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Antiprotozoal Activity and Selectivity Index of Organics Salts of Albendazole and Mebendazole

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Abstract: Infections caused by the protozoans *Entamoeba histolytica* (*E. histolytica), Giardia lamblia* (*G. lamblia*), and *Trichomonas vaginalis* (*T. vaginalis*) represent a global public health concern. Albendazole and mebendazole are drugs used to treat parasitosis resulting from protozoa. However, the low aqueous solubility of these compounds has led to the exploration of new strategies to enhance their solubility, with the formation of salts being a commonly employed strategy. The sulfonates **A1**, **A2**, and **A3** of albendazole, and **M1, M2**, and **M3** of mebendazole were synthesized, and their antiparasitic activity was determined in vitro against trophozoites of *E. histolytica*, *G. lamblia*, and *T. vaginalis*. Consequently, IC_{50} values ranged from 24.17 to 138.02 μM for the albendazole and mebendazole salts. For comparison, the IC_{50} values for the positive controls—albendazole, mebendazole, and metronidazole—ranged from 16.08 to 270.66 μM. Additionally, it was determined that salts **A1**, **A3**, **M2**, and **M3** do not exhibit cytotoxic effects at concentrations of 500 μM on the VERO cell line. Taken together, these findings indicate that the formation of these new solid saline phases enhances the antiparasitic effects in vitro, which is crucial in the current search for improved, safe, and effective antiparasitic agents.

Keywords: benzimidazole; antiparasitic activity; *Entamoeba histolytica*; *Giardia lamblia*; *Trichomonas vaginalis*; toxicity; selective index; sulfonate salts

1. Introduction

Parasitic infections caused by microaerophilic protozoa are a major problem affecting around 3 billion people globally. Among the main protozoa are *Entamoeba histolytica* (*E. histolytica*) and *Giardia lamblia* (*G. lamblia*), which are human pathogens of the gastrointestinal tract, and *Trichomonas vaginalis* (*T. vaginalis*), a pathogen of the human genitourinary tract [1–3].

Metronidazole (α-hydroxyethyl-2-methyl-5-nitroimidazole, **Mtz**) is the first choice for treating amoebiasis, giardiasis, and trichomoniasis [4,5]. However, overuse, indiscriminate over the counter (OTC) sales, and inadequate treatment regimens have resulted in the emergence of nitroimidazole-resistant strains with increased inhibitory concentrations of the drug, leading to treatment failures [6,7].

Resistance to **Mtz** in clinical isolates has been reported in *G. lamblia* [8,9], *E. histolytica* [10,11], and *T. vaginalis* [12,13]. Therefore, when treatment with Mtz is ineffective, it is recommended to use albendazole (methyl [5-(propylthio)-1H-benzimidazole-2-yl] carbamate, **Abz**) and mebendazole (methyl 5-benzoyl-1H-benzimidazole-2-yl-carbamate, **Mbz**) alone or in combination with Mtz [14–18].

The main mechanism of action of **Abz** and **Mbz** in protozoa is through their selective binding to β-tubulin dimers in the parasite, which prevents the polymerization of microtubules. Consequently,

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essential biological processes such as adhesion, motility, and cell division are directly affected [19–21]. In addition, benzimidazoles can induce oxidative stress by forming adducts in the parasite's deoxyribonucleic acid (DNA) and causing DNA double-strand breaks, leading to cell cycle dysregulation and ultimately the death of the parasite [22,23].

Abz and **Mbz** have low water solubility, which implies low intestinal absorption [24–26]. To improve water solubility, various methodologies have been described, such as the formation of nanocapsules [27], solid dispersions [28], nanocrystals [29], liposomes [30], cyclodextrins [31], inclusion complexes [32], self-emulsifying drug delivery systems [33], and pharmaceutical salts [34]. These methodologies do not involve chemical structure modification but rather the combination with other molecules through mixtures or acid-base reactions.

The formation of salts with pharmaceutically acceptable counterions is useful for modifying physicochemical, biopharmaceutical, or therapeutic properties. These salts are considered chemical entities with improved pharmacokinetic, toxicological, and potency characteristics [35,36]. The use of sulfonic acids for salt formation has increased over time because they substantially improve the physicochemical properties of active pharmaceutical ingredients (APIs) and, in some cases, are safer than alternative salts [37,38].

The aim of the present study is to evaluate the in vitro amebicidal, trichomonicidal, and giardicidal activity of albendazole (**A**) and mebendazole (**M**) salts with benzenesulfonic acid (**A1**, M1), methanesulfonic acid (**A2**, **M2**), and *p*-toluenesulfonic acid (**A3**, **M3**). Additionally, their mean cytotoxicity concentration (CC_{50}) on the VERO cell line and their selectivity index were assessed, aiming to select those with the best biological properties for subsequent in vivo pharmaceutical studies, which may serve as candidates for the treatment of parasitosis.

