Vol.64: e21200783, 2021
https://doi.org/10.1590/1678-4324-2021200783
ISSN 1678-4324 Online Edition

Article – Human and Animal Health

In vitro Antioxidant Activity and *In vivo* Anti-inflammatory Effect of *Ricinus communis* (L.) and *Withania somnifera* (L.) Hydroalcoholic Extracts in Rats

Asif Hussain[1]
https://orcid.org/0000-0002-8026-6110

Bilal Aslam*[1]
https://orcid.org/0000-0003-3383-8961

Faqir Muhammad[1]
https://orcid.org/0000-0001-9930-4204

Muhammad Naeem Faisal[1]
https://orcid.org/0000-0001-6448-9325

University of Agriculture, Faisalabad, Institute of Physiology and Pharmacology, Faisalabad, Punjab, Pakistan

Editor-in-Chief: Alexandre Rasi Aoki
Associate Editor: Acácio Antonio Ferreira Zielinski

Received: 2020.12.14; Accepted: 2021.10.18.

*Correspondence: cba933@gmail.com; Tel.: +92-41-9200161-71; Mob.: +92-300-5013800 (B.A.).

HIGHLIGHTS

- *Ricinus communis* and *Withania somnifera* extracts contain considerable polyphenol compounds.
- *R. communis* and *W. somnifera* extracts showed significant *in vitro* antioxidant activities.
- *R. communis* and *W. somnifera* extracts markedly inhibited xylene-induced ear edema in rats.
- Both herbal extracts significantly reduced paw edema induced by egg albumin and carrageenan.

Abstract: *Ricinus communis* L. and *Withania somnifera* L. have traditionally been used as analgesic and anti-inflammatory remedies. The current study was aimed to evaluate the *in vitro* antioxidant potential and anti-inflammatory activity of hydroalcoholic extract of *R. communis* leaves (RCE) and *W. somnifera* roots (WSE) in Wistar rats. Total phenolic and flavonoid contents were quantified and *in vitro* antioxidant activity of extracts was determined through DPPH* scavenging, superoxide anion scavenging and reducing power activities, while anti-inflammatory activity was observed by xylene-induced ear edema and paw edema induced by egg albumin and carrageenan. RCE and WSE demonstrated considerable antioxidant activity in DPPH* scavenging (IC_{50}: 250.10 and 309.42 µg/mL), superoxide anion scavenging (IC_{50}: 193.42 and 206.81 ug/mL), and reducing power (maximum absorbance: 1.47±0.01 O.D and 1.28±0.01 O.D at 500 ug/mL) activities, respectively, with high phenolic and flavonoid contents. Both extracts showed dose-dependent edema inhibition in inflammation models. A maximum ear edema inhibitions by RCE (51.49±2.54%) and WSE (49.28±1.90%) at 500 mg/kg were observed when compared to indomethacin (56.42±13.17%) in xylene-

induced ear edema. RCE and WSE showed a maximum percentage of paw edema inhibitions of 46.62±8.98% and 43.00±12.44%, respectively as compared to chlorpheniramine (62.02±12.21%) after 4 h in the egg albumin model. In carrageenan-induced paw edema, RCE (72.88±13.79%) significantly inhibited paw edema in comparison to WSE (57.81±17.43%) against diclofenac (89.93±18.53%). Conclusively, both plants have shown plausible antioxidant and anti-inflammatory activities that might be due to high phenolic and flavonoid contents. Moreover, RCE demonstrated more promising effects than WSE.

Keywords: *Ricinus communis*; *Withania somnifera*; Antioxidants; Inflammation; Carrageenan.

INTRODUCTION

Inflammation can be described as a complex body response to injurious stimuli like pathogens, irritants, or damaged cells [1]. Inflammatory responses are generated and maintained through the interactions of inflammatory mediators, such as histamine, kinins, cytokines, eicosanoids, calcitonin gene-related peptide, substance-P, and platelet-activating factor, derived from leukocytes and damaged tissues. The primary manifestations of inflammation are the infiltration of leukocytes and the formation of edema. Edema formation is a consequence of an inflammatory mediator's interaction that promotes vascular permeability and blood flow [1,2]. Various synthetic drugs such as NSAIDs have good efficacy in inflammatory conditions but also exert some deleterious effects including gastrointestinal problems [3]. Hence, herbal remedies have always been important in Asian countries.

