solutions are being sought as alternatives to currently used agents. There are many new ideas and solutions, especially compounds of natural origin, including essential oils. In the present study, the antiprotozoal activity of a mixture of essential oils (eucalyptus, lavender, cedar and tea tree), organic acids (acetic acid, propionic acid and lactic acid) and metal ions (Cu, Zn, Mn) were tested. As a model, protozoans were selected: *Euglena gracilis*, *Gregarina blattarum*, *Amoeba proteus*, *Paramecium caudatum*, *Pentatrichomonas hominis*. The tested concentrations of mixtures were in the range of 0.001–1.5%. The analyses show unexpected, very strong protozoicidal activity of combinations, presenting the synergy of compounds via determination of LD<sub>50</sub> and LD<sub>100</sub> values. Obtained mixtures showed significantly higher activity against protozoans, compared to chloramphenicol and metronidazole. Most of the analyzed samples show high antiprotozoal activity at very low concentration, in the range of 0.001–0.009%. The most effective combinations for all analyzed protozoans were the cedar essential oil and tea tree essential oil with a mixture of acids and manganese or zinc ions. Innovative combinations of essential oils, organic acids and metal ions are characterized by very high antiprotozoal activity at low doses, which, after further investigation, can be applicable for control of protozoan pathogens.

**Keywords:** antiprotozoal; antiparasitic activity; essential oils; LD<sub>50</sub>; phytochemistry; bioactivity; terpenes; phytoncides



**Citation:** Iwiński, H.; Łyczko, J.; Różański, H.; Szumny, A. Novel Formula of Antiprotozoal Mixtures. *Antibiotics* **2022**, *11*, 913. <https://doi.org/10.3390/antibiotics11070913>

Academic Editors: Riccardo Petrelli, Filippo Maggi and Marcello Iriti

Received: 2 June 2022

Accepted: 2 July 2022

Published: 7 July 2022

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## 1. Introduction

Each year, more than 15 million people die worldwide due to infectious diseases caused by various pathogens including bacteria, protozoa, viruses or fungi [1]. One of the main causes of death, besides bacterial and viral diseases, are diseases caused by protozoa such as *Plasmodium spp.*, *Cryptosporidium spp.*, *Leishmania spp.* and *Trypanosoma spp.* [2].

The most deadly diseases caused by protozoa undoubtedly include malaria, human African trypanosomiasis (HAT, sleeping sickness), Chagas disease, Visceral leishmaniasis (VL, kala azar), toxoplasmosis, Naegleriasis (PAM—primary amoebic meningoencephalitis), babesiosis or cryptosporidiosis [2–4]. The most common signs of protozoan infection include diarrhea, fever, malaise or hepatosplenomegaly [2,5].

Currently, there are three main methods of controlling protozoan parasites: prevention and containment of protozoan vectors, vaccination and antibiotic-based pharmacotherapy [2,6]. Based on the example of malaria, we know that the primary and so far most effective weapon in the fight against parasitic protozoa is prevention and elimination of vectors, which are most often mosquitoes, ticks or bedbugs. To reduce the risk of infection, many international and national guidelines recommend the use of insecticide-treated bed nets (ITNs), long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS) or both

methods together. Moreover, control of vector abundance also contributes significantly to reduce the spread of pathogens. The most commonly used method of controlling protozoa is the use of pharmaceuticals. Most often, these are single active substances that show antimicrobial activity. These include nifurtimox, pentamidine, quinine sulfate, fexinidazoles, benznidazoles, artemisinin derivatives and chloroquine, among others. However, most of them have been marketed for completely different purposes and medical indications. Mixed therapies combining two or more pharmacological agents, for example, nifurtimox-eflornithine (NECT), artemether-lumefantrine or quinine sulfate with doxycycline, tetracycline or clindamycin are also commonly used to optimize effects [5,7–11].

Mixed therapies are also a response to increasing resistance of microorganisms to available antibiotics. This is currently a significant problem that affects people all over the world. Its genesis is very complex and is influenced, among others, by high mobility and the possibility of movement all over the world, inadequate hygienic and sanitary conditions or the excessive use of antibiotics both in humans and animals [12,13]. As mentioned above, reducing pathogenic protozoa, especially those showing resistance, is through combination therapy. However, without clinical trials using similar combinations, it is very difficult to estimate their cytotoxicity and interaction in the body.

The lack of new and effective antibiotics causes the need for another alternative. This leads very often to developing an effective method to fight protozoa. The object of interest of researchers around the world are currently natural compounds extracted or obtained from plants [14–16]. Compounds showing antimicrobial activity include phenolic compounds, terpenes, sulfur glycosides or alkaloids [17].

Essential oils are characterized by a very high antimicrobial and antiprotozoal potential [18–20]. They are volatile mixtures, most of which are scented, of various substances of mainly plant origin. They are obtained via steam distillation from various fragments of plant leaves, flowers, fruits, buds, bark, seeds and even roots. They are usually stored in glandulars [21]. The growing interest in natural solutions, being an alternative to the currently used, generates new studies and reports in the literature. Papers or patents present that EOs are characterized by great antimicrobial potential. In vitro studies performed show very strong antiprotozoal activity of, e.g., tea tree oil (*Melaleuca alternifolia* (Maiden & Betche) Cheel) [22], lavender (*Lavandula angustifolia* Mill.) [23], thyme (*Thymus vulgaris* L.) [24], catnip (*Nepeta cataria* L.) [25], yarrow (*Achillea millefolium* L.), clove (*Syzygium aromaticum* L.), basil (*Ocimum basilicum* L.) [18], *Lippia* sp. [26], peppermint (*Mentha piperita* L.) [27] and rosemary (*Rosmarinus officinalis* L.) [28]. The current interest in natural alternatives to the available therapies is generating an increasing amount of scientific research. A growing number of studies are also investigating the antiprotozoal properties of individual components of essential oils, such as monoterpenoids and sesquiterpenoids as well as their alcohol, ester or ketone derivatives [29].

Organic acids are commonly known compounds used in food, cosmetic, feed and pharmaceutical markets as acidifiers, stabilizers, acidity regulators or preservatives. The last feature has been used widely through the ages for food prevention. That property is mainly based on the lowering of the pH, which results in the inhibition of microbials. Organic acids show very good results in broilers and fish production, by their antibacterial and antiprotozoal properties [30–33].

The combination of metals, e.g., iron, cobalt, nickel, gallium, copper, gold or silver with drugs are well known and very effective. Complex of Ru(II) chloroquine was one of the first tested antiprotozoal drugs combined with metal ions and showed a much better result than chloroquine itself. Other examples tested and evaluated for their antiprotozoal properties can be auranofin, triethylphosphine gold(I) chloride, cisplatin, 2-mercaptopyridine N-oxide complexes with Pt(II), Au(I) and Pd(II), or one of the most efficient for organometallic compounds, ferroquine [34–36]. Moreover, not only do the combining synthetic drugs and metals have great potential. Natural compounds such as essential oils, alkaloids and phenols in combination with metal ions are also investigated by researchers [37].

The mode of action of metal ions, mostly, is to impair the proper function of the cell membrane. They may be incorporated with the cell membrane, modulate ion channels, disrupt proton transfer or electrostatically interact with charges on the membrane surface. Metal ions can also affect various cell processes within cytoplasm such as inhibition of enzymes and proteins, catalyze the n of oxygen and hydroperoxide radicals and interrupt nutrient uptake [37–39].

The aim of this study was to investigate antiprotozoal properties of an innovative mixture of essential oils (eucalyptus, lavender, cedar and tea tree), organic acids (acetic, propionic, lactic) and metal ions (Cu, Zn and Mn). Previous research and scientific papers showed antiprotozoal properties of all components, but there were no scientific data about the proposed combinations.

## 2. Results

### 2.1. Antiprotozoal Activity

In the study, antiprotozoal properties were analyzed for single components of the mixtures: 48 combinations (4 essential oils with 3 metals and 3 organic acids and their mixtures). All obtained combinations are presented in Table 1. Chloramphenicol and metronidazole were used as standard substances. The obtained results are presented in Tables 2–9. They are characterized by variable efficiency depending on the combination used and the protozoan species. The combinations containing an essential oil, a single acid and one of the selected metals showed much stronger antiprotozoal properties than the single components, and in some cases, than the antibiotics used for comparison. Among these combinations, the combination of tea tree essential oil, with propionic acid and manganese (TPMn) showed the highest potential. TPMn showed LD<sub>50</sub>, at a very low amount—0.01%. That result is much better than the reference antibiotics—chloramphenicol and metronidazole. It was also observed that the strongest activity independently of the used oil was observed for propionic acid and manganese ions.

**Table 1.** Combinations obtained during the research.

Essential Oil	Acetic Acid (A)			Propionic Acid (P)			Lactic Acid (L)			Mixture of Acids (M)		
	Cu	Mn	Zn	Cu	Mn	Zn	Cu	Mn	Zn	Cu	Mn	Zn
Eucalyptus essential oil ( <i>Eucalyptus globulus</i> Labill.) (E)	EACu	EAMn	EAZn	EPCu	EPMn	EPZn	ELCu	ELMn	ELZn	EMCu	EMMn	EMZn
Tea tree essential oil ( <i>Melaleuca alternifolia</i> (Maiden & Betche) Cheel) (T)	TACu	TAMn	TAZn	TPCu	TPMn	TPZn	TLCu	TLMn	TLZn	TMCu	TMMn	TMZn
Cedar essential oil ( <i>Cedrus</i> sp.) (C)	CACu	CAMn	CAZn	CPCu	CPMn	CPZn	CLCu	CLMn	CLZn	CMCu	CMMn	CMZn
Lavender essential oil ( <i>Lavandula angustifolia</i> Miller) (L)	LACu	LAMn	LAZn	LPCu	LPMn	LPZn	LLCu	LLMn	LLZn	LMCu	LMMn	LMZn

**Table 2.** LD<sub>50</sub>, LD<sub>100</sub> values of eucalyptus essential oil (*Eucalyptus globulus* Labill.) and the components used in the study.

Protozoa	CH <sup>a</sup>	M <sup>b</sup>	Acetic Acid	Propionic Acid	Lactic Acid	Mixture of Acids <sup>c</sup>	Manganese (II) Chloride Solution <sup>d</sup>	Copper (II) Carbonate Hydroxide Solution <sup>e</sup>	Zinc Carbonate Solution <sup>f</sup>	Catalyst Solution <sup>g</sup>	Eucalyptus Essential Oil ( <i>Eucalyptus globulus</i> Labill.)
<i>Euglena gracilis</i>	LD <sub>50</sub> : 0.05% LD <sub>100</sub> : 0.09%	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.6% LD <sub>100</sub> : 1.3%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.9%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.7%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.1%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%
<i>Gregarina blattarum</i>	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.4% LD <sub>100</sub> : 0.7%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.4%	LD <sub>50</sub> : 0.2% LD <sub>100</sub> : 0.4%	LD <sub>50</sub> : 0.7% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.2% LD <sub>100</sub> : 0.5%
<i>Amoeba proteus</i>	LD <sub>50</sub> : 0.07% LD <sub>100</sub> : 0.15%	LD <sub>50</sub> : 0.3% LD <sub>100</sub> : 0.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.6% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.4%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.7%
<i>Paramecium caudatum</i>	LD <sub>50</sub> : 0.001% LD <sub>100</sub> : 0.006%	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.3%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 0.3% LD <sub>100</sub> : 0.5%	LD <sub>50</sub> : 0.3% LD <sub>100</sub> : 0.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%
<i>Pentatrichomonas hominis</i>	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 0.05% LD <sub>100</sub> : 0.14%	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.3%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.2% LD <sub>100</sub> : 0.4%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%

<sup>a</sup>—chloramphenicol, <sup>b</sup>—metronidazole, <sup>c</sup>—in rate 1:1:1, <sup>d</sup>—10% solution, <sup>e</sup>—10% solution, <sup>f</sup>—10% solution, <sup>g</sup>—5% solution, n.t—not tested.

**Table 3.** LD<sub>50</sub>, LD<sub>100</sub> values for the tested mixtures of eucalyptus essential oil (*Eucalyptus globulus* Labill.) (E), organic acids (Acetic acid—A, Propionic acid—P, Lactic acid—L, Mixture of acids—M) and metal ion against selected protozoa.