2. Materials and Methods

2.1. General Procedure to Prepare Abz and Mbz Sulfonic Salts

2.1.1. Reagents and Solvents

Albendazole (≥ 98%, CAS 54965-21-8), Mebendazole (≥ 98%, CAS 31431-39-7), *p*-toluensulfonic acid monohydrate (≥ 98.5% CAS 6192-52-5), benzenesulfonic acid monohydrate (97%, CAS 26158-00-9) and methanesulfonic acid (≥ 99%, CAS 75-75-2) were purchased from Sigma-Aldrich® and used without prior purification. Methanol (≥ 99.9%, CAS 67-56-1) and acetone (99.8%, CAS 67-64-1) were reagent grade and purchased from J.T. Baker.

2.1.2. Equipment and Conditions for Chemical Characterization

Powder X-ray diffraction (PXRD) was performed on a Bruker D2-Phaser. The data were collected in the interval 5–50° of 2θ angle and a stepsize of 0.02°. FT-IR spectra were recording using Thermo Scientific Spectrophotometer Nicolet iS10 and measured in the range of 4000–500cm^{-1} with diamond ATR accessory. NMR spectra were obtained on a Bruker AVANCE III HD 500 MHz (^{1}H) and 125 MHz (^{13}C) spectrometer using room temperature, samples of 30 mg, and DMSO-d_6 as solvent for all samples. The melting point was determined by using a Fisher-Johns Melting Point Apparatus 12-144 from Fisher Scientific® in the range 20 – 300 °C.

2.1.3. General Method for Salts Preparation

An equimolar mixture of the benzimidazole drug (**Abz**, 265 mg; or **Mbz**, 295 mg) and the sulfonic acid (methane sulfonic acid, 97 mg; benzenesulfonic acid, 159 mg; or *p*-toluenesulfonic acid, 158 mg) was placed into a 10 mL glass vial provided with magnetic stirrer. Then enough solvent (acetone or methanol, ca. 3 mL) is poured to form a solid suspension. The reaction mixture is stirred at room temperature for 24 h. At terminus the suspension is filtered, and the solid is rinsed with an aliquot of fresh solvent to remove the starting material. Finally, the solid is dried under vacuum for 2 h. The yield of solid salt was 60-75%.

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2.1.4. Chemical Characterization of Salt A1 (Albendazole Besylate)

^{1}H NMR spectrum (DMSO-d_6): δ (ppm) 7.61(d, 2H), 7.45(d, 1H), 7.37(dd, 1H), 7.31(m, 2H) 7.15(d, 2H), 3.78(s, 3H), 2.87(t, 2H), 1.53(sextet, 2H), 0.95(t, 3H). ^{13}C NMR spectrum (DMSO-d_6): δ (ppm) 154.33, 146.97, 135.3, 133.8, 128.3, 128.0, 127.6, 125.5, 124.1, 115.2, 114.0, 52.8, 36.3, 22.0, 13.1. XRPD (2θ angle): 7.0°, 7.5°, 11.0°, 11.4°, 18.0°, 18.7°, 19.5°, 20.8°, 24.9°, 25.8°, 27.3°, 29.4°. FT-IR: ν (cm^{-1}) 3321, 2958, 1711, 1615, 1585, 1441, 1263, 1092. M.p. 182-184°C. Yield 65%.

2.1.5. Chemical Characterization of Salt A2 (Albendazole Mesylate)

^{1}H NMR spectrum (DMSO-d_6): δ (ppm) 7.54(m, 2H) 7.36(dd, 1H), 3.88(s, 3H), 2.95(t, 2H), 2.43(s, 3H)1.57(sextet, 2H), 0.97(t, 3H). ^{13}C NMR spectrum (DMSO-d_6): δ (ppm) 152.6, 144.2, 132.3, 130.0, 127.8, 125.6, 113.7, 112.9, 52.8, 36.3, 22.0, 13.1. XRPD (2θ angle): 7.0°, 11.9°, 13.0°, 13.7°, 16.3°, 16.9°, 19.0°, 19.6°, 20.7°, 21.3°, 22.9°, 23.6°, 25.4°, 26.1°, 27.4°, 29.1°, 30.3°. FT-IR: ν (cm^{-1}) 3213, 1755, 1638, 1435, 1231, 1151, 1089. M.p. 154-155°C. Yield 71%.

