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Full Length Research Paper

***In vivo* and *in vitro* evaluation of antiplasmodial activity of *Amasonia campestris* (Aubl.) Moldenke**

Elizabeth Viana Moraes da Costa^{1,2}, Glenda Quaresma Ramos¹, Larissa Daniele Machado Góes¹, Cleydson Breno Rodrigues dos Santos¹, Valter Ferreira de Andrade Neto³ and José Carlos Tavares Carvalho^{1,2*}

¹Laboratório de Pesquisa em Fármacos, Departamento de Ciências Biológicas e da Saúde, Universidade Federal do Amapá, Macapá, Amapá, Brazil.

²Programa de Pós-graduação em Biodiversidade tropical, Universidade Federal do Amapá, Macapá, Amapá, Brasil.

³Laboratório de Biologia da Malaria e Toxoplasmose – LABMAT, Centro de Biociências, Universidade Federal do Rio Grande do Norte, Natal, Brasil.

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In this study, phytochemical screening and evaluation were done for the antiplasmodial activity using *in vitro* test in *Plasmodium falciparum* and *in vivo* test in murine model (*Plasmodium berghei*) of the crude ethanolic extract of roots of *Amasonia campestris* (Aubl.) Moldenke (CEEAc), popularly known as mendoca and according to surveys ethnobotanically performed in this study, it is used as antimalarial in the State of Amapá, Amazonia, Brazil. The results of the phytochemical screening of CEEAc showed the presence of organic acids, reducing sugars, phenols, tannins, alkaloids and anthocyanins. The CEEAc showed reduction of 96% of the parasitic infection with the dose of 90 µg/mL and moderate antiplasmodial activity, with IC₅₀ value of 42.94 µg/mL on the *in vitro* assay and partial antimalarial activity just on the highest dose tested (1000 mg/kg), with reduction of parasitic infection of 42.1% on the 5th day and of 37% on the seventh day after inoculation. The other doses were considered inactive. This is the first study reporting the use of *A. campestris*, to perform more detailed studies on its antiplasmodial and phytochemical activities with the aim to isolate bioactive compounds and elucidate the mechanisms of action.

Key words: Malaria, antiplasmodial activity, phytochemistry, *Amasonia campestris* (Aubl.) Moldenke.

INTRODUCTION

Amasonia campestris (Aubl.) Moldenke belongs to the Lamiaceae family and *Amazon* genus. It is widely distributed in the north, northeast, midwest and southeast regions of the Brazil, occurring from the Amazon to the Espírito Santo, and found in Amapá, Pará, Maranhão, Piauí, Ceará, Pernambuco, Bahia and Goiás, This species

inhabits the area Amazon in shady locations and edges of forests in latosols, and savanna environments; cerrado and rock field, and transition areas between savanna and cerrado. Mendoca, rabo de arara and bambã de arara, are listed as vernacular names for *A. campestris*. In folk medicine, the root is the main part of the plant used to

*Corresponding author. E-mail: farmacos@unifap.br.

treat uterine problems, bleeding and woman inflammation (Coelho-Ferreira, 2009).

Interest in the isolation and identification of chemical substances resulting from the secondary metabolism of plants and the growing search for new compounds that may have biological activity, increase every day. In this sense, the phytochemical covers more space combined with other branches of science such as botany, pharmacology and toxicology, which together contribute to the knowledge of the plant's chemistry building as well as their pharmacological and toxicological properties, thus for safe use of the species and to confirm or not the indication of the traditional medicinal use (Sasidharan et al., 2011; Carvalho et al., 2015).

According to Bero et al. (2009), the search for new drugs with antimalarial activity is necessary due to the increase in resistant parasites to the main antimalarials used in medicine. The search must be based mainly on the popular medicine of many countries, which use plants for the disease's treatment and its symptoms. The scientific studies related to the ethnopharmacology and the bioguided fractionations resulted in some of the major antimalarial, such as quinine and artemisinin, and yet have taken to the isolation of promising new antimalarial with a variety of structures.

The intensification of the studies related to natural substances to combat malaria resulted in many herbal extracts which have been tested to verify the antiplasmodial activity, but only a limited number of active compounds, belonging to different molecular classes, were isolated and identified (Burrows et al., 2013).

Although, many types of researches, reported on antiplasmodial activity *in vivo* and *in vitro* of several extracts and/or compounds isolated from plants, it is important to note that these activities are often considered moderate, suggesting that the species in question may have a limited effect in man and that the cure is improbable. However, this does not mean that the use of these species have no value, since many of them act as symptomatic in malaria treatment, for example, by reduction of anemia, relief of pain, fever and increasing immunity (Nogueira and Rosário, 2010; Veiga and Scudeller, 2015). It is noteworthy that, from the results obtained in these studies, the ethnopharmacology is a rich source for the discovery of new antimalarial compounds.

In general, evaluation of the antimalarial activity of the compounds is fulfilled by *in vivo* and *in vitro* tests, which may be complementary, as data obtained by *in vitro* tests require confirmation with live models and are fundamental to the development of new therapeutic agents. However, the analysis procedure and validation of antimalarial drugs are relatively time-consuming and complicated. The first phase of evaluation of a drug commonly consists of two subphases. The first one involves the use of assays for determining the effect of the compound on the growth of the human parasite covers analysis of the *in*

vivo efficiency of drugs selected from small malaria models of animals, mainly the rodent's parasites, *Plasmodium berghei*, *Plasmodium yoelii* and *Plasmodium chabaudi* in laboratory mice (Nogueira and Rosário, 2010).

In the State of Amapá, Amazon, Brazil, the traditional knowledge of medicinal plants is one of the greatest richness of the population. However, with the spread of the use of allopathic medicines, the natural medicine transmitted from generation to generation may end up forgotten. It is noteworthy that the majority of the cities in the state are considered a malaria-risk area and the resident population in these places has the habit of using medicinal plants to solve their health problems. Despite the constant and indiscriminate use, these species need to be widely researched and scientifically confirmed.

It is noteworthy that there is lack of studies on *Amasonia* genus, being described so far and there are only systematic and reproductive biology studies, which makes it a promising group for conducting chemical, pharmacological and toxicological studies. Therefore, the species, *Amasonia campestris* (Aubl.) Moldenke was investigated with regards to the chemical and pharmacological aspects of its crude ethanol extract through antimalarial tests, *in vivo* and *in vitro* and qualitative analysis through phytochemical screening of the main metabolites present in the extract.

MATERIALS AND METHODS

Collection and preparation of the CEEAc

The *A. campestris* (Aubl.) Moldenke samples (Figure 1) were collected in the city of Porto Grande (0.335043N, -51.641241W), in the state of Amapá, Amazonia, Brazil in August 2012, and identified in the Botany Laboratory of the Federal University of Amapá, and the voucher specimen was deposited in the Amapaense Herbarium (HAMAB) under number 018 681.

The ethanolic extract was obtained by cold maceration for 48 h. The process was repeated twice and the extraction solution was subjected to rotary evaporation to remove the solvent and obtain the CEEAc.

Phytochemical screening

A sample of CEEAc was submitted for the prospecting tests, according to the techniques adapted from Carrera et al. (2014). These analyses aimed at the qualitative analysis of the secondary metabolites present in the extract.

In this study, tests were done to analyze organic acids, reducing sugars, anthraquinones, polysaccharides, alkaloids, flavonoids, phenols and tannins, steroids and triterpenes, anthocyanin glycosides, saponins, depsides and depsidones.

Chromatographic profile

The CEEAc was subjected to column chromatography through the dry packaging technique. The adsorbent used was silica 60 gel, using the gradient eluents with increasing polarity gradient (hexane;



Figura 1. *Amasonia campestris* (Aubl.) Moldenke whole plant and root. Sample in the city of Porto Grande (0.335043N, -51.641241W), in the state of Amapá, Amazonia, Brazil.

hexane/ethyl acetate; ethyl acetate; ethyl acetate/methanol)

In thin layer chromatography, analytical (0.3 mm) stationary phase used was silica gel GF-254 from Merck. The chromatographic revelations were made through direct observation of the chromatoplates in ultraviolet light, with a wavelength of 254 and 399 nm and sputter of chromatoplates with methanol/sulfuric acid.

***In vitro* antimalarial tests**

The *P. falciparum* K1 strains used in the experiment were provided by Prof. Dr. Valter Ferreira de Andrade Neto (LABMAT/UFRN). The CEEAc was initially prepared in a stock solution of 5 mg/mL using the RPMI 1640 medium. From the stock solution, different dilutions of the CEEAc were prepared to be tested (90, 30, 10, 3.3, 1.1, 0.37 and 0.12 µg/mL).

To assess the response to *P. falciparum* samples to be tested, parasitized erythrocytes with approximately 1-2% parasitemia and predominance of young trophozoites (ring-shaped) were resuspended in a complete culture environment to a 3% hematocrit (Trager and Jensen 1976; 1977). This suspension was distributed in microplate (96 wells) of polyethylene, putting up 175 µl per well. Hereafter, approximately 2 h of incubation in a humidified CO₂ incubator at 37°C (time required for sedimentation of red blood cells), the medium was removed from all wells and blood smears were prepared and randomly chosen from 6 to 8 wells. The procedure was performed to ensure the uniformity of the distribution of parasites in microplates. After that, only the culture mediums were added to the control well containing different concentrations of the extract.

The test was performed according to Andrade-Neto et al. (2004). 25 µl of each dilution and the control with chloroquine were transferred to the microplate containing the parasitized erythrocytes. The concentrations were tested in duplicate. The plate was incubated for 48 h at 37°C. After 48 h incubation period, smears were prepared from all samples and examined under an optical

microscope to determine the percentage of parasitized erythrocytes. The growth inhibition of the parasites was determined by comparison of the growth without the samples according to the formula below:

$$\text{Inhibition (\%)} = \frac{(\text{Control of parasitaemia} - \text{parasitaemia with sample}) \times 100}{\text{Control of parasitaemia}}$$

In vitro activity criterion was established as: active sample (A) = parasites' inhibition of growth in 80 to 100%. Partially active sample (PA) = parasites' inhibition of growth in 50 to 79%. Inactive sample (I) = when the inhibition was less than 50%.

To evaluate the CEEAc antiplasmodial activity, the proposed scheme by Basco et al. (1994), modified by Dolabela (2008), was adopted, in which the extract was classified in accordance with the range of IC₅₀ values: active (IC₅₀ < 10.0 µg/mL); moderately active (IC₅₀ between 10.0 and 100.0 µg/mL); inactive (IC₅₀ > 100.0 µg/mL).

***In vivo* antimalarial tests**

This study was approved by the Ethics Committee of the Federal University of Amapá under the Protocol 002A/2012 August 6, 2012. The *P. berguei* from NNK-65 lineage was provided by Biology Laboratory of Malaria and Toxoplasmosis (LABMAT) of Federal University of Rio Grande do Norte (UFRN).

This study used 25 male black mice (age, 6 weeks; weight 18 ± 2g; lineage C57BL/6); in the experimental animal, the mice were maintained in room at a temperature of 22 ± 2°C, relative humidity and 12-h light/12-h dark cycle. They were acclimatized for two weeks to the experimental tests with commercial food and water. In the experiment, 0.2 mL with 1×10⁵ erythrocytes infected with *P. berghei* were inoculated intraperitoneally in each animal and then,

Table 1. Results of phytochemical screening tests, performed with CEEAc.

Phytochemicals compounds	Results
Organic acids	Positive
Reducing sugars	Positive
Polysaccharides	Negative
Phenols and Tannins	Positive
Flavonoids	Negative
Alkaloids	Positive
Purine	Positive
Steroids and triterpenoids	Positive
Anthocyanin heterosids	Positive
Saponins	Negative
Depsidies and Depsidonas	Positive

they were randomly divided into five groups with five animals per cage. The experiment used the following scheme: a) group I: treated with CEEAc, dose of 250 mg/kg; b) group II: treated with CEEAc, dose of 500 mg/kg; c) group III: treated with CEEAc, dose of 1000 mg/kg; d) group IV: control group treated with chloroquine, dose of 10 mg/kg; e) group V: negative control group, treated only with water.