Medicinal plants have a significant role in sustaining human health in developing countries due to lack of primary healthcare facilities, poverty, increased demand for inexpensive medicines, and drug-associated fewer side effects [4]. Plants provide a source of bioactive compounds that possess pharmacologically important properties [5]. Antioxidants neutralize free radical species that are generated as byproducts of normal biochemical processes in the body [6]. Excessive production of free radicals in cells causes oxidative stress, subsequently damages the essential macromolecules which lead to inflammatory and degenerative conditions [7]. Phytocompounds found in medicinal plants exert free radical scavenging and anti-inflammatory activities. Secondary metabolites of plants that exhibit biological activities are alkaloids, carotenoids, phenols, flavonoids, coumarins, quinones, tannins, and stilbenes [8].

Ricinus communis L., also known as Castor oil plant, is a part of spurge Euphorbiaceae family that can be found throughout Asian countries and is widely cultivated for castor oil in warm and tropical regions of the world. The perennial shrub can grow up to 6 m in height. The plant has been a therapeutic agent since ancient times due to its effectiveness in treating various diseases and disorders [9]. Various parts of *R. communis* (flower, fruit, seeds, leaves, roots, and stem) have versatile uses in different aspects of life. Seed oil is being used in the preparation of lubricants, greases, varnishes, enamels, paints, oiled fabrics, and printing inks. Oil derived from leaves effectively relieves flatulence in infants. Also, powdered leaves are externally applied in the form of poultice on boils, sores, and swellings. A decoction or infusion of leaves is used for relieving stomach ache, improve lactation, and has anti-bacterial and antifungal activities. A variety of phytochemicals such as gallic acid, quercetin, triterpenoids, ingenol, kaempferol, catechin, epicatechin, gentisic acid, ricinoleic acid, linoleic acid, camphor, athujone, and β-thujone have been reported in different parts of *R. communis* [10]. Various parts of *R. communis* have been used to treat liver disorders and inflammation. It is also employed to treat cold, constipation, and several types of tumors, and is used as a contraceptive and hypoglycemic herbal medicine. Also, several studies reported anti-microbial, antifertility, anti-diabetic, anti-arthritic, hepatoprotective, anti-carcinogenic, and laxative properties of *R. communis* leaves [11-13].

Withania somnifera L., (Solanaceae family) is widely cultivated in Asia, Africa, and the Mediterranean regions for its medicinal purposes. It is commonly known as Ashwagandha in the Unani medicine system. The plant is an evergreen shrub that can reach the height of 30-150 cm. The roots of *W. somnifera* are collected from January to March. The use of freshly collected and dried roots is preferred because it loses its potential within two years of collection [14]. Leaves and roots of *W. somnifera* are mainly used in crude or purified form to treat bronchitis, asthma, skin infections, gastrointestinal disorders, and inflammatory diseases. In addition, anti-diabetic, anti-tumor, anti-depressant, cardio-protective, hepatoprotective, neuroprotective, and immune-modulatory activities are also reported [13,15,16]. The pharmacological activities of *W. somnifera* are due to the presence of phytochemicals include alkaloids, anthocyanins, glycosides, carotenoids, flavonoids, lignins, steroids, phytosterols, tannins, amino acids, reducing sugars, and starch. Withanolides such as withanolide-A, withaferin-A, sitoinoside (IX, X) are the main reported alkaloidal constituents of this plant [14].

The scientific studies are mostly comprised of *in vitro* and/or *in vivo* experiments to investigate the potential therapeutic efficacy of medicinal plants based on their traditional uses [17]. The literature review

revealed the noteworthy traditional uses of *R. communis* and *W. somnifera* in several inflammatory conditions [9,10,13,14]. In this regard, this study was designed to evaluate their *in vitro* antioxidant activity using DPPH radical, superoxide anion scavenging and reducing power assays, and anti-inflammatory activity in xylene, egg albumin and carrageenan-induced inflammation in Wistar rats.