2.1.6. Chemical Characterization of Salt A3 (Albendazole Tosylate)

^{1}H NMR spectrum (DMSO-d_6): δ (ppm) 7.55-7.47(m, 4H), 7.36(dd, 1H), 7.12(d, 2H), 3.88(s, 3H), 2.95(t, 2H), 2.28(s, 3H), 1.57(sextet, 2H), 0.97(t, 3H). ^{13}C NMR spectrum (DMSO-d_6): δ (ppm) 157.3, 153.1, 144.6, 138.3, 132.7, 130.6, 126.1, 125, 114.2, 113.5, 54.2, 35.7, 22.3, 21.2, 13.6. XRPD (2θ angle): 6.7°, 18.6°, 18.9°, 19.7°, 21.6°, 22.7°, 25.3°, 29.7°. FT-IR: ν (cm^{-1}) 3063, 2927, 1758, 1644, 1601, 1442, 1237, 1155, 1096. M.p. 188-189°C. Yield 68%.

2.1.7. Chemical Characterization of Salt M1 (Mebendazole Besylate)

^{1}H NMR spectrum (DMSO-d_6): δ (ppm) 7.88(s, 1H), 7.72(d, 2H), 7.68-7.55(m, 6H), 7.34-7.27(m, 1H), 3.81(s, 3H). ^{13}C NMR spectrum (DMSO-d_6): δ (ppm) 195.35, 153.95, 148.44, 138.11, 132.03, 130.60, 129.37, 128.41, 128.32, 127.59, 125.47, 124.37, 115.75, 113.81, 52.97. XRPD (2θ angle): 6.4°, 7.0°, 7.5°, 11.0°, 11.4°, 18.1°, 18.8°, 20.7°, 22.4°, 23.6°, 24.8°, 25.7°, 27.1°. FT-IR: ν (cm^{-1}) 3368, 2947, 1755, 1731, 1635, 1593, 1456, 1257, 1227, 1179, 1090. M.p. decomposition occurs prior to the melting of sample. Yield 70%.

2.1.8. Chemical Characterization of Salt M2 (Mebendazole Mesylate)

^{1}H NMR spectrum (DMSO-d_6): δ (ppm) 7.96(d, 1H), 7.73(m, 5H), 7.59(t, 2H), 3.89(s, 3H), 2.44(s, 6H). ^{13}C NMR spectrum (120 MHz, DMSO-d_6): δ (ppm) 194.94, 152.75, 146.06, 137.36, 133.15, 132.95, 132.70, 129.63, 128.68, 126.32, 115.34, 113.37, 53.95. XRPD (2θ angle): 6.7°, 10.3°, 12.8°, 17.0°, 18.0°, 18.5°, 19.3°, 19.8°, 20.5°, 21.8°, 22.8°, 23.4°, 25.3°, 26.3°, 28.5°, 29.3°. FT-IR: ν (cm^{-1}) 3570, 3055, 1769, 1662, 1639, 1598, 1439, 1235, 1009. M.p. 207-208°C. Yield 65%.

2.1.9. Chemical Characterization of Salt M3 (Mebendazole Tosylate)

^{1}H NMR spectrum (DMSO-d_6): δ 7.95(d, 1H), 7.78-7.68(m, 5H), 7.58(t, 2H), 7.50(d, 2H), 7.12(d, 2H), 3.88(s, 3H), 2.28(s, 3H), 3H). ^{13}C NMR spectrum (DMSO-d_6): δ (ppm) 194.91, 152.89, 146.30, 145.34, 137.91, 137.43, 133.97, 133.97, 132.53, 130.19, 129.54, 128.58, 128.16, 125.98, 125.53, 115.35, 113.40, 53.75, 20.88. XRPD (2θ angle): 6.9°, 11.3°, 12.4°, 14.0°, 16.2°, 18.6°, 19.8°, 21.6°, 22.7°, 23.7°, 25.4°, 26.2°, 29.7°. FT-IR: ν (cm^{-1}) 3567, 3063, 2804, 1764, 1654, 1641, 1602, 1441, 1231, 1200, 1009. M.p. 180-181°C. Yield 60%.