Chloroquine was used as reference drug, and the CEEAc were diluted in distilled water. All samples in the volume of 200 μ L per animal (gavage) were administered orally. The mice were treated for four consecutive days. This test is called Peters Suppression Test (Peters, 1965), modified by Carvalho et al. (1991).

The CEEAc activity evaluation was accompanied from the 5th day after inoculation. The determination of the percentage of parasitemia was made through blood smears of the mice, made at 5th, 7th and 9th days. The smears were fixed and stained with dye Panoptic and examined by an optical microscope (1000x).

The parasitemia was determined by the reading of the smears by counting the number of parasitized erythrocytes among the 3000 total erythrocytes (Carvalho et al., 1991). The calculation of the parasitemia reduction, in percentage terms, was made in relation to the untreated control group and based on the following formula:

$$R = \frac{GC - GT}{GC} \times 100$$

In which, GC = average of parasitized erythrocytes of the negative control group and GT = average parasitized erythrocytes of the test group. It was established as activity criteria of CEEAc *in vivo* for the inhibition of 30% or more.

Statistical analysis

The assays *in vivo* and *in vitro* were realized in duplicate and express in percentage. Then, for the parasitemia and antimalarial trials analysis ANOVA following the Tukey's test were used. Results with $p < 0.05$ were considered significant. For the IC₅₀ calculation, the Microcal Origin 5.0 software was used, with the probit analysis.

RESULTS

The phytochemical test results are shown in Table 1. The

CEEAc column chromatography generated 25 fractions. The fractions collected with hexane/AcOEt 25% (B, C and D) were analyzed by thin layer chromatography (TLC- Hexane/AcOEt 9:1), and the chromatograms revealed with sulfuric acid/methanol showed purple chromatographic zones, typically of triterpenes class substances (Martelanc et al., 2009).

The D-fraction, apparently purest, appeared as a white solid, amorphous aspect and through the results of the TLC and of the NMR¹H spectrum of the fraction (Figure 2); it was possible to view the area of olefinic hydrogens and hydrogens linked to C-3 (4.1, 4.6 and 4.7 ppm) of pentacyclic triterpenes, and it is assumed to be a mixture of substances belonging to the class of terpenes.

The CEEAc showed *in vivo* antimalarial activity only at the highest dose tested (1000 mg), with 42.1% reduction in parasitemia on the 5th day and 37% on 7th day after inoculation (Table 2). The remaining doses were found inactive by the criteria established in this study, where the activity is considered from inhibition of 30% or more (Carvalho et al., 1991).

Regarding the treated animals mortality, it was observed that it was very close to the control untreated group, with survival average of 30 days for the control group and between 31 and 33 days for the treated groups. The small difference in mortality time between treated and untreated animals may be related by the half-life of the extract and metabolization in the organism, or due to immunological variations between the animals used in the study (Table 2).

A criterion was established for *in vitro* activity that the doses that inhibit the growth of parasites from 80 to 100% are considered active; 50 to 79% partially active and less than 50% inactive. After the experiment and the results analyses, it was observed that the CEEAc was considered active in the highest tested dose (90 μ g/mL), with reduction of parasite growth by 96% (Figure 3). The results indicate that the CEEAc has a moderate schizonticidal activity, with IC₅₀ value = 42.94 μ g/mL (Figure 4).

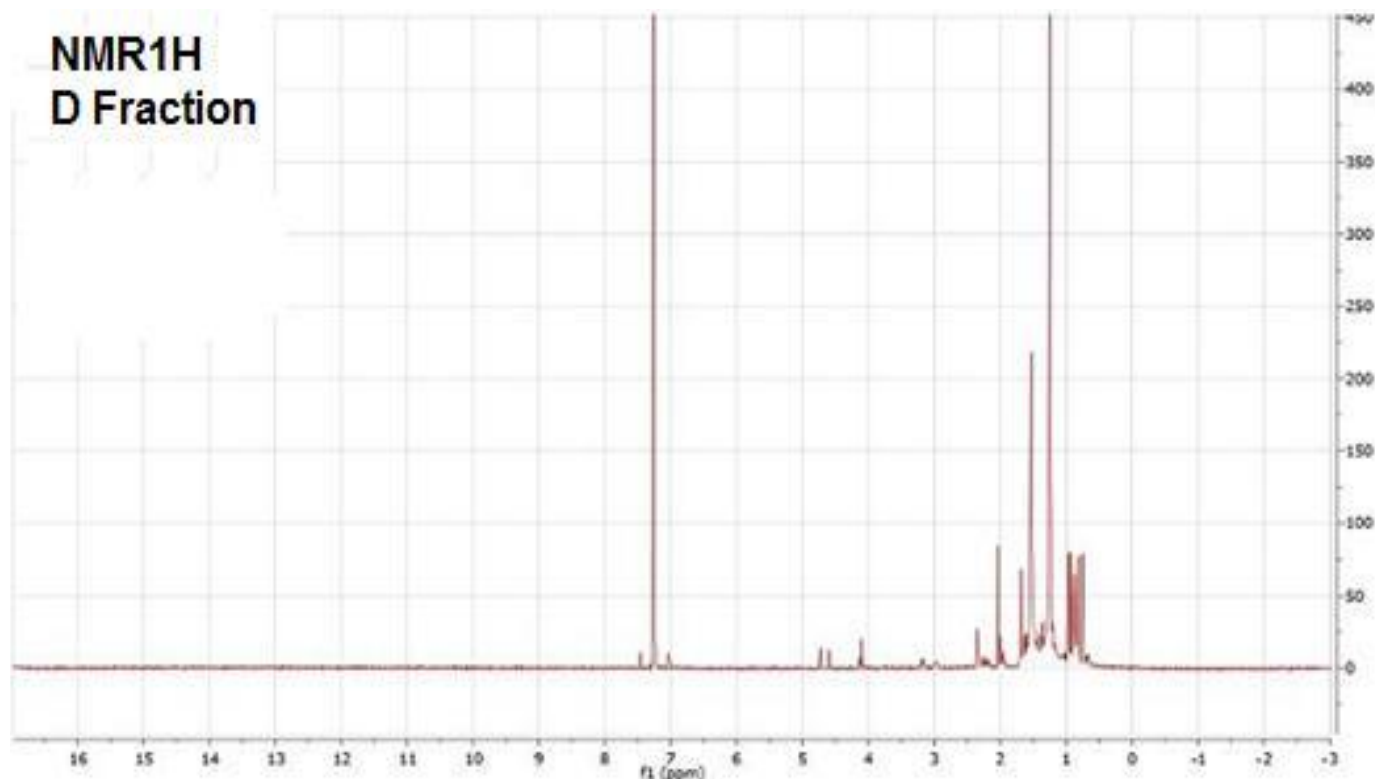


Figure 2. NMR¹H spectrum of the fraction D; it was possible to view the area of olefinic hydrogens and hydrogens linked to C-3 (4.1, 4.6 and 4.7 ppm), characteristic of pentacyclic triterpenes.

Table 2. Effect of the oral administration of CEEAc (250, 500 and 1000 mg/kg) on black mice infected with *P. berghei*.

Parameter	Doses (mg/kg)	Parasitaemia inhibition (%)		
		5th day	7th day	9th day
<i>A. campestris</i>	250	0	0	0
	500	0	28	0
	1000	42.1*	37*	10.9*

The numbers represent the media of the percentage (duplicate assays) of $n = 5/\text{group}$. * Significant in relation to other doses ($p < 0.05$, ANOVA, Tukey's test).

DISCUSSION

According to Sasidharan et al. (2011), the preliminary phytochemical analysis is used to identify the groups of secondary compounds, when chemical studies are not available on species of interest or may be relevant to direct research on isolating and structural elucidation of these substances. In this study, this preliminary information on the chemical composition of the species was extremely important, because despite the extensive literature search, chemical studies on the species of the *Amasonia* genus were not found.

Regarding the use of species as antimalarial treatment,

the positive result for the presence of alkaloids in the extract suggests that the species might have antiplasmodial activity (Basco et al., 1994). Saxena et al. (2003) reported that the antiplasmodial activity of plant-derived alkaloids has been widely reported in literature, and in 1990 and 2000, over a hundred of substances of this class are described, some of them are even more potent than chloroquine but in this study, a substance with characteristic of pentacyclic triterpenes was detected (Figure 2).

The mixture of substances with the class of terpenes, although preliminary results are fundamental, due to its huge structural diversity, have large numbers of biological

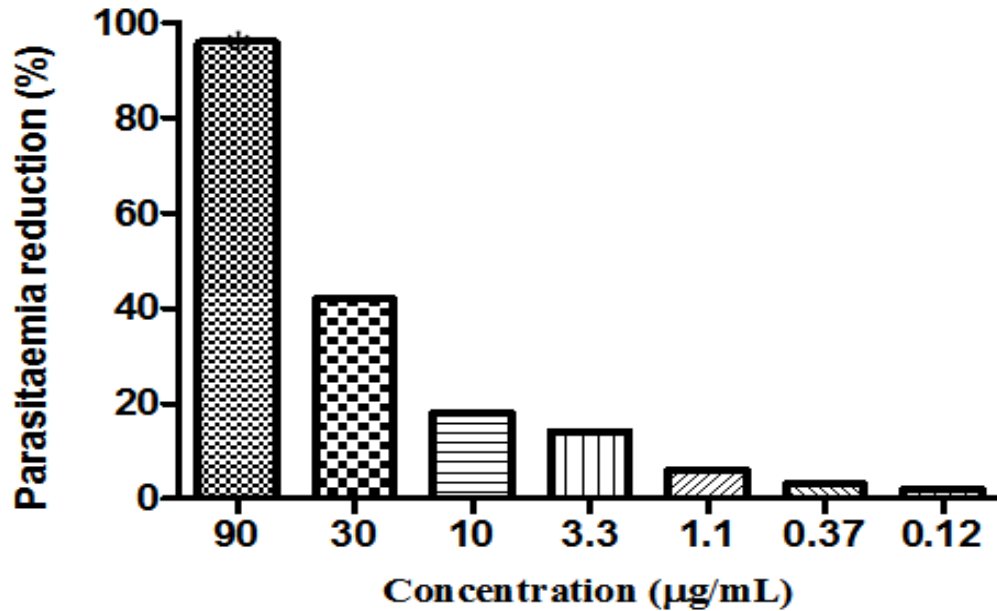


Figure 3. *In vitro* effect of CEEAc (90, 30, 10, 3.3, 1.1, 0.37 and 0.12 µg/mL) on *P. falciparum* parasitized erythrocytes with approximately 1-2% parasitemia. The bars represent the media of the parasitaemia reduction percentage (duplicate assays). *Significant in relation to other doses ($p < 0.05$, Anova, Tukey's test).