Protozoa	Eucalyptus Essential Oil ( <i>Eucalyptus globulus</i> Labill.)											
	Acetic Acid			Propionic Acid			Lactic Acid			Mixture of Acids <sup>a</sup>		
	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>
	EACu	EAMn	EAZn	EPCu	EP Mn	EP Zn	ELCu	EL Mn	EL Zn	EMCu	EM Mn	EM Zn
<i>Euglena gracilis</i> <sup>1</sup>	LD <sub>50</sub> : 0.04% ± 0.035 ab	LD <sub>50</sub> : 0.03% ± 0.032 ab	LD <sub>50</sub> : 0.03% ± 0.032 ab	LD <sub>50</sub> : 0.01% ± 0.015 b	LD <sub>50</sub> : 0.04% ± 0.038 ab	LD <sub>50</sub> : 0.03% ± 0.028 ab	LD <sub>50</sub> : 0.03% ± 0.032 ab	LD <sub>50</sub> : 0.01% ± 0.102 a	LD <sub>50</sub> : 0.04% ± 0.038 ab	LD <sub>50</sub> : 0.03% ± 0.032 ab	LD <sub>50</sub> : 0.03% ± 0.028 ab	LD <sub>50</sub> : 0.01% ± 0.102 a
	LD <sub>100</sub> : 0.08% ± 0.068 abc	LD <sub>100</sub> : 0.06% ± 0.058 abcd	LD <sub>100</sub> : 0.07% ± 0.065 abc	LD <sub>100</sub> : 0.03% ± 0.025 d	LD <sub>100</sub> : 0.08% ± 0.078 a	LD <sub>100</sub> : 0.06% ± 0.070 ab	LD <sub>100</sub> : 0.07% ± 0.069 abc	LD <sub>100</sub> : 0.03% ± 0.032 bcd	LD <sub>100</sub> : 0.08% ± 0.080 a	LD <sub>100</sub> : 0.06% ± 0.088 a	LD <sub>100</sub> : 0.07% ± 0.068 abc	LD <sub>100</sub> : 0.03% ± 0.030 cd
<i>Gregarina blattarum</i> <sup>1</sup>	LD <sub>50</sub> : 0.04% ± 0.038 a	LD <sub>50</sub> : 0.03% ± 0.034 a	LD <sub>50</sub> : 0.03% ± 0.032 a	LD <sub>50</sub> : 0.03% ± 0.034 a	LD <sub>50</sub> : 0.04% ± 0.040 a	LD <sub>50</sub> : 0.03% ± 0.031 a	LD <sub>50</sub> : 0.03% ± 0.028 a	LD <sub>50</sub> : 0.03% ± 0.028 a	LD <sub>50</sub> : 0.04% ± 0.042 a	LD <sub>50</sub> : 0.03% ± 0.035 a	LD <sub>50</sub> : 0.03% ± 0.025 a	LD <sub>50</sub> : 0.03% ± 0.035 a
	LD <sub>100</sub> : 0.05% ± 0.045 b	LD <sub>100</sub> : 0.07% ± 0.065 b	LD <sub>100</sub> : 0.06% ± 0.060 b	LD <sub>100</sub> : 0.04% ± 0.038 b	LD <sub>100</sub> : 0.05% ± 0.052 b	LD <sub>100</sub> : 0.07% ± 0.068 b	LD <sub>100</sub> : 0.06% ± 0.062 b	LD <sub>100</sub> : 0.04% ± 0.042 b	LD <sub>100</sub> : 0.05% ± 0.049 b	LD <sub>100</sub> : 0.07% ± 0.248 a	LD <sub>100</sub> : 0.06% ± 0.056 b	LD <sub>100</sub> : 0.04% ± 0.041 b
<i>Amoeba proteus</i> <sup>1</sup>	LD <sub>50</sub> : 0.03% ± 0.028 c	LD <sub>50</sub> : 0.04% ± 0.045 abc	LD <sub>50</sub> : 0.05% ± 0.052 abc	LD <sub>50</sub> : 0.07% ± 0.065 ab	LD <sub>50</sub> : 0.03% ± 0.032 c	LD <sub>50</sub> : 0.04% ± 0.042 bc	LD <sub>50</sub> : 0.05% ± 0.049 abc	LD <sub>50</sub> : 0.07% ± 0.072 a	LD <sub>50</sub> : 0.03% ± 0.031 c	LD <sub>50</sub> : 0.04% ± 0.045 abc	LD <sub>50</sub> : 0.05% ± 0.050 abc	LD <sub>50</sub> : 0.07% ± 0.070 ab
	LD <sub>100</sub> : 0.06% ± 0.062 a	LD <sub>100</sub> : 0.08% ± 0.076 a	LD <sub>100</sub> : 0.09% ± 0.082 a	LD <sub>100</sub> : 0.08% ± 0.075 a	LD <sub>100</sub> : 0.06% ± 0.062 a	LD <sub>100</sub> : 0.08% ± 0.080 a	LD <sub>100</sub> : 0.09% ± 0.085 a	LD <sub>100</sub> : 0.08% ± 0.082 a	LD <sub>100</sub> : 0.05% ± 0.055 a	LD <sub>100</sub> : 0.08% ± 0.082 a	LD <sub>100</sub> : 0.09% ± 0.078 a	LD <sub>100</sub> : 0.08% ± 0.075 a
<i>Paramecium caudatum</i> <sup>1</sup>	LD <sub>50</sub> : 0.02% ± 0.020 a	LD <sub>50</sub> : 0.02% ± 0.022 a	LD <sub>50</sub> : 0.04% ± 0.040 a	LD <sub>50</sub> : 0.03% ± 0.032 a	LD <sub>50</sub> : 0.02% ± 0.022 a	LD <sub>50</sub> : 0.02% ± 0.022 a	LD <sub>50</sub> : 0.04% ± 0.042 a	LD <sub>50</sub> : 0.03% ± 0.032 a	LD <sub>50</sub> : 0.02% ± 0.024 a	LD <sub>50</sub> : 0.02% ± 0.024 a	LD <sub>50</sub> : 0.04% ± 0.032 a	LD <sub>50</sub> : 0.03% ± 0.032 a
	LD <sub>100</sub> : 0.06% ± 0.062 a	LD <sub>100</sub> : 0.07% ± 0.070 a	LD <sub>100</sub> : 0.07% ± 0.072 a	LD <sub>100</sub> : 0.08% ± 0.075 a	LD <sub>100</sub> : 0.06% ± 0.062 a	LD <sub>100</sub> : 0.07% ± 0.072 a	LD <sub>100</sub> : 0.07% ± 0.071 a	LD <sub>100</sub> : 0.08% ± 0.075 a	LD <sub>100</sub> : 0.06% ± 0.065 a	LD <sub>100</sub> : 0.07% ± 0.069 a	LD <sub>100</sub> : 0.07% ± 0.065 a	LD <sub>100</sub> : 0.08% ± 0.078 a

Table 3. Cont.

Protozoa	Eucalyptus Essential Oil ( <i>Eucalyptus globulus</i> Labill.)											
	Acetic Acid			Propionic Acid			Lactic Acid			Mixture of Acids <sup>a</sup>		
	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>
	EACu	EAMn	EAZn	EPCu	EPMn	EPZn	ELCu	ELMn	ELZn	EMCu	EMMn	EMZn
<i>Pentatrichomonas hominis</i> <sup>1</sup>	LD <sub>50</sub> : 0.04% ± 0.035 a	LD <sub>50</sub> : 0.03% ± 0.032 a	LD <sub>50</sub> : 0.05% ± 0.050 a	LD <sub>50</sub> : 0.03% ± 0.032 a	LD <sub>50</sub> : 0.04% ± 0.040 a	LD <sub>50</sub> : 0.03% ± 0.030 a	LD <sub>50</sub> : 0.05% ± 0.045 a	LD <sub>50</sub> : 0.03% ± 0.035 a	LD <sub>50</sub> : 0.04% ± 0.040 a	LD <sub>50</sub> : 0.03% ± 0.048 a	LD <sub>50</sub> : 0.05% ± 0.024 a	LD <sub>50</sub> : 0.03% ± 0.052 a
	LD <sub>100</sub> : 0.07% ± 0.072 ab	LD <sub>100</sub> : 0.05% ± 0.045 b	LD <sub>100</sub> : 0.09% ± 0.085 ab	LD <sub>100</sub> : 0.04% ± 0.042 b	LD <sub>100</sub> : 0.07% ± 0.065 ab	LD <sub>100</sub> : 0.05% ± 0.050 b	LD <sub>100</sub> : 0.09% ± 0.085 ab	LD <sub>100</sub> : 0.04% ± 0.035 b	LD <sub>100</sub> : 0.07% ± 0.068 ab	LD <sub>100</sub> : 0.05% ± 0.052 b	LD <sub>100</sub> : 0.09% ± 0.125 a	LD <sub>100</sub> : 0.04% ± 0.040 b

<sup>1</sup> Values followed by the same letter within a row are not significantly different ( $p > 0.05$ , Tukey's test), <sup>a</sup>—in rate 1:1:1, <sup>b</sup>—10% solution, <sup>c</sup>—10% solution, <sup>d</sup>—10% solution, EACu, EAMn, EAZn—Eucalyptus essential oil (E) with acetic acid (A) and Cu, Mn, Zn ions, respectively; EPCu, EPMn, EPZn—Eucalyptus essential oil (E) with propionic acid (P) and Cu, Mn, Zn ions, respectively; ELCu, ELMn, ELZn—Eucalyptus essential oil (E) with lactic acid (L) and Cu, Mn, Zn ions, respectively; EMCu, EMMn, EMZn—Eucalyptus essential oil (E) with mixture of acids (M) and Cu, Mn, Zn ions, respectively.

Table 4. LD<sub>50</sub>, LD<sub>100</sub> values of tea tree essential oil (*Melaleuca alternifolia* (Maiden & Betche) Cheel) and the components used in the study.

Protozoa	CH <sup>a</sup>	M <sup>b</sup>	Acetic Acid	Propionic Acid	Lactic Acid	Mixture of Acids <sup>c</sup>	Manganese (II) Chloride Solution <sup>d</sup>	Copper (II) Carbonate Hydroxide Solution <sup>e</sup>	Zinc Carbonate Solution <sup>f</sup>	Catalyst Solution <sup>g</sup>	Tea Tree Essential Oil ( <i>Melaleuca alternifolia</i> (Maiden & Betche) Cheel)
<i>Euglena gracilis</i>	LD <sub>50</sub> : 0.05% LD <sub>100</sub> : 0.09%	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.6% LD <sub>100</sub> : 1.3%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.9%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.7%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.1%	LD <sub>50</sub> : 0.05% LD <sub>100</sub> : 0.1%
<i>Gregarina blattarum</i>	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.4% LD <sub>100</sub> : 0.7%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.4%	LD <sub>50</sub> : 0.2% LD <sub>100</sub> : 0.4%	LD <sub>50</sub> : 0.7% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.25% LD <sub>100</sub> : 0.3%
<i>Amoeba proteus</i>	LD <sub>50</sub> : 0.07% LD <sub>100</sub> : 0.15%	LD <sub>50</sub> : 0.3% LD <sub>100</sub> : 0.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.6% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.4%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.3% LD <sub>100</sub> : 0.5%
<i>Paramecium caudatum</i>	LD <sub>50</sub> : 0.001% LD <sub>100</sub> : 0.006%	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.3%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 0.3% LD <sub>100</sub> : 0.5%	LD <sub>50</sub> : 0.3% LD <sub>100</sub> : 0.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 0.2% LD <sub>100</sub> : 0.25%
<i>Pentatrichomonas hominis</i>	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 0.05% LD <sub>100</sub> : 0.14%	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.3%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.2% LD <sub>100</sub> : 0.4%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.08% LD <sub>100</sub> : 0.1%

<sup>a</sup>—chloramphenicol, <sup>b</sup>—metronidazole, <sup>c</sup>—in rate 1:1:1, <sup>d</sup>—10% solution, <sup>e</sup>—10% solution, <sup>f</sup>—10% solution, <sup>g</sup>—5% solution, n.t—not tested.

**Table 5.** LD<sub>50</sub>, LD<sub>100</sub> values of tea tree essential oil (*Melaleuca alternifolia* (Maiden & Betche) Cheel) (T), organic acids (Acetic acid—A, Propionic acid—P, Lactic acid—L, Mixture of acids—M) and metal ion against selected protozoa.