MATERIAL AND METHODS

Drugs and chemicals

Diclofenac sodium (Defnac®, Searle Pharmaceutical (Pvt.) Ltd., Pakistan), chlorpheniramine (Hamzivil®, Elite Pharma (Pvt.), Ltd., Pakistan), and indomethacin (Shirocin®, Wilshire Laboratories (Pvt.) Ltd.) were purchased. Chemicals include gallic acid, catechin, and ascorbic acid, and carrageenan (Sigma-Aldrich) were procured. All other chemicals of the analytical grade used in this research were purchased from a local scientific store.

Plant collection and extract preparation

Fresh leaves of *R. communis* and roots of *W. somnifera* were collected in March 2019 from the botanical garden of University of Agriculture, Faisalabad (UAF). The specimens were deposited to the Department of Botany, UAF for future reference (Herbarium no.; *Ricinus communis*: 212-1-19, *Withania somnifera*: 212-2-19). The extraneous parts of both plant materials were removed, rinsed thoroughly, shade dried for two weeks and pulverized to get uniform powder. Extracts were prepared by macerating 200 g of the powdered plant in 5 L of solvent mixture (water: ethanol, 30:70 v/v) at room temperature for 5 days with vigorous shaking at regular intervals [13]. Then, extracts were filtered (Whatman filter paper no. 1) and concentrated at 40-60^{o}C using a rotary evaporator (Heizbad Hei-VAP, Heidolph, Germany). The extraction yields of obtained *R. communis* (RCE) and *W. somnifera* (WSE) extracts were 19.3% and 13.8% (w/w), respectively.

Estimation of total phenolic content (TPC)

RCE and WSE were analyzed for the estimation of TPC using the Folin and Ciocalteu method [18]. About 50 mg of each concentrated extract was added to 0.5 mL of Folin-Ciocalteu reagent, diluted with 7.5 mL of double-distilled water, and incubated for 10 min at room temperature. Then, 1.5 mL of Na_2CO_3 (20% w/v) was mixed with the reaction mixture, heated for 20 min at 40^{o}C in a water bath, and immediately cooled in an ice bath. The appearance of a blue color complex in the reaction mixture was spectrophotometrically (Thermo Scientific Multiskan GO™) analyzed at 765 nm. The calibration curve (y=0.0055x+0.0987, R^2=0.9986) of gallic acid as standard was used for the quantification of TPC of extracts. The experiments were performed in triplicates and results were averaged.

Estimation of total flavonoid content (TFC)

The quantification of TFC of extracts was done using the spectrophotometric method [18]. In short, 1 mL of each extract was diluted with 4 mL of double distilled water and mixed with 0.3 mL of $NaNO_2$ solution (5%). About 0.3 mL of $AlCl_3$ (10%) was added at 5 min and 2 mL of 1M NaOH was added to the mixture after 6 min. Further, reaction mixtures were diluted with 2.4 mL of double-distilled water, and absorbencies were taken at 510 nm. The calibration curve (y=0.0038x+0.0285, R^2=0.9835) of catechin as standard was used to calculate the TFC of each extract. Triplicate readings were taken and averaged results were obtained.

In vitro antioxidant assays

DPPH free radical scavenging assay

A method previously described [19] was applied with some modification to estimate the free radical scavenging potential of RCE and WSE. Various concentrations (100, 200, 300, 400, and 500 µg/mL) of each plant extract and ascorbic acid as standard were prepared in methanol. About 0.5 mL of freshly prepared methanolic solution of DPPH (0.002%) was added to 2 mL of each test solution and incubated at room temperature for 15 min and subsequently, absorbencies were taken at 517 nm. Similarly, a blank solution was prepared by adding the same quantities of DPPH and methanol, except for plant extract. Experiments were performed in triplicates and free radical scavenging activity was determined by the given formula [20]:

$$\text{Scavenging activity (\%)} = [(\text{Abs.}_{\text{Control}} - \text{Abs.}_{\text{Sample}})/ \text{Abs.}_{\text{Control}}] \times 100 \quad [1]$$

The IC_{50} values were calculated by plotting scavenging activities (%) of various concentrations of extracts.