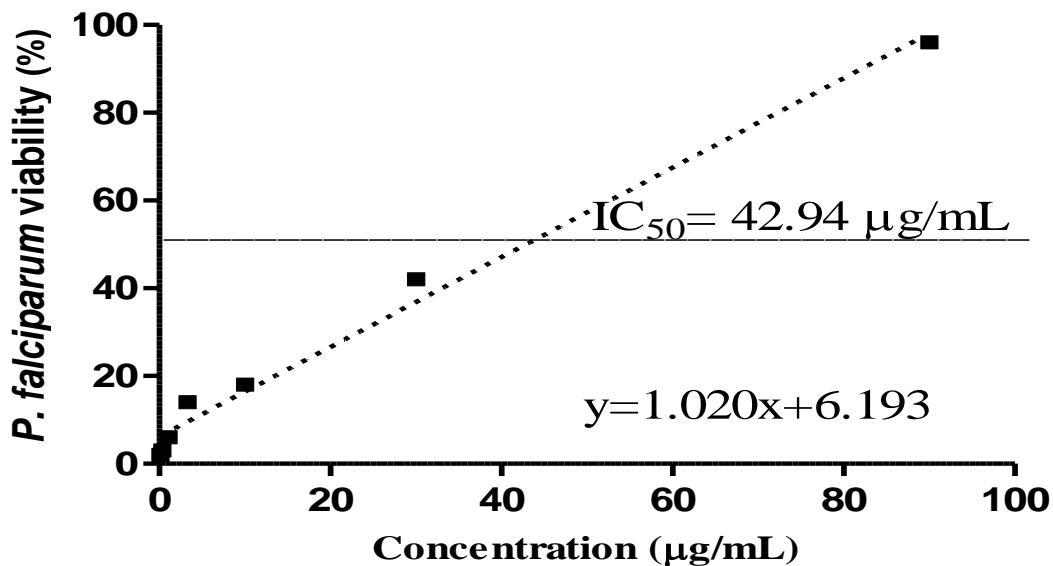


Figure 4. *In vitro* effect of CEEAc on *P. falciparum* parasitized erythrocytes with approximately 1-2% parasitemia. Each point represent media of the concentrations (90, 30, 10, 3.3, 1.1, 0.37 and 0.12 µg/mL) tested in duplicate. $IC_{50} = 42.94 \mu\text{g/mL}$ and $y = 1.020x + 6.193$ (Probit analysis).

properties as anti-cancer and anti-malarial activity (Maimore and Baran, 2007). They may be, therefore, associated with antimalarial activity related to the species.

The malaria treatment aims at blood schizogony interruption, which is the cause of pathogenesis and

clinical symptoms of the disease. According to Oliveira et al. (2009), the oral treatment prevents disease progression to severe conditions; in other words, if drugs are administered properly, there is a decrease in morbidity and mortality from disease. In this study, CEEAc samples

were administered orally (gavage), which is considered the appropriate choice in malaria treatment.

About the results, in which the CEEAc had partial antimalarial activity only at the highest tested dose (1000 mg), several factors must be considered (Table 2). The crude extracts obtained from plants contain many substances that can interfere with antagonistic form with the active substance, for example tannins, that was detected in the CEEAc (Table 1), often resulting in inactivity of them, facing the *Plasmodium* (Carvalho, 2016).

This is the first study that analyzed the antimalarial activity of *A. campestris*, and there is no data at this moment on the pharmacological studies or chemical compositions of the species and the *Amasonia* genus, and there is no data to compare this species with other species of the genus. However, *A. campestris* belongs to the family Lamiaceae, which in Brazil is represented by approximately 496 species (Harley, 2004), wherein some were already mentioned in ethnopharmacological survey as antimalarial, such as for invigorating antimalarial drugs used in Guiana French performed by Vigneron et al. (2005), which were cited as species *Leonotis nepetifolia* (L.) R.Br., *Ocimum campechianum* P. Mill. and *Plectranthus barbatus* Andrews. However, there are no guarantees that these species present compounds resulting from secondary metabolism that are similar and that may provide antimalarial activity, although they belong to the same family, but not to the same genus.

Despite the fact that value of IC₅₀ in this study ranks CEEAc as moderately active, it is observed that it reduced the parasitemia in 96% at a dose of 90 µg/mL (Figures 3 and 4), as preliminary results, as this is an initial screening, supporting at this moment the traditional use of *A. campestris* (Aubl.) Moldenke (Mendoca) as antimalarial, according to ethnodirected survey of plants used as antimalarials in the State of Amapá.

Conclusion

This study evaluated, for the first time, the antimalarial activity of the roots of *A. campestris*, and the results showed antimalarial activity both in *in vitro* tests with infected erythrocytes with strains of *P. falciparum* and *in vivo* tests with mice infected with strains of *P. berghei*; however, these tests are preliminary, requiring more detailed studies of their antiplasmodial activity and chemical composition. The results obtained for antimalarial tests, although preliminary, corroborate with the popular statement obtained by ethnodirected studies on antimalarial medicinal plants in the state of Amapá.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Evaluation of the colo-protective effects of tadalafil in an experimental model of ulcerative colitis in rats

Dina A. Aly Labib^{1*}, Walaa Yehia Abdelzaher², Olfat G. Shaker³ and Lobna O. Elfarouk⁴

¹Department of Medical Pharmacology, Faculty of Medicine, Cairo University, Egypt.

²Department of Pharmacology, Faculty of Medicine, Minia University, Egypt.

³Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University, Egypt.

⁴Department of Pathology, Faculty of Medicine, Cairo University, Egypt.

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Ulcerative colitis is a serious premalignant condition with a confusing multifactorial pathogenesis. It should warrant all attention by researchers, for exploration of new prophylactic or therapeutic drugs targeting its pathophysiology. The current study was conducted to study the possible role of tadalafil and its potential actions in ulcerative colitis rat model, induced by acetic acid. Forty eight male Wistar albino rats were classified into 6 groups: control group, acetic acid (AA)-ulcerated group, AA-ulcerated + tadalafil (1 mg/kg/day), AA-ulcerated + tadalafil (5 mg/kg/day), AA-ulcerated + tadalafil (10 mg/kg/day) and AA-ulcerated + sulfasalazine 100 mg/kg/day groups. Tissue malondialdehyde (MDA), superoxide dismutase (SOD) and myeloperoxidase (MPO) were determined. Also, caspase-3 gene expression was measured as an indicator of apoptosis. Histopathological examination of the colonic tissue was also done. In addition, serum levels of interleukin (IL)-1 β and tumour necrosis factor (TNF)- α were measured. In AA-ulcerated group, there was significant elevation in tissue MDA levels and MPO activity with upregulation of caspase 3 gene expression. Meanwhile, AA caused decreases in the SOD activities. Also, AA induced elevation in the serum IL-1 β and TNF- α levels. Pretreatment with tadalafil in doses of 1, 5 and 10 mg/kg/days guarded against changes in these parameters. Its effects were dose-dependent. According to the results, pretreatment with tadalafil in doses of 1, 5 and 10 mg/kg/day exerted dose-dependent beneficial effects against AA-induced damage of the colon, possibly by exerting anti-inflammatory and antioxidant effects. Also, reduction of apoptosis proved to be one of the contributing protective mechanisms of actions.

Key words: Tadalafil, ulcerative colitis, rats, acetic acid, anti-apoptotic.

INTRODUCTION

Inflammatory bowel disease (IBD) is a debilitating and life threatening disease affecting the colon and primarily including ulcerative colitis (UC) and Crohn's disease (CD).

UC is a premalignant disease, with both inflammatory and oxidative factors sharing fundamentally in its pathogenesis (Karakoyun et al. 2011). Transforming

*Corresponding author. E-mail: labibd5@gmail.com. Tel: 01273335134

growth factor beta 1 (TGF-B1) is a corner stone mediator in UC, which may induce production of proinflammatory cytokines such as tumour necrosis factor alpha (TNF- α). The pathogenesis of IBD also involves activated T-cells, which release various cytokines responsible for production of free radicals and destructive enzymes, with injurious effect on the gastrointestinal tract (Wan et al. 2014). These T cells are abnormal and contribute to the pathogenesis of IBD through apoptosis and can possibly cause the progression to colon cancer (Salari-Sharif and Abdollahi 2010). Defective apoptotic functions occur at the sites of inflammation in UC (Sturm et al. 2008). There is augmentation in intestinal epithelial cell apoptosis with reduction in inflammatory cell apoptosis. Caspase-3, a key enzyme in apoptosis, is activated in apoptotic cells through both extrinsic and intrinsic pathway. This causes colonic destruction with disturbed functions (Qiu et al. 2011).

Almost all drugs used in treatment of UC, aim at reducing symptoms or maintaining remission. These are aminosalicylates, corticosteroids, immunosuppressants and biologic medications, but still they can have side effects as hepatitis, nephritis, fluid retention, immunosuppression and others (Head and Jurenka 2003). Ongoing researches aim at exploring new remedies which may be of further benefit in ameliorating the disease or have additive or synergistic effects to the already used therapies for better disease prognosis.

In studying the anti-inflammatory drugs, phosphodiesterase inhibitors (PDEIs) were found to be effective in different inflammatory disorders and not just restricted to treating erectile dysfunction. Some of the PDE4I showed potential effects in animal studies. Salari-Sharif and Abdollahi (2010) showed their effectiveness in IBD. Their study was the most reliable study in this field. They concluded that PDE4Is benefited IBD by several mechanisms including reduction of inflammation, fibrosis and depression. Parallel to the discovery of PDE4Is and their anti-inflammatory properties, there are ongoing studies on other PDE isoenzymes in immune and proinflammatory cell (Salari and Abdollahi 2012). Thus, more attention is being paid to the effects and further mechanisms of action of PDE4 and PDE5 and their specific inhibitors that affect intestine.

The PDE enzymes are grouped into 11 subfamilies according to their properties, their amino acid sequence and susceptibility to pharmacological therapies (Anwar and Alchter, 2013). They metabolize cAMP and cGMP to 5'-GMP and 5'-AMP (Chung, 2006). PDEs as PDE4 and PDE7 are mainly selective on hydrolysis of cAMP whereas, PDE5, PDE6 and PDE9 are selective on cGMP. Since cGMP accumulation inhibits inflammation, the selective PDE5 inhibitors as sildenafil, tadalafil and vardenafil may seem good candidates for targeting UC diseases in which inflammation plays a central role (Keravis and Lugnier, 2012; Titus et al. 2014).

Tadalafil is a strong long-acting, highly selective

inhibitor of PDE5, which targets the enzyme PDE5 and causes smooth muscle relaxation and increases vascular blood supply (La Vignera et al. 2011). Moreover, tadalafil is superior to other well-known selective PDE5 inhibitors as sildenafil or vardenafil in its pharmacokinetic properties which allow for its sustained actions. Also, it has greater selectivity on PDE5 and is slowly metabolized thus could probably be used at lower doses for long-term management of patients. Tadalafil is the only PDE5I which is not affected by food, and therefore it could be given less frequently during long term therapies and it has a relatively rapid onset of action (16 to 17 min) (Kuan and Brock, 2002; Kouvelas et al. 2009).

The aim of this study was to extend the work of other researchers on PDE5I role in colitis and investigate the potential effects and mechanisms of action of tadalafil in a rat model of AA-induced UC.

MATERIALS AND METHODS

Drugs, chemicals and kits

Sulfasalazine (Pfizer, Australia) and Tadalafil (Pfizer, NY, USA) were used. Drugs were stored at 2 to 4°C and kept away from exposure to light. Acetic acid glacial (CID pharmaceutical Co, Egypt) 4% (volume/volume), myeloperoxidase (MPO) assay kit (Ray Bio, USA), interleukin (IL)-1 β and TNF- α enzyme-linked immunosorbent assay (ELISA) kits, malondialdehyde (MDA) and superoxide dismutase (SOD) assay kits (Nanjing jiancheng Bioengineering, China) were used for analyses.

Animals

A total of 48 adult healthy male Wistar-albino rats weighing 150 to 200 g were used in this study. Animals were harbored on a 12-h light/dark cycle (lights on from 08:00 am) at a constant temperature (24 \pm 1°C) and humidity with normal rat chow and water was available *ad libitum*. The study followed the guidelines for animal welfare and was approved by the Institutional Reviewer Board of Faculty of Medicine, Cairo University.

Induction of colitis

According to Mascolo (Mascolo et al. 1995), after fasting overnight, rats under ether anaesthesia were intrarectally infused with 2 ml AA (4%) using a lubricated paediatric catheter inserted 8 cm proximal to the anus. Rats were kept in a horizontal position for 30 s to avoid AA leakage. Rats in the normal control group received an equal volume of 0.9% saline instead of AA.

