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Effect of *Annona muricata* aqueous leaf extract on reactive oxygen and nitrogen species

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Abstract. – OBJECTIVE: *Annona muricata* extracts are used in traditional medicine due to their significant biological effects. Verification and elucidation of their mechanisms is beneficial in terms of the usefulness of these extracts in everyday life or in the context of disease treatment or prevention.

MATERIALS AND METHODS: The effectiveness of the extract was assessed from dried *A. muricata* leaves available for direct consumption. It is targeted against reactive oxygen and nitrogen species such as superoxide ($O_2^{\cdot-}$), hydroxyl ($^{\cdot}OH$), nitric oxide (NO) radicals, and peroxynitrite anion (ONO_2^-) at concentrations of 5, 10, 25, 50, 100 µg.ml^{-1}.

RESULTS: No significant inhibitory activity was measured against $O_2^{\cdot-}$ at the assessed concentrations of the extract. Conversely, substantial antioxidant properties were found towards $^{\cdot}OH$. Moreover, very efficient uptake was recorded at low concentrations of the extract, 5 µg.ml^{-1} (53.91%) and 10 µg.ml^{-1} (45.3%). The antioxidant effect decreased with increasing concentration. By indirect determination of NO oxidation derivatives it was found that, as the extract concentration increased, the nitrite concentration decreased. In contrast, even at low concentrations, the extract causes an increase in the peroxynitrite concentration.

CONCLUSIONS: The results themselves show that the effects of *A. muricata* leaf extract are mainly mediated by the activity against $^{\cdot}OH$, as well as the consequences of increased ONO_2^- formation.

Key Words:
Annona muricata, Free radicals, Hydroxyl radical, Nitric oxide, Peroxynitrite, Reactive oxygen species, Superoxide radical.

Introduction

Annona muricata L., family *Annonaceae*, also known as soursop or graviola, is native to the tropical regions of North and South America[1]. It is widely used in the food industry. However, all parts of the plant are used in ethnomedicine to treat various diseases, especially the bark, leaves and roots. Diseases treated include fever, rheumatism, inflammation, skin, parasitic infections, bacterial diseases, cancers, and diabetes mellitus, as well as being used for its sedative, insecticidal and immunosuppressive effects[2-8].

The fruits, bark, leaves and roots of *A. muricata* are rich in flavonoids, isoquinoline alkaloids and annonaceous acetogenins. The stem, leaves and seeds are reported to contain more than 70 acetogenins[9-11], which are also the most prevalent bioactive compounds. About 22 alkaloids detected in the leaves are another group of naturally occurring secondary metabolites[12]. In addition, vitamins, amides, and 80 essential oils, especially sesquiterpene derivatives, are present in the leaves[13]. However, of the phenolic compounds isolated from *A. muricata*, 34 are among the most important, they are mostly soluble in water and aqueous infusions are the basis of traditional medicine[5,10,14].

Besides other remarkable biological activities, antioxidant effects of various *A. muricata*-derived extracts have been described[15]. Administration of aqueous leaf extract to streptozotocin-induced diabetic rats led to an improvement in the levels of superoxide dismutase, catalase activities, malondialdehyde (MDA) and nitrites up to the level of nondiabetic rats[16]. Similarly, in another rat experiment[17], ethyl acetate leaf extract increased activities of catalase, glutathione peroxidase, superoxide dismutase and decreased MDA levels. Experiments assessing the antioxidant potential and reducing power of ethanolic extract demonstrated it to be superior to the aqueous extract[7]. One possible ex-

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planation for the increase antioxidant enzyme activities can be the findings of Son et al[18] regarding upregulation of Nrf2 upon introduction of 50% ethanolic leaf extract in Hep G2 cells. Similarly, free radical scavenging abilities, ferric reducing power, α, α-diphenyl-β-picrylhydrazyl free radical scavenging, hydroxyl scavenging, and DNA damage protective activities revealed more pronounced effects from methanolic extracts in comparison to aqueous[14]. Also, strong positive correlation was found between radical scavenging activity and the total phenolic content[14].

Our previous experiment assessing the effect of dried leaf extract on cancer cell lines and the antioxidant status of isolated rat liver mitochondria showed higher concentrations causing significant negative effects on the redox state of mitochondria[19]. Likewise, Florence et al[16] revealed higher doses of extract to cause up to 25% mortality in a treatment group of rats. Therefore, the aim of this study was to determine the ability of graviola aqueous leaf extract to scavenge four types of reactive oxygen and nitrogen species in a comprehensive way and to better explain how the properties of the aqueous leaf extract contribute to this phenomenon.