Protozoa	Tea Tree Essential Oil ( <i>Melaleuca alternifolia</i> (Maiden & Betche) Cheel)											
	Acetic Acid			Propionic Acid			Lactic Acid			Mixture of Acids <sup>a</sup>		
	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>
	TACu	TAMn	TAZn	TPCu	TPMn	TPZn	TLCu	TLMn	TLZn	TMCu	TMMn	TMZn
<i>Euglena gracilis</i> <sup>1</sup>	LD <sub>50</sub> : 0.03% ± 0.032 a	LD <sub>50</sub> : 0.04% ± 0.035 a	LD <sub>50</sub> : 0.03% ± 0.052 abc	LD <sub>50</sub> : 0.03% ± 0.026 a	LD <sub>50</sub> : 0.01% ± 0.0134 ab	LD <sub>50</sub> : 0.02% ± 0.020 ab	LD <sub>50</sub> : 0.02% ± 0.021 ab	LD <sub>50</sub> : 0.02% ± 0.022 ab	LD <sub>50</sub> : 0.02% ± 0.022 ab	LD <sub>50</sub> : 0.001% ± 0.001 b	LD <sub>50</sub> : 0.001% ± 0.001 b	LD <sub>50</sub> : 0.003% ± 0.002 b
	LD <sub>100</sub> : 0.06% ± 0.059 ab	LD <sub>100</sub> : 0.07% ± 0.065 a	LD <sub>100</sub> : 0.05% ± 0.052 abc	LD <sub>100</sub> : 0.04% ± 0.035 bcd	LD <sub>100</sub> : 0.02% ± 0.018 de	LD <sub>100</sub> : 0.03% ± 0.028 cde	LD <sub>100</sub> : 0.04% ± 0.038 bcd	LD <sub>100</sub> : 0.04% ± 0.042 abcd	LD <sub>100</sub> : 0.05% ± 0.052 abc	LD <sub>100</sub> : 0.003% ± 0.002 e	LD <sub>100</sub> : 0.002% ± 0.002 e	LD <sub>100</sub> : 0.004% ± 0.004 e
<i>Gregarina blattarum</i> <sup>1</sup>	LD <sub>50</sub> : 0.04% ± 0.038 b	LD <sub>50</sub> : 0.04% ± 0.036 b	LD <sub>50</sub> : 0.04% ± 0.035 b	LD <sub>50</sub> : 0.04% ± 0.036 b	LD <sub>50</sub> : 0.05% ± 0.048 b	LD <sub>50</sub> : 0.05% ± 0.051 b	LD <sub>50</sub> : 0.03% ± 0.031 b	LD <sub>50</sub> : 0.05% ± 0.049 b	LD <sub>50</sub> : 0.08% ± 0.075 a	LD <sub>50</sub> : 0.004% ± 0.004 c	LD <sub>50</sub> : 0.004% ± 0.004 c	LD <sub>50</sub> : 0.003% ± 0.002 c
	LD <sub>100</sub> : 0.07% ± 0.065 b	LD <sub>100</sub> : 0.05% ± 0.052 bcd	LD <sub>100</sub> : 0.06% ± 0.060 b	LD <sub>100</sub> : 0.06% ± 0.058 bc	LD <sub>100</sub> : 0.08% ± 0.078 b	LD <sub>100</sub> : 0.07% ± 0.068 b	LD <sub>100</sub> : 0.04% ± 0.038 bcd	LD <sub>100</sub> : 0.07% ± 0.065 b	LD <sub>100</sub> : 0.15% ± 0.138 a	LD <sub>100</sub> : 0.006% ± 0.006 d	LD <sub>100</sub> : 0.007% ± 0.007 cd	LD <sub>100</sub> : 0.005% ± 0.004 d
<i>Amoeba proteus</i> <sup>1</sup>	LD <sub>50</sub> : 0.04% ± 0.038 bc	LD <sub>50</sub> : 0.03% ± 0.035 cd	LD <sub>50</sub> : 0.04% ± 0.042 bc	LD <sub>50</sub> : 0.04% ± 0.042 bc	LD <sub>50</sub> : 0.01% ± 0.014 de	LD <sub>50</sub> : 0.07% ± 0.072 a	LD <sub>50</sub> : 0.06% ± 0.058 ab	LD <sub>50</sub> : 0.01% ± 0.012 e	LD <sub>50</sub> : 0.01% ± 0.016 de	LD <sub>50</sub> : 0.002% ± 0.002 e	LD <sub>50</sub> : 0.002% ± 0.002 e	LD <sub>50</sub> : 0.001% ± 0.001 e
	LD <sub>100</sub> : 0.06% ± 0.062 bc	LD <sub>100</sub> : 0.06% ± 0.064 bc	LD <sub>100</sub> : 0.07% ± 0.072 abc	LD <sub>100</sub> : 0.07% ± 0.072 abc	LD <sub>100</sub> : 0.01% ± 0.014 de	LD <sub>100</sub> : 0.09% ± 0.092 a	LD <sub>100</sub> : 0.08% ± 0.082 ab	LD <sub>100</sub> : 0.03% ± 0.028 d	LD <sub>100</sub> : 0.05% ± 0.052 c	LD <sub>100</sub> : 0.003% ± 0.004 de	LD <sub>100</sub> : 0.004% ± 0.003 e	LD <sub>100</sub> : 0.002% ± 0.002 e
<i>Paramecium caudatum</i> <sup>1</sup>	LD <sub>50</sub> : 0.02% ± 0.022 abcd	LD <sub>50</sub> : 0.02% ± 0.020 abcd	LD <sub>50</sub> : 0.03% ± 0.032 abcd	LD <sub>50</sub> : 0.05% ± 0.045 ab	LD <sub>50</sub> : 0.01% ± 0.012 bcd	LD <sub>50</sub> : 0.05% ± 0.050 a	LD <sub>50</sub> : 0.05% ± 0.049 a	LD <sub>50</sub> : 0.03% ± 0.035 abc	LD <sub>50</sub> : 0.02% ± 0.024 abcd	LD <sub>50</sub> : 0.002% ± 0.002 cd	LD <sub>50</sub> : 0.001% ± 0.001 d	LD <sub>50</sub> : 0.003% ± 0.003 cd
	LD <sub>100</sub> : 0.05% ± 0.052 bcd	LD <sub>100</sub> : 0.05% ± 0.045 cde	LD <sub>100</sub> : 0.06% ± 0.062 abc	LD <sub>100</sub> : 0.08% ± 0.075 ab	LD <sub>100</sub> : 0.02% ± 0.022 ef	LD <sub>100</sub> : 0.09% ± 0.085 a	LD <sub>100</sub> : 0.07% ± 0.068 abc	LD <sub>100</sub> : 0.04% ± 0.042 cde	LD <sub>100</sub> : 0.03% ± 0.032 de	LD <sub>100</sub> : 0.005% ± 0.005 f	LD <sub>100</sub> : 0.002% ± 0.002 f	LD <sub>100</sub> : 0.005% ± 0.005 f
<i>Pentatrachomonas hominis</i> <sup>1</sup>	LD <sub>50</sub> : 0.09% ± 0.085 a	LD <sub>50</sub> : 0.08% ± 0.075 a	LD <sub>50</sub> : 0.07% ± 0.065 ab	LD <sub>50</sub> : 0.03% ± 0.032 cd	LD <sub>50</sub> : 0.01% ± 0.012 de	LD <sub>50</sub> : 0.07% ± 0.065 ab	LD <sub>50</sub> : 0.025% ± 0.025 cde	LD <sub>50</sub> : 0.05% ± 0.045 bc	LD <sub>50</sub> : 0.02% ± 0.020 cde	LD <sub>50</sub> : 0.004% ± 0.004 e	LD <sub>50</sub> : 0.007% ± 0.007 de	LD <sub>50</sub> : 0.002% ± 0.002 e
	LD <sub>100</sub> : 0.35% ± 0.362 a	LD <sub>100</sub> : 0.2% ± 0.238 a	LD <sub>100</sub> : 0.25% ± 0.238 a	LD <sub>100</sub> : 0.05% ± 0.045 b	LD <sub>100</sub> : 0.04% ± 0.035 b	LD <sub>100</sub> : 0.09% ± 0.088 b	LD <sub>100</sub> : 0.045% ± 0.046 b	LD <sub>100</sub> : 0.07% ± 0.072 b	LD <sub>100</sub> : 0.05% ± 0.045 b	LD <sub>100</sub> : 0.004% ± 0.004 b	LD <sub>100</sub> : 0.009% ± 0.008 b	LD <sub>100</sub> : 0.004% ± 0.002 b

<sup>1</sup> Values followed by the same letter within a row are not significantly different ( $p > 0.05$ , Tukey's test), <sup>a</sup>—in rate 1:1:1, <sup>b</sup>—10% solution, <sup>c</sup>—10% solution, <sup>d</sup>—10% solution, TACu, TAMn, TAZn—Tea tree essential oil (T) with acetic acid (A) and Cu, Mn, Zn ions, respectively; TPCu, TPMn, TPZn—Tea tree essential oil (T) with propionic acid (P) and Cu, Mn, Zn ions, respectively; TLCu, TLMn, TLZn—Tea tree essential oil (T) with lactic acid (L) and Cu, Mn, Zn ions, respectively; TMCu, TMMn, TMZn—Tea tree essential oil (T) with mixture of acids (M) and Cu, Mn, Zn ions, respectively.

**Table 6.** LD<sub>50</sub>, LD<sub>100</sub> values of cedar essential oil (*Cedrus sp.*) and the components used in the study.

Protozoa	CH <sup>a</sup>	M <sup>b</sup>	Acetic Acid	Propionic Acid	Lactic Acid	Mixture of Acids <sup>c</sup>	Manganese (II) Chloride Solution <sup>d</sup>	Copper (II) Carbonate Hydroxide Solution <sup>e</sup>	Zinc Carbonate Solution <sup>f</sup>	Catalyst Solution <sup>g</sup>	Cedar Essential Oil ( <i>Cedrus sp.</i> )
<i>Euglena gracilis</i>	LD <sub>50</sub> : 0.05% LD <sub>100</sub> : 0.09%	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.6% LD <sub>100</sub> : 1.3%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.9%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.7%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.1%	LD <sub>50</sub> : 0.7% LD <sub>100</sub> : 0.9%
<i>Gregarina blattarum</i>	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.4% LD <sub>100</sub> : 0.7%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.4%	LD <sub>50</sub> : 0.2% LD <sub>100</sub> : 0.4%	LD <sub>50</sub> : 0.7% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.7% LD <sub>100</sub> : 0.9%
<i>Amoeba proteus</i>	LD <sub>50</sub> : 0.07% LD <sub>100</sub> : 0.15%	LD <sub>50</sub> : 0.3% LD <sub>100</sub> : 0.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.6% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.4%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.4% LD <sub>100</sub> : 0.6%
<i>Paramecium caudatum</i>	LD <sub>50</sub> : 0.001% LD <sub>100</sub> : 0.006%	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.3%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 0.3% LD <sub>100</sub> : 0.5%	LD <sub>50</sub> : 0.3% LD <sub>100</sub> : 0.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.25%
<i>Pentatrichomonas hominis</i>	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 0.05% LD <sub>100</sub> : 0.14%	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.3%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.2% LD <sub>100</sub> : 0.4%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%

<sup>a</sup>—chloramphenicol, <sup>b</sup>—metronidazole, <sup>c</sup>—in rate 1:1:1, <sup>d</sup>—10% solution, <sup>e</sup>—10% solution, <sup>f</sup>—10% solution, <sup>g</sup>—5% solution, n.t—not tested.

**Table 7.** LD<sub>50</sub>, LD<sub>100</sub> values of cedar essential oil (*Cedrus sp.*) (C), organic acids (Acetic acid—A, Propionic acid—P, Lactic acid—L, Mixture of acids—M) and metal ion against selected protozoa.