Experimental design

Six groups of animals were studied (eight animals in each). Group I: Normal control group that received only distilled water; Group II: AA-ulcerated, non-treated group, animals that received AA for UC induction; Group III: Tadalafil (1 mg/kg/day)- treated group + AA; Group IV: Tadalafil (5 mg/kg/day)-treated group + AA; Group V: Tadalafil (10 mg/kg/day)-treated group + AA; Group VI: sulfasalazine (100 mg/kg/day)-treated group + AA (Sener et al. 2014; Thippeswamy et al. 2011).

From previous studies, the dose of tadalafil which achieved therapeutic effects varied from 0.5 to 10 mg/kg/day in different experimental works (Oh et al. 2008; Ko et al. 2009; Sawamura et al. 2009). It was found that doses of 0.5 and 10 mg/kg tadalafil in rats simulate human doses of 2.5 and 40 mg/day, respectively (Sawamura et al. 2009). Therefore, the doses of 1, 5 and 10 mg/kg per day of tadalafil were chosen in the study.

The pretreated groups received tadalafil or sulfasalazine orally daily for 7 days before induction of colitis and for another 3 days following colitis-induction. Blood samples were collected from the tail vein. Serum was separated by centrifugation and stored at -80°C until used for measuring serum IL-1 β and TNF- α . Then, the animals were sacrificed by cervical dislocation under deep anesthesia (Kannan and Jain, 2000). After dissection, the colonic specimens were kept in 10% formalin for histopathological examination. The remaining colonic tissues were maintained at -80°C (ultra-low freezer, Environmental Equipment, Ohio, USA) till homogenized and used for assessment of MDA levels, MPO, SOD activities and caspase 3 gene expression.

Assessment of colitis

Macroscopic colonic damage scoring

According to the scoring system of Millar (Millar et al. 1996), mucosal damage was assessed using the microscope. Inflammation scores were assigned using a scale ranging from 0 to 4: 0 indicates no macroscopic changes, 1 indicates mucosal erythema only, 2 indicates mild mucosal edema, slight bleeding, or small erosions, 3 indicates moderate edema, bleeding ulcers or erosions, and 4 indicates severe ulceration, erosions, edema and tissue necrosis.

Histopathological study

Cross sections of colonic tissues were fixed in 10% formaldehyde, embedded in paraffin blocks, and cut into fine sections. Samples were collected on glass slides, stained with hematoxylin and eosin (H&E) and examined under the microscope by a pathologist in a blinded manner. Additional sections from the paraffin wax blocks were stained with Alcian blue dye. Histopathological slides were examined for destruction of the epithelium and glands, dilatation of glandular crypts, depletion and loss of goblet cells, inflammatory cells infiltration, edema, hemorrhagic mucosa and crypt abscesses using parameters scored from 0 to 3. The colitis score of each rat was assessed by the sum of the subscores of different parameters (Gaudio et al. 1999).

Biochemical studies

Sera stored at -80°C was used to determine TNF- α and IL-1 β levels. Homogenized tissue samples were used for the measurement of MDA, MPO and SOD and caspase 3 gene expression.

Measurement of tissue MDA

According to Balasubramanian (Balasubramanian et al. 1988), the colonic content of MDA was measured using the assay kit of TBARS. The results were expressed as nmol/g.

Measurement of tissue MPO activity

MPO activity was measured as described by Krawisz et al. (1984).

The results were expressed in ng/g.

Measurement of tissue SOD activity

The enzymatic activity of SOD was measured as described by Kono (1978). The results were expressed in u/g.

Gene expression of caspase 3

According to the manufacturer instructions, isolation of RNA from 100 mg of tissue was done by the aid of an RNA extraction kit (Qiagene, USA). After synthesis of first-strand complementary DNA from 2 μ g total RNA (Invitrogen Inc., Carlsbad, California, USA) and denaturing the template RNA and primers (25 pmol of each reverse oligonucleotide primer) at 70°C for 10 min, 40 U reverse transcriptase was added in the presence of RT buffer, 4 μ l dNTP mix (250 μ mol/l each), 40 U RNase inhibitor, and RNase-free water to achieve the final volume. Incubation of the mixture (50 μ l) was done for 1 h at 43°C, then stopped at 4°C, and used on the spot for polymerase chain reaction (PCR) or kept at -80°C until use. Reactions were carried out in triplicate. Conditions of the reaction were: an initial 15 min at 95°C, followed by 40 cycles of 15 s at 94°C, 30 s at 55 to 60°C, and 30 s at 72°C. Real-time PCR was carried out in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, California, USA). Calculation of relative gene expression was done using the comparative threshold cycle (Ct) method (Livak and Schmittgen, 2001).

Measurement of serum levels of TNF- α and IL-1 β

Serum levels of IL-1 β and TNF- α were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. Values were expressed as pg/ml.

Statistical methods

Data were coded and entered using the statistical package SPSS version 24. Data was described using mean and standard deviation for quantitative variables. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test. P-values less than 0.05 were considered as statistically significant.

RESULTS

Macroscopic examination

AA caused severe edematous inflammation in the colon, with a high macroscopic scoring of colonic damage as compared to the control group ($p < 0.05$). Sulfasalazine (100 mg/kg/day) significantly reduced the severity of gross lesion scores as compared to the AA group ($p < 0.05$). There was an improvement in colonic damage score by tadalafil (1, 5 and 10 mg/kg/day). The improvement occurred in a dose-related manner (Table 1). Significant difference was found between tadalafil 5 and 10 mg/kg. The effect of sulfasalazine was comparable to that of tadalafil 10 mg/kg.

Table 1. The effect of tested drugs on macroscopic and histopathological scoring of colonic tissues in AA-induced ulcerative colitis in albino rats.

Groups	Macroscopic scoring	Histopathological scoring
Group I: Normal, control	0.0±00	0.0±00
Group II: AA, positive control	3.8±0.5*	17.5±0.5*
Group III: Tadalafil (1 mg/kg/day) +AA	3.2±0.5#	15.6±0.7#
Group IV: Tadalafil (5mg/kg/day) +AA	2.3±0.7#\$	9.5±0.5#\$
Group V: Tadalafil (10 mg/kg/day) +AA	1.4±0.5#@	3.5 ±0.5#@
Group VI: Sulfasalazine (100 mg/kg)+ AA	1±00#	2.5±0.5#&

n=8; Values are presented as mean ±SD; AA, acetic acid; *statistically significant as compared to corresponding value in control group (P<0.05); #, statistically significant as compared to corresponding value in AA group (P<0.05); \$, statistically significant as compared to corresponding value in Tadalafil 1+AA group (P<0.05); @, statistically significant as compared to corresponding value in Tadalafil 5+AA group (P<0.05); &, statistically significant as compared to corresponding value in Tadalafil 10+AA group V (P<0.05).

Histopathological changes

The histopathological examination is shown in Figure 1. The normal control group showed normal colonic mucosa with preserved mucigenic activity. AA group showed ulceration of the colonic mucosa with inflammation. Rats pretreated with tadalafil 1 mg/kg showed mucosa with minimal residual goblet cell preservation. Tadalafil 5 mg/kg showed focal fibrosis with incomplete ulcer healing whereas, tadalafil 10 mg/kg showed near normal mucosa, with ulcer healing and preserved mucigenic activity. The drug showed a dose-dependent protective effect against AA-colitis, whereas, sulfasalazine treated group showed near normal results. The histopathological scoring of all groups is illustrated in Table 1. The scoring of AA group was significantly increased as compared to the control group (p<0.05), whereas, tadalafil 1, 5 and 10 mg/kg and sulfasalazine significantly decreased as compared to AA (p<0.05). The effects of tadalafil were dose-dependent and those of sulfasalazine were significant as compared to tadalafil 10 mg/kg.

Tissue MDA concentration

In the disease model of UC (AA-ulcerated group), the colonic MDA levels showed significant elevation as compared to the control group (p<0.05). Pretreatment of rats with tadalafil 1, 5 and 10 mg/kg/day showed dose-dependent improvements in each level. Sulfasalazine significantly inhibited elevation of MDA as compared to AA group (p<0.05) meanwhile, no significant difference was found between treatment by sulfasalazine and treatment by tadalafil 10 mg/kg/day (p>0.05) (Figure 2a).

Tissue MPO concentrations

In the AA group, colonic MPO measurements proved to be significantly elevated as compared to control group

(p<0.05). Tadalafil (1, 5 and 10 mg/kg/d) pre-treatment and sulfasalazine significantly prevented the increase in MPO activity as compared to the AA group (p<0.05). The effects of tadalafil were dose-dependent meanwhile, the effect of sulfasalazine was significant as compared to tadalafil 10 mg/kg (Figure 2b).

Colonic SOD activity

Activity of colonic SOD was significantly decreased in AA group as compared to control animals (p<0.05). Three doses of tadalafil and sulfasalazine used showed significant increase in SOD activity as compared to AA group (p<0.05). In addition, the effect of sulfasalazine was significant as compared to tadalafil 10 mg/kg (Figure 2c).

Caspase 3 gene expression

Caspase 3 proteins were highly upregulated in the AA group when compared with the control group (P<0.05). Tadalafil pretreatment by the 3 doses: 1, 5 and 10 mg/kg showed normal levels of caspase 3. In the sulfasalazine-treated group, results were significantly lower as compared to the AA group (P<0.05). Meanwhile, no significant difference was found between sulfasalazine and tadalafil 10 mg/kg (p>0.05) (Figure 2d)

Serum level of TNF-α

The levels of IL-1β and TNF-α in the serum of AA group significantly increased as compared to the control group (p<0.05). But they were significantly lower in the tadalafil (1, 5 and 10 mg/kg) and sulfasalazine pretreated groups as compared to AA-induced colitis group, respectively (p<0.05). The effect of sulfasalazine was significant as compared to tadalafil 10 mg/kg (Figure 2e and f).