Materials and Methods

Crushed leaves were used as the plant material, as described in a previous study Liliána et al[19]. The material, a product intended for direct consumption, was provided by Dr. Rafael Alvis Pizzaro from Perù in collaboration with Huminnet Ltd. later EKS-Granite Kft. (Hungary). For the experiment, an aqueous extract of the leaves (1 $mg.ml^{-1}$) was prepared over 24 hours, and determinations in the range of final concentrations of 5, 10, 25, 50 and 100 $\mu g.ml^{-1}$ were provided. The study represents part of task No. 1A/2016 approved by the Ethics committee of the Faculty of Medicine, Pavol Jozef Šafárik University in Košice.

Activity towards the superoxide radical ($O_2^{\bullet-}$) was examined using the pyrogallol autoxidation method[20]. The essence is the autooxidation of pyrogallol by atmospheric oxygen in an alkaline medium to give purpurogallin, which is spectrometrically detectable at a wavelength of 325 nm. The rate of decrease in absorbance in the presence of the test extract indicated the rate of $O_2^{\bullet-}$ inhibition. (%) = [(Acontrol-Asample)/Acontrol] × 100. Hydroxyl radical ($^{\bullet}OH$) scavenging properties were determined by 2-deoxy-D-ribose oxidation[21]. The principle of the method is the oxidation of 2-deoxy-D-ribose by $^{\bullet}OH$ generated in the Fenton reaction, forming various reactive aldehydes, which react with thiobarbituric acid (TBA) to form colour adducts of TBA-TBARS (TBA-ThioBarbituric Acid-Reactive species). The amount of adducts formed over a certain period of time (measured at 532 nm) was linearly proportional to the concentration of $^{\bullet}OH$ in the reaction mixture. A decrease in absorbance in the presence of the extract compared to the control (without extract) indicated the inhibition of $^{\bullet}OH$, the degree of antioxidant capacity of the extract, and was calculated as: (%) $= [A_0 - (A_1 - A_2)] \times 100/A_0$. A_0 was the absorbance of the control, A_1 was the absorbance of sample and deoxyribose, A_2 was the absorbance of the sample without deoxyribose. Nitrogen monoxide (NO) was indirectly measured by detecting nitrite, according to Beda and Nedospasov[22]. The principle of the determination is that sodium nitroprusside produces nitric oxide at physiological pH, which reacts with oxygen to form nitrite in the Griess reaction. After diazotization with sulphanilamide and subsequent coupling of the resulting diazonium salt with *N*-(1-naphthyl) ethylenediamine dihydrochloride, absorbance of the resulting chromophore was monitored at 546 nm. The peroxynitrite anion (ONO_2^-) was measured according to Beckman et al[23]. The mixture was frozen at -20°C overnight. The absorbance was measured at 302 nm.

Statistical Analysis

Measurements were performed in triplicate, and results were expressed as mean ± standard deviation (SD). The results of the action of tested concentrations were compared by Student's *t*-test with the activity of the standard antioxidant in complex mixtures, trolox (6-hydroxy-2, 5, 7, 8-tetramethyl-2-carboxylic acid; Fluka, Buchs, Switzerland) measured under the same conditions and published in Žatko et al[24].

An analysis of variance, followed by Tukey post-hoc test, was employed to determine statistical significance within different concentrations for the same parameter. Values of $*p<0.05$, $**p<0.01$, $***p<0.001$ were statistically significant, and $^{a}p<0.001$, $^{c}p<0.05$ for Tukey post-hoc test.

Results

Very weak activity of leaf extract was found at all tested concentrations against $O_2^{\bullet-}$ (Figure 1). Despite this, at none of the measured concen-

