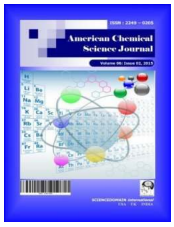

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Electron Withdrawing Groups and Steric Effects on the Methanogenic Toxicity

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Authors' contributions

This work was carried out in collaboration between all authors. Authors BMN and KK designed the study, author PVT performed the statistical analysis, author DTM wrote the protocol and author PTM wrote the first draft of the manuscript. Authors SOM and EKA managed the analyses of the study. Authors NLB and DSTT managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This work highlights the influence of the additivity of withdrawing electronic groups attached to the aromatic ring on the inhibition of the production of methane gas by the methanogenic bacteria acetoclastic.

Study Design: Anaerobic digestion of pig manure, anaerobic toxicity essay, Effect of additivity and the number of electron withdrawing groups on the methanogenic toxicity, relationship between the methanogenic toxicity, electron and steric effects.

Place and Duration of Study: Department of Chemistry, University of Kinshasa, DR Congo, from March 2012 to January 2013.

Methodology: The toxicity to acetoclastic Methanogenic archaea was performed with the standard method of serum bottles, digested pig manure was utilized as inoculums and acetate as substrate.

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The methane gas volume produced was measured by serum bottles liquid displacement systems (Mariotte flask system).

Results: The obtained results show that the increase of the number of electron withdrawing groups on the aromatic ring does not imply by force the increase of the Methanogenic toxicity (MT) of these aromatic compounds, in comparison to those which has only one electron withdrawing group. We notice that Nitrobenzene and Chlorobenzene have shown higher toxicity than their *ortho-, meta-* and *para*-Nitro and Chloro groups isomers, respectively. Moreover for these groups, the *meta*-isomers show more toxicity than the *para*-isomers, which in their turns are more toxic than the *ortho*-isomers.

Conclusion: The overall obtained results indicate that as for electronic effects of various withdrawing substituents on the aromatic ring, the steric constraints of those substituents also have a considerable effect on MT.

Keywords: Methanogenic toxicity; electron withdrawing groups; inhibition; biogas; methanogenic archaea; biomethanization.

1. INTRODUCTION

Access to energy is an essential component of sustainable economic, social and political development of a continent. Despite a huge potential in fossil and renewable energy, Africa has significant energy deficits: the continental resources are underexploited, so exported in raw form, or even often wasted [1].

With population's increasing that was always higher on average than on others continents, Africa is currently faced with an increased demand of energy. It is also noticed that about 77% of the population of sub-Saharan Africa has no access to electricity or clean form of energy because of generally ancient and vulnerable electric equipment.

However biomass, especially firewood, presents 60 to 80% on average of the consumed energy, so causing serious deforestation problems and respiratory diseases. Among which are added greenhouse effect due to gas emission because of the use of fossil fuel (oil), which contributes to almost 35 % of the energy consumed in this part of the continent [1-3].

Clean and affordable energy Supply, population rising and effective management of waste: many crucial issues which arouse attention of all searches conducted this recent decade in the environmental and energy field in Africa [1,2].

Anaerobic digestion (Biomethanisation) involves the degradation and stabilization of organic matter by microbial organisms which leads to the formation of biogas (a mixture of carbon dioxide and methane, a renewable energy source) and microbial biomass (digestat used as organic fertilizer) [4-7]. This technology is one of the most effective and least expensive treatments of waste for developing countries and offers many significant advantages, such as low sludge production, the production of organic fertilizer, energy recovery and reducing deforestation [8-10].

However, the aromatic compounds founded in agricultural and industrial organic waste constitute a limiting process factor [11-13].

In our previous work [14], we studied the nature, number and position (isomers) effects of substituents attached to the aromatic rings on the MT of the latter, without the possibility to reach this same aspect for electron withdrawing substituents.

In this work, we continued on that same line to assess the effect of the number and position (isomers) electron withdrawing substituents on the MT of aromatic compounds.

2. MATERIALS AND METHODS

2.1 Biomass

Pig manure from Domaine Agro-Industriel de la N'sele (DAIPN)/ Kinshasa (DR Congo) was digested in laboratory scale digester for about six months. The digested pig manure (sludge), not previously acclimated to any aromatic compounds, was utilized as inoculums in anaerobic toxicity tests.