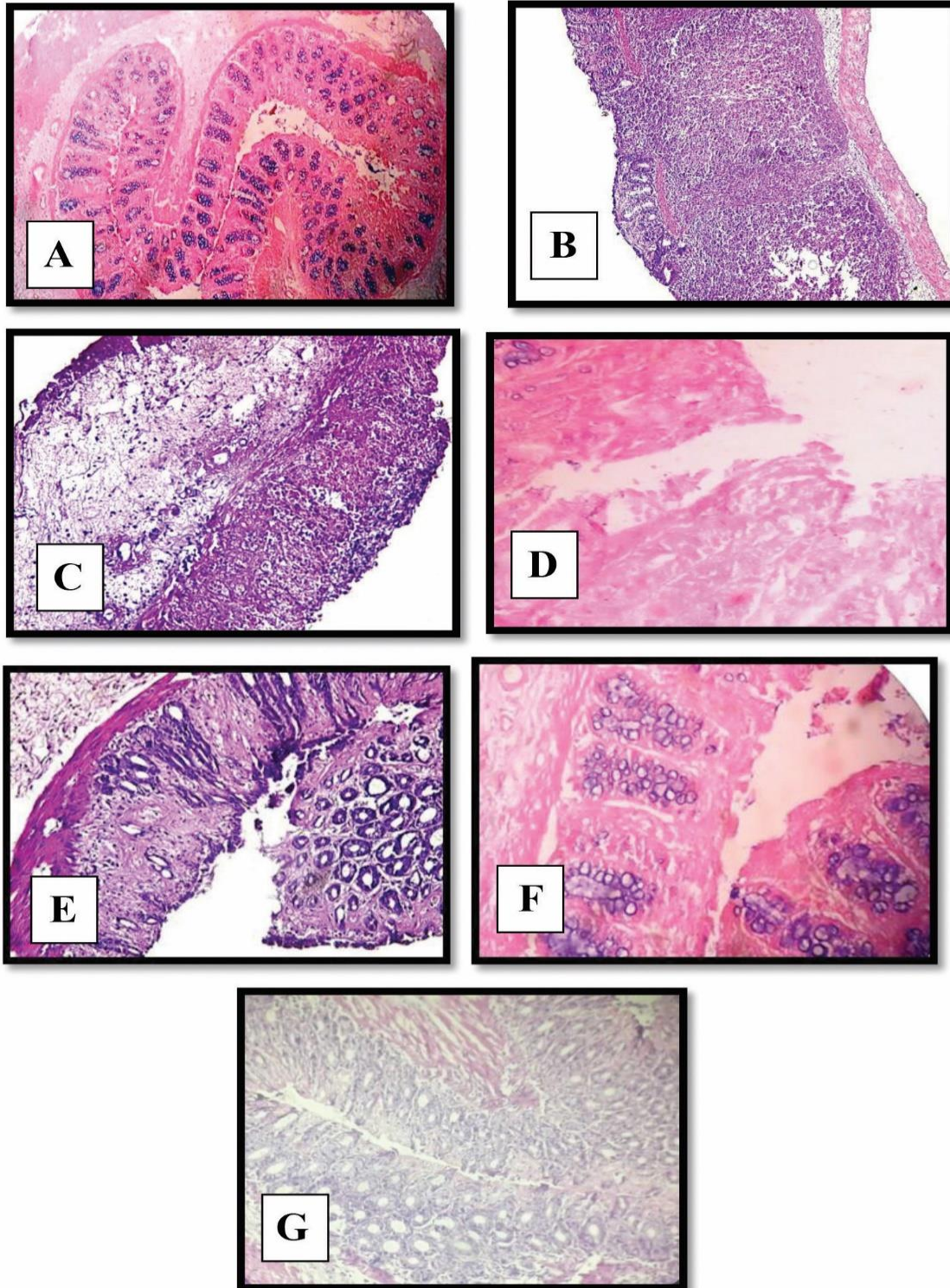


Figure 1. Histopathological sections of colons from different studied groups (each group= 8 rats). A, Group I (normal control group): showing normal colon with preserved mucigenic activity (Alcian blue 40 x). B and C, Group II (AA-ulcerated, non-treated group), B shows central ulceration and transmurial inflammation (H&E 40x), while C shows complete ulceration (H&E 100x). D, Group III (Tadalafil (1 mg/kg/day)- treated group +AA): showing denuded mucosa with minimal residual goblet cell preservation in uppermost left portion. E, Group IV (Tadalafil (5 mg/kg/day)- treated group +AA): showing focal fibrosis with incomplete ulcer healing (H&E 100x). F, Group V (Tadalafil (1 mg/kg/day)- treated group +AA): showing near normal mucosa, with ulcer healing and almost preserved mucigenic activity (Alcian blue x200). G, Group VI (Sulfasalazine (100 mg/kg/day)-treated group): showing the colon which is near normal (H&E 100x).

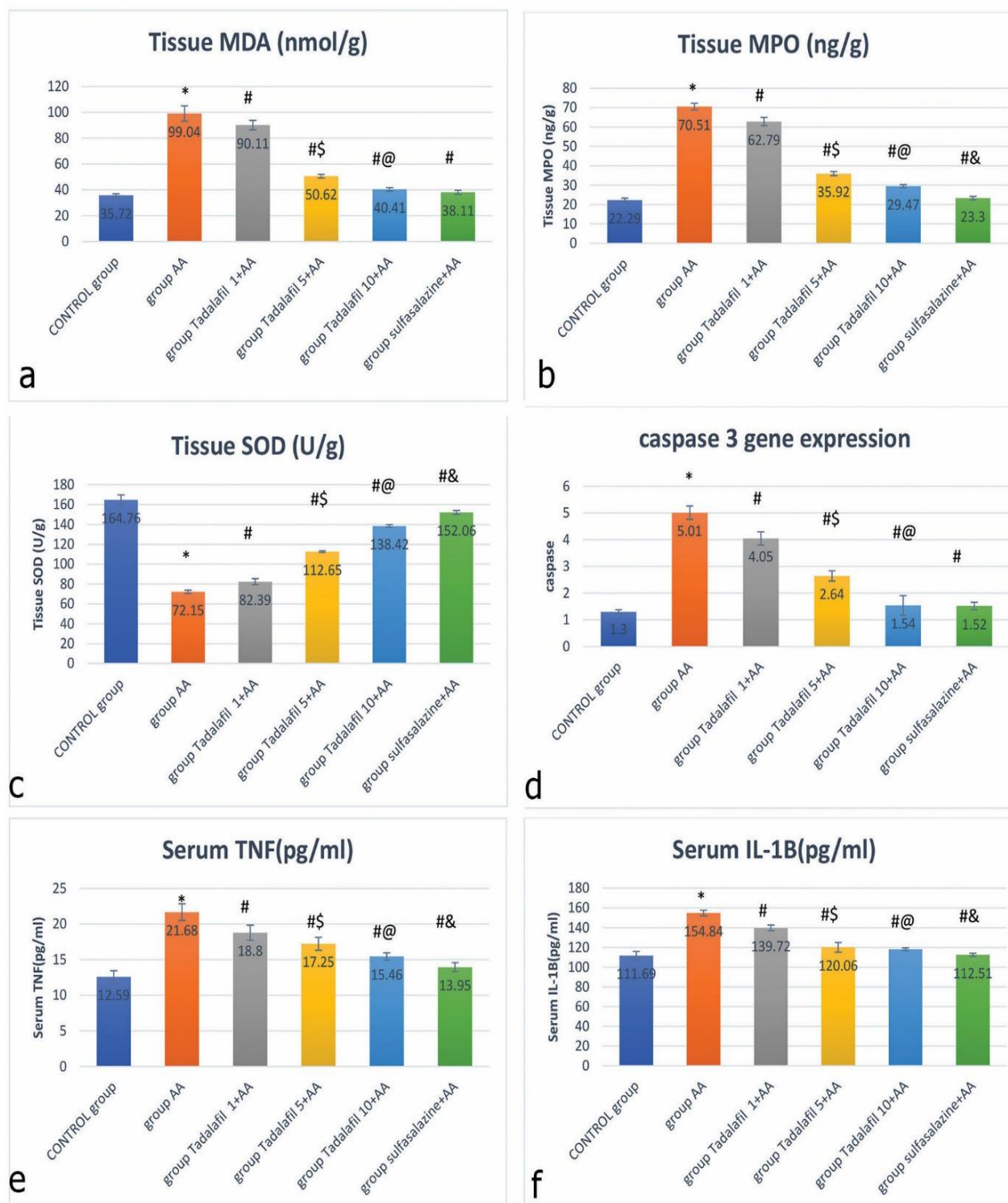


Figure 2. Effects of tadalafil (1, 5 and 10 mg/kg/d) and sulfasalazine on (a) tissue MDA in albino rats; (b) tissue MPO in albino rats; (c) tissue SOD in albino rats; (d) tissue caspase 3 gene expression in albino rats; (e) serum TNF- α in albino rats; (f) serum IL-1B in albino rats; n= 8. Values are presented as mean \pm SD; AA, acetic acid; SOD, superoxide dismutase; MDA, malondialdehyde; TNF- α , tumor necrosis factor-alpha; IL-1 β , interleukin-1 beta. *statistically significant as compared to corresponding value in control group (P<0.05); #, statistically significant as compared to corresponding value in AA group (P<0.05); \$, statistically significant as compared to corresponding value in Tadalafil 1+AA group (P<0.05); @, statistically significant as compared to corresponding value in Tadalafil 5+AA group (P<0.05); &, statistically significant as compared to corresponding value in Tadalafil 10+AA group (P<0.05).

DISCUSSION

IBD is a chronic inflammatory disease with highly expressed inflammatory cytokines such as TNF- α , IL-1 β , IFN- γ and enzymes such as inducible nitric oxide synthase (iNOS) and cyclo-oxygenase 2 (COX-2). Reactive oxygen metabolites (ROM) are responsible in part, for tissue injury in all inflammatory conditions including colitis. Toxic oxidants can cause damage if the capacity of the endogenous antioxidant enzymes as SOD, catalase and glutathione peroxidase cannot cope with their excess production. Thus, increased oxidative stress and impairment of the antioxidant defenses by the deleterious effect of ROMs contributes to the pathogenesis of colitis (Iseri et al. 2009). It was previously found that oxidative stress and its consequent lipid peroxidation results in increased colonic MDA contents. This in part, is responsible for impaired defensive mechanism (Girgin et al. 2000; Ek et al. 2007). The current study showed that colonic MDA levels were significantly increased in the AA control group and this data is in agreement with the study of Cetinkaya (Cetinkaya et al. 2005). Meanwhile, the administration of tadalafil 1, 5 and 10 mg showed a dose-dependent decrease in MDA levels. Similarly, in the study of Wu et al. (2015), the increased MDA levels in testicular tissues, following I/R injury, were reversed with tadalafil treatment.

Furthermore, there is a strong relation between the status of antioxidant enzymes e.g., SOD and the systemic protection against inflammation. SOD is responsible for the conversion of superoxide to peroxide. This guards against lipid peroxidation in colon by eliminating free-radicals. Decreasing SOD activity in the colonic tissue leads to mucosal injury due to decreased ability of scavenging oxidative radicals (Barazzone and White 2000; Krieglstein et al. 2001). The current study showed that SOD activity was significantly decreased in the AA control group and this data is in agreement with others such as Al-Rejaie (Al-Rejaie et al. 2013). The data demonstrated that administration of tadalafil 1, 5 and 10 mg ameliorated alterations induced by AA in SOD levels. This improvement was dose-related. Similarly, Adeneye and Benebo (2016) proved that tadalafil pretreatment succeeded to restore SOD levels near normal.

The deleterious effects of activated neutrophils lie mainly in production of oxygen metabolites and activation of MPO (Kettle and Winterbourn, 1997). The results of this study showed that AA group was associated with an increase in MPO activity. This was previously confirmed by Mannasaheb (Mannasaheb et al. 2015). Pretreatment with tadalafil in the 3 doses effectively reduced this enzyme activity, suggesting that tadalafil could exert anti-inflammatory effects. Tadalafil inhibited tissue neutrophil accumulation and the associated MPO activity. Similarly, tadalafil succeeded to reverse the increase in renal MPO activity following I/R injury (Küçük et al. 2012). A study by Santos et al. (2005) showed that Sildenafil, another

prototype of PDE5I, provided effective protection against indomethacin-induced gastropathy in rats by decreasing the MPO activity.

Serum TNF- α and IL-1 β levels significantly increased in the AA control group. Tadalafil limited the up-regulation of pro-inflammatory cytokines, TNF- α and IL-1 β which are believed to play a significant role in the pathogenesis of IBD. These authors provided evidence that tadalafil therapy decreased the levels of circulating inflammatory cytokines in the studied animals (Varma et al. 2012). In accordance with the current findings, another study demonstrated that pretreatment with PDE5I, zaprinast, inhibited the increase in serum TNF- α level in mice (Iric et al. 2001). Similarly, Sildenafil, a prototype of PDE5I has shown good effects in experimental colitis by balancing oxidant-antioxidant status and inhibiting ROM production and release of cytokines (Salari-Sharif and Abdollahi, 2010). In a rat model of colitis, Sildenafil prevented lipid peroxidation, cytokine production and neutrophil accumulation, Sildenafil reversed TNF- α and IL-1 β in the colitis back to the control value (Iseri et al. 2009). A recent study confirmed the anti-inflammatory effect of a PDE5I in a colitis model in rats (Margonis et al. 2015). Also, Sildenafil was found to be beneficial in AA-induced colitis in rats by preventing lipid peroxidation, cytokine release and maintaining oxidant anti-oxidant status (Ahmed et al. 2012).

