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Free Radical Scavenging and Antioxidant effects of Tolfenamic Acid in L-NAME-Induced Hypertensive Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Objectives: The aim of this study was to determine the antioxidant and free radical scavenging properties of tolfenamic acid (TA) against N-Nitro-L-arginine methyl ester hydrochloride (L-NAME) induced hypertension.
Materials and Methods: The rats were divided into five groups at random: Group I (control rats), Group II (control TA), Group III (L-NAME), Group IV (L-NAME+TA), and Group V (L-NAME+Enalapril). For four weeks, rats were given L-NAME (40mg/kg body weight) dissolved in drinking water to induce hypertension. Intraperitoneal injections of TA (50mg/kg body weight) and enalapril (0.7 mg/kg body weight) were given to rats.
Results: The results showed that the *In vitro* free radical scavenging effect of TA on DPPH and ABTS was concentration dependent. In vivo studies with L-NAME resulted in increases in blood pressure, lipid hydroperoxides (LOOH), and thiobarbituric acid reactive substances (TBARS). In

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addition, the level of the non-enzymatic antioxidant reduced glutathione (GSH) and other enzyme antioxidant activities in the heart and aorta, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Gpx), are reduced. The level of nitric oxide metabolism in the erythrocyte aorta of L-NAME rats was increased.
Conclusions: These findings imply that tolfenamic acid acts as an antihypertensive and antioxidant agent in L-NAME-induced hypertension.

Keywords: Tolfenamic acid; L-NAME; hypertension; free radicals; antioxidants.

1. INTRODUCTION

"Hypertension is the most common risk factor for cardiovascular disease (CVD) and a leading cause of mortality and morbidity globally. These illnesses impact almost 600 million people, and hypertension is expected to afflict 29 percent of the global population by 2025" [1]. "This condition suggests that hypertension management should be given top priority. The imbalance between reactive oxygen species (ROS) formation and antioxidant defense systems is referred to as oxidative stress. ROS are involved in a variety of damage pathways in the development of many diseases" [2].

"It is widely documented that oxidative stress promotes to hypertension development via nitric oxide (NO) deficit [3-5]. NO, a product of vascular endothelial cells, is a potent vasodilator that plays a crucial role in blood vessel development and resistance" [3]. "By lowering NO activity, NO synthase inhibitors cause endothelial dysfunction and oxidative stress. Chronic hypertension is caused by subchronic treatment of NO synthase inhibitors such as N-nitro-Larginine methyl ester (L-NAME) to laboratory mice" [4]. "As a result, the substance is frequently used in experimental models to induce hypertension. Tolfenamic acid, also known as 2-[(3-Chloro-2-methylphenyl) amino] benzoic acid, is an analgesic that belongs to the nonsteroidal anti-inflammatory medication (NSAID) class (Fig. 1). Tolfenamic acid is a fenamic acid derivative with a chemical structure similar to that of mefenamic acid, flufenamic acid, and other fenamate NSAIDs. It was discovered by Medica Pharmaceutical Company experts in Finland" [5]. "Despite being an NSAID, fenamate-derived medicines are commonly utilized to treat migraine attacks. Tolfenamic acid and other fenamate NSAIDs cure migraine by reducing prostaglandin production and blocking prostaglandin receptors" [6]. In this context, the current work sought to examine the effects of TA on hypertension-related oxidative stress in a pharmacological model of nitric oxide-deficient hypertensive rats.

2. MATERIALS AND METHODS

2.1 Sample Collection

Male albino Wistar rats weighing between 150 and 200 grams were acquired from Annamalai University's Central Animal House. The animals were housed at 22 C in 12-hour light/dark cycles, with unlimited access to food and water.

2.2 Chemicals

Tolfenamic acid (TA), Enalapril, and Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) were acquired from Sigma-Aldrich (St. Louis, Missouri, USA). The analytical grade chemicals utilized in this investigation were procured from Merck and Himedia in India.

