

Article

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

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Abstract: Infections from the protozoa *Entamoeba histolytica* (*E. histolytica*), *Giardia lamblia* (*G. lamblia*), and *Trichomonas vaginalis* (*T. vaginalis*) pose a public health issue, with albendazole and mebendazole serving as the second-line medications for treating these parasitic infections. 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, along with **M1**, **M2**, and **M3** of mebendazole, were synthesized. The antiparasitic activity in vitro was assessed against the trophozoites of *E. histolytica*, *G. lamblia*, and *T. vaginalis*. The salts **A2**, **A3**, **M2**, and **M3** demonstrated a greater antiparasitic effect (IC₅₀ 37.95–125.53 µM) compared to the positive controls albendazole and mebendazole. The 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; toxicity; selective index; sulfonate salts



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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]. *E. histolytica* colonizes the large intestine, leading to an infection called amoebiasis, which can present as either asymptomatic or invasive, potentially invading the liver, heart, lungs, and brain [2,3]. *G. lamblia* ranks as the second most common cause of infectious diarrhea in humans. Giardiasis may occur as an asymptomatic infection or can be linked to diarrhea, stomach pain, nausea, bloating, gas, malabsorption, weight loss, and growth delays in children [4,5]. *T. vaginalis* is responsible for the sexually transmitted infection known as trichomoniasis, resulting in 248 million cases reported globally each year [6]. In men, it can present as either an asymptomatic or mild condition, whereas in women, it might be asymptomatic or lead to intense vaginitis (characterized by vaginal discharge with a foul smell and yellow–green hue, dysuria, itching, and abdominal pain) [7].

Metronidazole (α-hydroxyethyl-2-methyl-5-nitroimidazole, **Mtz**) is the first choice for treating amoebiasis, giardiasis, and trichomoniasis [8]. 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 [9]. Resistance to **Mtz** in clinical isolates has been reported in *G. lamblia* [10], *E. histolytica* [11], and *T. vaginalis* [12].

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** [13,14]. 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, essential biological processes such as adhesion, motility, and cell division are directly affected [15].

Although the resistance related to **Abz** and **Mbz** has been documented, it occurs less frequently than has been observed for **Mtz** [10]. Conversely, unlike the significant water solubility of **Mtz**, the main limitation for the use of **Abz** and **Mbz** is their poor water solubility, impacting the total amount of drug absorbed [16,17].

To improve water solubility, various methodologies have been described, such as the formation of nanocapsules [18], solid dispersions [19], nanocrystals [20], liposomes [21], cyclodextrins [22], inclusion complexes [23], self-emulsifying drug delivery systems [24], and pharmaceutical salts [25]. 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 [26,27]. 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 [28].

It has also been reported that the counterion used for the formation of drug salts impacts the values of inhibitory concentration in vitro antibacterial studies [29,30]. Therefore, the aim of the present study is to evaluate the in vitro amebicidal, trichomonocidal, 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**) (Figure 1). This will determine whether the modulation of activity occurs for antiparasitic drug salts by analyzing their IC₅₀ values, their cytotoxic concentration (CC₅₀) on the Vero cell line, and their selectivity index. Finally, the goal is to select those with the best biological properties for subsequent in vivo pharmaceutical studies, which may serve as candidates for the treatment of parasitic infections.