Protozoa	Cedar Essential Oil ( <i>Cedrus sp.</i> )											
	Acetic Acid			Propionic Acid			Lactic Acid			Mixture of Acids <sup>a</sup>		
	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>
	CACu	CAMn	CAZn	CPCu	CPMn	CPZn	CLCu	CLMn	CLZn	CMCu	CMMn	CMZn
<i>Euglena gracilis</i> <sup>1</sup>	LD <sub>50</sub> : 0.04% ± 0.035 a	LD <sub>50</sub> : 0.03% ± 0.033 a	LD <sub>50</sub> : 0.04% ± 0.035 a	LD <sub>50</sub> : 0.03% ± 0.026 a	LD <sub>50</sub> : 0.01% ± 0.014 ab	LD <sub>50</sub> : 0.02% ± 0.020 ab	LD <sub>50</sub> : 0.01% ± 0.014 ab	LD <sub>50</sub> : 0.02% ± 0.023 ab	LD <sub>50</sub> : 0.03% ± 0.025 a	LD <sub>50</sub> : 0.001% ± 0.001 b	LD <sub>50</sub> : 0.002% ± 0.002 b	LD <sub>50</sub> : 0.003% ± 0.003 b
	LD <sub>100</sub> : 0.08% ± 0.079 a	LD <sub>100</sub> : 0.07% ± 0.065 abc	LD <sub>100</sub> : 0.07% ± 0.068 ab	LD <sub>100</sub> : 0.05% ± 0.048 bcd	LD <sub>100</sub> : 0.02% ± 0.018 fg	LD <sub>100</sub> : 0.03% ± 0.028 def	LD <sub>100</sub> : 0.02% ± 0.023 efg	LD <sub>100</sub> : 0.04% ± 0.043 cde	LD <sub>100</sub> : 0.06% ± 0.055 bc	LD <sub>100</sub> : 0.002% ± 0.002 g	LD <sub>100</sub> : 0.004% ± 0.004 g	LD <sub>100</sub> : 0.006% ± 0.007 fg
<i>Gregarina blattarum</i> <sup>1</sup>	LD <sub>50</sub> : 0.03% ± 0.032 a	LD <sub>50</sub> : 0.02% ± 0.022 ab	LD <sub>50</sub> : 0.04% ± 0.035 a	LD <sub>50</sub> : 0.03% ± 0.034 a	LD <sub>50</sub> : 0.03% ± 0.033 a	LD <sub>50</sub> : 0.03% ± 0.031 a	LD <sub>50</sub> : 0.03% ± 0.031 a	LD <sub>50</sub> : 0.02% ± 0.022 ab	LD <sub>50</sub> : 0.04% ± 0.043 a	LD <sub>50</sub> : 0.003% ± 0.003 b	LD <sub>50</sub> : 0.002% ± 0.002 b	LD <sub>50</sub> : 0.004% ± 0.004 b
	LD <sub>100</sub> : 0.06% ± 0.062 abc	LD <sub>100</sub> : 0.05% ± 0.052 bc	LD <sub>100</sub> : 0.08% ± 0.082 a	LD <sub>100</sub> : 0.04% ± 0.042 c	LD <sub>100</sub> : 0.07% ± 0.070 ab	LD <sub>100</sub> : 0.05% ± 0.052 bc	LD <sub>100</sub> : 0.04% ± 0.038 c	LD <sub>100</sub> : 0.05% ± 0.049 bc	LD <sub>100</sub> : 0.05% ± 0.045 bc	LD <sub>100</sub> : 0.004% ± 0.004 d	LD <sub>100</sub> : 0.005% ± 0.005 d	LD <sub>100</sub> : 0.005% ± 0.005 d
<i>Amoeba proteus</i> <sup>1</sup>	LD <sub>50</sub> : 0.04% ± 0.038 ab	LD <sub>50</sub> : 0.05% ± 0.052 a	LD <sub>50</sub> : 0.06% ± 0.062 a	LD <sub>50</sub> : 0.05% ± 0.045 ab	LD <sub>50</sub> : 0.05% ± 0.045 ab	LD <sub>50</sub> : 0.04% ± 0.038 ab	LD <sub>50</sub> : 0.06% ± 0.058 a	LD <sub>50</sub> : 0.05% ± 0.045 ab	LD <sub>50</sub> : 0.02% ± 0.019 bc	LD <sub>50</sub> : 0.006% ± 0.006 c	LD <sub>50</sub> : 0.005% ± 0.005 c	LD <sub>50</sub> : 0.002% ± 0.002 c
	LD <sub>100</sub> : 0.05% ± 0.052 b	LD <sub>100</sub> : 0.08% ± 0.076 ab	LD <sub>100</sub> : 0.01% ± 0.014 c	LD <sub>100</sub> : 0.07% ± 0.072 ab	LD <sub>100</sub> : 0.08% ± 0.078 ab	LD <sub>100</sub> : 0.06% ± 0.058 ab	LD <sub>100</sub> : 0.08% ± 0.082 a	LD <sub>100</sub> : 0.07% ± 0.072 ab	LD <sub>100</sub> : 0.05% ± 0.052 b	LD <sub>100</sub> : 0.008% ± 0.008 c	LD <sub>100</sub> : 0.007% ± 0.007 c	LD <sub>100</sub> : 0.005% ± 0.005 c

Table 7. Cont.

Protozoa	Cedar Essential Oil ( <i>Cedrus</i> sp.)											
	Acetic Acid			Propionic Acid			Lactic Acid			Mixture of Acids <sup>a</sup>		
	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>
	CACu	CAMn	CAZn	CPCu	CPMn	CPZn	CLCu	CLMn	CLZn	CMCu	CMMn	CMZn
<i>Paramecium caudatum</i> <sup>1</sup>	LD <sub>50</sub> : 0.03% ± 0.025 ab LD <sub>100</sub> : 0.08% ± 0.082 a	LD <sub>50</sub> : 0.04% ± 0.040 a LD <sub>100</sub> : 0.07% ± 0.072 a	LD <sub>50</sub> : 0.05% ± 0.048 a LD <sub>100</sub> : 0.08% ± 0.075 a	LD <sub>50</sub> : 0.04% ± 0.042 a LD <sub>100</sub> : 0.08% ± 0.075 a	LD <sub>50</sub> : 0.04% ± 0.042 a LD <sub>100</sub> : 0.06% ± 0.060 abc	LD <sub>50</sub> : 0.04% ± 0.040 a LD <sub>100</sub> : 0.07% ± 0.068 ab	LD <sub>50</sub> : 0.03% ± 0.030 ab LD <sub>100</sub> : 0.04% ± 0.042 bc	LD <sub>50</sub> : 0.03% ± 0.035 a LD <sub>100</sub> : 0.08% ± 0.078 a	LD <sub>50</sub> : 0.02% ± 0.024 ab LD <sub>100</sub> : 0.04% ± 0.035 cd	LD <sub>50</sub> : 0.003% ± 0.002 b LD <sub>100</sub> : 0.004% ± 0.004 e	LD <sub>50</sub> : 0.003% ± 0.002 b LD <sub>100</sub> : 0.008% ± 0.008 de	LD <sub>50</sub> : 0.002% ± 0.002 b LD <sub>100</sub> : 0.004% ± 0.004 e
<i>Pentatrachomonas hominis</i> <sup>1</sup>	LD <sub>50</sub> : 0.09% ± 0.085 a LD <sub>100</sub> : 0.15% ± 0.150 a	LD <sub>50</sub> : 0.02% ± 0.020 bc LD <sub>100</sub> : 0.04% ± 0.042 bcde	LD <sub>50</sub> : 0.04% ± 0.040 b LD <sub>100</sub> : 0.07% ± 0.072 b	LD <sub>50</sub> : 0.04% ± 0.035 b LD <sub>100</sub> : 0.06% ± 0.058 bc	LD <sub>50</sub> : 0.03% ± 0.025 bc LD <sub>100</sub> : 0.04% ± 0.040 bcde	LD <sub>50</sub> : 0.03% ± 0.025 bc LD <sub>100</sub> : 0.05% ± 0.048 bcd	LD <sub>50</sub> : 0.03% ± 0.032 b LD <sub>100</sub> : 0.04% ± 0.042 bcde	LD <sub>50</sub> : 0.02% ± 0.022 bc LD <sub>100</sub> : 0.03% ± 0.025 cde	LD <sub>50</sub> : 0.025% ± 0.026 bc LD <sub>100</sub> : 0.07% ± 0.068 b	LD <sub>50</sub> : 0.003% ± 0.002 c LD <sub>100</sub> : 0.004% ± 0.004 e	LD <sub>50</sub> : 0.002% ± 0.002 c LD <sub>100</sub> : 0.003% ± 0.002 e	LD <sub>50</sub> : 0.002% ± 0.002 c LD <sub>100</sub> : 0.007% ± 0.006 de

<sup>1</sup> Values followed by the same letter within a row are not significantly different ( $p > 0.05$ , Tukey's test), <sup>a</sup>—in rate 1:1:1, <sup>b</sup>—10% solution, <sup>c</sup>—10% solution, <sup>d</sup>—10% solution; CACu, CAMn, CAZn—Cedar essential oil (C) with acetic acid (A) and Cu, Mn, Zn ions, respectively; CPCu, CPMn, CPZn—Cedar essential oil (C) with propionic acid (P) and Cu, Mn, Zn ions, respectively; CLCu, CLMn, CLZn—Cedar essential oil (C) with lactic acid (L) and Cu, Mn, Zn ions, respectively; CMCu, CMMn, CMZn—Cedar essential oil (C) with mixture of acids (M) and Cu, Mn, Zn ions, respectively.

Table 8. LD<sub>50</sub>, LD<sub>100</sub> values of lavender essential oil (*Lavandula angustifolia* Miller) and the components used in the study.

Protozoa	CH <sup>a</sup>	M <sup>b</sup>	Acetic Acid	Propionic Acid	Lactic Acid	Mixture of Acids <sup>c</sup>	Manganese (II) Chloride Solution <sup>d</sup>	Copper (II) Carbonate Hydroxide Solution <sup>e</sup>	Zinc Carbonate Solution <sup>f</sup>	Catalyst Solution <sup>g</sup>	Lavender Essential Oil ( <i>Lavandula angustifolia</i> Miller)
<i>Euglena gracilis</i>	LD <sub>50</sub> : 0.05% LD <sub>100</sub> : 0.09%	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.6% LD <sub>100</sub> : 1.3%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.9%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.7%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.1%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 0.9%
<i>Gregarina blattarum</i>	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.4% LD <sub>100</sub> : 0.7%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.4%	LD <sub>50</sub> : 0.2% LD <sub>100</sub> : 0.4%	LD <sub>50</sub> : 0.7% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.7% LD <sub>100</sub> : 0.8%
<i>Amoeba proteus</i>	LD <sub>50</sub> : 0.07% LD <sub>100</sub> : 0.15%	LD <sub>50</sub> : 0.3% LD <sub>100</sub> : 0.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.6% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.4%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.2%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.5% LD <sub>100</sub> : 0.8%
<i>Paramecium caudatum</i>	LD <sub>50</sub> : 0.001% LD <sub>100</sub> : 0.006%	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.3%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 0.3% LD <sub>100</sub> : 0.5%	LD <sub>50</sub> : 0.3% LD <sub>100</sub> : 0.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.2%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.25%
<i>Pentatrachomonas hominis</i>	LD <sub>50</sub> : n.t LD <sub>100</sub> : n.t	LD <sub>50</sub> : 0.05% LD <sub>100</sub> : 0.14%	LD <sub>50</sub> : 1.0% LD <sub>100</sub> : 1.5%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.3%	LD <sub>50</sub> : 0.8% LD <sub>100</sub> : 1.0%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.1% LD <sub>100</sub> : 0.3%	LD <sub>50</sub> : 0.2% LD <sub>100</sub> : 0.4%	LD <sub>50</sub> : 0.9% LD <sub>100</sub> : 1.1%	LD <sub>50</sub> : 0.2% LD <sub>100</sub> : 0.4%

<sup>a</sup>—chloramphenicol, <sup>b</sup>—metronidazole, <sup>c</sup>—in rate 1:1:1, <sup>d</sup>—10% solution, <sup>e</sup>—10% solution, <sup>f</sup>—10% solution, <sup>g</sup>—5% solution, n.t—not tested.



**Table 9.** LD<sub>50</sub>, LD<sub>100</sub> values of lavender essential oil (*Lavandula angustifolia* Miller) (L), organic acids (Acetic acid—A, Propionic acid—P, Lactic acid—L, Mixture of acids—M) and metal ion against selected protozoal.