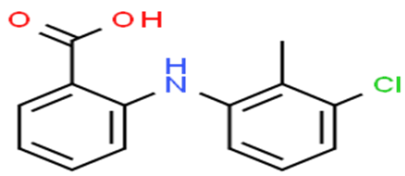

Fig. 1. Structure of tolfenamic acid

2.3 L-NAME hypertensive animal model and tolfenamic acid treatment

For four weeks, L-NAME at a dose of 40 mg/kg body weight (b.w.) was given to the animals in drinking water [7]. For four weeks, TA was given orally through an intragastric tube after being dissolved in 5% DMSO. There were seven animals in each of the following groups. Group I consisted of control rats; Group II treated the rats with TA (50 mg/kg b.w.); Group III treated the rats with L-NAME (40 mg/kg b.w.); Group IV treated the rats simultaneously with L-NAME (40

mg/kg b.w.) and TA (50 mg/kg b.w.); Group V treated the rats simultaneously with L-NAME (40 mg/kg b.w.) and Enalapril (0.7 mg/kg b.w.). The vehicle was given orally via an intragastric tube on a daily basis for four weeks.

2.4 *In Vitro* free radical scavenging assay

The DPPH• was measured using the method Mensor et al. (2001), [8.9], and the total antioxidant potential was assessed using the ABTS+ test, as reported by Miller et al. (1996). The study examined the concentration-dependent free radical scavenging potential of TA at concentrations of 20, 40, 60, 80, and 100 µM. By comparing the absorbance readings of the test and control samples, the percentage of inhibition was computed. Each test was run thrice, and the average of the three observations was used to put the results on the graph.

2.5 Antioxidants and Lipid Peroxidation

Slices of the heart and aorta were obtained, and 20% homogenate (w/v) was obtained by homogenizing the tissues in 0.1 M TrisHCl buffer under chilling conditions (pH 7.4). 10 minutes at 4˚C were spent centrifuging the homogenate at 560 6 g. For a variety of biochemical calculations, the supernatant was isolated and employed.

2.6 Superoxide Dismutase (SOD)

The heart and aorta tissues' levels of superoxide dismutase (SOD) activity were measured using the Kakkar et al. technique [10]. In a total volume of 3 mL, the assay combination included 1.2 mL of sodium pyrophosphate buffer, 0.1 mL of phenazine methosulphate, 0.3 mL of nitroblue tetrazolium, and suitably diluted enzyme preparation. The addition of 0.2 mL of nicotinamide adenine dinucleotide (NADH) initiated the process. 1.0 mL of glacial acetic acid was added to stop the reaction after it had been incubated at 30˚C for 90 seconds. The reaction mixture was shaken and given a thorough stir using 4.0 milliliters of n-butanol. After letting the mixture stand for ten minutes, the n-butanol layer was separated by centrifugation. The colour density of the chromogen in n-butanol was measured at 510 nm against butanol blank.

2.7 The activity of Catalase

Sinha's method [11] was used to measure the catalase activity in the tissues. Tissue homogenate (0.1 mL) and H2O2 (0.4 mL) were added to 0.9 mL of phosphate buffer solution. Adding 2.0 mL of the dichromate–acetic acid mixture stopped the reaction after 60 seconds. 620 nm was used to measure the color developed in the tubes after they were submerged in boiling water for ten minutes.

2.8 The Activity of Glutathione Peroxidase (GPx)

Using Rotruck et al.'s method [12], the tissues' glutathione peroxidase (GPx) activity was determined. 0.2 mL of ethylene diamine tetraacetic acid (EDTA), 0.1 mL of sodium azide, 0.5 mL of tissue homogenate, and 0.1 mL of Tris buffer were added. Glutathione (0.2 mL) and H2O2 (0.1 mL) were added to the mixture. After thoroughly combining the contents, they were incubated for 10 minutes at 37˚C alongside a tube that held all of the reagents save the sample. 0.5 mL of 10% TCA was added to stop the reaction after 10 minutes. Glutathione was estimated using the supernatant obtained from centrifuging the tubes.