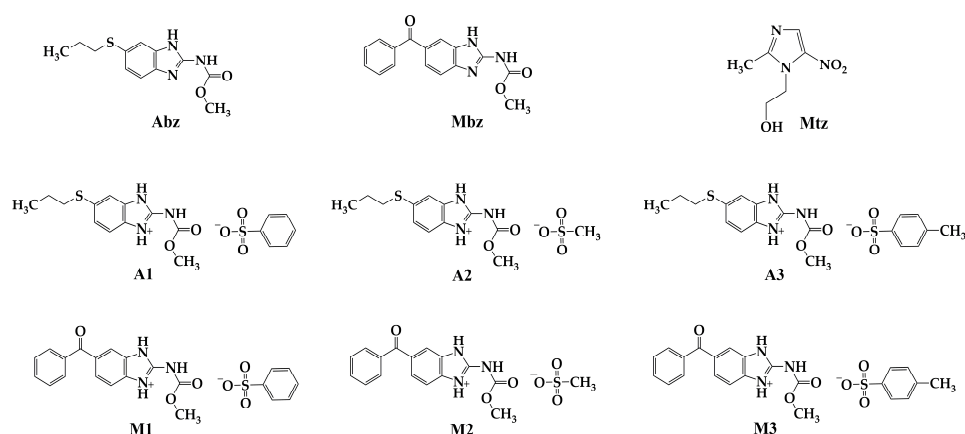


Figure 1. Chemical structure of **Abz** and **Mbz** salts, as well as reference controls. **A1**: albendazole with benzenesulfonic acid; **A2**: albendazole with methanesulfonic acid; **A3**: albendazole with *p*-

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. Materials and Methods

2.1. General Procedure to Prepare Abz and Mbz Sulfonic Salts

2.1.1. Reagents and Solvents

Albendazole ($\geq 98\%$, CAS 54965-21-8), mebendazole ($\geq 98\%$, CAS 31431-39-7), *p*-toluenesulfonic acid monohydrate ($\geq 98.5\%$ CAS 6192-52-5), benzenesulfonic acid monohydrate (97%, CAS 26158-00-9), and methanesulfonic acid ($\geq 99\%$, CAS 75-75-2) were purchased from Sigma-Aldrich® (St. Louis, MO, USA) and used without prior purification. Methanol ($\geq 99.9\%$, CAS 67-56-1) and acetone (99.8%, CAS 67-64-1) were reagent-grade and purchased from J.T. Baker (Monterrey, NL, México).

2.1.2. Equipment and Conditions for Chemical Characterization

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

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 a magnetic stirrer. Then, enough solvent (acetone or methanol, ca. 3 mL) was poured to form a solid suspension. The reaction mixture was stirred at room temperature for 24 h. At the terminus, the suspension was filtered, and the solid was rinsed with an aliquot of fresh solvent to remove the starting material. Finally, the solid was dried under vacuum for 2 h. The yield of solid salt was 60–75%.

2.1.4. Chemical Characterization of Salt **A1** (Albendazole Besylate)

^1H 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. $182\text{--}184^\circ\text{C}$. Yield 65%.

2.1.5. Chemical Characterization of Salt **A2** (Albendazole Mesylate)

^1H 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)

¹H 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). ¹³C 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 (2 θ 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)

¹H 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). ¹³C 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 (2 θ 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 occurred prior to the melting of the sample. Yield 70%.

2.1.8. Chemical Characterization of Salt **M2** (Mebendazole Mesylate)

¹H 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). ¹³C 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 (2 θ 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)

¹H 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). ¹³C 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 (2 θ 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%.

2.2. In Vitro Antiprotozoal Activity Assays

2.2.1. Stock Solutions

Stock solutions of **Abz** salts, **Mbz** salts, **Abz**, **Mbz**, and **Mtz** were prepared by dissolving in 0.1% (*v/v*) dimethyl sulfoxide (DMSO, AppliChem, Ottoweg, Darmstadt, Germany) at the level at which no inhibition of trophozoites occurs [31]. 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 in 100 μ L of culture medium. Each test included **Mtz**, **Abz**, and **Mbz** as reference antiparasitic drugs, grow culture tubes (culture medium with trophozoites only), and blank 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 were 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 Diamond's 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 [32] was used. A total of 2×10^4 trophozoites of *G. lamblia* and 1×10^4 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 (2922–2.85 µM). As positive inhibition controls, the drugs **Abz**, **Mbz**, and **Mtz** were included under the same evaluation concentrations (2922–2.85 µM), and as negative controls, trophozoite cultures without treatment and trophozoites with 0.1% DMSO (AppliChem, Ottoweg, Darmstadt, Germany) were used. After the exposure time, the percentage of cell viability and the percentage (%) of inhibition of trophozoite growth (% inhibition = $100 - \% \text{ viability}$) 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 (IC₅₀) was 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*) [31,33]). 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 2 mM, and 1% penicillin/streptomycin/amphotericin B (In Vitro, Mexico City, Mexico) at 37 °C with a 5% CO₂ 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 1×10^4 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 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphenyl)-2H-tetrazolium)(Roche Diagnostics GmbH, Mannheim, Germany), which allows for 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 (CC₅₀) 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, 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) [31,33,34]. Normally, an index greater than 1 is considered good since the in vitro antiparasitic activity is more selective toward the parasite, while an index lower than 1 is considered more selective toward mammalian cells according to what was reported [34,35].