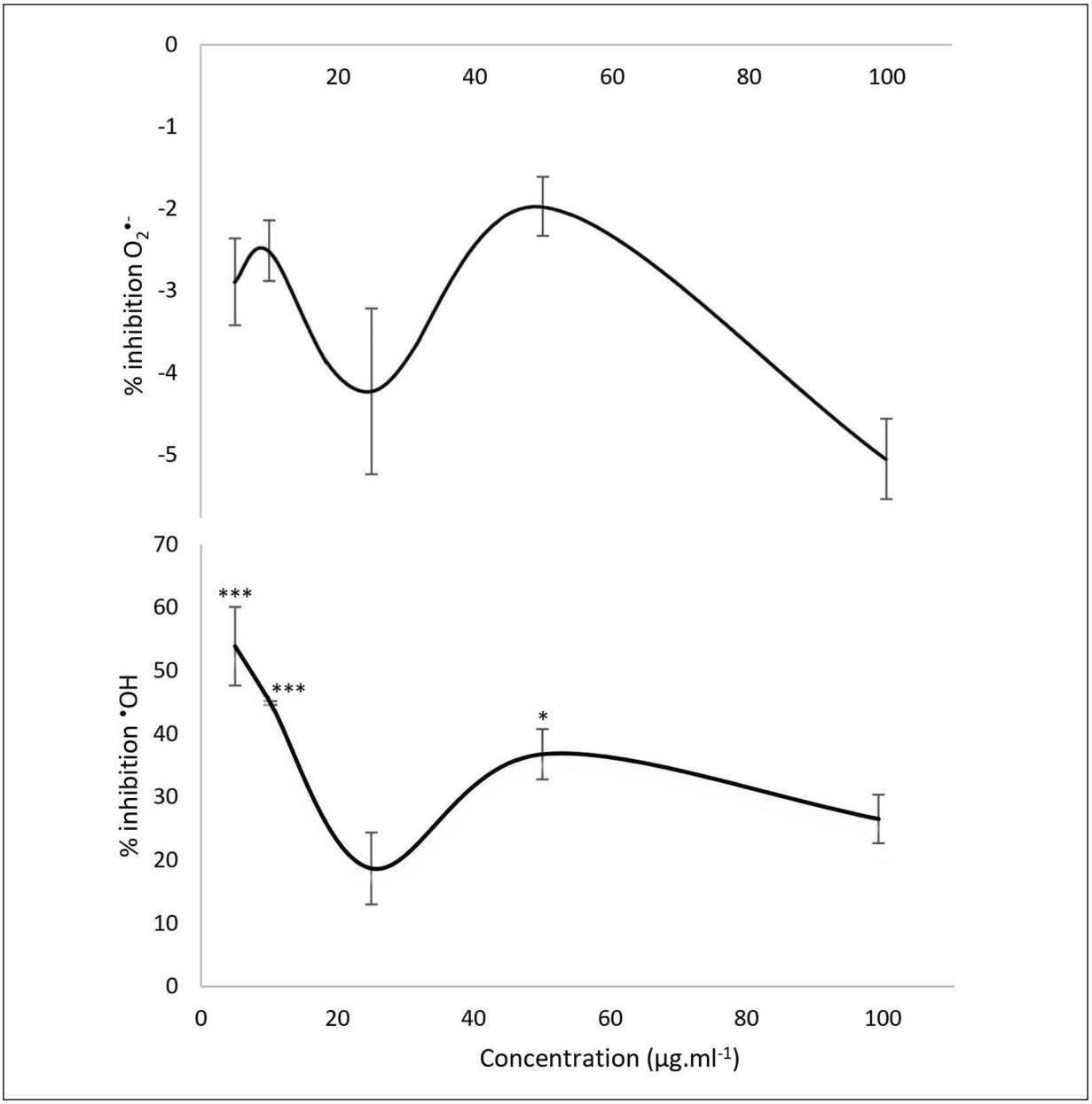

Figure 1. Percentage inhibition of the superoxide radical ($O_2^{\bullet-}$), hydroxyl radical (•OH), Statistical significance at *p< 0.05; ***p< 0.001.

trations it could be unambiguously stated that it was the antioxidant activity of the extract, as no inhibition of $O_2^{\bullet-}$, expressed as activity over zero, was recorded in any group. However, they did not differ from the Trolox used as a standard antioxidant in determining the antioxidant properties of complex compounds.

In the measurement of •OH inhibition, it was found that the antioxidant effect of *A. muricata* extract decreased with the increase of the extract concentration. At a concentration of 5 µg.ml^{-1}, the inhibition of •OH was measured at a level of 53.91%, while at 10 µg.ml^{-1} this activity decreased to 45.3% (Figure 1). A more significant decrease in the antioxidant effect of the extract of 18.71% was recorded when diluting the extract to 25 µg.ml^{-1}. When using the extract at higher concentrations of 50 and 100 µg.ml^{-1}, the inhibition of •OH was recorded higher than at a concentration of 25 µg.ml^{-1}, but corresponded to a gradual decrease in antioxidant activity compared to lower concentrations of the tested extract. Specifically, at 50 µg.ml^{-1} of