The characteristics of inoculums were: total suspended solids (TSS, 94.20 g/L), volatile suspended matter (VSM, 58.18 g/L), and specific acetoclastic methanogenic activity: 159.74-205.83 mgCOD-CH_4/g VSS.

2.2 Stock Solutions

2.2.1 Stock substrate solution

The stock solution of the substrate consisted of a solution of sodium acetate (pH=7) obtained by reacting acetic acid with NaOH. A concentration of 100g COD-CH_3COOH/L (chemical oxygen demand per liter) was used in this study.

2.2.2 Stock solution 1

The used macro-nutrients compounds in that study are the following:

> NH_4Cl (170g/L), KH_2PO_4 (37g/L), $MgSO_4.4H_2O$ (37g/L), and $CaCl_2.2H_2O$ (10 g/L) [15-17].

2.2.3 Stock solution 2

The precursor compounds of trace elements are: $FeCl_3.4H_2O$ (200 mg/L), $CoCl_2.6H_2O$ (2000 mg/L), $MnCl_2.4H_2O$ (50 mg/L), $CuCl_2.2H_2O$ (50 mg/L), $ZnCl_2$ (50 mg/L), H_3BO_3 (50 mg/L), $(NH_4)_3MO_7O_2.4H_2O$ (90 mg/L), $Na_2SeO_3.5H_2O$ (100 mg/L), EDTA (1000 mg/L), HCl 36% (1 mg/L), $NiCl_2.6H_2O$ (50 mg/L), yeast extract (200 mg/L), and resazurin (500 mg/L) [15-17].

2.3 Aromatic Compounds

A total of 12 compounds studied with one or more than two electron withdrawing groups are shown in the Fig. 1. Benzene was used as reference for comparison purposes. All aromatic compounds were pure for analysis products supplied by MERCK VWR (Leuven, Belgium).

2.4 Anaerobic Toxicity Assay [15-17]

Specific acetoclastic methanogenic activity measurements were performed with 1L glass serum bottles sealed with rubber septa. These measurements were done based on the following procedure:

> Add to each serum bottle from the digester 1.5 g VSM of digested pig manure; 2 mL stock solution 1; 1 mL stock solution 2 and 40 mL stock substrate solution. The serum bottle was filled to about 1000 mL with oxygen free tap water (tap water flushed with nitrogen gas for at least 15 minutes). The flask was sealed with rubber septum cap and shook at room temperature for a few minutes.

The required quantity of test compounds (toxicants) was added to provide the concentrations to be investigated. No toxicant was added to the control samples. The toxicant concentrations were chosen as to induce an inhibition (0-100%) of the acetoclastic methanogenic activity.

The concentrations of inhibitors used in the anaerobic toxicity assay are given in the Table 1.

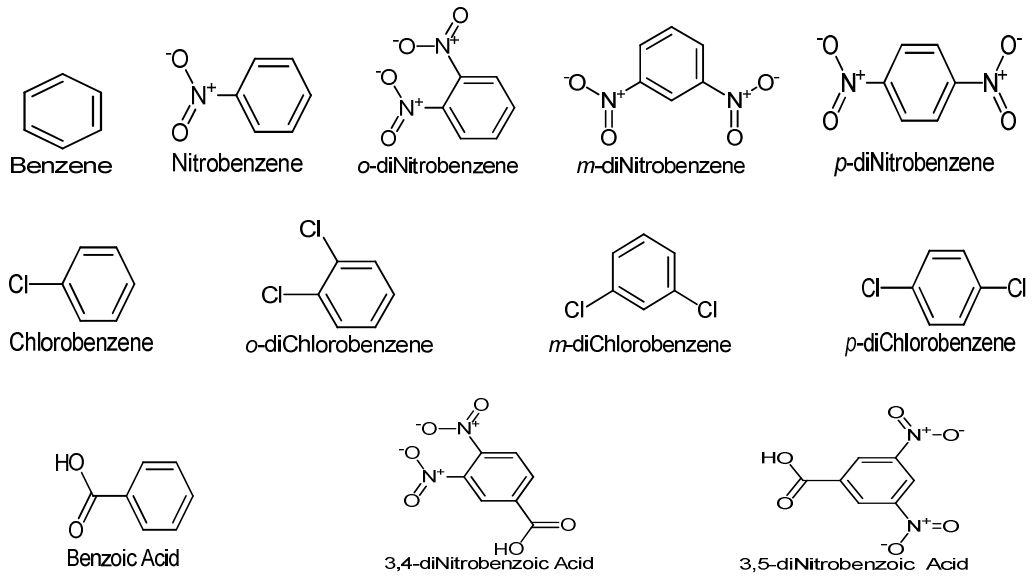

Fig. 1. Structures of studied compounds

Table 1. The inhibitory concentration of compounds used in anaerobic toxicity assay