2.4. Statistical Analysis

All data are shown as mean $\pm$ standard deviation (SD). The mean inhibitory concentration (IC_{50}) of each salt or compound was determined through linear regression, and 95% confidence intervals were also calculated. The mean cytotoxic concentration (CC_{50}) was also calculated using linear regression. For the comparison of multiple groups, a one-way analysis of variance (ANOVA) was performed, followed by Dunnett's post hoc test, considering a p value < 0.05 to be statistically significant. The analysis was performed using GraphPad Prism software version 8.0.2 (San Diego, CA, USA).

3. Results

3.1. In Vitro Antiparasitic Activity

The antiparasitic activity of the **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.

The salts **A2** and **A3** exhibited activity against *E. histolytica* that was 1.9 and 1.8 times greater than **Abz**, with IC_{50} values of 37.95 and 39.93 μ M, respectively, whereas salt **A1** displayed no antiparasitic activity. All **Mbz** salts inhibited the growth of *E. histolytica*, with salt **M3** showing the lowest IC_{50} (44.34 μ M), rendering it 1.3 times more effective than **Mbz**. All three **Abz** salts were active against *G. lamblia*, with IC_{50} values ranging between 51.31 and 38.02 μ M. Salt **A3** increased its potency by 5.2 times compared to **Abz**. The salts **M2** and **M3** were 3.2- and 3.3-times more active than **Mbz**, whereas **M1** showed no effect on *G. lamblia*. Regarding *T. vaginalis*, only salt **A2** demonstrated an antiparasitic effect, enhancing its potency by 1.68 times in relation to **Abz**. Conversely, all **Mbz** salts exhibited antiparasitic effects against *T. vaginalis*, with salt **M1** demonstrating the lowest IC_{50} value (24.17 μ M). Finally, the **Mtz** control presented IC_{50} values of 16.08 and 16.16 μ M 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 for *G. lamblia* was 97.63 μ M, while the IC_{50} value obtained for salts **A2**, **A3**, **M2**, and **M3** ranged from 51.31 to 79.62 μ M.

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

Antiparasitic Activity		
<i>E. histolytica</i>	<i>G. lamblia</i>	<i>T. vaginalis</i>
Salt/Compound		
IC_{50} [μ M]		
Confidence interval		
Mtz ¹	A3 ^b	Mtz ³
16.08 $\pm$ 0.69	51.31 $\pm$ 1.23	16.16 $\pm$ 0.66
(15.40–16.77) ^a	(50.09–52.52) ^a	(15.50–16.81) ^a

Table 1. Cont.

Antiparasitic Activity		
<i>E. histolytica</i>	<i>G. lamblia</i>	<i>T. vaginalis</i>
A2 ^b 37.95 ± 0.72 (37.24–38.66) ^a	M3 ^c 77.98 ± 0.70 (77.28–78.67) ^a	M1 ^c 24.17 ± 0.15 (24.01–24.33) ^a
A3 ^b 39.93 ± 0.81 (39.14–40.73) ^a	A2 ^b 78.05 ± 0.43 (77.62–78.05) ^a	Mbz ² 36.59 ± 0.69 (35.91–37.28) ^a
M3 ^c 44.34 ± 0.45 (43.89–44.79) ^a	M2 ^c 79.62 ± 0.99 (78.64–80.59) ^a	M2 ^c 49.86 ± 0.80 (49.07–50.64) ^a
M2 57.72 ± 0.70 (57.03–58.41) ^a	Mtz ³ 97.63 ± 1.43 (96.22–99.04) ^a	M3 ^c 62.59 ± 1.44 (61.18–64.01) ^a
Mbz ² 59.81 ± 1.18 (58.65–60.98) ^a	A1 ^b 138.02 ± 1.41 (136.64–139.41) ^a	A2 ^b 125.53 ± 1.80 (123.76–127.30) ^a
Abz ³ 73.51 ± 1.09 (72.44–74.59) ^a	Mbz ² 262.74 ± 2.49 (260.29–265.19) ^a	Abz ¹ 211.71 ± 2.18 (209.57–213.85) ^a
M1 ^c 128.05 ± 2.69 (125.41–130.69) ^a	Abz ³ 270.66 ± 1.89 (263.89–277.44) ^a	A1 N. E. ⁴
A1 N. E. ⁴	M1 N. E. ⁴	A3 N. E. ⁴