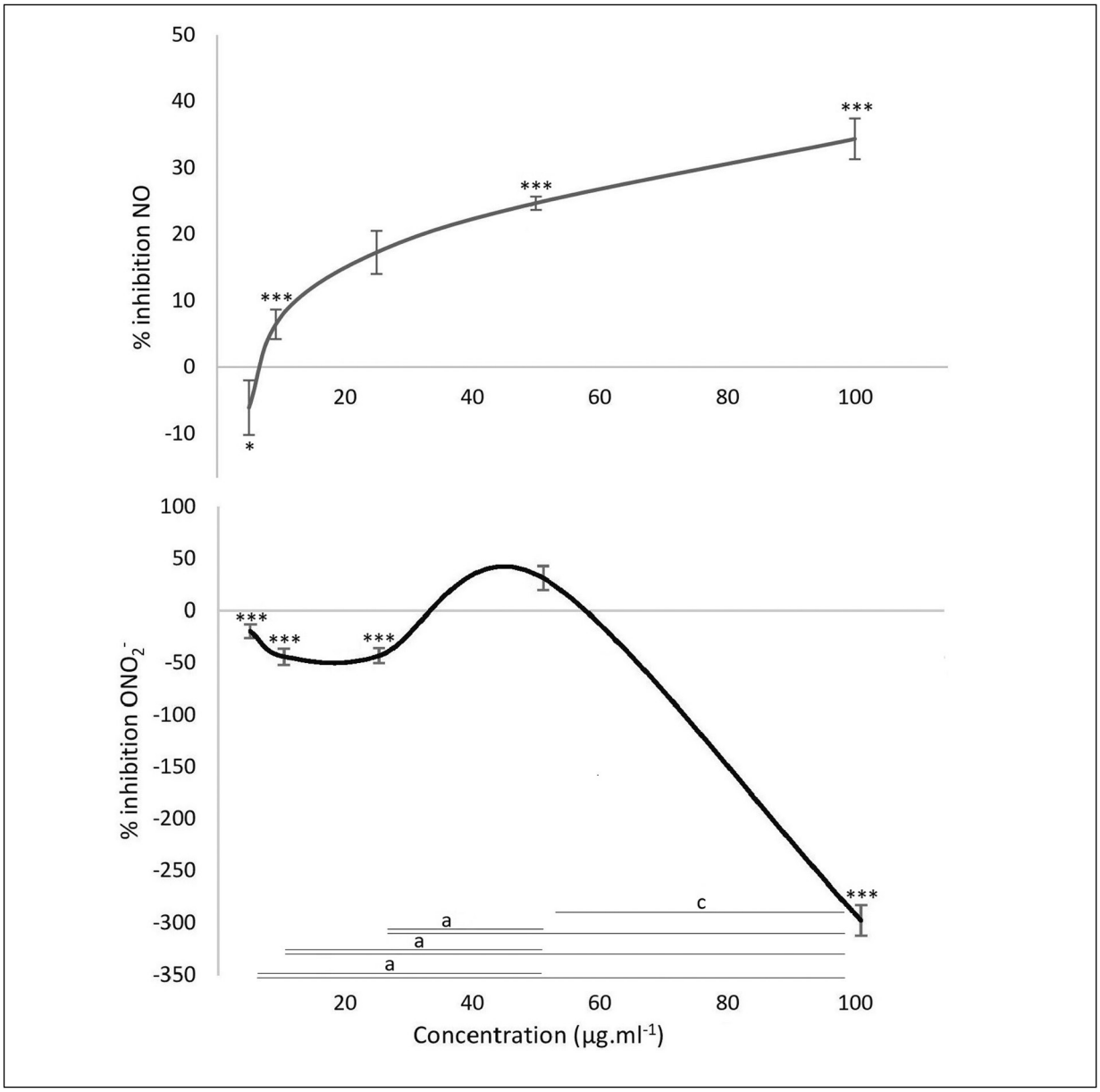

Figure 2. Percentage inhibition of the nitric oxide (NO), and peroxynitrite (ONO_2^-). Statistical significance at ***p< 0.001, *p< 0.05; [a]p< 0.001, [c]p< 0.05.

extract, a 36.79% inhibition of •OH was recorded and •OH inhibition of 26.44% at a concentration of 100 µg.ml^{-1}. Scavenging activities of extract at concentrations of 5, 10 and 50 µg.ml^{-1} were significantly higher (p<0.001 and p<0.05) than trolox.