In the AA group, caspase 3 protein expression was upregulated as previously described by Kaushal et al. (2001). The current study results showed that tadalafil pretreatment by either 1, 5 or 10 mg/kg reduced caspase 3 protein expression. Similarly, Tavukçu et al. (2014) in their study found that tadalafil is able to reduce caspase 3 activity, as an index of apoptosis.

Furthermore, histological data also match the biochemical changes. Microscopical examination confirmed the previous results, where AA group showed ulceration of the colonic mucosa with inflammation. Rats pretreated with tadalafil 1, 5 and 10 mg/kg/day showed a dose-dependent amelioration of AA-colitis. The scoring of AA group significantly increased as compared to the control group, whereas that of tadalafil 1, 5 and 10 mg/kg and sulfasalazine significantly decreased as compared to AA ($p < 0.05$). The effect of sulfasalazine was significant as compared to tadalafil 10 mg/kg.

Conclusion

Overall, the findings of the present study demonstrated that tadalafil pretreatment in doses of 1, 5 and 10 mg/kg exerted significant ameliorating effect against AA-induced UC model. This is most probably attributed to its antioxidant, anti-inflammatory and anti-apoptotic effects. The improvement was dose-dependent. Patients treated with tadalafil will benefit from its additional colo-protective effect. A further comparative study on the effects of

tadalafil and other selective PDE5I such as sildenafil and vardenafil in UC is recommended. It would be of benefit to point out the one that is most potent and can relatively achieve the best results.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Ozonation process for hazardous drugs molecules in aqueous media

Atoosa Haghighizadeh¹ and Omid Rajabi^{2*}

¹Department of Research and Development, Dr Rajabi Pharmaceutical Company, Mashhad, Iran.

²Department of Drug and Food control, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

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This study presents results of ozonation of some different aromatic rings of medicines in aqueous solution. The aim of the research was to investigate the influence of ozone on ring cleavage of drug molecules with an aromatic structure. In this regard, phenobarbital, naphazoline, doxycycline, isoniazid and salbutamol were chosen for exposure to ozone gas overnight. It is proved that ozone can attack the OH groups, opening the aromatic ring to form unsaturated acids, aldehydes and linear alkyl/cycloalkyl groups which then appear as stable products. It is suggested that the first step in these reactions includes penetration of ozone at the C-H bond with the formation of a quinone and a subsequent attack of the aromatic ring T-bond system by the ozone with the formation of ozonides. With this process, ozonation can produce simple, biodegradable and less carcinogenic compounds from complex aromatic ones.

Key words: Ozone, aromatic compound, oxidation, toxicity.

INTRODUCTION

In recent years one of the most significant concerns of the research-based pharmaceutical industry is environmental contamination and, more specifically for pharmaceutical agents, safety. Among the organic compounds, soluble aromatics have been especially studied because of their biological stability (Dore et al., 1980). Aromatic rings are found in most drugs and have been studied widely due to their toxicity. Their potent toxicity or carcinogenesis is a major issue for human populations, as are their potential impact as environmental pollutants and in emissions from diesel

and gasoline engines. Relative to their importance, the toxicity, carcinogenicity and their metabolic pathways in organisms have been examined (Marie, 2009). They also preferred to be replaced with linear and branched alkyl/cycloalkyl groups.

Ozonation has been known for more than a century. In the 1920s, this phenomenon was studied while ozone-induced cracking was developed during and immediately after World War II; the research led to the discovery of the first chemical antiozonants. Ozone, a triatomic allotrope of oxygen, is a tremendously strong oxidant

*Corresponding author. E-mail: rajabio@mums.ac.ir. Tel/Fax: +985138843483.

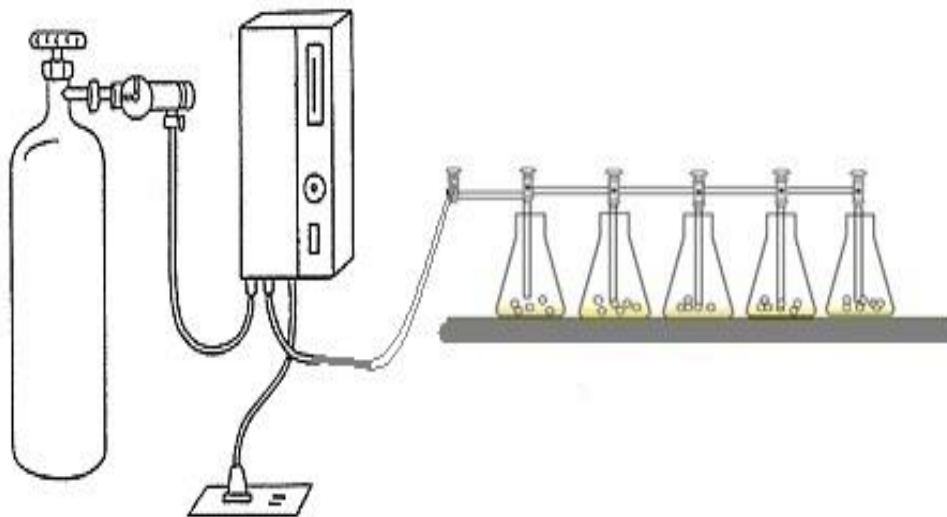


Figure 1. Schematic representation of batch reactor.

agent that can easily react with the unsaturated chemical bonds in organics. It also can cleave and oxidize benzene and other aromatic rings (Gong et al., 2008) (Sahu, 2011). The term “ozonolysis” as applied in this study specifically refers to the cleavage of bonds between sp^2 or sp carbon atoms. The reaction between ozone and the C=C bond is assumed to follow a bimolecular law, where each C=C bond functions as an independent kinetic unit. The concepts behind this mechanism had been summarized previously (Anachkov et al., 2000; Anachkov et al., 1996; Razumovsky et al., 1986). Moreover, it also has been observed that ozonation can convert large molecules into smaller ones and increase the ratio of hydrophilic organics in water (Gong et al., 2008). Ozone is widely used in Europe for the removal of colors, tastes, odors and pathogens in drinking water.

Two mechanisms were considered for the reaction between the ozone molecule and an organic: (1) Direct O_3 molecule oxidization (electrophilic addition) or (2) Indirect oxidization through $\cdot OH$ (nucleophilic addition). Comparatively, the electron-donating groups (-OH, -NH etc.) react faster with O_3 than the electron-withdrawing groups (-COOH, $-NO_2$ etc.) when ozone attacks the ortho and para positions primarily. In the other words, O_3 reacts easily with electron-donor with aromatic compounds resulting in ortho and para intermediates which can be further oxidized, consequently to quinonoid compounds and later to aliphatic compounds concluding with hydroxide radical and carboxy compounds. The following findings demonstrate the manner in which radicals like $\cdot OH$ may be produced and the manner in which they function (Hui-xiang et al., 2005; Staehelin and Hoigne, 1982).

The literature concerning the kinetics and mechanism

of ozonation of such macromolecular compounds is scarce. Most qualitative opinions on the kinetics have been published (Marie, 2009; Sweeney, 1981). The problem is that ozonation is multistage process, and a wide variety of intermediates are formed that are difficult to identify.

MATERIALS AND METHODS

Sample preparation

All of the selected drugs were used as model to investigate the ability of the ozonation process to eliminate the aromatic rings of these compounds, with an ultimate goal of reducing their toxicity. Experiments were carried out in a glass reactor with jacket at room temperature, as illustrated in Figure 1. Ozone was produced for the test using an ozone generator, model CD- 0013.5. This unit produces 13.5 g of ozone per hour embedded in a flow of medical oxygen at 3 L min^{-1} . Depending on a solubility of the compounds, 50 mg of phenobarbital, naphazoline, doxycycline, isoniazid and salbutamol in deionized water in a volume of 50 ml were tested. The top of each glass reactor had five connections providing for the collection of materials with the same flow rate of the ozone bubbling. After ozonation, samples were collected, lyophilized and kept under refrigeration until characterization.

Laboratory determinations

Fourier transform infrared spectroscopy (FTIR) analysis using a Perkin-Elmer spectrum GX spectrometer was used to determine the FTIR spectra of samples before and after ozonation. Lyophilized powders of the sample were mixed with spectrophotometric grade KBr at 1:5 ratios and pressed into pellet form. FTIR spectra were recorded in the frequency range of 400 to 4000 cm^{-1} with 32 scans and a resolution of 4 cm^{-1} . The functional groups in the compounds were identified by comparisons of the resultant IR spectra before and after ozonation. This technique also was deployed for studying various stretching and bending vibrations of the aromatic functional

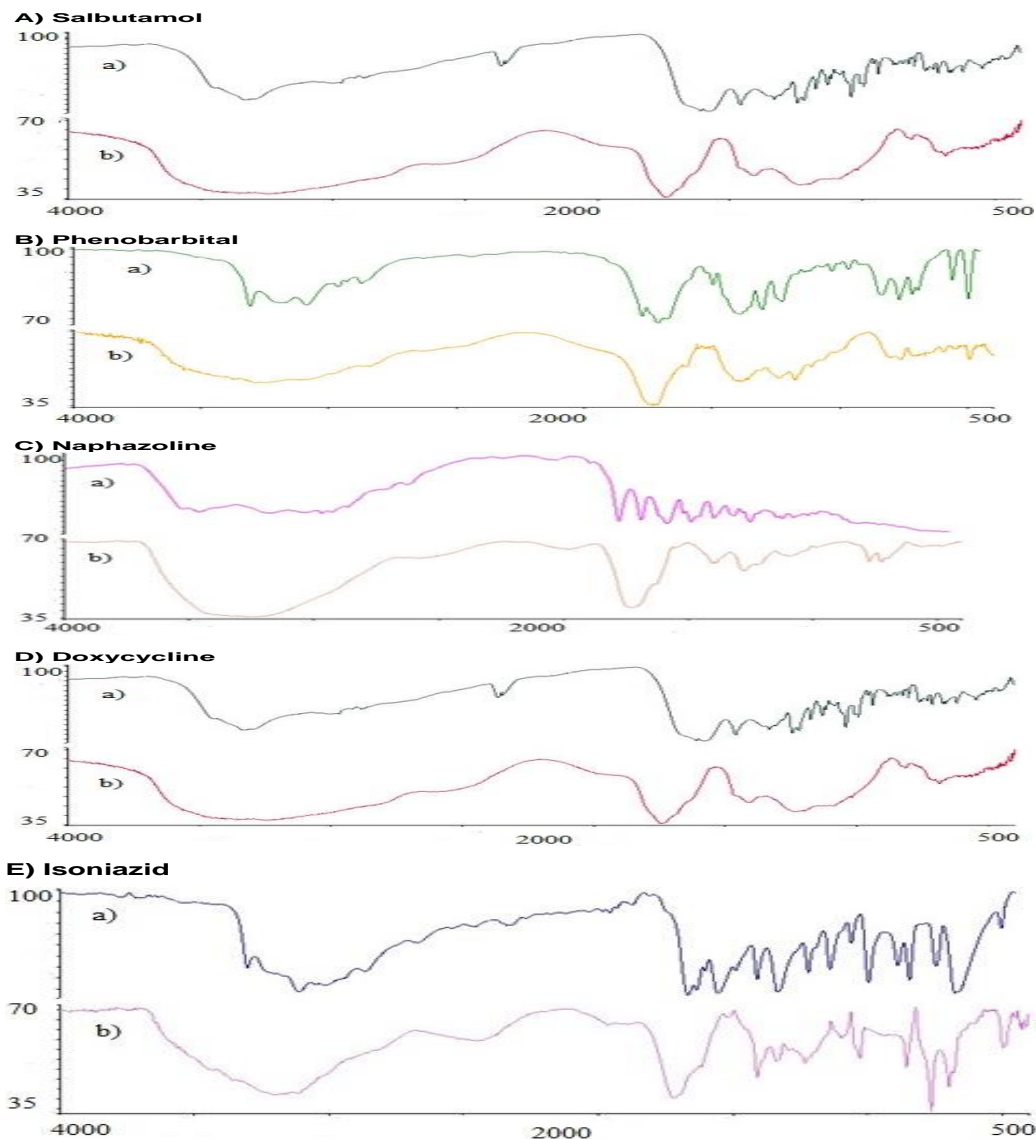


Figure 2. IR spectra of different drug molecules. (a) Before ozonation, (b) After ozonation.

groups. UV/Vis absorption spectra were recorded with a Shimadzu 160A UV/Vis spectrometer. A 1 cm quartz cuvette was used for measuring absorbance and deionized water was used as a blank. The Nuclear Magnetic Resonance (NMR) spectra were recorded in D₂O using Bruker Drx-500 Avance spectrometers at 296 K. Approximately 50 mg of each dried sample were added to 0.5 ml D₂O in a 10 mm NMR tube. The signal for D₂O was used as a reference and set to 4.8 ppm chemical shift. Chemical shifts are given in parts per million (δ -scale).