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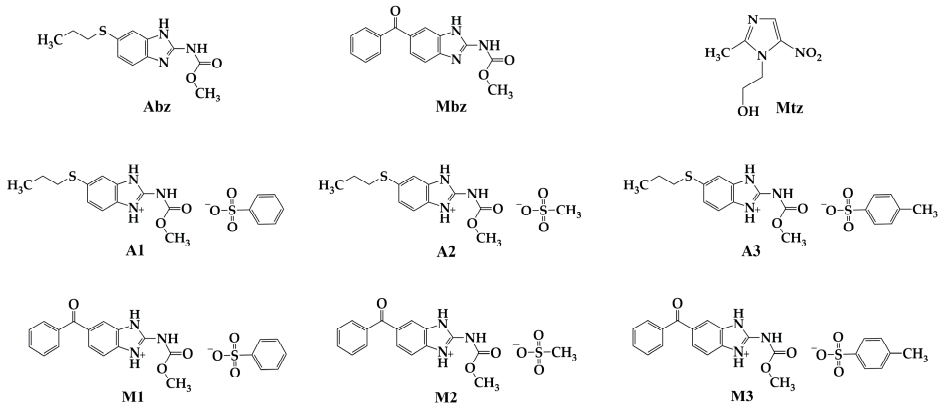

Figure 1. Chemical structure of **Abz** and **Mbz** salts, as well as reference controls. **A1**: albendazole with benzenesulfonic acid; **A2**: albendazole with methanesulfonic acid; **A3**: albendazole with *p*-toluenesulfonic acid; **M1**: mebendazole with benzenesulfonic acid; **M2**: mebendazole with methanesulfonic acid; **M3**: mebendazole with *p*-toluenesulfonic acid; **Abz**: albendazole; **Mbz**: mebendazole and **Mtz**: metronidazole.

2.2. In Vitro Antiprotozoal Activity Assays

2.2.1. Stock Solutions

Stock solutions of **Abz** salts, **Mbz** salts, **Abz**, **Mbz** and **Mtz** where prepared by dissolving in 0.1 % (v / v) dimethyl sulfoxide (DMSO, AppliChem, Ottoweg, Darmstadt, Germany) at level at which no inhibition of trophozoites occurs[39]. The solutions were further diluted to 1 mL by adding freshly prepared culture medium to reach a concentration of 2 mg/mL. Two-fold serial dilutions were made in eppendorf tubes (Eppendorf) in 100 µL of culture medium. Each test included **Mtz**, **Abz** and **Mbz** as reference antiparasitic drugs, grow culture tubes (culture medium with trofozoites only) and blanck tubes (culture medium only).

2.2.2. Trophozoite Culture Conditions

Antiprotozoal activity was evaluated against trophozoites of *E. histolytica* (strain HM1: IMSS), *G. lamblia* (isolate J10) and *T. vaginalis* (isolate MB: FF09). *E. histolytica* trophozoites maintained axenically in BI-S-33 pH 6.5 medium supplemented with 10% calf serum (previously inactivated at 56 °C for 30 min (Microlab Laboratories, Mexico City, Mexico). *G. lamblia* trophozoites were maintained axenically in TYI-S-33 medium pH 6.8 supplemented with bovine bile and 10% fetal bovine serum previously inactivated (Microlab Laboratories, Mexico City, Mexico). *T. vaginalis* trophozoites were maintained axenically in modified Diamonds medium pH 7.0 supplemented with 10% calf serum previously inactivated (Microlab laboratories, Mexico City, Mexico). To determine the viability of the cultures and the number of trophozoites per milliliter, 0.4% trypan blue dye (In Vitro, Mexico City, Mexico) was used in a Neubauer chamber to guarantee viable cultures.

2.2.3. In Vitro Antiparasitic Activity Tests

To evaluate the antiparasitic effect of **Abz** and **Mbz** salts, the tube microdilution technique modified from Hernández-Ochoa [40] was used. 2 x 10^4 trophozoites of *G. lamblia* and 1 x 10^4 trophozoites of *E. histolytica* or *T. vaginalis* were used. The trophozoites were exposed for 48 h at 37 °C, with different concentrations of **Abz** and **Mbz** salts (2922- 2.08 µM). As positive inhibition controls, the drugs **Abz**, **Mbz** and **Mtz** were included under the same evaluation concentrations, and as negative controls, trophozoite cultures without treatment and trophozoites with 0.1% DMSO (AppliChem, Ottoweg, Darmstadt, Germany) were used. After exposure time, the percentage of cell viability and the percentage (%) of inhibition of trophozoite growth (% inhibition = 100 - % viability) were determined by microscopic counting, using 0.4% trypan blue staining (1:1, v / v) (In Vitro, Mexico City, Mexico) in a Neubauer chamber. Finally, the mean inhibitory concentration (IC_{50}) was

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determined by linear regression using the concentration of compounds and the % inhibition. Three independent assays were performed in triplicate.