When comparing the effect of leaf extract on •OH with $O_2^{\bullet-}$ significant differences were determined. More than 90% difference was detected between individual ROS in samples with extract concentrations of 5, 10 and 50 µg.ml^{-1}. At a concentration of 100 µg.ml^{-1}, a difference was recorded of over 80% and the smallest difference was recorded at a concentration of 25 µg.ml^{-1} at a level under 80%, which also indicates a significant difference in the effectiveness of the extract against two different ROS.

It was found that, as the concentration of the extract in the mixture increased, the amount of NO oxidation derivatives decreased (Figure 2). At a concentration of 100 µg.ml^{-1}, the efficiency of the extract reached up to 34%. The extract had an efficiency of almost 25% at a concentration of 50

μg.ml^{-1}. Still, the activities were significantly lower (p<0.001) in comparison to Trolox. The efficacy at lower concentrations was very weak. At the lowest concentration (5 μg.ml^{-1}), a higher amount of nitrite was actually found (p<0.05).

Even at the lowest tested concentration, the extract showed no uptake of ONO_2^- and at concentrations of 10 and 25 μg.ml^{-1}, its presence caused an almost 44% increase in ONO_2^- concentration (Figure 2). At the highest tested extract concentration of 100 μg.ml^{-1}, an almost 300% increase in ONO_2^- concentration was recorded. Activity of extract towards ONO_2^- differed significantly from Trolox (p<0.001). Only at a concentration of 50 μg.ml^{-1}, a scavenging activity similar to Trolox was detected. Extract activities against ONO_2^- were also significantly different amongst the concentrations tested.

Discussion

The ability of phytoactive substances in the extract to affect the concentration of reactive oxygen species and nitrogen can be considered as one of the basic mechanisms of their action. These were then used as the basis for determining the radical scavenging activities of the aqueous leaf extract, which is the natural, common and most readily available resource for humans. These effects are mediated by flavonoids, tannins, glycosides, alkaloids, anthocyanins, leuco-anthocyanins, triterpenoids, steroids, mucilage, reducing compounds and coumarins, which were mostly found in the aqueous extracts of *A. muricata*[25,26].

$O_2^{\bullet-}$ is naturally formed in the body by one electron reduction of oxygen, and the most potent sources are within the respiratory chain on complexes I and III, and respiratory burst within phagocytic cells of the immune system as the defence mechanisms against pathogens. We have found very low pro-oxidant and very weak scavenging activity at all tested concentrations. Thus, while there is a very low pro-oxidant activity of extract related to $O_2^{\bullet-}$ this can in principle lead to a desirable phenomenon, depending on momentary conditions. In response to $O_2^{\bullet-}$, uncoupling proteins can activate to cause mild uncoupling of oxidative phosphorylation leading to lowered proton motive force and decreased $O_2^{\bullet-}$ production on complex I[27]. Similarly, Pineda-Ramírez et al[28] proved that ethanolic extracts from various members of the *Annonacea* family, *Annona muricata* included, achieved approximately 50% $O_2^{\bullet-}$ trapping even using high doses of extract (3 mg.ml^{-1}). In doing so, the scavenging activity of ethanolic extract was confirmed to be even higher than aqueous[7]. Our finding was that the effect of aqueous extract could be a possible mechanism of *Annonaceae* ability to inhibit $O_2^{\bullet-}$ production[29].

Much more dangerous is $^{\bullet}OH$, arising from hydrogen peroxide or hydroperoxides decomposition, whose reactivity is quite high. Moreover, the organism does not have its own defence or regulatory mechanisms against it. We observed the highest inhibition of $^{\bullet}OH$ by low tested concentrations of extract. These scavenging activities were even higher than those of reference antioxidant, Trolox. Ilango et al[30] reported aqueous leaf extract of *A. muricata*, increasing $^{\bullet}OH$ scavenging activity reaching a maximum of 93.50% at 500 μg.ml^{-1}. Very similarly to our results, leaf and fruit pulp essential oils of *A. squamosa* manifested moderate (below 50%) hydroxyl radical scavenging activity[31]. George et al[14] proved that aqueous and methanolic leaf extracts exhibited over 50% scavenging activity from concentration 100 μg.ml^{-1}; however, methanolic extract exceeded this level by 50 μg.ml^{-1}. Gavamukulya et al[7] pointed to the relatively high and dose-dependent $^{\bullet}OH$ scavenging activity, with the highest being of that of *A. muricata* ethanolic extract between other examined annonaceae. Several above-mentioned studies, as well as Ahalya et al[2], Agu and Okolie[32], Bryan-Thomas[33] confirmed that methanolic and ethanolic extracts exhibit more pronounced antioxidant and scavenging ability due to higher total phenol and alkaloid contents.