RESULTS AND DISCUSSION

Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of the compounds were prepared, before and after ozonation. All the samples (Figure 2)

showed band shifting and reductions in the functional groups of the aromatic rings. For comparison the vibration of the O-H bond in hydroxyl groups in the region of ~ 3200 to 3500 cm^{-1} after ozonation for all compounds are shifted to lower frequencies at ~ 3076 to 3291 cm^{-1} . This may imply that more OH groups are involved in intermolecular bonds (Allen et al., 2001). In all compounds except naphazoline, there is a broad carbonyl peak in aromatic rings at $\sim 1730\text{ cm}^{-1}$ due to lactone, followed by a maximum at $\sim 1739\text{ cm}^{-1}$ from the formation of aliphatic esters. In phenobarbital the peak splits to one additional data at 1752 cm^{-1} that is related to ketones. In doxycycline and isoniazid, the main peak in the region of $\sim 1620\text{ cm}^{-1}$ is related to carbonyl groups which disappear after ozonation. This suggests that the carbonyl group is

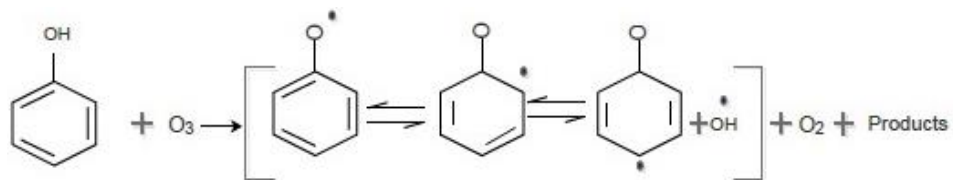


Figure 3. Ozonation process.

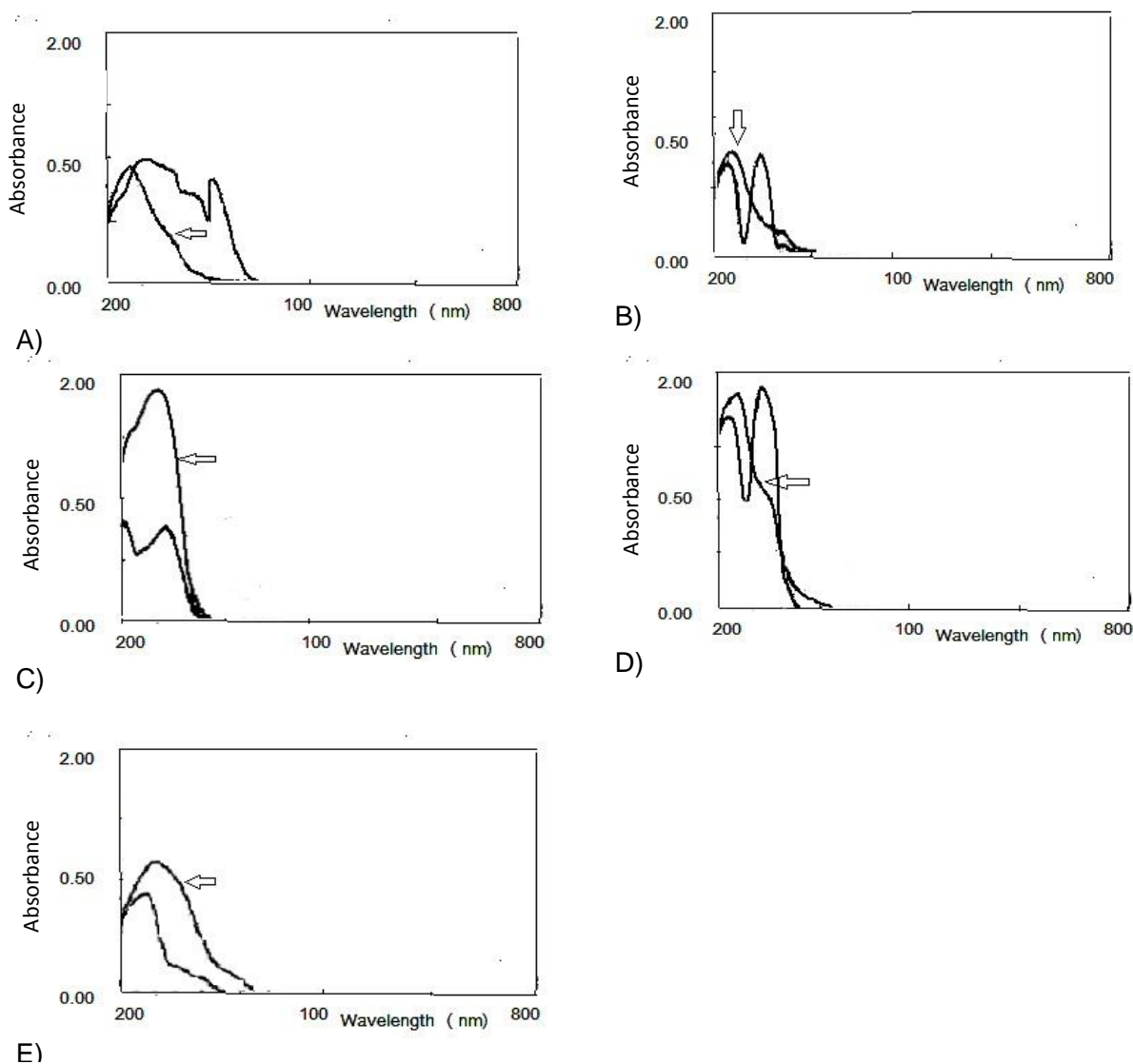


Figure 4. UV-Vis spectra of: (A) Doxycycline, (B) Salbutamol, (C) Isoniazid, (D) Naphazoline and (E) Phenobarbital before and after ozonation.

changed to α, β -unsaturated aliphatic groups (Sahu, 2011).

The appearance of two absorption bands in phenobarbital and isoniazide at ~ 1599 to 1523 cm^{-1} are consistent with the formation of an unusual but novel $\text{C}=\text{C}$ -stretch of an enol tautomer of α, β -diketone

product. The band at ~ 1407 cm^{-1} is synonymous with the formation of methyl ketones or aldehydes. The bands that appeared at ~ 1174 cm^{-1} are related with a mixture of aliphatic esters and ethers. The two shoulders at 1050 to 1000 cm^{-1} typically are due to residual ozonides. Mono substituted terminal vinyl groups also appear to be

formed at $\sim 900\text{ cm}^{-1}$. FTIR bands decreasing in phenobarbital and doxycycline are the aromatic in-chain di and tri-substituted vinyl groups absorbing at $\sim 810\text{ cm}^{-1}$ (Allen et al., 2001).

Some peaks rose due to the treatment of the residue with ozone. In other words, a number of functional groups were apparent in the ozone-treated samples that were elusive before treatment. The application of small doses of ozone may result in polymerization processes (Amy, 1988). It is seen that all the spectra are quite similar. Specifically, the O-H in-plane bending vibration is observed in the region $1398\text{ to }1413\text{ cm}^{-1}$. The bands centered in the region $\sim 2970\text{ to }2872\text{ cm}^{-1}$ in naphazoline, phenobarbital, salbutamol correspond to the stretching mode of asymmetrical CH_3 and CH_2 vibrations mainly of aliphatic carbonyl-containing functional groups. The peak centered at 2794 cm^{-1} in salbutamol may correspond to the stretching mode of symmetrical C-H vibrations of cyclic ether. The absorption peaks detected in the region of $1500\text{ to }1800\text{ cm}^{-1}$ are typical for the overlapping of the (C=C) stretching vibration mode of the aromatic ring, quinone, with the (C=O) absorption peaks of free carboxylic, ester and carbonyl groups. The most remarkable feature of the spectra is the appearance of a broad carbonyl (C=O) peak in the region $1709\text{ to }1712\text{ cm}^{-1}$ which is asymmetrical to higher frequencies, and displays a shoulder at 1770 cm^{-1} .

Ultraviolet spectrometry (UV visible)

A further literature search on reaction mechanisms shows that most authors are in agreement that the first step consists in hydroxylation of the aromatic ring. The absorption bands for phenol and quinone, and the yields resulting from ring opening, are strong and separate. Some organic impurities also have absorption at 200 to 300 nm. At the same time, there was a split parallel increase in the intensity of absorption at 230 to 240 nm region of the spectrum associated with absorption of the unsaturated carbonyl and carboxylic acids formed in aromatic ring opening. The spectrum of quinone in aqueous solution shows a maximum at 260 nm. The fact that there was no obvious increase in the band at 260 nm ruled out the possibility of the formation of quinone as an intermediate in the interaction of O_3 with aromatic ring in aqueous solution. The ozone attacks the O-H bond of the aromatic, giving a mixture of several tautomeric radicals which then break down to form the mixed reaction yields (Razumovskii et al., 1979).

A study of the change in UV spectra after ozonation, suggests the progressive disappearance of absorbance maxima which corresponds to bands of aromatic rings (transition $\pi \rightarrow \pi^*$). As for salbutamol, appearance and Disappearance of a band was observed and the absorbance maximum was 245 nm. This band

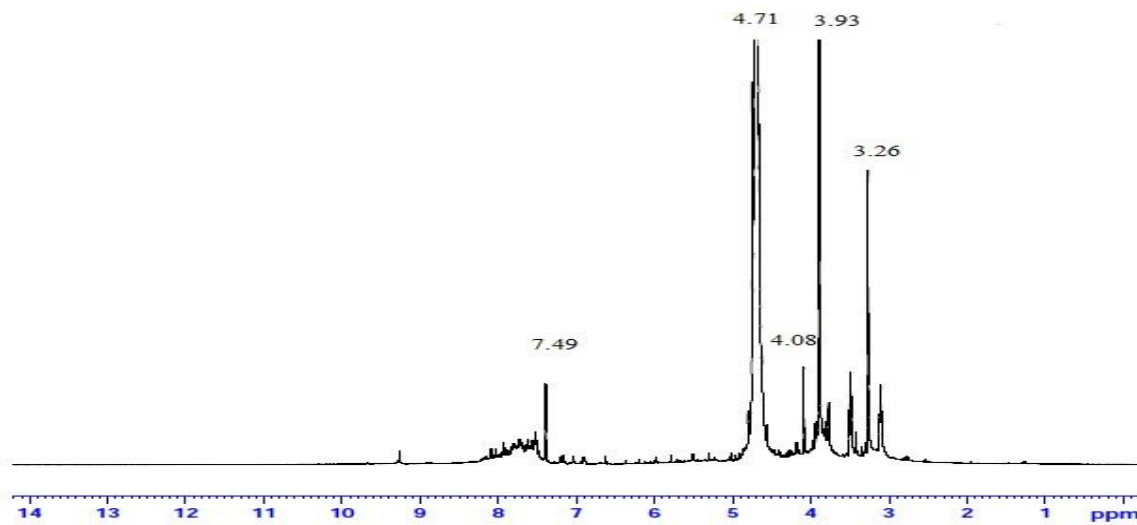
corresponds to the formation of *p*-quinone.

