Investigation of the Effect of Toluene on Nitric Oxide Production and Protective Properties of Resveratrol

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ABSTRACT

Aim: Toluene is the most preferred organic solvent. Prolonged exposure to toluene, causes serious health problems. The International Agency for Research on Cancer (IARC) has classified toluene as "possibly carcinogenic to humans" (Group 2B). This classification states that toluene may have potential carcinogenic effects. Toluene exposure has been linked to the formation of reactive nitrogen species and reactive oxygen species, resulting in direct tissue damage and alteration of various antioxidant systems. Resveratrol, which has a polyphenol structure, is a molecule that has important effects on plants. It is known to have positive effects on humans as well. In this study, the effect of exposure to toluene on nitric oxide production, which has an important role as a biological regulator in cardiovascular, neurological, immunological and many other systems, and the protective properties of resveratrol were investigated.
Materials and Methods: While 900mg/kg dose of toluene was administered intraperitoneally to male rats (250-350gr Wistar-Albino), 5, 10 and 20mg/kg doses of resveratrol were administered the same way for six days. Nitric oxide levels and Nitric oxide synthase activities were investigated in liver tissue and serum.
Findings: The data showed an increased nitric oxide level in the liver tissue and serum and a high nitric oxide synthase activity following toluene administration. Significant reductions in nitric oxide levels and nitric oxide synthase activity in the liver were observed after the administration of various dosages of resveratrol.
Results: Our results suggested that high doses of toluene induce nitric oxide production, whereas resveratrol possesses protective properties.
Keywords: Toluene, resveratrol, nitric oxide, nitric oxide synthase

Toluenin Nitrik Oksit Üretimine Etkisinin ve Resveratrolün Koruyucu Özelliklerinin Araştırılması

ÖZET

Amaç: Organik çözücü olarak en çok tercih edilen Toluendir. Toluene uzun süre maruz kalmak ciddi sağlık sorunlarına neden olur. Uluslararası Kanser Araştırmaları Birliği (IACR), tolüeni "insanlar için muhtemelen kanserojen" (Grup 2B) olarak sınıflandırmıştır. Bu sınıflandırma, toluenin potansiyel kanserojen etkilere sahip olabileceğini belirtir. Toluene maruz kalma, reaktif nitrojen türlerinin ve reaktif oksijen türlerinin oluşumuyla bağlantılı olup, doğrudan doku hasarına ve çeşitli antioksidan sistemlerin değişmesine neden olur. Polifenol yapısında olan resveratrol, bitkilerde önemli etkilere sahip bir moleküldür. İnsanlarda da olumlu etkilere sahip olduğu bilinmektedir. Bu çalışmada, kardiyovasküler, nörolojik, immünolojik ve diğer birçok sistemde biyolojik düzenleyici olarak önemli bir role sahip olan nitrik oksit üretimine toluene maruz kalmanın etkisi ve resveratrolün koruyucu özellikleri araştırılmıştır.
Yöntem: Tolüenin 900mg/kg dozu erkek sıçanlara (250-350gr Wistar-Albino) intraperitoneal yolla uygulanırken resveratrol 5, 10 ve 20mg/kg dozları aynı yolla altı gün boyunca uygulanmıştır. Karaciğer dokusunda ve serumda nitrik oksit seviyeleri ve nitrik oksit sentaz aktiviteleri araştırılmıştır.
Bulgular: Veriler, karaciğer dokusunda ve serumda nitrik oksit seviyesinin arttığını ve toluen uygulamasını takiben yüksek nitrik oksit sentaz aktivitesini gösterdi. Resveratrolün çeşitli dozlarının uygulanmasından sonra karaciğerde nitrik oksit seviyelerinde ve nitrik oksit sentaz aktivitesinde önemli azalmalar gözlendi.
Sonuçlar: Sonuçlarımız yüksek dozda toluenin nitrik oksit üretimini tetiklediğini, resveratrolün ise koruyucu özelliklere sahip olduğunu gösterdi.
Anahtar Kelimeler: Toluen, resveratrol, nitrik oksit, nitrik oksit sentaz