2.3. In Vitro Cytotoxicity Assays

2.3.1. Cell Line Culture Conditions

The cytotoxic activity was carried out on the VERO cell line (green monkey kidney cells: *Cercopithecus aethiops*) [39,41]). The cells were cultured in RPMI – 1640 medium (In Vitro, Mexico City, Mexico), supplemented with 10% fetal bovine serum previously inactivated (Microlab Laboratories, Mexico City, Mexico), L- glutamine 2Mm and 1% penicillin/streptomycin/amphotericin B (In Vitro, Mexico City, Mexico) at 37 °C with a 5% CO_2 atmosphere. Cells were allowed to grow to a density of 85% and were subsequently harvested using a 0.05% trypsin-versene solution (In Vitro, Mexico City, Mexico) prior to each experiment. The viability and number of cells per milliliter were determined using 0.4% trypan blue dye (1:1, v / v) (In Vitro, Mexico City, Mexico) and a Neubauer chamber.

2.3.2. Cytotoxicity Assays

For the assay, cells were seeded in 96-well plates at concentrations of $1x10^4$ cells/ well and incubated for 24 h at 37 °C. After incubation, cells were treated with different concentrations (500 µM - 0.97 µM) of **Abz** and **Mbz** salts, in each assay the following were included as controls: cells with culture medium, cells with 0.1% DMSO (AppliChem, Ottoweg, Darmstadt, Germany), as well as the positive controls with the drugs **Abz**, **Mbz** and **Mtz**. Cells were incubated for 48 h at 37 °C. The cytotoxic effect of the salts evaluated on Vero cells was determined using the colorimetric technique with the WST–1 reagent (Roche Diagnostics GmbH, Mannheim, Germany), which allows measuring cell proliferation and viability by reducing the tetrazolium salt WST-1 to formazan by cellular dehydrogenases, the amount of formazan formed is directly correlated with the number of metabolically active cells in the culture, in this way the viability of the cells was measured with the optical density of the formazan products. With the results obtained, the Average Cytotoxic Concentration (CC_{50}) was determined by linear regression with the percentage of inhibition and logarithm of the concentration. Three independent assays were performed in duplicate.

2.3.3. Selectivity Index (SI)

To determine the selectivity of the antiprotozoal activity, the cytotoxicity profile of the evaluated compounds was used, by calculating the SI: which is the quantitative relationship that exists between the cytotoxic activity against a mammalian cell line and the antiprotozoal activity of a certain compound (CC_{50} of the compounds evaluated on the VERO cell line / IC_{50} of the compounds evaluated on trophozoites)[39,41,42]. Normally an index greater than 1 is considered good since the in vitro antiparasitic activity is more selective towards the parasite, while a lower index than 1 is considered more selective towards mammalian cells according to what was reported [42,43].

3. Results

3.1. In Vitro Antiparasitic Activity

The antiparasitic activity of **Abz** and **Mbz** salts was evaluated in vitro against *E. histolytica*, *G. lamblia*, and *T. vaginalis*. The results are shown in Table 1 as IC_{50} values. **Abz**, **Mbz**, and **Mtz** were used as reference drugs.

Salt **A1** did not present antiparasitic effects on *E. histolytica*, while salts **A2** and **A3** were 1.9 and 1.8 times more active than the **Abz** reference control, with IC_{50} values of 37.95 and 39.93 µM, respectively. All **Mbz** salts inhibited the growth of *E. histolytica*, with salt **M3** having the lowest IC_{50} value (44.34 µM), being 1.3 times more active than **Mbz**. All three **Abz** salts were active against *G. lamblia*, with IC_{50} values ranging between 51.31 and 38.02 µM. Salt **A3** increased its potency 5.2 times

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compared to the reference control **Abz**. Regarding **Mbz** salts, **M1** had no antiparasitic effect on *G. lamblia*, while **M2** and **M3** salts were 3.2 and 3.3 times more active compared to **Mbz**. As for *T. vaginalis*, salts **A1** and **A3** did not present antiparasitic effects; however, salt **A2** presented better antiparasitic activity, increasing its potency 1.68 times compared to **Abz**. All **Mbz** salts exhibited antiparasitic activity against *T. vaginalis*, with salt **M1** having the lowest IC_{50} value (24.17 μM). Finally, the **Mtz** control presented IC_{50} values of 16.08 and 16.16 μM for *E. histolytica* and *T. vaginalis*, respectively, values that were lower than those obtained for the **Abz** and **Mbz** salts (Table 1). However, the IC_{50} value on *G. lamblia* was 97.63 μM, while the IC_{50} value obtained for salts **A2**, **A3**, **M2**, and **M3** ranged from 51.31 to 79.62 μM.

Even though **Abz** and **Mbz** salts have good antiprotozoal activity, **A1** does not inhibit the growth of *E. histolytica* and *T. vaginalis*. Nonetheless, the rest of the salts evaluated behaved as potent antiprotozoal agents, in almost all cases performing even better than the reference controls **Abz** and **Mbz**.