Superoxide anion scavenging activity

The antioxidant capacity of RCE and WSE was determined by superoxide anion scavenging activity assay [21]. Briefly, different concentrations (100-500 µg/mL) of each plant extract were prepared and 1 mL of each sample was well mixed with PO_4 buffer (500 µL, 50 mM, pH 7.6), nitroblue tetrazolium (100 µL, 0.5 mM), phosphor-methazine sulfate (250 µL, 20 mM) and riboflavin (300 µL, 50 mM). The reaction mixtures were kept under a fluorescent lamp for 20 min to initiate the reaction and absorbance was taken at 560 nm using a spectrophotometer. The absorbance of ascorbic acid as standard was measured. The percentages of scavenging activity were determined and used to calculate IC_{50} (inhibition concentration).

$$\text{Scavenging activity (\%)} = [(1 - \text{Abs.}_{\text{Sample}})/ \text{Abs.}_{\text{Control}}] \times 100 \quad [2]$$

Reducing power assay

Reducing power capacity of RCE and WSE was estimated [22]. Antioxidants reduce potassium ferricyanide (Fe^{3+}) to potassium ferricyanide (Fe^{2+}), which forms a ferric-ferrous complex by reacting with ferric chloride. The increased reducing capacity of the sample can be indicated by increase conversion of Fe^{3+} to Fe^{2+} ion and an increase in the corresponding absorbance. In short, 2 mL of different concentrations (100-500 µg/mL) of RCE and WSE were added to 2 mL of potassium ferricyanide (1%), 2 mL of phosphate buffer (0.2 M, pH 6.6), and incubated at 45°C for 30 min. Further, 2 mL of TCA was mixed and reaction mixtures were centrifuged at 3000 xg for 10 min. About 2 mL of supernatant from each tube was further mixed with 0.4 mL of ferric chloride (0.1% w/v) and 2 mL of distilled water. The absorbance of samples and ascorbic acid as standard were taken at 700 nm using a spectrophotometer.

In vivo **anti-inflammatory studies**

Animals

Female Wistar rats (body weight ranging from 160 to 200 g) were purchased and kept at an animal house facility located at the Institute of Microbiology, University of Agriculture, Faisalabad-Pakistan. Before conduct *in vivo* study, all animals were acclimatized for one week giving a standard feed, freshwater *ad-libitum*, and maintaining 25±2°C room temperature, 40-60% humidity and 12 h light/dark cycle. Approval was granted by the Institutional Biosafety Committee (IBC) of University of Agriculture, Faisalabad-Pakistan (D. No. 3498/ORIC) to conduct this study. All animals were cared for according to the National Institute of Health guidelines (NIH publication no. 85-23, revised 1996).

Xylene-induced ear edema

A method devised by Hosseinzadeh [23] was applied with some modifications to observe the anti-inflammatory activity of RCE and WSE in xylene-induced ear edema. All rats were allocated to eight groups (n=6) and treated orally as control (3 mL/kg of normal saline), indomethacin (10 mg/kg), and extracts administered at 150, 250, and 500 mg/kg, respectively. After 30 min, the left ear of each animal was set as control while 30 µL of xylene was injected into the inner surface of the right ear. All rats were decapitated after 2 h and spherical sections of 7 mm diameter of both ears were collected using a cork borer. The effect of treatments was determined by finding the difference between both ears and percentages of inhibition were calculated by the given formula:

$$\text{Edema inhibition (\%)} = [(\text{Wt. difference}_{\text{Control}} - \text{Wt. difference}_{\text{Treated}})/\text{Wt. difference}_{\text{Control}}] \times 100 \quad [3]$$

Egg albumin-induced paw edema

The anti-inflammatory effect of RCE and WSE was observed in egg albumin-induced paw edema in Wistar rats [24]. Briefly, experimental rats were randomly divided into eight groups (n=6) and treated as control (3 mL of normal saline), chlorpheniramine (60 mg/kg), and with three doses (150, 250, and 500 mg/kg) of each extract. After 1 h of oral administration of treatments, 0.1 mL of 20% egg albumin solution prepared in normal saline was injected into the sub-plantar surface of the hind paw of all experimental

rats. Pre- and post-injection (0.5, 1, 2, 3, 4 h) paw thickness was measured using a digital vernier caliper, and percentages of inhibition were calculated as:

$$\text{Change in paw edema (E)} = \text{Paw thickness}_{\text{After}} - \text{Paw thickness}_{\text{Initial}} \quad [4]$$

$$\text{Edema inhibition (\%)} = [(E_{\text{Control}} - E_{\text{Treated}})/ E_{\text{Control}}] \times 100 \quad [5]$$

Where E_{Control} is a change in paw edema of the control group and E_{Treated} is a change in paw edema of the treated group.