¹ Metronidazole; ² mebendazole; ³ albendazole; ⁴ no effect at the evaluated concentrations; ^a 95% confidence intervals, α : 0.05; ^b statistical significance between the salts and the positive control (**Abz**) at $p < 0.05$; ^c statistical significance between the salts and the positive control (**Mbz**) at $p < 0.05$. Results are represented as the mean ± standard deviation of three independent assays performed in duplicate.

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**.

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 with protozoans. Cell viability was evaluated by colorimetric assay with the WST-1 reagent. The results are expressed in Figures 2 and 3 as the 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—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%.

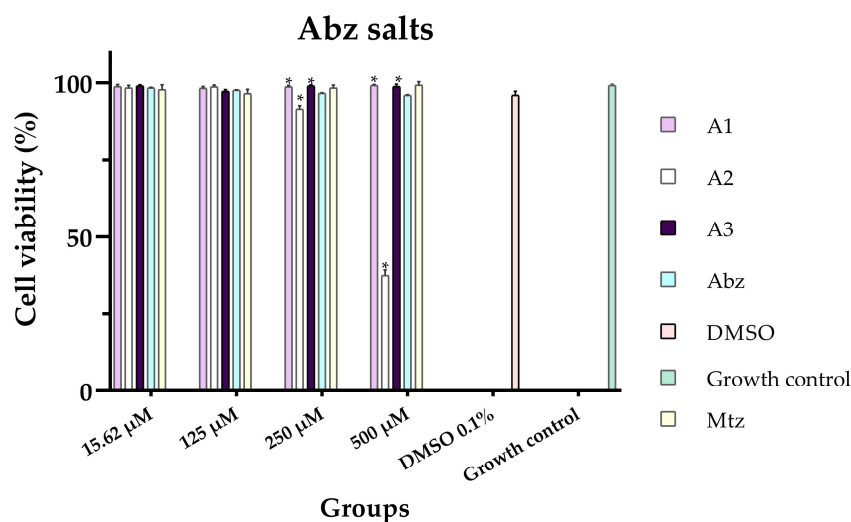


Figure 2. Effects on cell viability of **Abz** salts on Vero cell line after 48 h of exposure. Cells without treatment: 0.1% DMSO solvent control. Results are presented as the mean $\pm$ standard deviation of three independent assays performed in duplicate. The asterisk above the bar represents statistical significance between the salts and the positive control (**Abz**) at $p < 0.05$.

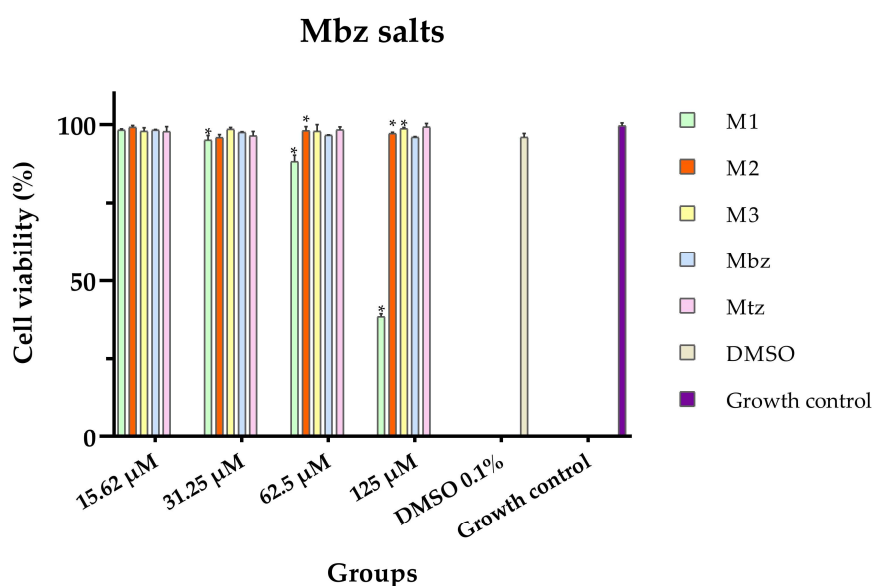


Figure 3. Effect on cell viability of **Mbz** salts on Vero cell line after 48 h of exposure. Cells without treatment: 0.1% DMSO solvent control. Results are presented as the mean $\pm$ standard deviation of three independent assays performed in duplicate. The asterisk above the bar represents statistical significance between the salts and the positive control (**Mbz**) at $p < 0.05$.