Protozoa	Lavender Essential Oil ( <i>Lavandula angustifolia</i> Miller)											
	Acetic Acid			Propionic Acid			Lactic Acid			Mixture of Acids <sup>a</sup>		
	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>	Cu <sup>b</sup>	Mn <sup>c</sup>	Zn <sup>d</sup>
	LACu	LAMn	LAZn	LPCu	LPMn	LPZn	LLCu	LLMn	LLZn	LMCu	LMMn	LMZn
<i>Euglena gracilis</i> <sup>1</sup>	LD <sub>50</sub> : 0.04% ± 0.035 ab	LD <sub>50</sub> : 0.02% ± 0.024 bcd	LD <sub>50</sub> : 0.05% ± 0.045 a	LD <sub>50</sub> : 0.03% ± 0.026 abc	LD <sub>50</sub> : 0.01% ± 0.014 cde	LD <sub>50</sub> : 0.03% ± 0.028 abc	LD <sub>50</sub> : 0.02% ± 0.016 bcde	LD <sub>50</sub> : 0.03% ± 0.025 abc	LD <sub>50</sub> : 0.03% ± 0.025 abc	LD <sub>50</sub> : 0.002% ± 0.002 e	LD <sub>50</sub> : 0.001% ± 0.001 e	LD <sub>50</sub> : 0.004% ± 0.004 de
	LD <sub>100</sub> : 0.06% ± 0.059 ab	LD <sub>100</sub> : 0.04% ± 0.035 cd	LD <sub>100</sub> : 0.08% ± 0.078 a	LD <sub>100</sub> : 0.045% ± 0.046 bcd	LD <sub>100</sub> : 0.03% ± 0.025 de	LD <sub>100</sub> : 0.05% ± 0.050 bc	LD <sub>100</sub> : 0.04% ± 0.042 bcd	LD <sub>100</sub> : 0.05% ± 0.045 bcd	LD <sub>100</sub> : 0.05% ± 0.052 bc	LD <sub>100</sub> : 0.004% ± 0.004 e	LD <sub>100</sub> : 0.005% ± 0.004 e	LD <sub>100</sub> : 0.006% ± 0.006 e
<i>Gregarina blattarum</i> <sup>1</sup>	LD <sub>50</sub> : 0.03% ± 0.032 abc	LD <sub>50</sub> : 0.01% ± 0.011 cd	LD <sub>50</sub> : 0.03% ± 0.032 abc	LD <sub>50</sub> : 0.03% ± 0.034 ab	LD <sub>50</sub> : 0.03% ± 0.032 abc	LD <sub>50</sub> : 0.04% ± 0.036 ab	LD <sub>50</sub> : 0.05% ± 0.049 a	LD <sub>50</sub> : 0.02% ± 0.022 bcd	LD <sub>50</sub> : 0.05% ± 0.045 a	LD <sub>50</sub> : 0.003% ± 0.003 d	LD <sub>50</sub> : 0.002% ± 0.002 d	LD <sub>50</sub> : 0.004% ± 0.004 d
	LD <sub>100</sub> : 0.06% ± 0.062 ab	LD <sub>100</sub> : 0.04% ± 0.040 b	LD <sub>100</sub> : 0.08% ± 0.082 a	LD <sub>100</sub> : 0.045% ± 0.044 b	LD <sub>100</sub> : 0.04% ± 0.060 ab	LD <sub>100</sub> : 0.05% ± 0.052 b	LD <sub>100</sub> : 0.07% ± 0.065 ab	LD <sub>100</sub> : 0.04% ± 0.042 b	LD <sub>100</sub> : 0.09% ± 0.085 a	LD <sub>100</sub> : 0.004% ± 0.004 c	LD <sub>100</sub> : 0.005% ± 0.005 c	LD <sub>100</sub> : 0.006% ± 0.006 c
<i>Amoeba proteus</i> <sup>1</sup>	LD <sub>50</sub> : 0.05% ± 0.052 ab	LD <sub>50</sub> : 0.05% ± 0.052 ab	LD <sub>50</sub> : 0.06% ± 0.062 a	LD <sub>50</sub> : 0.04% ± 0.042 abc	LD <sub>50</sub> : 0.05% ± 0.045 ab	LD <sub>50</sub> : 0.04% ± 0.038 abc	LD <sub>50</sub> : 0.02% ± 0.016 cd	LD <sub>50</sub> : 0.04% ± 0.042 abc	LD <sub>50</sub> : 0.03% ± 0.026 bcd	LD <sub>50</sub> : 0.003% ± 0.003 d	LD <sub>50</sub> : 0.002% ± 0.002 d	LD <sub>50</sub> : 0.003% ± 0.002 d
	LD <sub>100</sub> : 0.06% ± 0.055 b	LD <sub>100</sub> : 0.07% ± 0.074 ab	LD <sub>100</sub> : 0.09% ± 0.085 a	LD <sub>100</sub> : 0.06% ± 0.060 ab	LD <sub>100</sub> : 0.07% ± 0.070 ab	LD <sub>100</sub> : 0.07% ± 0.068 ab	LD <sub>100</sub> : 0.05% ± 0.049 b	LD <sub>100</sub> : 0.07% ± 0.072 ab	LD <sub>100</sub> : 0.06% ± 0.055 b	LD <sub>100</sub> : 0.004% ± 0.004 c	LD <sub>100</sub> : 0.004% ± 0.004 c	LD <sub>100</sub> : 0.005% ± 0.005 c
<i>Paramecium caudatum</i> <sup>1</sup>	LD <sub>50</sub> : 0.05% ± 0.050 a	LD <sub>50</sub> : 0.05% ± 0.048 ab	LD <sub>50</sub> : 0.03% ± 0.032 abc	LD <sub>50</sub> : 0.05% ± 0.045 ab	LD <sub>50</sub> : 0.03% ± 0.032 abc	LD <sub>50</sub> : 0.03% ± 0.030 bc	LD <sub>50</sub> : 0.02% ± 0.018 cd	LD <sub>50</sub> : 0.04% ± 0.039 ab	LD <sub>50</sub> : 0.04% ± 0.035 abc	LD <sub>50</sub> : 0.006% ± 0.006 d	LD <sub>50</sub> : 0.005% ± 0.005 d	LD <sub>50</sub> : 0.004% ± 0.004 d
	LD <sub>100</sub> : 0.08% ± 0.082 a	LD <sub>100</sub> : 0.08% ± 0.075 a	LD <sub>100</sub> : 0.07% ± 0.072 a	LD <sub>100</sub> : 0.08% ± 0.075 a	LD <sub>100</sub> : 0.06% ± 0.060 ab	LD <sub>100</sub> : 0.08% ± 0.080 a	LD <sub>100</sub> : 0.04% ± 0.042 b	LD <sub>100</sub> : 0.07% ± 0.072 a	LD <sub>100</sub> : 0.06% ± 0.062 ab	LD <sub>100</sub> : 0.008% ± 0.008 c	LD <sub>100</sub> : 0.008% ± 0.008 c	LD <sub>100</sub> : 0.006% ± 0.006 c
<i>Pentatrichomonas hominis</i> <sup>1</sup>	LD <sub>50</sub> : 0.07% ± 0.070 ab	LD <sub>50</sub> : 0.07% ± 0.072 ab	LD <sub>50</sub> : 0.06% ± 0.040 cd	LD <sub>50</sub> : 0.08% ± 0.075 ab	LD <sub>50</sub> : 0.06% ± 0.025 de	LD <sub>50</sub> : 0.08% ± 0.080 a	LD <sub>50</sub> : 0.05% ± 0.048 bcd	LD <sub>50</sub> : 0.08% ± 0.075 ab	LD <sub>50</sub> : 0.055% ± 0.054 abc	LD <sub>50</sub> : 0.003% ± 0.002 e	LD <sub>50</sub> : 0.003% ± 0.002 e	LD <sub>50</sub> : 0.004% ± 0.004 e
	LD <sub>100</sub> : 0.1% ± 0.125 a	LD <sub>100</sub> : 0.09% ± 0.085 bc	LD <sub>100</sub> : 0.08% ± 0.075 bc	LD <sub>100</sub> : 0.1% ± 0.098 abc	LD <sub>100</sub> : 0.09% ± 0.092 abc	LD <sub>100</sub> : 0.1% ± 0.110 ab	LD <sub>100</sub> : 0.07% ± 0.072 c	LD <sub>100</sub> : 0.1% ± 0.110 ab	LD <sub>100</sub> : 0.09% ± 0.088 bc	LD <sub>100</sub> : 0.005% ± 0.005 d	LD <sub>100</sub> : 0.007% ± 0.006 d	LD <sub>100</sub> : 0.008% ± 0.008 d

<sup>1</sup> Values followed by the same letter within a row are not significantly different ( $p > 0.05$ , Tukey's test), <sup>a</sup>—in rate 1:1:1, <sup>b</sup>—10% solution, <sup>c</sup>—10% solution, <sup>d</sup>—10% solution; LACu, LAMn, LAZn—Lavender essential oil (L) with acetic acid (A) and Cu, Mn, Zn ions, respectively; LPCu, LPMn, LPZn—Lavender essential oil (L) with propionic acid (P) and Cu, Mn, Zn ions, respectively; LLCu, LLMn, LLZn—Lavender essential oil (L) with lactic acid (L) and Cu, Mn, Zn ions, respectively; LMCu, LMMn, LMZn—Lavender essential oil (L) with mixture of acids (M) and Cu, Mn, Zn ions, respectively.

The lowest antiprotozoal values against analyzed protozoa were obtained for innovative combinations of essential oil with the mixture of organic acids and selected metal. They showed an activity almost ten times stronger than that of analogous mixtures containing a single organic acid and an activity almost a hundred times stronger than that of single essential oils. Many of the proposed combinations showed activity against protozoa in the lowest tested concentration—0.001%. Most of the mixtures were very effective against *Euglena gracilis*. This protozoa was the most vulnerable form of all tested organisms. The concentration 0.001% was equal LD<sub>50</sub> for 4 combinations, tea tree essential oil, mixture of acids and copper (TMCu); tea tree essential oil, mixture of acids and manganese (TMMn); cedar essential oil, mixture of acids and copper (CMCu); lavender essential oil, mixture of acids and manganese (LMMn). The highest concentration for the essential oil, mixture of acids and metal ions was obtained for the tea tree essential oil, mixture of acids and manganese (TMMn)—0.009%. Nevertheless, all the combinations with manganese ions showed the best results for all protozoans. However, it should be noted that the worst result for the combination of essential oil, mixture of acids and metal, is more than 10 times better than for the reference substance, which in this case is metronidazole. The values obtained for the remaining combinations were characterized by significantly lower effective doses than the reference substances, chloramphenicol or metronidazole. The mixtures with highest potential and lowest LD<sub>50</sub> and LD<sub>100</sub> values can be found in Table 10. Comparison between the most effective compositions, analysis of variance (ANOVA) using Tukey's test, are presented in the Supplementary Materials.

**Table 10.** The most efficient combination against tested protozoan.

Protozoa	LD <sub>50</sub>	LD <sub>100</sub> :
<i>Euglena gracilis</i>	TMCu, TMMn, CMCu, LMMn	TMMn, CMCu
<i>Gregarina blattarum</i>	CMMn, LMMn	CMCu, LMCu
<i>Amoeba proteus</i>	TMZn	TMZn
<i>Paramecium caudatum</i>	TMMn	TMMn
<i>Pentatrichomonas hominis</i>	TMZn, CMMn, CMZn	CMMn

## 2.2. GC-MS Analysis of the Compositions

The chromatograms (presented in Supplementary Materials Figures S1–S4) show the chemical composition of the four essential oils used. Presented in Tables S1–S4 proves the typical mixtures for these essential oils' composition. Predominated compounds for lavender EOs are linalyl acetate, linalool, 1-terpinen-4-ol and eucalyptol. In case of eucalyptus oil, 80.7% of the sum of the investigated compounds is an 1,8-cineol (eucalyptol) together with p-cymene 9.8%. Tea tree oil was rich in terpineols (mainly 1-terpinen-4-ol ~40%) with corresponding terpinenes (respectively  $\gamma$ -15.4% and  $\alpha$ -9%). Finally, cedar oil was himachalene chemotype ( $\beta$ -isomer, 27.3% and  $\alpha$ , with 9.1% content) together with sesquiterpenoid atlantone (isomer Z, 12.3% and trans- $\alpha$  9.6%).

Detailed composition of investigated EOs are presented in Tables S1–S4.

## 3. Discussion

The results of the in vitro studies presented below confirm the antiprotozoal properties of blends which contained in their composition essential oils (eucalyptus, cedar, lavender or tea tree) with organic acids (acetic, propionic, lactic) and metal ions (Cu, Zn, Mn).

The combination containing essential oils, organic acids and metal ions was a concept that occurred after several years of research on natural alternatives for antibiotics. Moreover, and what is very important, all of the used components are allowed to be used in food and feed. The idea corresponds strongly with the scientific results obtained by other researchers. Components used in this study were chosen by their antiprotozoal activity and different polarity [20,40–42].