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INTRODUCTION

Toluene is a commercially used substance. It is a volatile, water-insoluble compound in gasoline, cigarette smoke, and benzene production. It is used as a solvent in various substances, including paints, paint thinners, adhesives, and nail polish (Agency for Toxic Substances and Disease Registry, 2017). Furthermore, toluene is one of the most commonly abused solvents. Because it is used in the manufacture of adhesives, it is an easy product to obtain among children and adolescents (Balster et al., 2009). Toluene is quickly absorbed through the lungs and readily distributed via the blood to numerous tissues, particularly the brain, liver, kidney, and adipose tissue (Gotohda et al., 2009). Most inhaled toluene is metabolized in the liver by cytochrome p-450 (CYP) and Glutathione-S-transferase (GST). As a result, metabolic intermediates and a by-product reactive oxygen species (ROS) are formed (Kim et al., 2015). Toluene is regarded as a potentially harmful chemical (Pelletti et al., 2018).

Resveratrol, also called 3,5,4'-trihydroxy-trans-stilbene, is a polyphenol compound naturally synthesized in many plant species, including Japanese knotweed, black grapes, peanuts and mulberries (Gupta et al., 2011). Plants synthesize resveratrol as a defense mechanism against stress, injury, excessive sunlight, ultraviolet radiation, infections, and fungi (Chitarrini et al., 2017). Resveratrol is also present in many commercial grape products, such as grape juices and wine, particularly red wine (Hasan and Bae, 2017). Resveratrol exists in two isoforms a cis and trans. According to reports, the trans-isoform is more prevalent and biologically active (Navarro-Cruz et al., 2017). Trans-resveratrol has been reported to exhibit a wide range of benefits, including anti-oxidative (Truong et al., 2018), anti-inflammatory, anti-cancer (Espinoza et al., 2019), anti-aging (Li et al., 2018), cardioprotective and neuroprotective properties (Villaflores et al., 2012).

Nitrite oxide (NO), which is formed from the amino acid L-Arginine by the catalysis of the nitric oxide synthase (NOS) enzyme, is a gaseous free radical (Yu et al., 2018). Three isomers of NOS have been identified in mammalian biochemistry. Inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) (Pradhan et al., 2018). The reason why NO is a very reactive radical is that it has an unpaired electron in its last orbital. For this reason, it can react immediately with many reagents. It has been suggested that NO can regulate signalling pathways in this way (Fang et al., 2021). In a study, it was observed that NO levels and different types of NOS-dependent reagents increased in the fluid taken by bronchoscopy from rats that had inhaled toluene. Therefore, it has been stated that exposure to toluene increases the free radical level of NO synthesized by alveolar macrophages (Huffman et al., 1997). Another study found that exposure to toluene diisocyanate (TDI) causes oxidative stress, which leads to the formation of ROS and reactive nitrogen species (RNS), resulting in direct tissue damage and the change of several antioxidant systems (Choi et al., 2018).

Aim: The aim of this article is to reveal the effect of toluene exposure on NO production in blood and liver tissue and to explain how much this effect can be protected by resveratrol.

METHODS

Animals and study design

This study used 36 male Wistar-Albino rats weighing 250-350g supplied by Bursa Uludağ University Faculty of Medicine Experimental Animal Breeding and Research Center Unit. The ethics committee decision number is 2019/04-03. All rats were randomized equally to six groups (n=6) as follows: saline, %10 ethanol, toluene (900mg/kg), and resveratrol (5, 10, and 20mg/kg). All animals were maintained at room temperature at 22-24ºC with free access to food and water (*Ad libitum*). All studies with rats were carried out in accordance with the ARRIVE guidelines. Experiments with animals comply with EU directive 2010/63/EU.

Toluene and resveratrol treatment

One hour after giving rats 900mg/kg of toluene intraperitoneally (i.p) for six days, resveratrol doses of 5, 10, and 20mg/kg were administered in the same manner (resveratrol was dissolved in 10% ethanol). The control groups received saline and 10% ethanol using the same method. On the sixth day, the liver and blood tissues of rats euthanized by cervical dislocation were quickly collected and stored at -20°C until further analysis.

Liver tissue and blood samples collection

Blood samples obtained directly from the heart were collected in Eppendorf tubes and kept at room temperature for 15 minutes, then centrifuged at 4ºC in 1000g for 15 minutes to obtain serum. The sera were stored at -80ºC until further analysis. The liver tissues were washed with pH=7.4 cold phosphate buffer, homogenized, and centrifuged according to the instructions of the measurements kits and made ready for analysis (Elabscience, 2018).