The CC_{50} was calculated using linear regression, determining the values to be 435.46 μ M for salt **A2** and 104.04 μ M for salt **M1** (Table 2). For the rest of the **Abz** and **Mbz** salts, as well as the controls, it was not possible to calculate the specific CC_{50} because they were not cytotoxic to Vero cells at the concentrations tested; therefore, it was assumed to be greater than 500 μ M (Figures 1 and 2).

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 μ M was used as the basis for the SI calculation. The SI exceeded 1 [34,35], indicating that the **Abz** and **Mbz** salts preferentially target protozoans.

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

Cytotoxic Activity	
Salt/Compound	CC ₅₀ (μM) Confidence Interval
A1	>500
A2	435.46 ± 2.17 (433.33–437.59) ^a
A3	>500
M1	104.04 ± 3.85 (100.26–107.82) ^a
M2	>500
M3	>500
Abz	>500
Mbz	>500
Mtz	>500

^a 95% confidence intervals, α: 0.05. The results are represented as the means of three independent tests performed in duplicate, ± SD: standard deviation.

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

Selectivity Index			
Salt/Compound	<i>E. histolytica</i>	<i>G. lamblia</i>	<i>T. vaginalis</i>
A1	N. E.	3.62	N. E.
A2	11.47	5.57	3.46
A3	12.52	9.74	N. E.
M1	0.81	N. E.	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

N.E. 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) [36], 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*.

In a similar way, Silva et al. [37] prepared mefloquine salts combined with sulfonic acids and assessed their antibacterial properties against *Mycobacterium tuberculosis*, emphasizing mesylate and tosylate for having lower minimum inhibitory concentration (MIC) values (26.3 and 22.7 μM, respectively) compared to the commercial salt mefloquine hydrochloride (MIC: 30.1 μM). Similarly, Madeira et al. [38] prepared organic salts of ciprofloxacin and norfloxacin and assessed their antibacterial effect on *Staphylococcus*

aureus, *Bacillus subtilis*, and *Klebsiella pneumoniae*. The mesylate enhanced the antibacterial efficacy of ciprofloxacin against the three tested strains, leading to a better IC_{50} value (14.38–55.07 μ M) in comparison to the reference drug (IC_{50} = 196.50 μ M). Meanwhile, norfloxacin mesylate enhanced the antibacterial activity solely against *Klebsiella pneumoniae* (IC_{50} = 203.8 μ M) when compared to norfloxacin (IC_{50} = 255.2 μ M). These writers ascribe these impacts to enhancements in the physicochemical characteristics, enabling a higher quantity of the drug to penetrate the microbe's interior to exert its action or even to influence the drug's buildup within the microorganism.

Conversely, the entry of salt moiety into the protozoan should be the rate-limiting step since the cell membrane is selectively permeable, and the flow through it is mediated by ion channels and transmembrane proteins that move molecules across the lipid barrier, facilitating the exchange of ions and other molecules from the extracellular environment to the interior [39]. Therefore, it is possible that the salts present unique discrete interactions with the cell membrane due to interactions with lipids, carbohydrates, and membrane proteins, depending on the characteristics of the counterion, as outlined by Ferraz et al. [30,40].

In this instance, *E. histolytica*, *G. lamblia*, and *T. vaginalis* possess a plasma membrane with distinct composition, fluidity, and permeability, indicating that the interaction of our sulfonates with the cell surface varies for each salt. Nonetheless, suitable permeability investigations are necessary to clarify the combined effect of the counterion, the drug, and the membrane.

Moreover, it is crucial to consider the toxicity of counterions when formulating a pharmaceutical salt [41]. Our findings indicated that the salts do not exhibit cytotoxicity toward 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 [37,42]. 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 SI > 1 considered the minimum threshold to demonstrate a preference for the parasites [34,35,43].

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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