2.9 Reduced Glutathione (GSH)

Using Ellman's method [13], the reduced glutathione (GSH) in the tissues was estimated. After pipetting out 0.5 mL of homogenate, 2.0 mL of 5% TCA was used to precipitate it. After centrifugation, 4.0 mL of 0.3 M disodium hydrogen phosphate and 1.0 mL of Ellman's reagent were added to the 2.0 mL of supernatant that was collected. At 412 nm, the developed yellow color was measured. Utilizing the Lowry et al. method [14], total protein was measured.

2.10 The level of Thiobarbituric Acid Reactive Substances (TBARS)

Using Niehaus and Samuelson's method [15], the amount of thiobarbituric acid reactive substances (TBARS) in tissues was determined. TBA–TCA hydrochloric acid (HCL) reagent was added after 0.5 mL of tissue homogenate was diluted with 0.5 mL of double-distilled water and thoroughly mixed. The final volume added was 2.0 mL. The mixture spent fifteen minutes in a bath of boiling water. The tubes were centrifuged for ten minutes after cooling, and the supernatant was removed for measurement. At 535 nm, the absorbance was measured using a reagent blank.

2.11 Estimation of Tissue Lipid Hydroperoxides (LOOH)

Using Jiang et al.'s method [16], tissue lipid hydroperoxides (LOOH) were estimated. After mixing 0.9 mL of Fox reagent with 0.1 mL of tissue homogenate, the mixture was allowed to sit at room temperature for 30 minutes. At 560 nm, the color developed was measured.

2.12 Aortic Nitric Oxide Metabolites Level

Since free radicals readily degrade nitric oxide (NO), aortic nitrite levels were determined as a measure of NO that has been inactivated by superoxide radical (O2-). In aortic homogenate, nitrite was measured colorimetrically using the Griess reagent. The Griess reagent (sulfanilamide 1% w/v, naphthylethylenediamine dihydrochloride 0.1% w/v, and orthophosphoric acid 2.5% v/v) and aortic homogenate were combined in roughly equal volumes, incubated for 10 minutes at room temperature, and the absorbance was measured at 540 nm wavelength [17]. Using sodium nitrite as the standard, the nitrite was calculated from the standard curve. The amount of nitrite that was produced was compared to the corresponding aorta's protein content.

2.13 Statistical Analysis

ANOVA and Duncan's multiple range test (DMRT) were used to examine the data, and version 14.0 of the statistical package for the social sciences (SPSS) software was used. The average ± standard deviation was reported for every six rats in each group. $P < 0.05$ was defined as the statistically significant value.

3. RESULTS

Fig. 2 depicts TA's free radical scavenging efficiency. The antioxidant potential of TA was investigated *in vitro* using DPPH• and ABTS+ scavenging effects at various doses (20, 40, 60, 80, and 100 M). The radicals DPPH• and ABTS+ were scavenged by TA in a concentration-dependent manner.

Table 1 displays the amounts of GSH, a non-enzymatic antioxidant, and lipid peroxidation products like LOOH and TBARS, along with the activities of enzymatic antioxidants like SOD, CAT, and GPx. The aforementioned enzymes' activity was significantly (P, 0.05) reduced by L-NAME induction, which also raised the synthesis of lipid peroxidation products and lowered GSH levels. GSH levels rose, lipid peroxidation products were reduced, and enzymatic antioxidant activity was significantly (P,0.05) restored by TA. Aortic nitrite/nitrate levels were significantly (P, 0.05) lower in L-NAME animals, while TA therapy significantly (P, 0.05) restored the above (Fig. 3). Enalapril's and TA's effects were contrasted.