Spectroscopy

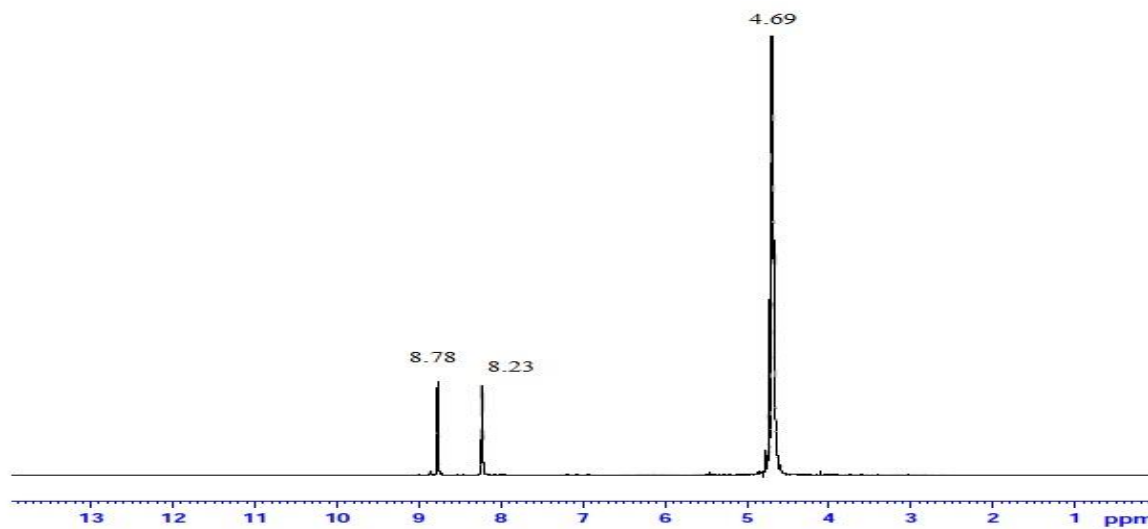
The $^1\text{H NMR}$ spectrum for all samples confirmed the presence of seven strong proton signals (1.26, 4.08, 4.69, 4.71, 2.56, 3.93 and 3.26 ppm) (Figure 5). The signals at 5.85 and 6.02 ppm are attributable to aromatic hydrogen, respectively. The twin signals at 4.08 and 4.71 ppm for salbutamol and another signal at 4.69 ppm for phenobarbital indicated the presence of three amino groups (NH). These findings are in complete agreement with data obtained from literature reviews (Fotouhi et al., 2006). A small signal between 8.00 and 9.00 ppm for isoniazid is assigned to amide proton. This cleavage seems to be favored due to the possible resonance stabilized carbanion formed from deprotonation of methine carbon. In doxycycline and salbutamol at 0 to 1.6 ppm, protons on methyl and methylene carbons directly bonded to other carbons. The small signal at 1.6 ppm in salbutamol is due to protons on methyl and methylene carbons in alpha position to aromatic rings and the 3.2 ppm signal in phenobarbital, naphazoline and doxycycline is related to carboxyl and carbonyl groups. At 3.2 to 4.3 ppm, protons on methyl, methylene or methine carbons directly bond to oxygen or nitrogen, including carbohydrate and amino acid protons. The small signals at 8.07, 7.49 and 8.23 ppm in doxycycline, naphazoline and isoniazid are assumed to be from protons attached to unsaturated carbons or aromatic protons (Kim and Yu, 2007). Similar signals in doxycycline and phenobarbital were observed at 0.9 to 1.3 ppm, assigned mainly to saturated CH_2 groups. Within the region of 2.8 to 4.4 ppm, an increased signal was noticed, which indicates an increase in oxygen-containing functional groups. The signal at 3.07 ppm in doxycycline and salbutamol is due to H and CH_3 groups and the 3.93 ppm in naphazoline and salbutamol is for CH_2 ; it implies the 6.82 to 7.74 CH aromatic disappeared. In addition, the 4.65 ppm for all samples is related to CH_2 (Grinhut et al., 2011, Ohlenbusch et al., 1998). Thus, the obtained $^1\text{H NMR}$ data obtained support the hypothesis that the ozonation of aromatic compounds can be the result of cleavage of the aromatic rings. Comparatively, the protons of the products are much more than aromatics counterparts. However, comparison of the NMR and optical data provides evidence for a complex mechanism of interactions of substrates with ozone, and does allow us to state with certainty the cleavage of aromaticity direct coordination.

Conclusions

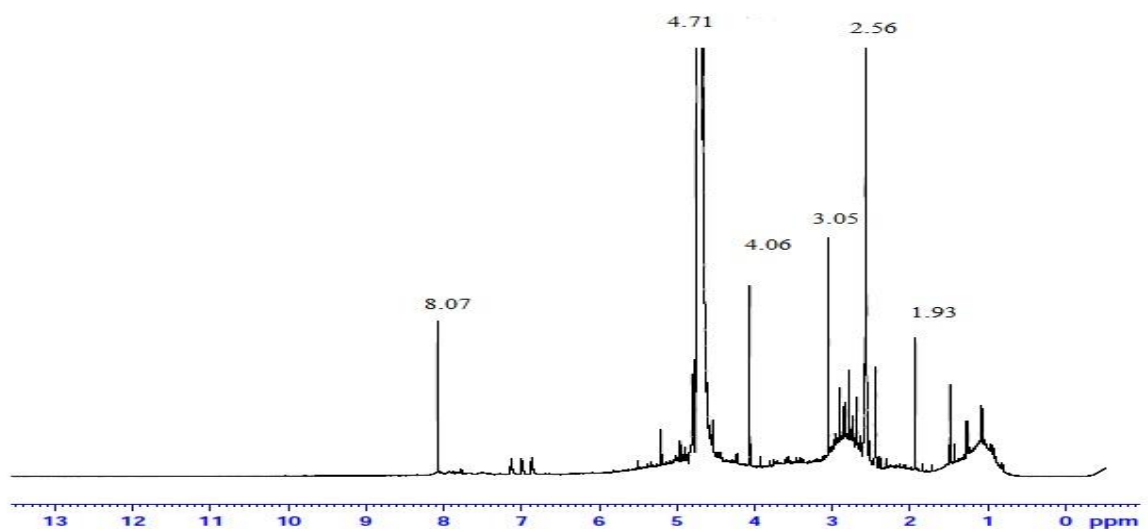
The data demonstrates exposure of some drugs with aromatic rings to ozone gas resulted in the consistent



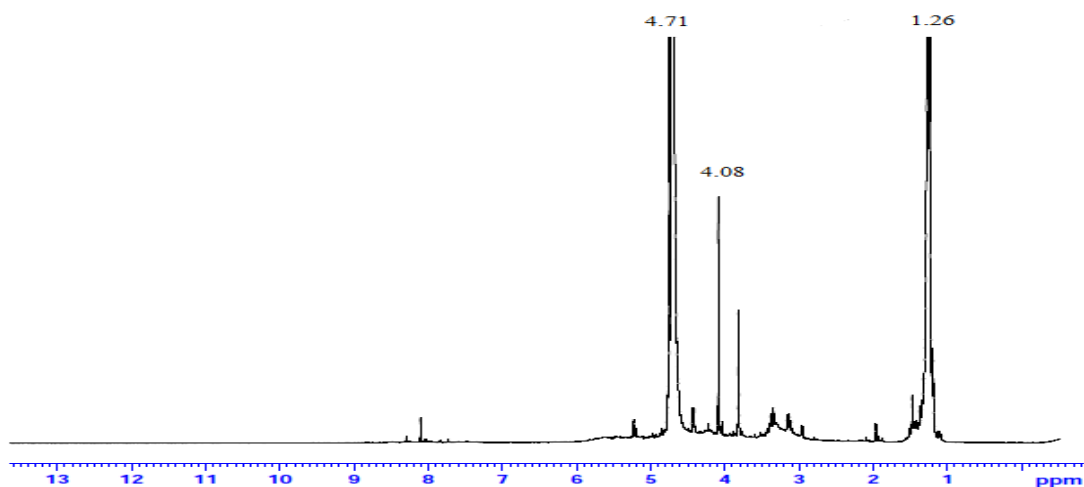
A)



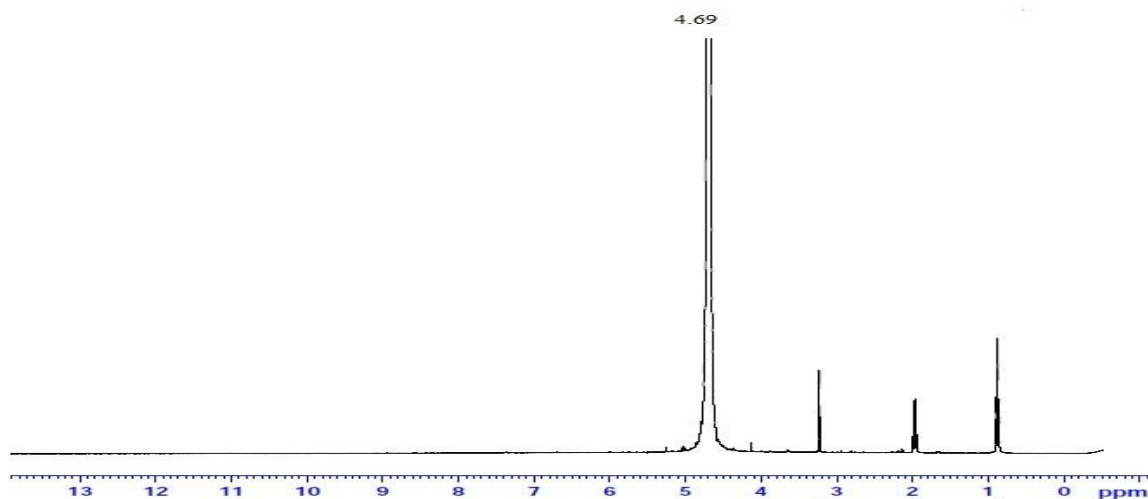
B)



C)



D)



E)

Figure 5. NMR spectra of: (A) Naphazoline, (B) Isoniazid, (C) Doxycycline, (D) Salbutamol and (E) Phenobarbital after ozonation.

formation of unsaturated yields based on aliphatic esters, ketones, and lactones as well as aromatic cleavage. Results obtained during the ozonation of aqueous solutions of aromatic compounds have shown that the reaction occurs in two steps: A first phase leading to the opening of the aromatic ring and a second phase in which the sub-products, resulting from the first phase, are stable towards further ozonation. They do not, consequently, consume ozone; moreover they are biodegradable (Evison, 1977; Hann, 1956). Since ozone is such a powerful oxidizer, this suggests this process may be used for the destruction of organic substances. It is evident that ozone preferably oxidizes in electron-rich

sites (Hoighe and Bader, 1983). As a result, a dramatic decrease in UV absorbance at 254 to 280 nm is observed during ozonation of the aromatic compounds. It is known that during ozonation in water solutions, hydroxyl radicals ($\text{OH}\cdot$) proceed along with direct ozonation. $\text{OH}\cdot$ radicals react relatively unselectively (Hoighe, 1982; Staehelin and Hoigne, 1985). In its turn, $\text{OH}\cdot$ radicals produce organic radicals as a result of their reactions with organic compounds. The reactions with $\text{OH}\cdot$ radicals may bring about considerable structural changes in macromolecules including hydroxylation, decarboxylation, and depolymerisation of the initial materials, producing oxidized structures that are hydrophobic and less aromatic.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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