N°	Aromatic compounds	Concentrations (mg/L)				
		1	2	3	4	5
1	Benzene	0	150	300	450	600
2	Nitrobenzene	0	2	3	6	9
3	o-diNitrobenzene	0	1	2	6	9
4	m-diNitrobenzene	0	1	2	6	9
5	p-diNitrobenzene	0	1	2	6	9
6	Chlorobenzene	0	10	15	30	50
7	o-diChlorobenzene	0	10	30	50	70
8	m-diChlorobenzene	0	10	30	50	70
9	p-diChlorobenzene	0	10	30	50	70
10	Benzoic Acid	0	900	1500	2500	3000
11	Acid 3,4-diNitrobenzoic	0	15	25	50	75
12	Acid 3,5-diNitrobenzoic	0	15	25	50	75

The specific methanogenic activity was calculated from the slope of the cumulative methane production versus time curve and the quantity of VSM. The compound concentration that caused 50% inhibition of the methanogenic activities of the control and samples containing inhibitory compounds were determined.

2.5 Methane Gas Measurement

The volume of methane gas produced was measured by serum bottle liquid displacement systems (Mariotte flask system) as previously described [10-12]. The liquid used was a solution of NaOH (15 mg/L). As the biogas passes through these high pH solutions, the CO_2 of biogas is converted to carbonate and absorbed into the liquid. Only methane gas passes through the solution and an equivalent volume is pushed out of the mariotte flask. The volume of the displaced liquid is then measured in a graduated cylinder [14,17].

3. RESULTS AND DISCUSSION

3.1 Inhibition of Specific Methanogenic Activity

The inhibitory effect of the test compounds and the reference (Benzene) on the activity of acetoclastic methanogenic bacteria was studied at different concentrations ranging from 1-3000 mg/L. This is exemplified by the experiment with Chlorobenzene as shown in Fig. 2 below, from which the IC_{50} was calculated as the concentration of Chlorobenzene corresponding to 50% of inhibition.

The method used to calculate the IC_{50} which is illustrated in the Fig. 2 was extended to all test compounds.

Table 2 below summarizes the inhibitory concentrations (IC_{50}) and the partition coefficient of test compounds (Partition coefficient taken in n-Octanol/water system) [18].

3.2 The Mechanism of the MT of Aromatic Compounds

The mechanism of action of aromatic compounds on the MT had been explained by their absorption on the cellular membrane of methanogenic bacteria and the modification of cellular diffusion that can lead to superficial tension change [13,17]. However, recently [14,19], It has been reported that this mechanism of action could also be explained by the disturbance of interactions between Coenzyme M (within the methanogenic bacteria) and the acetate substrate (from the decomposition of organic matter). The interactions are established by the formation of a complex between Coenzyme M and acetate substrate, leading to the production of methane. This involves charge transfer between Coenzyme M and the aromatic ring. In fact, the disruption of interactions between Coenzyme M and the acetate substrate is known to be greatly dependent on the nature (electron donating groups, electron withdrawing groups, or both) of the substituents on the aromatic ring [14,19].

3.3 Effect of the Electron Withdrawing Group Number and their Position on the MT

With reference to the Table 2 which carries obtained IC_{50} values during our toxicity tests, we noticed that the studied MT of aromatic compounds varied in the following ranking: Nitrobenzene > m- diNitrobenzene > p-

dinitrobenzene > o-diNitrobenzene > Chlorobenzene > m-diChlorobenzene > p-diChlorobenzene > o-diChlorobenzene > 3,5-diNitrobenzoicacid > 3,4-diNitrobenzoic acid > Benzene and Benzoic acid.

This sequence shows that Nitrobenzene is the most toxic studied aromatic compounds (IC_{50}=4.19 mg/L) followed by m-diNitrobenzene (IC_{50}=5.44 mg/L), whereas Benzene (IC_{50}=208.78 mg/L) and Benzoic acid (IC_{50}=2515.20 mg/L) are the least toxic.