Determination of NO level

NO amounts were calculated by determining Nitrite (NO_2^-) concentrations at 550 nm wavelength. NO colorimetric assay kit was utilized in the experiments (Elabscience, 2018).

Determination NOS activity

300mg of liver tissue was taken into the homogenization tube. 3ml of cold buffer solution (RIPA Lysis Buffer) was added and homogenized. Protein concentrations within each lysate were measured using a BCA protein assay kit. Samples containing 25µg protein were loaded into separate wells of the SDS-PAGE gel (NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gels) and transferred to a nitrocellulose membrane (iBlot® Transfer Stack, nitrocellulose). The membrane was blocked in TBS-T (Tris-buffered saline Tween20) for 1 h at room temperature to block the nonspecific binding site. The membrane was incubated with the following polyclonal primary antibodies at 4ºC overnight: iNOS (1:1000 dilution), eNOS (1:1000 dilution), nNOS (1:500 dilution), and a monoclonal β-actin antibody (1:2000 dilution).

The membrane was then washed and incubated with secondary antibodies at a dilution of 1:2000 for 1 hour. The specific band was detected using Fusion FX-7 chemiluminescent substrate by exposing them to X-ray film. β-actin was used as an internal control, and NOS activity was expressed relative to β-actin bands. Provided from nNos (elabscience ab70065), iNos (biolegend 5cb52), b actin (cell signaling).

Statistical analysis

Statistics were made on a computer using GraphPad Prism 9.3.0 software. Differences between experimental and control groups were determined using ANOVA, one-way analysis of variance and post-hoc Tukey tests. Differences between mean values were considered significant when $P<0.05$ and $P<0.001$. Standard deviation (SD) values were calculated by averaging the data obtained from the experiment repetitions.

FINDING AND DISCUSSION

NO level

Toluene administration significantly increased the NO level in the liver by 32% compared to the control groups ($p<0.05$). However, resveratrol doses of 5, 10, and 20mg/kg resulted in a statistically significant decrease in the level of NO in the liver compared to the toluene-treated group ($p<0.001$). While the 5 and 10mg/kg doses of resveratrol decreased by 83%, higher doses of 20mg/kg decreased by 68% (Table 1 and Figure 1A). When the NO level in serum samples was measured, the toluene group showed a statistically significant increase compared to the control groups ($p<0.05$). In contrast, resveratrol administration at 5 and 10mg/kg doses reduced the NO level in the serum by 73% compared to the toluene group ($p<0.001$). Although there was a decrease in 20mg/kg resveratrol compared to the toluene group, it was found to be statistically insignificant ($p>0.05$) (Table 1 and Figure 1B).

NOS activity

The results of western blot analysis of iNOS, eNOS, and nNOS activities of toluene and resveratrol are shown in Figure 2, whereas the histograms obtained as a result of toluene and resveratrol administration are shown in Figure 3. Toluene's effect showed the highest NOS activity in iNOS 1.96, eNOS 1.37, and nNOS 0.84 compared to the corresponding densities of β-actin bands. While iNOS increased approximately twofold compared to the saline control group, it remained nearly constant with the ethanol group. After resveratrol treatment, significant reductions in iNOS activity were observed.

After resveratrol treatment, eNOS activities at all three doses were calculated to be nearly equal to toluene-treated eNOS activity values. It is worth noting that all three dosages of resveratrol boosted eNOS activity compared to the ethanol group. With the effect of toluene, no change in the nNOS activity was observed compared to the ethanol control. However, all three doses of resveratrol reduced nNOS activity (Figure 4.3 C). This drop was assessed as 24.21% on average.