Table 1. Antiparasitic activity of **Abz** and **Mbz** salts.

	Antiparasitic activity		
Compound	***E. histolytica*** **IC_{50} [μM]**	***G. lamblia*** **IC_{50} [μM]**	***T. vaginalis*** **IC_{50} [μM]**
A1	N. E.[4]	138.02 ± 1.41	N. E.[4]
A2	37.95 ± 0.72	78.05 ± 0.43	125.53 ± 1.80
A3	39.93 ± 0.81	51.31 ± 1.23	N. E.[4]
M1	128.05 ± 2.69	N. E.[4]	24.17 ± 0.15
M2	57.72 ± 0.70	79.62 ± 0.99	49.86 ± 0.80
M3	44.34 ± 0.45	77.98 ± 0.70	62.59 ± 1.44
Abz[1]	73.51 ± 1.09	270.66 ± 1.89	211.71 ± 2.18
Mbz[2]	59.81 ± 1.18	262.74 ± 2.49	36.59 ± 0.69
Mtz[3]	16.08 ± 0.69	97.63 ± 1.43	16.16 ± 0.66

[1]Albendazole; [2]mebendazole; [3]metronidazole; [4]No effect at the evaluated concentrations. Results are represented as the mean ± standard deviation of three independent assays in duplicate.

3.2. In Vitro Cytotoxicity

Biological assays were performed on the VERO cell line to better understand the cytotoxic effects of these salts on human cells, compared to the effects observed in protozoans. Cell viability was evaluated by the colorimetric assay with the WST-1 reagent (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphenyl)-2H-tetrazolium). The results are expressed in Figures 2 and 3 as percentage of cell viability (%CV). It is observed that the viability of VERO cells was not affected after being exposed to salts **A1**, **A2**, **M2**, and **M3** at the highest concentration evaluated (500 μM) since a viability percentage greater than 98% was maintained. However, with salt **A2**, a decrease in cell viability was observed from the concentration of 250 μM (91%), and at the highest concentration evaluated, the viability was reduced to 37%.

On the other hand, with salt **M1**, a behavior similar to that of **A2** is observed, since as the concentration of the salt increases, cell viability decreases. Starting at 31.25 μM, a decrease (95%) in viability is observed, reaching 38% at the concentration of 125 μM. As expected, 0.1% of DMSO did not alter cell growth; similarly, this occurred with the controls **Abz**, **Mbz**, and **Mtz**, which presented viability percentages greater than 95%

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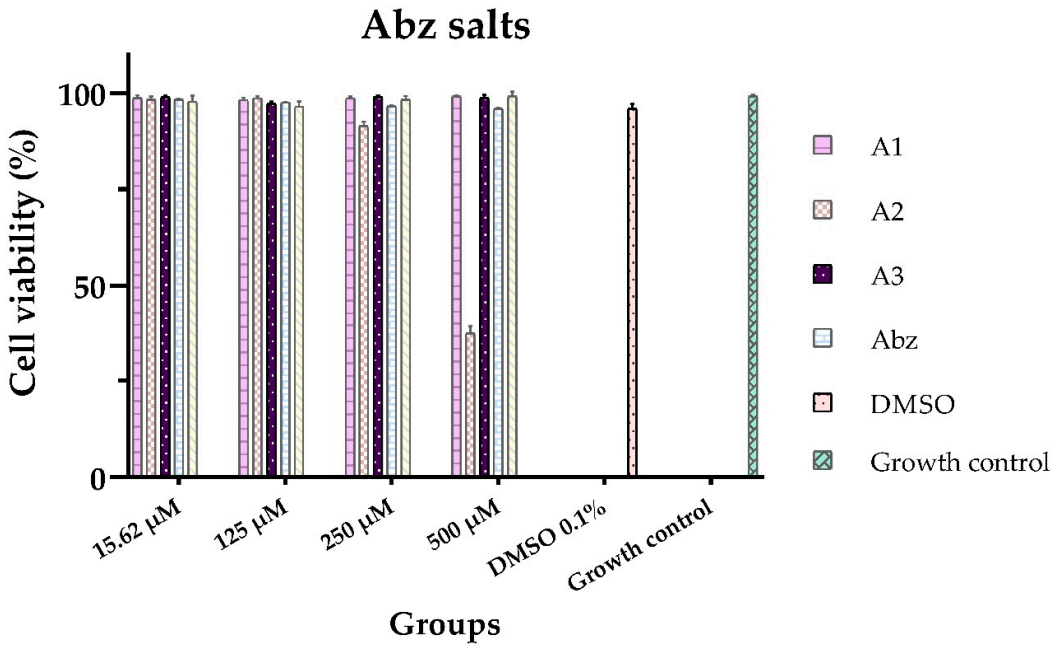

Figure 2. Effects on cell viability of **Abz** salts on VERO cell line, after 48 h of exposure. Cells without treatment, 0.1% DMSO solvent control. Results are presented as the mean ± standard deviation of three independent assays in duplicate.