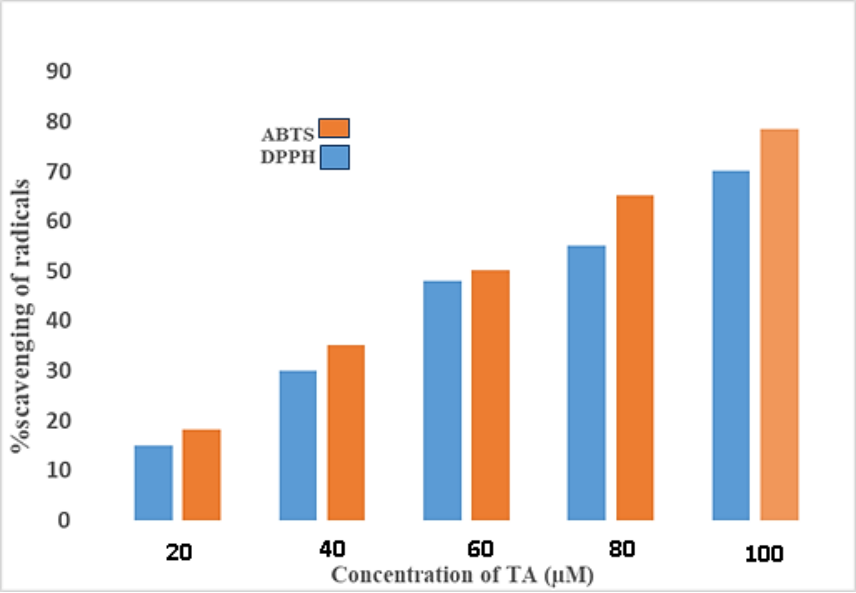

Fig. 2. *In vitro* assay for antioxidants. average of three experiments measuring the percentage of TA's scavenging activity on DPPH• and ABTS+.

Table 1. Effects of TA on lipid peroxidation and antioxidant status in L-NAME induced hypertensive rats

Parameter	Sample	Control	Control+TA	L-NAME	L-NAME+TA	L-NAME+Enalapril
SOD (U/mg protein)	**Erythrocyte**	6.96±0.59	7.39±0.63	3.12±0.26	6.33±0.52	7.01±0.59
	Heart	7.14±0.60	7.19±0.61	3.69±0.31	6.10±0.51	6.13±0.52
	Aorta	13.01±1.10	12.81±1.09	7.96±0.67	11.26±0.94	12.03±1.01
	Kidney	15.01±1.27	16.10±1.37	9.96±0.84	12.31±1.03	11.96±1.01
	liver	8.29±0.70	9.01±0.77	5.89±0.50	8.28±0.69	8.13±0.69
CAT(U/mg protein)	**Erythrocyte**	171.96±14.64	175.37±15.0	102±8.71	154±12.94	152±12.94
	Heart	50±4.25	51.15±4.37	33.29±2.83	45.19±3.78	43.96±3.74
	Aorta	54.96±4.67	55.25±4.72	35.45±3.01	47.29±3.95	47.01±3.01
	Kidney	33.56±2.77	33.97±2.90	18.96±1.61	28.65±2.39	29.26±2.49
	liver	73.01±6.21	71.77±6.14	56.13±4.77	66.66±5.57	64.96±5.53
GPX(U/mg protein)	**Erythrocyte**	13.96±1.18	15.02±1.28	7.36±0.62	12.07±1.01	12.01±1.022
	Heart	7.01±0.59	6.45±0.55	4.28±0.36	5.33±0.44	6.23±0.53
	Aorta	8.96±0.47	8.25±4.45	5.45±0.54	7.29±0.39	7.01±0.38
	Kidney	8.17±0.69	8.95±0.76	5.40±0.45	8.19±0.68	7.96±0.67
	liver	7.96±0.67	7.26±0.62	4.12±0.35	7.06±0.59	7.01±0.59
GSH(µg/mg protein)	**plasma**	35.01±2.98	36.71±3.14	20.96±1.78	31.86±2.66	32.16±2.73
	Heart	9.01±0.76	9.13±0.78	6.13±0.52	8.19±0.68	8.24±0.70
	Aorta	7.91±0.67	8.05±0.68	5.36±0.45	7.17±0.60	6.96±0.59
	Kidney	12.03±1.02	13.16±1.12	7.12±0.60	10.40±0.87	10.03±0.93
	liver	14.25±1.21	15.27±1.30	7.13±0.60	12.62±1.05	13.25±1.12
TABRS (mmol/100 g wet tissue)	**Plasma**	0.14±0.01	0.13±0.01	0.43±0.03	0.21±0.01	0.24±0.02
	Heart	0.61±0.05	0.57±0.04	2.51±0.21	0.93±0.07	0.93±0.07
	Aorta	0.52±0.04	0.52±0.44	1.85±0.15	0.73±0.06	0.73±0.06
	Kidney	1.40±0.12	1.31±0.11	3.79±0.32	1.80±0.15	1.83±0.15
	liver	0.87±0.07	0.81±0.06	2.4±0.20	1.24±0.10	1.25±0.10
LOOH (mmol/100 g wet tissue)	**Plasma**	9.10±0.77	9.58±0.82	21.24±1.80	13.13±1.12	12.96±1.10
	Heart	61.35±5.22	60.41±5.17	122.45±10.42	78.51±6.72	79.01±6.72
	Aorta	73.21±6.23	71.29±6.10	108.21±9.21	83.51±7.15	83.79±7.13
	Kidney	61.31±5.22	65.24±5.58	141.7±12.06	90.16±7.54	87.01±7.40
	liver	78.78±6.70	80.19±6.86	107.15±9.12	93.83±7.85	90.71±7.72