Table 1. Liver and serum NO levels

Groups	**NO (µmol/L)**	
	Liver	**Serum**
	Mean ± SD	Mean ± SD
Control saline	192.17 ± 46.52	484.67 ± 166.45
Control ethanol	194.67 ± 48.56	558.00 ± 135.00
Toluene	283.00 ± 47.34*	1013.00 ± 268.51*
Toluene+Resveratrol (5mg/kg)	48.00 ± 21.21***	280.50 ± 59.09***
Toluene+Resveratrol (10mg/kg)	46.75 ± 19.31***	268.00 ± 120.00***
Toluene+Resveratrol (20mg/kg)	90.50 ± 27.23***	623.00 ± 20.00

SD: standard deviation
Mean ± Standard Deviation
*p <0.05 toluene groups are statistically significant compared to control groups.
***p<0.001 resveratrol groups are statistically significant compared to toluene groups.

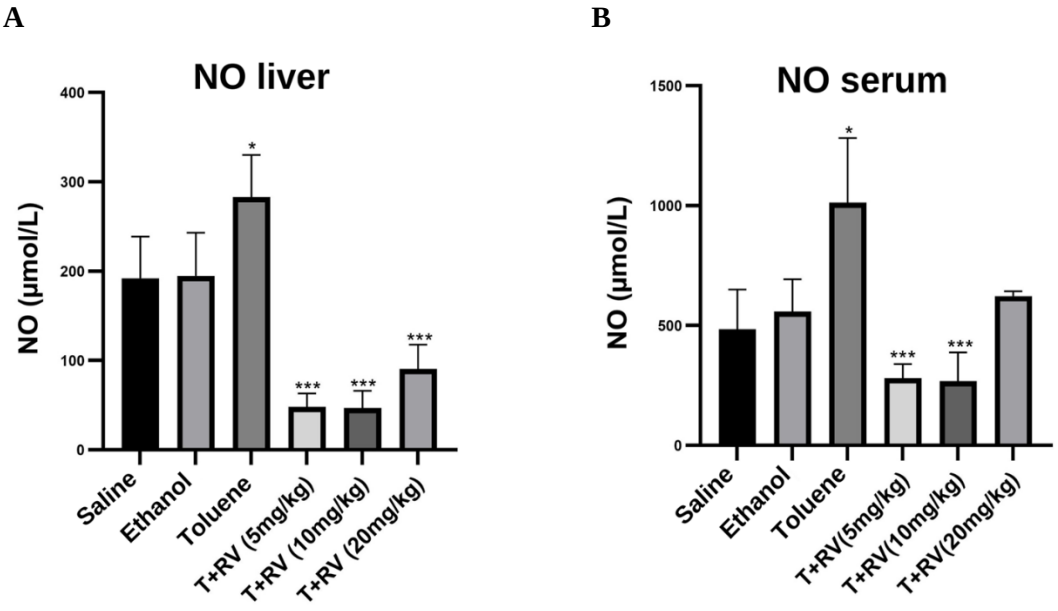

Figure 1. Liver (A) ve Serum (B) NO level

*p<0.05 toluene groups are statistically significant compared to control groups.
***p<0.001 resveratrol groups are statistically significant compared to toluene groups.

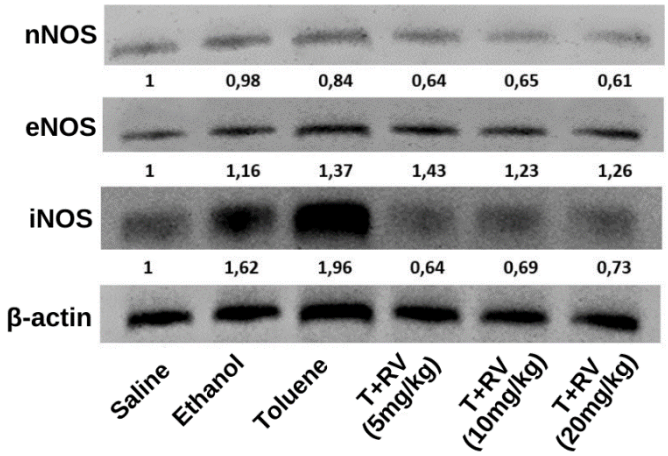

Figure 2. Effect of toluene and resveratrol on iNOS, eNOS and nNOS activities. Western blot was performed using an equal amount of protein (25µg) from liver tissue.