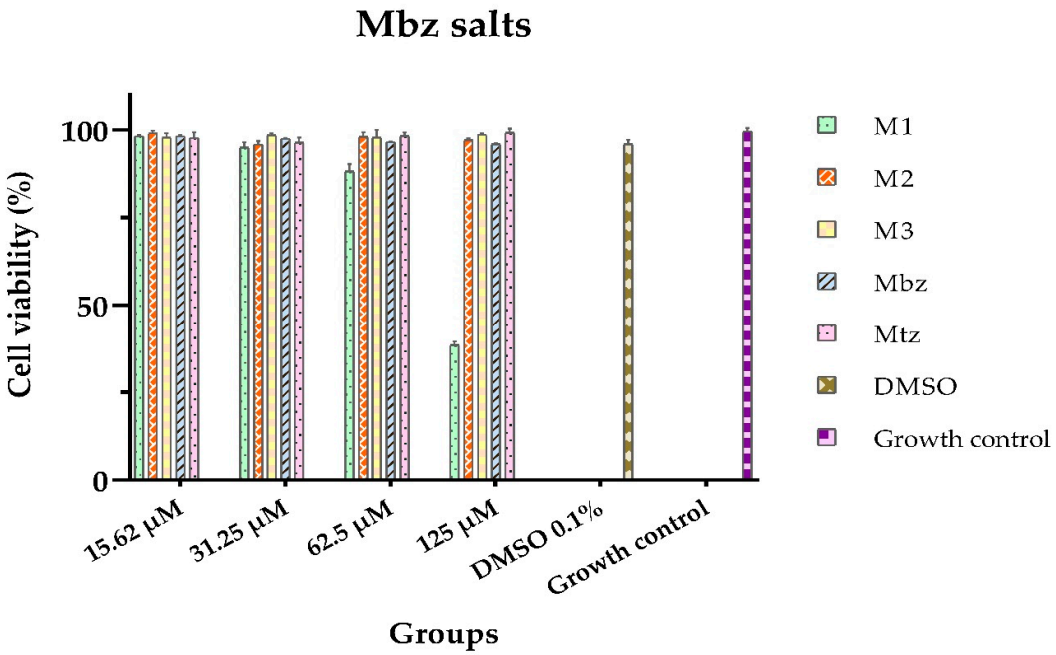

Figure 3. Effect on cell viability of **Mbz** salts on VERO cell line, after 48 h of exposure. Cells without treatment, 0.1% DMSO solvent control. Results are presented as the mean ± standard deviation of three independent assays in duplicate.

The CC_{50} was calculated using linear regression, determining the values to be 435.46 µM for salt **A2** and 104.04 µM for salt **M1** (Table 2). For the rest of the **Abz** and **Mbz** salts, as well as the controls,

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it was not possible to calculate the specific CC_{50} because they were not cytotoxic to VERO cells at the concentrations tested, so it was assumed to be greater than 500 μM (Figures 1 and 2)

Table 2. In vitro cytotoxicity values of **Abz** and **Mbz** salts in VERO cell line.

Cytotoxic activity	
Salt	**CC_{50} (μM)**
A1	> 500
A2	435.46 ± 2.17
A3	> 500
M1	104.04 ± 3.85
M2	> 500
M3	> 500
Abz	> 500
Mbz	> 500
Mtz	> 500

The results are represented as the means of three independent tests in duplicate, ± SD: standard deviation.

The selectivity index (SI) results for the three evaluated protozoans are presented in Table 3. For the salts that exhibited no cytotoxic effect at the tested concentrations on the VERO cell line, a CC_{50} of 500 μM was used as the basis for the SI calculation. SI exceeded 1 [42,43], indicating that the **Abz** and **Mbz** salts preferentially target protozoans.

Table 3. Selectivity index of **Abz** and **Mbz** salts.

Selectivity Index			
Salt	***E. histolytica***	***G. lamblia***	***T. vaginalis***
A1	N. E.[1]	3.62	N. E.[1]
A2	11.47	5.57	3.46
A3	12.52	9.74	N. E.[1]
M1	0.81	N. E.[1]	4.30
M2	8.66	6.27	10.02
M3	11.27	6.41	7.98
Abz	6.80	1.84	2.36
Mbz	8.35	1.90	13.66
Mtz	31.09	5.12	30.94

[1]No effect on the evaluated concentrations.