To the best of our knowledge, presented in this paper, combinations of EOs, acids and metal ions were tested for the first time. The object of investigations that have been conducted, are usually combinations of two or more essential oils [43], metals [38,42], antibiotics [44,45], organic acids [46], active compounds occurring in essential oil [22], essential oils with other compounds of natural origin [47] or other compounds of plant origin e.g., alkaloids [48–50], triterpene saponins [51] or chalcones and flavonoids [41]. The proposed combination is characterized by innovation and, most importantly, high efficiency.

Eucalyptus oil is very well known and used worldwide. Its properties have been widely described in the literature, with particular emphasis on antibacterial and anti-inflammatory properties [52,53]. However, very little information can be found on its antiprotozoal activity. These properties were proved in the following study. Eucalyptus oil showed the same properties as the other tested combinations. It does not change the fact that its effectiveness in combination with acids and metal ions was very high at low concentrations.

Another essential oil analyzed was tea tree essential oil. For years, it has not only been the subject of research, but also an ingredient in many antifungal products [54,55]. It is also undoubtedly effective against *Trichomonas vaginalis* [56]. A human trial showed that a dose of 0.4% tea tree essential oil was as effective as metronidazole treatment [22]. Similar results were obtained in the following work. However, another study showed a 100% antiprotozoal efficacy of tea tree oil at 455  $\mu\text{L}/\text{L}$  [57]. Similar results were obtained in the following work. Baldissera et al. showed a much higher effective concentration, 1–2%, in their study [58]. Other tests also showed its high antiprotozoal efficacy, but also high selectivity of the main component of tea tree oil—terpinen-4-ol [59]. The composition containing tea tree oil, together with a mixture of organic acids and metal ions, had the best antiprotozoal properties among the combinations tested. It exhibited a mostly lethal effect, against 50% of the population, at concentrations in the range of 0.001–0.004%.

Cedar essential oil shows very strong antibacterial, antiviral, insecticidal and antiprotozoal properties [60–63]. Studies have shown that in its pure form, it has efficacy levels of  $\text{LD}_{50} = 0.04\text{--}1\%$  and  $\text{LD}_{100} = 0.06\text{--}0.25\%$  against analyzed protozoa. Obtained concentrations were very low. Unlike the *C. deodara* species, *Cedrus libani* did not show antiprotozoal properties against *Leishmania major* [64]. Nisha et al. showed similar activity and effective concentration against the adult form of *Setaria digitata* [65]. In their study, Kar et al. showed a significant enhancement of the effect of cedrol, one of the major components of cedar essential oil, as a cedrol-loaded nano-structured lipid carrier [66]. In our studies, the combination of cedar oil with a mixture of acids and metal ions showed very good antiprotozoal properties in the range of 0.001–0.008% against the analyzed protozoa. Compared to the values obtained for cedar essential oil, it is 40 times and more than 30 times more potent,  $\text{LD}_{50}$  and  $\text{LD}_{100}$ , respectively.

Many studies present the high activity effects of lavender oil against microorganisms [67]. However, there are few reports in the literature on its antiprotozoal activity. The results presented in this paper allow us to conclude that lavender oil, especially in the proposed combinations, show very good antiprotozoal properties. The values for pure lavender oil were promising, while the combinations performed only confirmed this. All analyzed variants of lavender essential oil, mixture of acids and Cu, Mn, Zn ions (LMCu, LMMn and LMZn) showed strong protozoicidal activity at the level of 0.001–0.008%.

This article shows the very good antiprotozoal properties of the innovative combinations. The results obtained in this research are often even a hundred times stronger than those of standard antibiotics.

It is necessary to conduct further tests with using the analyzed mixtures. The obligatory element is undoubtedly the analysis of the toxicity of the combination and its direct influence on organs, as well as accumulation in tissues. However, the mixtures obtained have a very high potential and can be used not only in medicine and pharmacy, but also in the prophylaxis of diseases caused by protozoa, both in humans and animals.

## 4. Materials and Methods

### 4.1. Maintenance of Parasite Cultures and Evaluation of Antiprotozoal Activity

Five organisms representing the taxonomic groups to which the pathogenic protozoa belong were selected for in vitro studies of the antiprotozoal activity of the mixtures:

1. *Amoeba proteus*—*Chaos diffluens*—a protozoan of the order *Euamoebida*, belonging to the phylum *Amoebozoa*, living in water.
2. *Paramecium caudatum*—a paramecium representing aquatic ciliates.
3. *Gregarina blattarum*—gregarines were isolated from cockroaches, representing the type *Apicomplexa*, living in the digestive tracts or body cavities of invertebrates.
4. *Euglena gracilis*—a protozoan living in water, representing the flagellates—*Mastigophora*, family *Euglenaceae*.
5. *Pentatrichomonas hominis*—a protozoan that lives in the human colon, representing the *Trichomonadidae*.

The *Amoeba*, *Paramecium* and *Euglena* studied in this work were isolated from the freshwater river in Krosno (river Bado, 49°39'59.8' N 21°46'28.1' E, Krosno, Subcarpathian Voivodeship, Poland).

*Amoeba proteus* was cultivated in the Prescott medium and was fed with ciliates, for example, *Tetrahymena* and *Chilomonas* [68,69]. *Paramecium* was cultivated in hay infusion [70–72]. *Euglena* was cultivated in solution according to Wu [73]. *Pentatrichomonas hominis* was isolated from stool samples and kept in Pahm solution according to Chomicz et al. [74]

The gregarines were isolated from cockroaches and treated with the mixtures at different concentrations after being placed on a watch glass in Ringer's solution. Each sample included ten individuals. The isolation of gregarine from cockroaches was carried out according to the method of isolation of gregarines from beetles proposed by J. Moraczewski [75].

*Amoeba*, *Paramecium* and *Euglena* were observed microscopically on a watch glass with viscose wool fibers (to facilitate observation) in a drop of culture water, from which they originated. Different concentrations of the combinations were introduced into the test samples, establishing an LD<sub>50</sub> dose (50% mortality) and an LD<sub>100</sub> dose (100% mortality). For determining LD<sub>50</sub>, the Reed–Muench method was used. In all cases, four-fold replicates of the test were used along with a blank test. The lethal concentration of the substance LD<sub>50</sub> and LD<sub>100</sub> within 3 and 5 min was determined.

Identification of individual protozoa was made on the basis of their descriptions and drawings after W. A. Dogiel [76] and J. Hempel-Zawitkowska [35,77].

The obtained mixtures of phytoncides with metals and single phytoncides were dissolved in an aqueous solution of polysorbate 80 (0.05%) before being applied to a watch glass. No biocidal effect of polysorbate 80 was observed at the above concentrations. Chloramphenicol and metronidazole were used as standard substances to control protozoa. Concentration of the antibiotics were 5 mg/mL and the dilutions were prepared from the stock to reach LD<sub>50</sub> and LD<sub>100</sub>.

### 4.2. Essential Oils

Essential oils were ordered from two companies. Eucalyptus and tea tree were purchased from Food Base Kft. (Gödöllő, Hungary), cedar essential oil from Synthite Industries Pvt., Ltd. (Kolenchery, Kerala, India) and lavender essential oil from De Monchy Aromatics Ltd. (Poole, Dorset, UK)

### 4.3. Chemicals and Reagents

Organic acids (acetic acid 99%, propionic acid 99.5% and lactic acid 85%) and other chemical reagents purchased from Sigma-Aldrich (St. Louis, MO, USA) comply with FCC and FG standards. The purity and percentage composition, according to the supplier's specification, was minimum  $\geq 95\%$ .

#### 4.4. Phytoncides Mixture Preparation

Essential oils (100 mL) were added in the same amount to organic acids (100 mL) or mixture of acids (ratio 1:1:1) and 5 g of copper (II) carbonate hydroxide (2.87 g of ions  $\text{Cu}^{2+}$ ) or 5 g zinc carbonate (2.61 g of ions  $\text{Zn}^{2+}$ ) or 5 g manganese (II) chloride (2.18 g of ions  $\text{Mn}^{2+}$ ). The entire mixture was heated until the color changed. The mixture was then allowed to cool to obtain a clear solution (one, two or three phases). After this time, the mixture was filtered through a paper filter. The combination was diluted: 1.5% to 0.001%; after that, the protozoa were placed in each dilution.

#### 4.5. GC-MS Analysis

The profile of the essential oils investigated was assessed using the GC-MS technique according to the protocol [78]. Identification of all volatile constituents was based on comparison of experimentally obtained compound's mass spectra with mass spectra available in the NIST20 database. Additionally, the retention indices (RI) obtained experimentally, calculated using macro [79], were compared with the RI available in the NIST20 database and the data from the literature [80]. Shimadzu software GCMS Postrun Analysis (Shimadzu Company, Kyoto, Japan) and ACD/Spectrum Processor (Advanced Chemistry Development, Inc., Toronto, ON, Canada) were used to process the data. The quantification of identified constituents was performed by calculation based on the amount of added internal standard and expressed as a percentage of integrated peaks' area. Analysis was performed using the Shimadzu 2020 apparatus (Varian, Walnut Creek, CA, USA) equipped with a Zebron ZB-5 MSI (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ ) column (Phenomenex, Torrance, CA, USA). The temperature of the GC oven was programmed from 50 °C to 250 °C at a rate of 3.0 °C and kept for 3 min. Scanning was performed from 35 to 550 m/z in electronic impact (EI) at 70 eV and ion source temperature 250 °C. Samples were injected at split ratio 1:10 and gas helium was used as the carrier gas at a flow rate of 1.0 mL/min.

#### 4.6. Statistical Analysis

The data, from LD<sub>50</sub> and LD<sub>100</sub> evaluation, were subjected to the analysis of variance (ANOVA) using Tukey's test ( $p < 0.05$ ) using the STATISTICA 13.3 software for Windows (StatSoft, Krakow, Poland).

### 5. Conclusions

In the presented work, a hitherto unused combination of three types of compounds: essential oils, organic acids and metal ions, was used. It has been proved that the proposed combinations show very strong antiprotozoal activity. Studies conducted so far allow to conclude the synergistic effect of these combinations and obtain protozoicidal results much better than standard antibiotics—chloramphenicol or metronidazole. Very high effectiveness against all of the analyzed protozoans was found in the combinations of tea tree, cedar and lavender essential oils, mixture of acids and all of the ions. The LD<sub>50</sub> and LD<sub>100</sub> values were in the range 0.001–0.009%. The highest antiprotozoal properties were obtained in the combination with cedar and tea tree essential oils, mixture of acids and manganese or zinc ions. The proposed combinations may find application in eradication of protozoan diseases both in humans and animals. However, further steps should be taken to analyze the antiprotozoal effect on model protozoa such as *Cryptosporidium spp.*, *Leishmania spp.* and *Trypanosoma spp.* as well as toxicological studies of the effective concentrations.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antibiotics11070913/s1>, Figure S1: GC-MS chromatogram of tea tree essential oil, Figure S2: GC-MS chromatogram of cedar essential oil, Figure S3: GC-MS chromatogram of eucalyptus essential oil, Figure S4: GC-MS chromatogram of lavender oil, Table S1: Composition of tea tree essential oil, Table S2: Composition of cedar essential oil, Table S3: Composition of eucalyptus essential oil, Table S4: Composition of lavender essential oil.