Values are means ± S.D. for six rats in each group. Values not sharing a common superscript are significance with each other at P<0.05 (Duncan's Multiple Range Test)

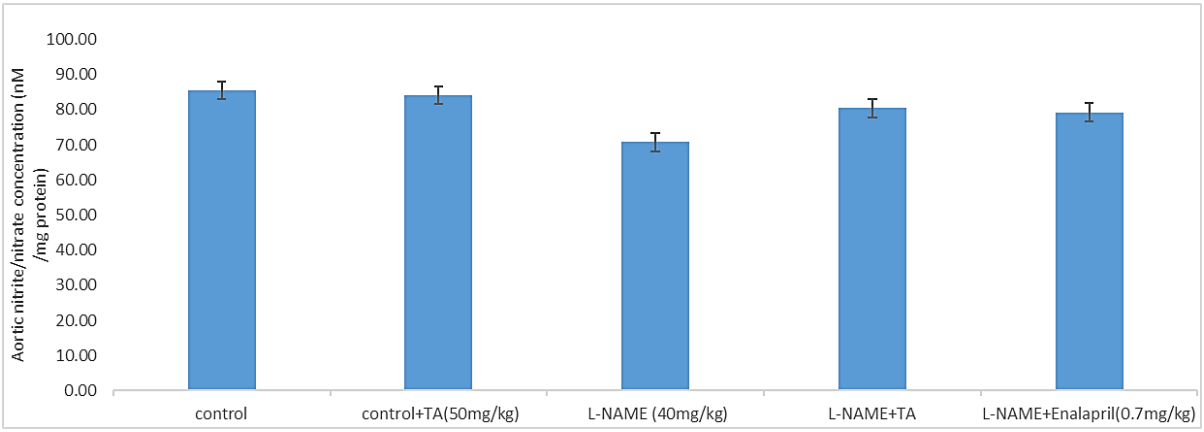

Fig. 3. In rats with experimental hypertension, tolfenamic acid restores the level of nitric oxide metabolites. Nitric oxide metabolite levels in different experimental groups were estimated. The values are given as mean ± SD for each group of six. P<0.05 in comparison to L-NAME and control

4. DISCUSSION

Mechanisms of oxidation are essential to cell survival. Organisms that use aerobic cellular respiration to produce energy from organic molecules like glucose also release free radicals during metabolism, which can harm cells [17]. An unpaired (free) electron with spin—a quantum-mechanical property—makes up a free radical. Such an entity has a high reactivity due to its open shell structure [18]. Nevertheless, a lot of free radicals are now stable in lab settings, meaning they can exist in the air and at room temperature [19]. Oxidative stress and free radicals have long been associated [20].

In the medical sciences, oxidative stress is a relatively new concept that is currently regarded as mainstream [21]. It happens when there is an overabundance of reactive oxygen species (ROS) produced by a cell's mitochondria. Free radicals are produced in biological systems ineluctably, and they are linked to a number of degenerative diseases, including atherosclerosis, mutagenesis, aging, cardiovascular disorders, preeclampsia, acute renal failure, high blood pressure, diabetes, and carcinogenesis [22]. Oxidative stress is caused by a variety of elements, including pollution and UV radiation, and it affects human health on a daily basis [23].