4. Discussion

Our findings indicate that the selection of the chosen counterion for forming **Abz** and **Mbz** salts influences the antiparasitic activity against the tested parasites. Although the literature states that forming pharmaceutical salts does not alter the biological activity of the active pharmaceutical ingredient (API) [44], our in vitro findings contradict this assertion. We observed that *G. lamblia* **A3** and **M3** displayed the strongest antiparasitic effects, with **A2** and **M2** following, when compared to our references **Mtz**, **Mbz**, and **Abz**, indicating that a lower dosage is needed to achieve the equivalent antiparasitic effect (Table 1). A comparable behavior was noted with *E. histolytica*, as **A2**, **M2**, **A3**, and **M3** demonstrated the most effective antiparasitic action in relation to **Abz** and **Mbz**; nevertheless, **Mtz** is the compound that showed the highest antiparasitic effect (Table 1). In conclusion, for *T. vaginalis*, the salt that exhibited the most effective antiparasitic properties was **M1**, which enhanced its effect compared to **Mbz**, whereas the other salts showed reduced or no activity against *T. vaginalis*. These findings suggest that the mesylate and tosylate ions increase the antimicrobial activity, akin to

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previous reports involving bacteria (*Mycobacterium tuberculosis* [45], *Staphylococcus aureus*, *Bacillus subtilis*, and *Klebsiella pneumoniae* [46]. Mesylate salts improve the antibacterial effectiveness when compared to the antibiotic in its unbound state. Comparable research has indicated that the antibacterial effectiveness of salts featuring different counterions and antimicrobials [47–50] varies based on the counterion utilized and the microorganism assessed. Nonetheless, the exact mechanism through which this behavior occurs is not completely clarified, and these researchers propose that enhancements in the physicochemical characteristics of salts facilitate specific interactions with the cell membrane of microorganisms, engaging with lipids, carbohydrates, and membrane proteins [48], thereby permitting a larger quantity of drug to penetrate the microorganism's interior and be accessible to perform its biological function. Based on these assumptions, *E. histolytica*, *G. lamblia*, and *T. vaginalis* have distinct plasma membrane compositions, fluidities, and permeabilities [51–53], indicating that the interactions between the sulfonates **Abz** and **Mbz** with the cell surface vary among the parasites, influencing the uptake or expulsion of the drug by the cell. Our findings back this up, as the influence of one salt benefits one parasite but diminishes or reduces its effect on another (Table 1). Nonetheless, sufficient permeability research is required to clarify the synergistic interaction between the counterion and the medication (**Abz** and **Mbz**). Moreover, it is crucial to consider the toxicity of counterions when formulating a pharmaceutical salt [54]. Our findings indicated that the salts do not exhibit cytotoxicity towards VERO cells, except for **A2** and **M1** (Figures 2 and 3). This phenomenon has been noted before, indicating that antibiotic sulfonate salts do not influence cell viability [45,55]. Nonetheless, it is essential to compare these values according to their antiparasitic effects; hence, we calculated the selectivity index (SI) to reflect the preference between parasites and host cells, with an IS > 1 considered the minimum threshold to demonstrate a preference for the parasites [42,43,56].

5. Conclusions

In conclusion, our findings showed that tosylate salts effectively inhibit the growth of *E. histolytica* and *G. lamblia*, outperforming the control drugs **Abz**, **Mbz,** and **Mtz**, all while preserving selectivity for parasites. Mesylate salts also effectively hinder the growth of *E. histolytica* and *G. lamblia*; however, **A2** exhibited cytotoxic effects on kidney cells. Moreover, besylate salts reduce their effectiveness against *G. lamblia* and *E. histolytica* and are not an appropriate substitute for curtailing the proliferation of *T. vaginalis*. Therefore, it is crucial to emphasize that **A3** and **M3** are potential drug salts effective against protozoan parasitic infections. Ultimately, our study is consistent with others that examine how the use of sulfonates as counterions influences the antimicrobial efficacy of the parent compound, either positively or negatively.

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Abbreviations

The following abbreviations are used in this manuscript:

ABZ	Albendazole
API	Active Pharmaceutical Ingredient
CC_{50}	Cytotoxic Concentration 50%
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
IC_{50}	Inhibitory Concentration 50%
MBZ	Mebendazole
MTZ	Metronidazole
NMR	Nuclear Magnetic Resonance
OTC	Over the counter
PXRD	Powder X-ray Diffraction
SI	Selectivity Index
%CV	Cell Viability percentage

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