Carrageenan-induced paw edema

Anti-inflammatory activity of different doses of RCE and WSE was further assessed in carrageenan-induced paw edema in Wistar rats [25]. Experimental rats were allocated to eight groups (n=6) and pre-treated as control (normal saline; 3 mL/kg), diclofenac (50 mg/kg), and RCE and WSE given at 150, 250 and, 500 mg/kg. All treatments were given orally 30 min before subplantar injection of 100 µL of carrageenan (1% w/v) in the hind paw of all groups. Post-injection increase in paw thickness was measured at 1, 2, 3, 4, and 6 h using a digital vernier caliper, and percentages of edema inhibition were calculated as previously described [26].

Statistical analysis

The obtained data were statistically analyzed by GraphPad Prism® 6 software and expressed as mean±SD values. *In vitro* assays were performed in triplicates (n=3) while *in vivo* studies were conducted on six animals per group. One-way and two-way ANOVA and post-hoc Tukey's tests were applied to determine the statistical difference (p<0.05).

RESULTS AND DISCUSSION

Total phenolic and flavonoid contents

Polyphenols are natural phytocompounds that can be categorized into phenolic acids, flavonoids, lignins, and stilbenes [27]. Chronic inflammation is considered to be an underlying cause of obesity, diabetes type-2, arthritis, cancer, and cardiovascular and neurodegenerative diseases. In this regard, the anti-inflammatory property of polyphenols could be attributed to their antioxidant activity, in addition to their potential to modulate the expression of pro-inflammatory factors such as cyclooxygenase, lipoxygenase, and nitric oxide synthases, multiple cytokines, and immune cell population [28]. Previous studies reported that polyphenol-rich medicinal plants and isolated phytochemicals have shown antioxidant, anti-inflammatory, immunomodulatory, hepatoprotective, and anti-diabetic activities possibly through reducing oxidative stress [29-32]. Therefore, the determination of total phenolic and flavonoid contents of extracts is worthwhile in this study. A considerably high phenolic and flavonoid contents were quantified in RCE in contrast to WSE. Total phenolic content found in RCE was 197.43±2.86 mg GAE/g and 183.37±1.86 mg GAE/g in WSE. The RCE and WSE showed the presence of 68.82±1.59 mg CE/g and 44.02±1.23 mg CE/g, respectively of total flavonoid content.

In vitro antioxidant activity

DPPH radical scavenging potential of extracts*

DPPH* is among various free radicals that are commonly used to test the free radical scavenging activity of plant extracts as well as pure compounds [33]. The ability to scavenge DPPH radical is an estimation of lipid peroxidation inhibition. Antioxidants neutralize the free radical character of DPPH* by either transferring an electron or a hydrogen atom [34]. The reducing potential of compounds could indicate the possible antioxidant property [35]. Results of the present study indicated a gradual increase in scavenging activity with the increase of extract concentration. The RCE and WSE demonstrated concentration-dependent DPPH* radical scavenging activity from 100 to 500 µg/mL in DPPH assay with significantly (p<0.001) higher scavenging activity of RCE than WSE as shown in Figure 1. The inhibitory percentages were 26.64±0.71 at 100 µg/mL to 77.12±0.70 at 500 µg/mL for RCE, and for WSE these were 21.22±0.73 at 100 µg/mL to 70.09±0.68 at 500 µg/mL. The respective IC_{50} values of RCE and WSE were 250.10 µg/mL and 309.42 µg/mL in comparison to 25.60 µg/mL of ascorbic acid.