It can be noticed that the substitution of -H benzene with an electron withdrawing substituent (functional group) generally rends it more toxic than benzene ring in the case of unsubstituted benzene. This confirms that the nature of the substituent has a great influence on MT.

The compounds generally having the Nitro substituent ($-NO_2$) were more toxic than compounds with substituting Chloro (-Cl), this is due to very strong electron nature of the Nitro group (σ=0.60) compared to Chloro group (σ=0.28). With σ, the Hamett's parameter characterizing electron withdrawing strength of a group [20].

According to the MT mechanism, this marked electron withdrawing effect of the Nitro group is responsible of the stabilization of the complex formed by charge transfer between coenzyme M (electrons donating) and the aromatic ring (electron withdrawing). In addition to this, there is the reactive nature of the Nitro group opposite to the cellular components of methanogenic bacteria, which justifies the toxicity of the aromatic ring substituted by the Nitro group increases more than the one substituted by chloro group.

Benzoic acid (IC_{50}=2515.20 mg/L) was found less toxic than benzene (IC_{50}=208.78 mg/L) taken as a control, therefore, the least toxic of all studied aromatic compounds and thus making an exception despite the nature of the electron withdrawing acid group (-COO, σ=0.32). This is justified by the acid group dissociation in solution ($-COOH \rightleftharpoons -COO^- + H^+$) thus rendering the aromatic ring polar (hydrophilic) than unsubstituted benzene, therefore difficult to cross the lipid bilayers of cell membrane to complex Coenzyme M. Thus the $LogP_{oct.}$ value of Benzoic acid is weak ($LogP_{oct.}$=1.87) compared to that of Benzene ($LogP_{oct.}$=2.13).

By comparing the IC_{50} values of 3,5-diNitrobenzoic acid (IC_{50}=63.17mg/L) and the one of 3,4-diNitrobenzoic acid (IC_{50}=67.20mg/L), It can be noticed that with respect to the Benzoic Acid, the –H substituting by two Nitro groups on the aromatic ring, has increased the toxicity of the latter thanks to the electron withdrawing effect and reactive nature of the Nitro group.

But 3,5-diNitrobenzoic acid has been found more toxic than 3,4-diNitrobenzoic acid. This is explained by the steric constraints due to two large Nitro groups in very close position (ortho-position) in 3,4-diNitrobenzoic acid thus preventing them to coexist in the same plane, thus causing widening of the size of the molecule through the offset of the two substituents on either side of the plane due to their repulsion. Over there is the steric constraints (molecule width), less the molecule will easily cross the cell membrane of methanogenic bacteria.

Fig. 3 shows IC_{50} of diNitrobenzene different isomers compared to benzene.

It is noticed that m-, p-, o-diNitrobenzene compounds have been less toxic than Nitrobenzene (IC50=4.19 mg/L) despite the presence of a second attractor Nitro group. This is justified by the steric constraints due to this second Nitro group width on the aromatic rings, which decreases their facility to pass through the cell membranes of methanogenic bacteria. But among the three isomers, m-diNitrobenzene (1,3-diNitrobenzene) has been the most toxic followed by p-diNitrobenzene (1,4-diNitrobenzene), and then o-diNitrobenzene (1,2-diNitrobenzene), respectively, due to the more marked steric constraints in this latter isomer. The above statement has been also established in the case of diChlorobenzene isomers as shown in Fig. 4.

As It can be seen, the *m*-diChlorobenzene (1,3-diChlorobenzene , IC_{50}=41.22 mg/L) was found the most toxic followed by *p*-diChlorobenzene (1,4-diChlorobenzene , IC_{50}=45.05 mg/L) and finally the *o*- diChlorobenzene (1,2-diChlorobenzene, IC_{50}=47.42 mg/L). But the three isomers were less toxic than Chlorobenzene (IC_{50}=30.08 mg/L) following steric constraints due to two Chloro substituents on the aromatic ring, which make difficult for the aromatic compound to be transported through the lipidic bacteria membranes and establish a complex with the bacteria Coenzyme M.