NO is a ubiquitous intracellular messenger that regulates blood flow, blood clotting, and neuronal activity. It is also important for non-specific cellular immunity. NO itself is not harmful to the organism[34]. Nitrosation, nitrosylation, and induction/suppression of NO-mediated apoptosis results from its relatively long biological half-life, ability to passively pass through membranes, and especially its effective concentration. NO is able to react with various components, such as metals, thiols and O_2, but especially $O_2^{\bullet-}$. This forms various secondary products, such as nitrates and reactive forms of nitrogen, e.g., nitrosonium ion, peroxynitrite (ONO_2^-), nitrosothiols, nitroxyl anion, dinitrogen trioxide and nitrogen dioxide[35]. The activity of *A. muricata* extract against NO was indirectly determined, through the detection of nitrite. The activity was concentration dependent, however still not reaching activities of Trolox. Son et al[18] observed ethanolic extracts of *Annona muricata* leaves to be more effective than steam extracts

in NO scavenging. Baskar et al[36] determined NO scavenging activity of *Annona muricata* as being the strongest (compared to *A. squamosa* and *A. reticulata*) reaching 72.6% at 500 μg.ml^{-1}. In various *A. squamosa* extracts, NO scavenging activity was moderate[37]. It is therefore interesting that the hypotensive effects, which are a basic biological effect of NO, were not confirmed to be mediated through NO pathway in the rat model after intravenous administration of an aqueous leaf extract of *A. muricata*[38].

ONO_2^- is a very stable and unusually selective oxidant. It is effectively used in the body for non-specific cellular immune response, but outside of phagocytic cells it causes lipid oxidation and, under physiological conditions, reacts with sulfhydryls, iron-sulphur centres, zinc-thiolates[34], and with CO_2 (mostly in the form of bicarbonates) to form a strong oxidant, peroxynitrosocarbonate anion (but also carbonate radical), which leads to nitration of proteins and porphyrins[39]. Detected activities of an aqueous *Annona* leaf extract are significant in terms of increasing concentration of ONO_2^-, especially at the highest 100 μg.ml^{-1}. Due to the implication of peroxynitrite in the pathophysiology of many diseases and chronic inflammatory conditions, it is questionable whether comprehensive biological properties of the extract will actually lead to ONO_2^- formation in the body at such a level to have negative consequences. NO reacts violently with $O_2^{\bullet-}$ to form a ONO_2^-[36]. In our experiment on *in vitro* conditions, activities of extract alone towards NO were moderately scavenging and softly promoting $O_2^{\bullet-}$ formation. What are the conditions for the fastest reaction of $O_2^{\bullet-}$ with ONO_2^- without enzyme catalysis and the formation of peroxynitrite. Therefore, it is necessary to know whether the aqueous *Annona* extracts induce increased formation of NO by induction of nitric oxide synthase (NOS). Nwokocha et al[38], as well Shukry et al[40] found *Annona* extract to downregulate inducible nitric oxide synthase (iNOS). This was not the case in activated macrophages, where *Annona* leaf extract was found to have upregulated inducible iNOS[41].

Conclusions

Knowledge about the effects of aqueous extracts of *Annona* leaves is less known, despite the natural availability of this form. The study provides a comprehensive view of its anti-radical activity. The $O_2^{\bullet-}$ activity itself is slightly inductive, but the extract is effective against $^{\bullet}OH$. This would confirm the antioxidant properties and efficiency of *Annona* extract in many diseases. The extract showed moderate and dose-dependent scavenging activity against NO, but clearly promoted the formation of ONO_2^-. Considering conditions in the body in comparison with *in vivo* studies, just the ability to selectively promote the formation of ONO_2^- is probably the basis of biological efficiency in terms of cytotoxicity.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

MH, JV, IB made the study conception and design. Data collection and measurements were performed by MH, LČ, DO, MVU. The manuscript was written by JV, MVU and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics Approval

The study was approved by the Ethics committee of the Faculty of Medicine, Pavol Jozef Šafárik University in Košice (No. 1A/2016).

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Availability of Data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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