**Author Contributions:** Conceptualization, H.I., H.R. and A.S.; methodology, H.I., H.R. and A.S.; investigation, H.I., H.R., A.S. and J.L.; data curation, H.I., H.R. and A.S.; validation H.I., H.R. and A.S.; writing—original draft preparation, H.I., H.R. and A.S.; writing—review and editing, H.I., J.L. and A.S.; visualization, H.I. and A.S.; supervision, H.R. and A.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study is supported by Ministry of Education and Science (Implementation Doctorates Project 2nd edition—Doktorat Wdrożeniowy).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available in the Supplementary Materials.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

## References

1. Kalt, M.-M.; Schuehly, W.; Saf, R.; Ochensberger, S.; Solnier, J.; Bucar, F.; Kaiser, M.; Presser, A. Palladium-catalysed synthesis of aryl naphthoquinones as antiprotozoal and antimycobacterial agents. *Eur. J. Med. Chem.* **2020**, *207*, 112837. [CrossRef]
2. Korpe, P.S.; Ravdin, J.I.; Petri, W.A. 271—Introduction to Protozoal Diseases. In *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*, 8th ed.; Bennett, J.E., Dolin, R., Blaser, M.J., Eds.; W.B. Saunders: Philadelphia, PA, USA, 2020; pp. 3270–3272. [CrossRef]
3. Mitra, A.K.; Mawson, A.R. Neglected Tropical Diseases: Epidemiology and Global Burden. *Trop. Med. Infect. Dis.* **2017**, *2*, 36. [CrossRef] [PubMed]
4. Benítez, J.; Guggeri, L.; Tomaz, I.; Arrambide, G.; Navarro, M.; Costa Pessoa, J.; Garat, B.; Gambino, D. Design of vanadium mixed-ligand complexes as potential anti-protozoa agents. *J. Inorg. Biochem.* **2009**, *103*, 609–616. [CrossRef] [PubMed]
5. Fairhurst, R.; Wellems, T. Plasmodium species (Malaria). *Princ. Pract. Infect. Dis.* **2014**, *2*, 3437–3462. [CrossRef]
6. Andrews, K.T.; Fisher, G.; Skinner-Adams, T.S. Drug repurposing and human parasitic protozoan diseases. *Int. J. Parasitol. Drugs Drug Resist.* **2014**, *4*, 95–111. [CrossRef]
7. CDC. How Can Malaria Cases and Deaths Be Reduced? Available online: [https://www.cdc.gov/malaria/malaria\\_worldwide/reduction/index.html](https://www.cdc.gov/malaria/malaria_worldwide/reduction/index.html) (accessed on 3 May 2022).
8. WHO. *Guideline WHO Guidelines for Malaria, 16 February 2021*; World Health Organization: Geneva, Switzerland, 2021.
9. WHO. *WHO Interim Guidelines for the Treatment of Gambiense Human African Trypanosomiasis*; World Health Organization: Geneva, Switzerland, 2019.
10. PAHO. *Guidelines for the Diagnosis and Treatment of Chagas Disease*; Pan American Health Organization: Washington, DC, USA, 2019.
11. Lindner, A.K.; Lejon, V.; Chappuis, F.; Seixas, J.; Kazumba, L.; Barrett, M.P.; Mwamba, E.; Erphas, O.; Akl, E.A.; Villanueva, G.; et al. New WHO guidelines for treatment of gambiense human African trypanosomiasis including fexinidazole: Substantial changes for clinical practice. *Lancet. Infect. Dis.* **2020**, *20*, e38–e46. [CrossRef]
12. Aslam, B.; Wang, W.; Arshad, M.I.; Khurshid, M.; Muzammil, S.; Rasool, M.H.; Nisar, M.A.; Alvi, R.F.; Aslam, M.A.; Qamar, M.U.; et al. Antibiotic resistance: A rundown of a global crisis. *Infect Drug Resist* **2018**, *11*, 1645–1658. [CrossRef]
13. Rolta, R.; Sharma, A.; Sourirajan, A.; Mallikarjunan, P.K.; Dev, K. Combination between antibacterial and antifungal antibiotics with phytochemicals of *Artemisia annua* L: A strategy to control drug resistance pathogens. *J. Ethnopharmacol.* **2021**, *266*, 113420. [CrossRef]
14. Abou Baker, D.H.; Al-Moghazy, M.; ElSayed, A.A.A. The in vitro cytotoxicity, antioxidant and antibacterial potential of *Satureja hortensis* L. essential oil cultivated in Egypt. *Bioorganic Chem.* **2020**, *95*, 103559. [CrossRef]
15. Gavanji, S.; Zaker, S.R.; Nejad, Z.G.; Bakhtari, A.; Bidabadi, E.S.; Larki, B. Comparative efficacy of herbal essences with amphotericin B and ketoconazole on *Candida albicans* in the in vitro condition. *Integr. Med. Res.* **2015**, *4*, 112–118. [CrossRef]
16. Derda, M.; Hadaś, E. The use of phytotherapy in diseases caused by parasitic protozoa. *Acta Parasitol.* **2014**, *60*, 1–8. [CrossRef] [PubMed]
17. Van Zyl, R.L.; Seatlholo, S.T.; Viljoen, A.M. Pharmacological interactions of essential oil constituents on the in vitro growth of *Plasmodium falciparum*. *S. Afr. J. Bot.* **2010**, *76*, 662–667. [CrossRef]
18. Santoro, G.F.; Cardoso, M.G.; Guimarães, L.G.L.; Mendonça, L.Z.; Soares, M.J. Trypanosoma cruzi: Activity of essential oils from *Achillea millefolium* L., *Syzygium aromaticum* L. and *Ocimum basilicum* L. on epimastigotes and trypomastigotes. *Exp. Parasitol.* **2007**, *116*, 283–290. [CrossRef] [PubMed]
19. Rodrigues Goulart, H.; Kimura Emília, A.; Peres Valnice, J.; Couto Alicia, S.; Aquino Duarte Fulgencio, A.; Katzin Alejandro, M. Terpenes Arrest Parasite Development and Inhibit Biosynthesis of Isoprenoids in *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* **2004**, *48*, 2502–2509. [CrossRef]

20. Monzote, L.; Alarcón, O.; Setzer, W. Antiprotozoal Activity of Essential Oils. *Agric. Conspec. Sci.* **2012**, *77*, 167–175.
21. Carson, C.F.; Hammer, K.A. Chemistry and Bioactivity of Essential Oils. In *Lipids and Essential Oils as Antimicrobial Agents*; Thormar, H., Ed.; John Wiley & Sons, Ltd.: Hoboken, NJ, USA, 2011; pp. 203–238. [[CrossRef](#)]
22. Lam, N.S.; Long, X.; Su, X.-Z.; Lu, F. *Melaleuca alternifolia* (tea tree) oil and its monoterpene constituents in treating protozoan and helminthic infections. *Biomed. Pharmacother.* **2020**, *130*, 110624. [[CrossRef](#)]
23. Moon, T.; Wilkinson, J.; Cavanagh, H. Antiparasitic activity of two *Lavandula* essential oils against *Giardia duodenalis*, *Trichomonas vaginalis* and *Hexamita inflata*. *Parasitol. Res.* **2006**, *99*, 722–728. [[CrossRef](#)]
24. Behnia, M.; Haghighi, A.; Komeylizadeh, H.; Tabaei, S.J.; Abadi, A. Inhibitory effects of Iranian *Thymus vulgaris* extracts on in vitro growth of *Entamoeba histolytica*. *Korean J. Parasitol.* **2008**, *46*, 153–156. [[CrossRef](#)]
25. Saeidnia, S.; Gohari, A.; Hadjiakhoondi, A. Trypanocidal oil of the young leaves of *Nepeta cataria* L., obtained by solvent extraction. *J. Med. Plants* **2008**, *7*, 54–57.
26. De Melo, A.R.B.; Maciel Higino, T.M.; da Rocha Oliveira, A.D.P.; Fontes, A.; da Silva, D.C.N.; de Castro, M.C.A.B.; Dantas Lopes, J.A.; de Figueiredo, R.C.B.Q. *Lippia sidoides* and *Lippia organoides* essential oils affect the viability, motility and ultrastructure of *Trypanosoma cruzi*. *Micron* **2020**, *129*, 102781. [[CrossRef](#)]
27. Ferreira, L.C.; Cruz, M.G.d.; Lima, T.B.C.; Serra, B.N.V.; Chaves, F.C.M.; Chagas, E.C.; Ventura, A.S.; Jerônimo, G.T. Antiparasitic activity of *Mentha piperita* (Lamiaceae) essential oil against *Piscinoodinium pillulare* and its physiological effects on *Colossoma macropomum* (Cuvier, 1818). *Aquaculture* **2019**, *512*, 734343. [[CrossRef](#)]
28. Anacarso, I.; Sabia, C.; de Niederhäusern, S.; Iseppi, R.; Condò, C.; Bondi, M.; Messi, P. In vitro evaluation of the amoebicidal activity of rosemary (*Rosmarinus officinalis* L.) and cloves (*Syzygium aromaticum* L. Merr. & Perry) essential oils against *Acanthamoeba polyphaga* trophozoites. *Nat. Prod. Res.* **2019**, *33*, 606–611. [[CrossRef](#)] [[PubMed](#)]
29. van Zyl, R.L.; Seatlholo, S.T.; van Vuuren, S.F.; Viljoen, A.M. The Biological Activities of 20 Nature Identical Essential Oil Constituents. *J. Essent. Oil Res.* **2006**, *18*, 129–133. [[CrossRef](#)]
30. Hauser, C.; Thielmann, J.; Muranyi, P. Chapter 46—Organic Acids: Usage and Potential in Antimicrobial Packaging. In *Antimicrobial Food Packaging*; Barros-Velázquez, J., Ed.; Academic Press: San Diego, CA, USA, 2016; pp. 563–580. [[CrossRef](#)]
31. Meira-Filho, M.R.C.; Ramirez, J.R.B.; Vianna, R.T.; Júnior, J.P. Efficacy of glacial acetic acid in the control of *Trichodina* sp. and *Apiosoma* sp. associated with *Mugil liza*. *Aquaculture* **2017**, *479*, 7–12. [[CrossRef](#)]
32. Surve, A.N.; Sherikar, A.T.; Bhilegaonkar, K.N.; Karkare, U.D. Preservative effect of combinations of acetic acid with lactic or propionic acid on buffalo meat stored at refrigeration temperature. *Meat Sci.* **1991**, *29*, 309–322. [[CrossRef](#)]
33. Aljumaah, M.R.; Alkhulaifi, M.M.; Abudabos, A.M.; Alabdullatifb, A.; El-Mubarak, A.H.; Al Suliman, A.R.; Stanley, D. Organic acid blend supplementation increases butyrate and acetate production in *Salmonella enterica* serovar Typhimurium challenged broilers. *PLoS ONE* **2020**, *15*, e0232831. [[CrossRef](#)]
34. Marcelino, P.R.F.; Moreira, M.B.; Lacerda, T.M.; da Silva, S.S. Metal-Based Drugs for Treatment of Malaria. *Biomed. Appl. Met.* **2017**, 167–193. [[CrossRef](#)]
35. Navarro, M.; Gabbiani, P.; Messori, L.; Gambino, D. Metal-based drugs for malaria, trypanosomiasis and leishmaniasis: Recent achievements and perspectives. *Drug Discov. Today* **2010**, *15*, 1070–1078. [[CrossRef](#)]
36. Wani, W.A.; Jameel, E.; Baig, U.; Mumtazuddin, S.; Hun, L.T. Ferroquine and its derivatives: New generation of antimalarial agents. *Eur. J. Med. Chem.* **2015**, *101*, 534–551. [[CrossRef](#)]
37. Low, W.-L.; Kenward, K.; Britland, S.T.; Amin, M.C.; Martin, C. Essential oils and metal ions as alternative antimicrobial agents: A focus on tea tree oil and silver. *Int. Wound J.* **2017**, *14*, 369–384. [[CrossRef](#)]
38. Low, W.L.; Martin, C.; Hill, D.J.; Kenward, M.A. Antimicrobial efficacy of silver ions in combination with tea tree oil against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*. *Int. J. Antimicrob. Agents* **2011**, *37*, 162–165. [[CrossRef](#)] [[PubMed](#)]
39. Kiss, T.; Osipenko, O. Metal ion-induced permeability changes in cell membranes: A minireview. *Cell. Mol. Neurobiol.* **1994**, *14*, 781–789. [[CrossRef](#)] [[PubMed](#)]
40. Ed-Dra, A.; Filali, F.R.; Lo Presti, V.; Zekkori, B.; Nalbone, L.; Bouymajane, A.; Trabelsi, N.; Lamberta, F.; Bentayeb, A.; Giuffrida, A.; et al. Chemical composition, antioxidant capacity and antibacterial action of five Moroccan essential oils against *Listeria monocytogenes* and different serotypes of *Salmonella enterica*. *Microb. Pathog.* **2020**, *149*, 104510. [[CrossRef](#)]
41. Sen, R.; Chatterjee, M. Plant derived therapeutics for the treatment of Leishmaniasis. *Phytomedicine* **2011**, *18*, 1056–1069. [[CrossRef](#)] [[PubMed](#)]
42. Ahmad, A.; Viljoen, A. The in vitro antimicrobial activity of *Cymbopogon essential* oil (lemon grass) and its interaction with silver ions. *Phytomedicine* **2015**, *22*, 657–665. [[CrossRef](#)]
43. Zenner, L.; Callait, M.P.; Granier, C.; Chauve, C. In vitro effect of essential oils from *Cinnamomum aromaticum*, *Citrus limon* and *Allium sativum* on two intestinal flagellates of poultry, *Tetratrichomonas gallinarum* and *Histomonas meleagridis*. *Parasite* **2003**, *10*, 153–157. [[CrossRef](#)]
44. Jugreet, B.S.; Mahomoodally, M.F. Reprint of: Essential oils from 9 exotic and endemic medicinal plants from Mauritius shows in vitro antibacterial and antibiotic potentiating activities. *S. Afr. J. Bot.* **2021**, *140*, 478–485. [[CrossRef](#)]
45. Mazoir, N.; Dakir, M.; Tebbaa, M.; Loughzail, M.; Benharref, A. Aromatization of (Z) and (E)- $\alpha$ -atlantones isolated from *Cedrus atlantica* essential oil followed by condensation with thiosemicarbazide: Synthesis of new thiadiazolines derivatives. *Tetrahedron Lett.* **2016**, *57*, 278–280. [[CrossRef](#)]