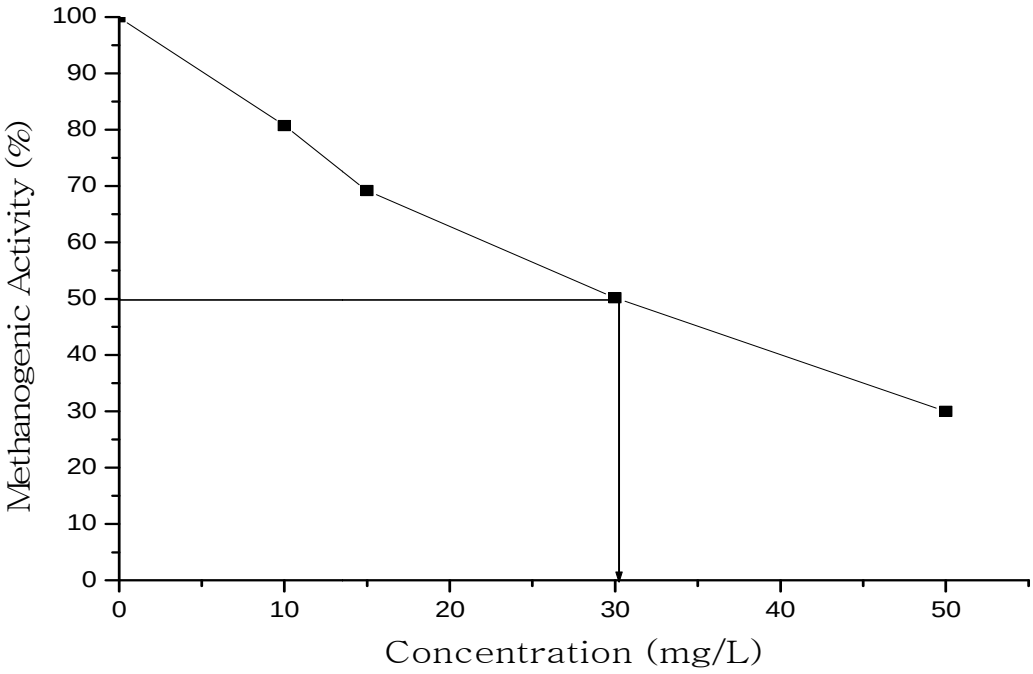

Fig. 2. Methanogenic activity of digested pig manure exposed to Chlorobenzene as a function of Chlorobenzene concentration

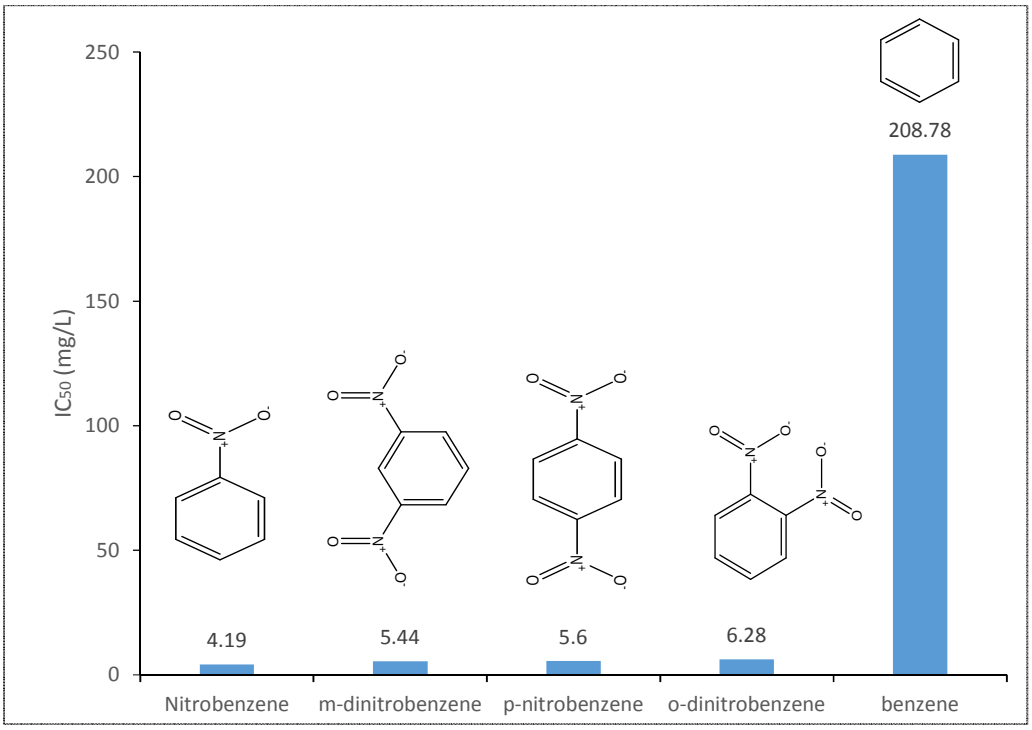

Fig. 3. Effect of the nature of Nitro aromatic compounds on Methanogenic toxicity