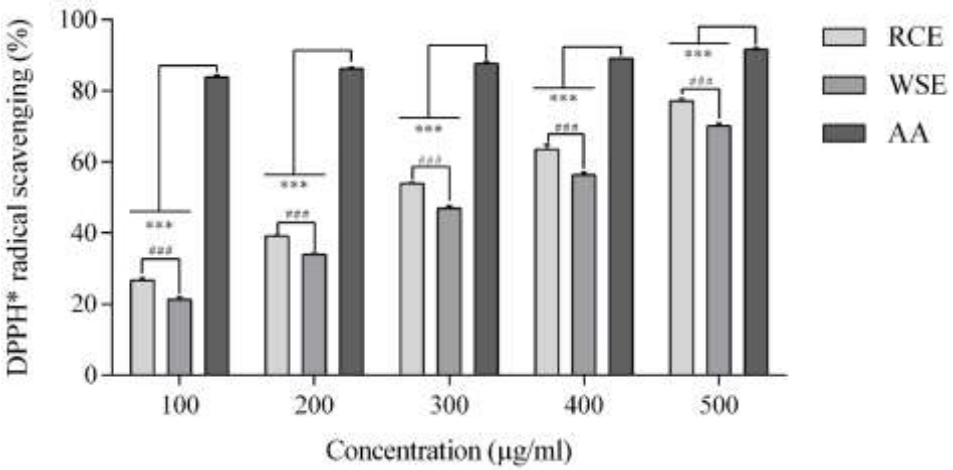

Figure 1. Antioxidant activity of RCE and WSE evaluated by DPPH* radical scavenging assay. Values are presented as mean±SD (n=3). ***p<0.001 significant difference of RCE and WSE from AA; ###p<0.001 significant difference between RCE and WSE. AA: ascorbic acid, RCE: *R. communis* extract, WSE: *W. somnifera* extract.

Superoxide anion scavenging activity of extracts

Superoxide anions are well known to damage biomolecules either directly or indirectly by producing $^{-}$OH, H_2O_2, singlet oxygen, or peroxynitrite during aging and pathological conditions like ischemic reperfusion injury. It has also been observed that superoxides directly involve lipid peroxidation [36,37]. The antioxidant potential of RCE and WSE was assessed. A marked increase in superoxide anion scavenging activity of both extracts was seen with the increase in concentrations of extracts. The increased scavenging activity observed as concentration-dependent inhibition from 17.46±0.23 at 100 µg/mL to 59.76±0.24 at 500 µg/mL for RCE and 13.97±0.18 at 100 µg/mL to 54.23±0.57 at 500 µg/mL for WSE (Figure 2). RCE demonstrated significantly (p<0.001) high scavenging activity as compared to WSE. The IC_{50} value of RCE (193.42 µg/mL) and WSE (206.81 µg/mL) were found in comparison to ascorbic acid (40.36 µg/mL). The higher inhibitory activity of both extracts on superoxide anion production observed in the present study could render them potent antioxidants.

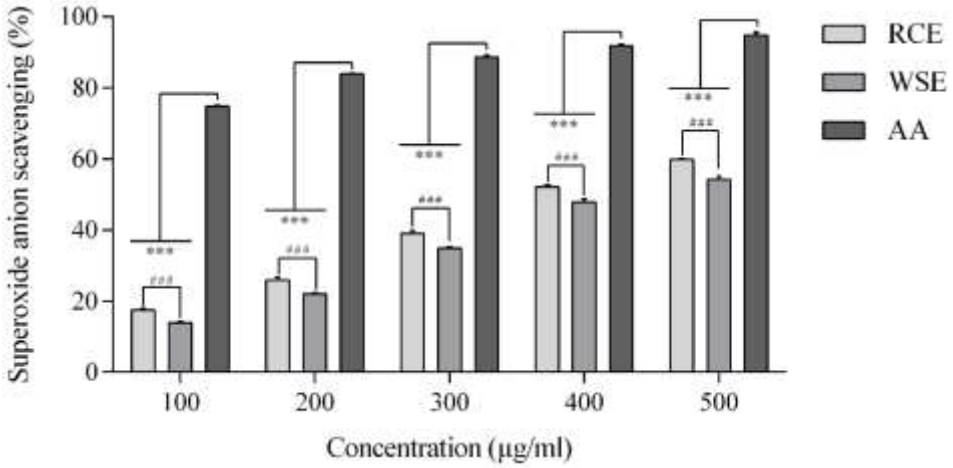

Figure 2. Antioxidant activity of RCE and WSE determined by superoxide anion scavenging assay. Values are presented as mean±SD (n=3). ***p<0.001 significant difference of RCE and WSE from AA; ###p<0.001 significant difference between RCE and WSE. AA: ascorbic acid, RCE: *R. communis* extract, WSE: *W. somnifera* extract.