46. Zhang, S.; Shen, Y.R.; Wu, S.; Xiao, Y.Q.; He, Q.; Shi, S.R. The dietary combination of essential oils and organic acids reduces *Salmonella enteritidis* in challenged chicks. *Poult. Sci.* **2019**, *98*, 6349–6355. [[CrossRef](#)]
47. Katopodi, A.; Detsi, A. Solid Lipid Nanoparticles and Nanostructured Lipid Carriers of natural products as promising systems for their bioactivity enhancement: The case of essential oils and flavonoids. *Colloids Surf. A Physicochem. Eng. Asp.* **2021**, *630*, 127529. [[CrossRef](#)]
48. Sahu, A.; Agrawal, R.K.; Pandey, R. Synthesis and systemic toxicity assessment of quinine-triazole scaffold with antiprotozoal potency. *Bioorganic Chem.* **2019**, *88*, 102939. [[CrossRef](#)]
49. Nair, J.J.; van Staden, J. Antiprotozoal alkaloid principles of the plant family Amaryllidaceae. *Bioorganic Med. Chem. Lett.* **2019**, *29*, 126642. [[CrossRef](#)] [[PubMed](#)]
50. Cimanga Kanyanga, R.; Kikweta Munduku, C.; Nsaka Lumpu, S.; Tshodi Ehata, M.; Makila Bool-Miting, F.; Kambu Kabangu, O.; Mbamu Maya, B.; Cos, P.; Maes, L.; Vlietinck, A.J.; et al. Isolation and structure elucidation of two antiprotozoal bisbenzylisoquinoline alkaloids from *Triclisia gillettii* stem bark. *Phytochem. Lett.* **2018**, *28*, 19–23. [[CrossRef](#)]
51. Mostafa, A.E.; El-Hela, A.A.; Mohammad, A.-E.I.; Cutler, S.J.; Ross, S.A. New triterpenoidal saponins from *Koeleruteria paniculata*. *Phytochem. Lett.* **2016**, *17*, 213–218. [[CrossRef](#)] [[PubMed](#)]
52. Bello, M.; Jiddah-kazeem, B.; Fatoki, T.H.; Ibukun, E.O.; Akinmoladun, A.C. Antioxidant property of *Eucalyptus globulus* Labill. Extracts and inhibitory activities on carbohydrate metabolizing enzymes related to type-2 diabetes. *Biocatal. Agric. Biotechnol.* **2021**, *36*, 102111. [[CrossRef](#)]
53. Aleksic Sabo, V.; Knezevic, P. Antimicrobial activity of *Eucalyptus camaldulensis* Dehn. plant extracts and essential oils: A review. *Ind. Crop. Prod.* **2019**, *132*, 413–429. [[CrossRef](#)] [[PubMed](#)]
54. Zhang, J.; Wu, H.; Jiang, D.; Yang, Y.; Tang, W.; Xu, K. The antifungal activity of essential oil from *Melaleuca leucadendra* (L.) L. grown in China and its synergistic effects with conventional antibiotics against *Candida*. *Nat. Prod. Res.* **2019**, *33*, 2545–2548. [[CrossRef](#)]
55. Murray, M.T. 91—*Melaleuca alternifolia* (Tea Tree). In *Textbook of Natural Medicine*, 5th ed.; Pizzorno, J.E., Murray, M.T., Eds.; Churchill Livingstone: St. Louis, MO, USA, 2020; pp. 693–696. [[CrossRef](#)]
56. Carson, C.F.; Hammer, K.A.; Riley, T.V. *Melaleuca alternifolia* (Tea Tree) oil: A review of antimicrobial and other medicinal properties. *Clin. Microbiol. Rev.* **2006**, *19*, 50–62. [[CrossRef](#)]
57. Valladão, G.M.R.; Gallani, S.U.; Ikefuti, C.V.; da Cruz, C.; Levy-Pereira, N.; Rodrigues, M.V.N.; Pilarski, F. Essential oils to control ichthyophthiriasis in pacu, *Piaractus mesopotamicus* (Holmberg): Special emphasis on treatment with *Melaleuca alternifolia*. *J. Fish Dis.* **2016**, *39*, 1143–1152. [[CrossRef](#)]
58. Baldissera, M.D.; Da Silva, A.S.; Oliveira, C.B.; Santos, R.C.V.; Vaucher, R.A.; Raffin, R.P.; Gomes, P.; Dambros, M.G.C.; Miletti, L.C.; Boligon, A.A.; et al. Trypanocidal action of tea tree oil (*Melaleuca alternifolia*) against *Trypanosoma evansi* in vitro and in vivo used mice as experimental model. *Exp. Parasitol.* **2014**, *141*, 21–27. [[CrossRef](#)]
59. Mikus, J.; Harkenthal, M.; Steverding, D.; Reichling, J. In vitro effect of essential oils and isolated mono- and sesquiterpenes on *Leishmania major* and *Trypanosoma brucei*. *Planta Med.* **2000**, *66*, 366–368. [[CrossRef](#)] [[PubMed](#)]
60. Ainane, A.; Khammour, F.; Charaf, S.; Elabboubi, M.; Elkouali, M.; Talbi, M.; Benhima, R.; Cherroud, S.; Ainane, T. Chemical composition and insecticidal activity of five essential oils: *Cedrus atlantica*, *Citrus limonum*, *Rosmarinus officinalis*, *Syzygium aromaticum* and *Eucalyptus globules*. *Mater. Today Proc.* **2019**, *13*, 474–485. [[CrossRef](#)]
61. Chaudhary, A.K.; Ahmad, S.; Mazumder, A. *Cedrus deodara* (Roxb.) Loud.: A Review on its Ethnobotany, Phytochemical and Pharmacological Profile. *Pharmacogn. J.* **2011**, *3*, 12–17. [[CrossRef](#)]
62. Saab, A.M.; Gambari, R.; Sacchetti, G.; Guerrini, A.; Lampronti, I.; Tacchini, M.; El Samrani, A.; Medawar, S.; Makhlof, H.; Tannoury, M.; et al. Phytochemical and pharmacological properties of essential oils from *Cedrus* species. *Nat. Prod. Res.* **2018**, *32*, 1415–1427. [[CrossRef](#)] [[PubMed](#)]
63. Bisht, A.; Jain, S.; Misra, A.; Dwivedi, J.; Paliwal, S.; Sharma, S. *Cedrus deodara* (Roxb. ex D.Don) G.Don: A review of traditional use, phytochemical composition and pharmacology. *J. Ethnopharmacol.* **2021**, *279*, 114361. [[CrossRef](#)]
64. Motazedian, M.H.; Mikaeili, F.; Mohebbi, M.; Miri, R.; Habibi, P.; Kamarloie, S. The antileishmanial effects of *Lawsonia inermis* and *Cedrus libani* on *Leishmania major* promastigotes: An in vitro study. *J. Parasit. Dis.* **2017**, *41*, 375–379. [[CrossRef](#)]
65. Nisha, M.; Kalyanasundaram, M.; Paily, K.P.; Abidha; Vanamail, P.; Balaraman, K. In vitro screening of medicinal plant extracts for macrofilaricidal activity. *Parasitol. Res.* **2007**, *100*, 575–579. [[CrossRef](#)] [[PubMed](#)]
66. Kar, N.; Chakraborty, S.; De, A.K.; Ghosh, S.; Bera, T. Development and evaluation of a cedrol-loaded nanostructured lipid carrier system for in vitro and in vivo susceptibilities of wild and drug resistant *Leishmania donovani* amastigotes. *Eur. J. Pharm. Sci.* **2017**, *104*, 196–211. [[CrossRef](#)]
67. Kazemi, M.; Mohammadifar, M.; Aghadavoud, E.; Vakili, Z.; Aarabi, M.H.; Talaie, S.A. Deep skin wound healing potential of lavender essential oil and licorice extract in a nanoemulsion form: Biochemical, histopathological and gene expression evidences. *J. Tissue Viability* **2020**, *29*, 116–124. [[CrossRef](#)]
68. Yudin, A.L. Amoeba and Other Protozoa. In *Animal Species for Developmental Studies: Volume 1 Invertebrates*; Dettlaff, T.A., Vassetzky, S.G., Eds.; Springer: Boston, MA, USA, 1990; pp. 1–11. [[CrossRef](#)]
69. Demin, S.Y.; Berdieva, M.A.; Podlipaeva, Y.I.; Goodkov, A.V. Karyotypic instability of endoprophase and mitotic cells of *Amoeba* sp. strain Cont from the “proteus-type” group (*Amoebozoa*, *Euamoebida*, *Amoebida*). *Eur. J. Protistol.* **2020**, *74*, 125691. [[CrossRef](#)]



70. Pawlaczyk-Szpilowa, M. *Ćwiczenia z Mikrobiologii Wody i Ścieków*; PWN: Warszawa, Poland, 1980.
71. Sonneborn, T.M. Chapter 12 Methods in Paramecium Research. In *Methods in Cell Biology*; Prescott, D.M., Ed.; Academic Press: New York, NY, USA, 1970; Volume 4, pp. 241–339.
72. Feng, J.; Zhu, H.; Lukeš, J.; Korabečná, M.; Fohlerová, Z.; Mei, T.; Chang, H.; Neužil, P. Nanowatt simple microcalorimetry for dynamically monitoring the defense mechanism of *Paramecium caudatum*. *Sens. Actuators A Phys.* **2021**, *323*, 112643. [[CrossRef](#)]
73. Wu, M.; Qin, H.; Deng, J.; Liu, Y.; Lei, A.; Zhu, H.; Hu, Z.; Wang, J. A new pilot-scale fermentation mode enhances *Euglena gracilis* biomass and paramylon ( $\beta$ -1,3-glucan) production. *J. Clean. Prod.* **2021**, *321*, 128996. [[CrossRef](#)]
74. Chomicz, L.; Padzik, M.; Laudy, A.; Kozłowska, M.; Pietruczuk, A.; Piekarczyk, J.; Godineau, N.; Olędzka, G.; Kazimierczuk, Z. Anti-Pentatrichomonas hominis activity of newly synthesized benzimidazole derivatives—In vitro studies. *Acta Parasitol.* **2009**, *54*, 165–171. [[CrossRef](#)]
75. Moraczewski, J. *Ćwiczenia z zoologii bezkręgowców. Wydanie I*; PWN: Warszawa, Poland, 1974; pp. 285–292.
76. Dogiel, W.A. *Zoologia Bezkręgowców. Wydanie III*; Państwowe Wydawnictwo Rolnicze i Leśne: Warszawa, Poland, 1972.
77. Hempel-Zawitkowska, J. *Zoologia dla uczelni rolniczych*; Wydawnictwo Naukowe PWN: Warszawa, Poland, 2006.
78. Łyczko, J.; Pawlak, A.; Augustyński, I.; Okińczyc, P.; Szperlik, J.; Kulma, A.; Różański, H.; Obmińska-Mrukowicz, B.; Szumny, A. Chemical profiling and cytotoxic activity of 150-year old original sample of Jerusalem Balsam. *Food Chem. Toxicol.* **2020**, *138*, 111183. [[CrossRef](#)]
79. Lucero, M.; Estell, R.; Tellez, M.; Fredrickson, E. A retention index calculator simplifies identification of plant volatile organic compounds. *Phytochem. Anal.* **2009**, *20*, 378–384. [[CrossRef](#)]
80. Adams, R.P. *Identification of Essential Oils by Ion Trap Mass Spectroscopy*; Academic Press: San Diego, CA, USA, 2012.