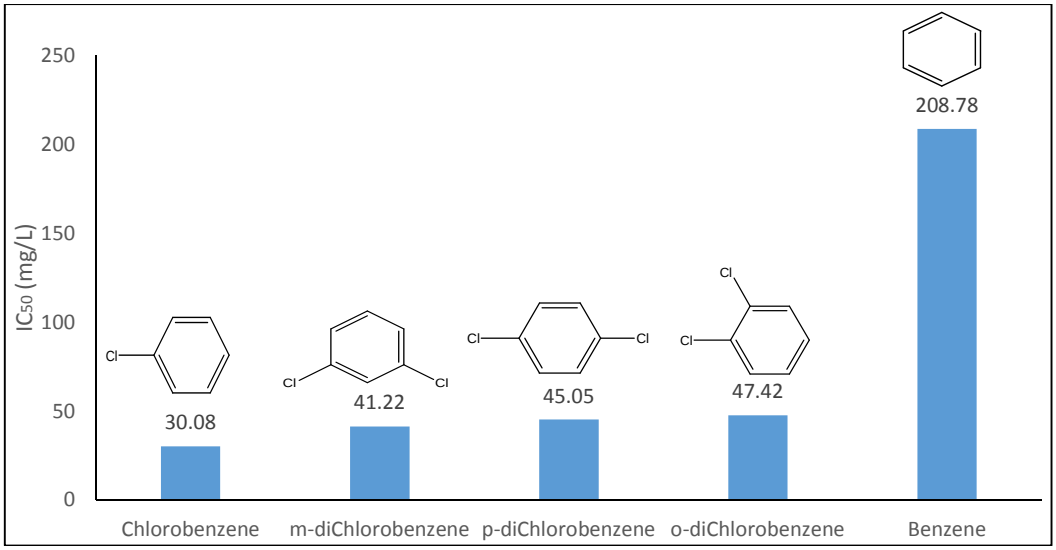

Fig. 4. Effect of the nature of Chloro aromatic compounds on Methanogenic toxicity

Table 2. Inhibitory concentrations (IC_{50}) and the partition coefficient values (Log $P_{oct.}$) of the test compounds

Compounds' group	Test compounds	Inhibitory concentrations (IC_{50}) mg/L	Log $P_{oct.}$
Unsubstituted benzene compound	Benzene	208.78±6.33	2.13
Nitro-monosubstituted benzene	Nitrobenzene	4.19±0.17	1.85
Nitro-substituted benzene compounds	o-diNitrobenzene	6.28±0.39	1.69
	m-diNitrobenzene	5.44±0.01	1.49
	p-diNitrobenzene	5.60±0.19	1.37
Chloro-monosubstituted benzene	Chlorobenzene	30.08±2.01	3.35
	o-diChlorobenzene	47.42±0.11	3.43
Chloro-substituted benzene compounds	m-diChlorobenzene	41.22±0.14	3.53
	p-diChlorobenzene	45.05±1.03	3.40
Acid compound	Benzoic acid	2515.20±31.94	1.87
Nitro-substituted acid compounds	3,4-diNitrobenzoic acid	67.20±0.95	1.71
	3,5-diNitrobenzoic acid	63.17±0.68	1.75

4. CONCLUSION

The results of this study indicated that increasing the number of electron-withdrawing group on the aromatic ring, not necessarily guarantee the increase in MT of the aromatic ring. Steric constraints due to substituents also have a considerable effect on the MT of aromatic compounds, as well as their electronic effects. In addition, we still noticed for different isomers studied that meta-isomers were more toxic than the para- and ortho-isomers, this thanks to steric constraints; therefore, the aromatic rings monosubstituted (Chlorobenzene and Nitrobenzene) have been found more toxic than those disubstituted isomers (diNitrobenzene and diChlorobenzene).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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