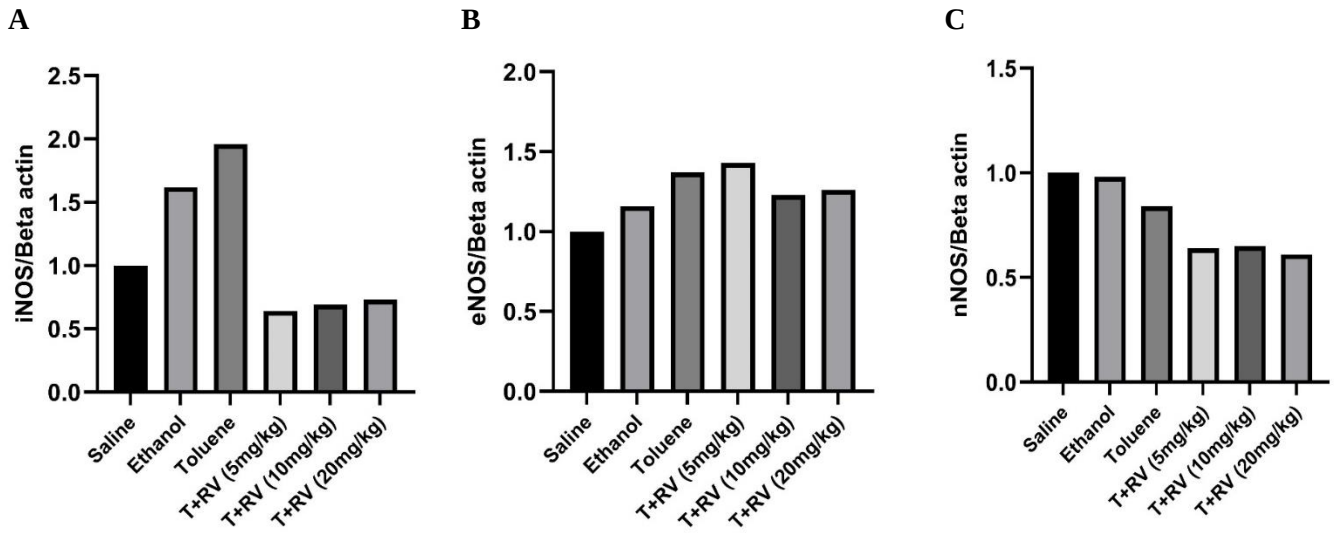

Figure 3. iNOS (A), eNOS (B), and nNOS (C) protein activities. The results were expressed relative to the density of the β-actin bands. T+RV: Toluene + Resveratrol

Our study showed that intraperitoneal injection of toluene increased NO levels in the liver and serum. The increased NO generation mediated by the toluene effect is due to increased iNOS activity in these tissues. This is substantiated by the strong iNOS/Beta-actin bands observed in Western blot analysis following toluene delivery (Figure 3A). Huffman et al. (1997) reported that toluene diisocyanate (TDI) inhalation exposure increased NO production due to lung upregulation of the iNOS isoform, primarily in bronchoalveolar lavage cells. It has been suggested that NO occurring in alveolar macrophages after TDI exposure increases the amount of free radicals produced in alveolar macrophages. Another explanation for the higher NO levels caused by the toluene effect could be that free radicals released by the toluene effect boosted NO production. Mattia et al. (1993) demonstrated that in vivo exposure to various doses of toluene (0.5, 1, and 1.5 g/kg i.p) increased ROS formation in the liver and blood tissues. Another study found that TDI exposure induces oxidative stress leading to

the formation of ROS and RNS, causing direct tissue damage and alteration in various antioxidant systems (Choi et al., 2018).

The rise in NO caused by toluene could also be attributed to liver injury. In one study, exposure to TDI caused an inflammatory response in the lungs, an increase in lymphocyte count in the airways, interferon-gamma release, and high NO production from alveolar macrophages (Huffman et al. 1997). In the resultant liver damage, It has been suggested that pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 1β (IL1β) induce iNOS expression by stimulating kupffer cells and hepatocytes (Miki et al., 2016).