The rate at which oxidants are eliminated under typical circumstances balances the rate and amplitude of oxidant formation [24]. On the other hand, an imbalance between pro- and antioxidants leads to oxidative stress [25]. One of the main ways that oxidative stress contributes to the development of hypertension is through the production of reactive oxygen species (ROS) [26]. Volume-dependent blood pressure measurement is produced by chronic NO suppression, and its clinical and physiological features resemble essential hypertension. L-NAME, a L-arginine analog, administered in vivo causes acute suppression of NO production, which in turn causes vasoconstriction and arterial hypertension [27]. In conjunction with L-NAME-induced hypertension, the purpose of the current study was to evaluate the antioxidant and free radical scavenging properties of TA.

The effects of antioxidant activity are said to extend to whitening, anti-inflammation, and hypertension, among other bioactivities. Stabilizing harmful free radicals in the human body is largely dependent on the DPPH and ABTS radical-scavenging assays, which provide a redox-functioned proton ion for unstable free radicals. The common method for achieving this is to take advantage of the fact that unstable free radicals like ABTS and violet DPPH can become stable yellow DPPH free radicals by accepting a hydrogen ion from antioxidants. With a characteristic long wave length absorption spectrum, the radical cation ABTS is the basis of the ABTS assay, which is based on inhibiting its absorbance [28]. An accurate method of assessing TA's antioxidant activity is to look for the decolorization of the ABTS cation radical.

Enzymes that scavenge free radicals, such as SOD, CAT, and GPx, serve as the initial defense against oxidative damage by breaking down superoxide and hydrogen peroxide, which would

otherwise combine to produce the more hazardous hydroxyl radical [29]. The efficient removal of oxidative stress intracellular organelles depends on the balance between free radicals and enzymatic antioxidants. This study demonstrated significant reductions in the levels of the enzymes SOD, CAT, and GPx in the heart and aorta of L-NAME rats. By eliminating lipid peroxidase and hydrogen peroxide, GPx shields cellular and subcellular membranes from peroxidative damage; its lowered activity could be related to a reduction in GSH availability [30].

Lipid peroxidation, which occurs when a free radical reacts with a lipid, has been related to altered membrane structure and enzyme deactivation. Its end products measured as thiobarbituric acid reactive substances and lipid hydroxides were seen to be highly increased oxidative stress by enhancing antioxidants prevents membrane damage through its antioxidant efficiency was already reported. Furthermore, the *in vitro* radical scavenging actions of TA via its antioxidant potential have already been found [31]. Based on these results, we conclude that the prodective activity of TA is at least significantly linked to its antioxidant capacity.

TA's ability to preserve vascular function was one of the study's significant findings. A crucial part in the pathophysiology of the cardiovascular system is played by vascular endothelium. In order to maintain vascular homeostasis, nitric oxide (NO), which is produced by eNOS, is physiologically significant. It quickly activates guanylyl cyclase and boosts the synthesis of cyclic GMP in vascular smooth muscle [32]. Peroxynitrite (ONOO-), which is created when endogenous NO combines with superoxide, has the ability to nitrate or oxidize a variety of biological substrates [33].
Conclusion

Finally, we believe that the current study's primary findings indicate that TA supplementation may conceivably and successfully improve hypertension and ROS production in L-NAME rats, as seen by TA's beneficial effect on antioxidant levels. The current study clearly demonstrates that TA has antihypertensive effects in nitric oxide-deficient hypertensive rats, as seen by a considerable decrease in lipid peroxidation, better antioxidant state, and free radical scavenging. A 50 mg/kg dosage of TA exhibited no deleterious effects in this investigation. Our findings imply that TA could be effective in the future treatment of hypertension and concomitant cardiovascular disease.

ETHICAL APPROVAL

The Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalainagar, approved the experimental protocols (IAEC PROPOSAL NO. AU-IAEC/1323/6/22).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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