In the present study, we discovered that resveratrol significantly decreased NO levels. This is because resveratrol reduces NO synthesis by decreasing or inhibiting iNOS expression. Several intracellular signalling pathways, including nuclear factor kappa B (NF-κB), protein kinase B/Akt, and c-Jun N-terminal kinase (JNK), have been shown to regulate hepatocyte iNOS expression (Zhang et al., 2004; Hong et al. al., 2010). Resveratrol is an NF-kB transcription inhibitor (Manna et al., 2000). A study reported that resveratrol inhibited iNOS expression by blocking NF-κB binding activity (Tsai et al., 1999). In another study, resveratrol was reported to decrease cytokine-induced iNOS expression and activation in hepatocytes by upregulating the JNK signalling pathway (Kimbrough et al., 2015).

NO increased by toluene's effect, can react with the superoxide radical to generate peroxynitrite ($ONOO^-$), a powerful and long-lasting compound. Peroxynitrite can damage various cellular molecules, including DNA, lipids, and proteins, and can also promote protein nitration by affecting the structure and function of many target proteins (Kurutas, 2016). Resveratrol may also have reduced these peroxynitrites. It is claimed that resveratrol, which has a polyphenol structure, has very strong antioxidant properties and eliminates free radicals (McGill et al., 2015). Resveratrol has been demonstrated to scavenge several oxidants directly, including superoxide, hydroxyl radicals, hydrogen peroxide and peroxynitrite (Xia et al., 2017).

In our study, we observed that resveratrol administration decreases NOS activities (Figure 3 ABC), with the considerable drop in iNOS activity possibly due to differences in properties from the other two NOS (eNOS and nNOS). iNOS is transcriptionally regulated and does not require intracellular calcium for its activation. Different inflammatory agents such as TNF-α, interferon, interleukin-1, endotoxin, hypoxia, oxidized LDL, and lipopolysaccharides stimulate its expression in the cell. One of the essential features of resveratrol is its anti-inflammatory effect. Resveratrol mediates the anti-inflammatory response by down-regulating pro-inflammatory cytokines. Das and Das (2007) found that resveratrol inhibits the pro-inflammatory enzyme cyclooxygenase-1 (COX-1), thereby suppressing pro-inflammatory eicosanoid formation. It has also been suggested that the anti-inflammatory effects may be mediated by SIRT1, suppressing the main inflammatory transcription factor NF-kB via deacetylation (Yeung et al., 2004). Another reason for the decrease in iNOS activity is that resveratrol has the ability to remove a variety of free radicals. Resveratrol inhibits ROS production via NADPH and down-regulates the expression and activity of oxidase. Additionally, it has been suggested that resveratrol

accelerates the detoxification of ROS by increasing the expression of various antioxidant enzymes (Xia et al., 2017).

Resveratrol administration is expected to affect eNOS activity (Figure 3B) slightly. It is worth noting that resveratrol boosts eNOS activity, particularly at 5mg/kg. One potential reason for the rise in eNOS activity is that resveratrol upregulates eNOS activity. Wallerath et al. (2002) reported that trans-resveratrol stimulates acute NO release from vascular endothelial cells and induces up-regulation of eNOS gene expression after 24 to 72 hours of incubation. In another study, Wallerath et al. (2003) and Wallerath et al. (2005) showed that resveratrol treatment increases the mRNA and protein expression of eNOS in cultured human endothelial cells. Resveratrol has been reported to boost eNOS promoter activity and mRNA stability through transcriptional and posttranscriptional pathways. Resveratrol can also exert its effect on NOS through its metabolites. Although not as powerful as the parent molecule, several resveratrol metabolites are biologically active (Lu et al., 2013). According to Cai et al. (2015), resveratrol and its metabolites exert their effects by accumulating in tissues.

CONCLUSION AND RECOMMENDATIONS

This study concluded that exposure to toluene increased NO levels in liver tissue and serum. The decrease in NO levels with resveratrol administration supports our view that resveratrol acts as a protector. More detailed research is needed to determine the mechanism of action of resveratrol in regulating the expressions of NOS enzymes in the liver following toluene exposure in rats.

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Ethics

Ethics Committee Approval: This study was conducted with the ethics committee decision (2019-04/03) of Bursa Uludağ University's local ethics committee for animal experiments

Conflict of Interest

We declare that there is no personal or financial conflict of interest between the authors in the preparation and presentation of the article.

Author Contributions

Egemen DERE created the concept and design of the study and contributed to the discussion section of the article. Raissa SOAMANJARY performed the experiments and wrote the findings. All authors read and approved the submitted version.

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