Reducing power capacity of extracts

The ability of natural antioxidants to donate electrons is usually determined by reducing power activity [38]. It is previously established that the reducing power of plants and their antioxidant activities is directly correlated [39]. In this study, the reducing power activity of RCE and WSE consistently increased with the increase in the concentration of extracts from 100 µg/mL to 500 µg/mL as shown in Figure 3. As compared to WSE (1.28±0.01 O.D), RCE (1.47±0.01 O.D) exhibited higher absorbance at 500 µg/mL. Meanwhile RCE showed significantly (p<0.001) increased reducing potential than WSE.

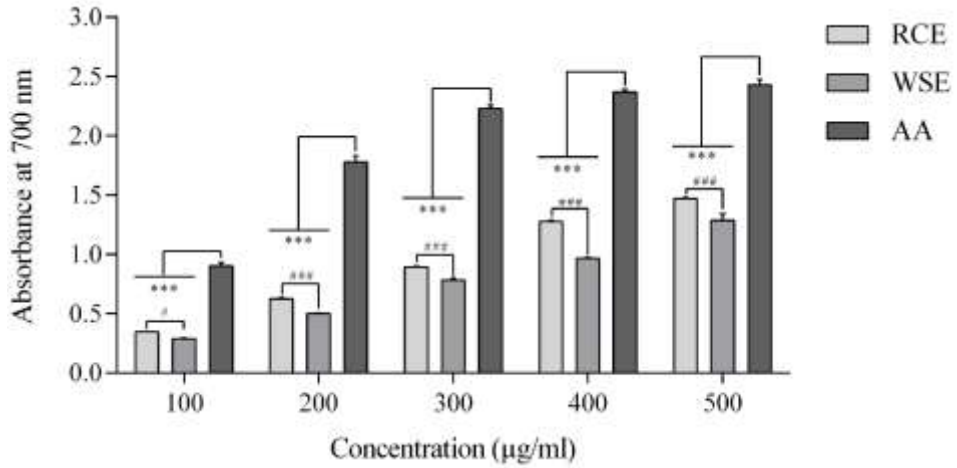

Figure 3. Antioxidant activity of RCE and WSE evaluated by reducing power assay. Values are presented as mean±SD (n=3). ***p<0.001 significant difference of RCE and WSE from AA; $^{\#}p$<0.05, $^{\#\#\#}p$<0.001 significant difference between RCE and WSE. AA: ascorbic acid, RCE: *R. communis* extract, WSE: *W. somnifera* extract.

In vivo anti-inflammatory activity of extracts

Animal models are commonly employed strategies to understand the pathophysiological features of any disease in an attempt to develop new drugs for their treatment [40]. In the present study, the anti-inflammatory activities of RCE and WSE were evaluated using xylene-induced ear edema and paw edema induced by egg albumin and carrageenan models in Wistar rats.

Effect of extracts on xylene-induced ear edema

Ear edema induced by xylene is commonly used as an acute inflammation model. The release of inflammatory mediators promotes vasodilation and increases vascular permeability, consequently produces ear edema [23]. Results of the present study showed the anti-inflammatory activity of RCE and WSE in xylene-induced ear edema (Table 1). It was noticed that treatment with graded doses of both extracts markedly inhibited ear edema in a dose-dependent manner. Both plants demonstrated significant (p<0.001) edema inhibition at 500 mg/kg comparable to indomethacin. Xylene-induced edema partially involves the release of substance-P, increase in capillary permeability, and infiltration of leukocytes, suggesting the possible inhibitory effect of plant extracts on the release of bradykinins and substance-P during the initial neurogenic phase. Therefore, anti-inflammatory activity of RCE and WSE might be referring to neurogenic inflammation [41].

Table 1. Anti-inflammatory effect of RCE and WSE on xylene-induced ear edema.

Treatment	Dose	Difference (mg)	Inhibition (%)
Control (vehicle)	3 mL/kg	7.71±0.28	---
Indomethacin	10 mg/kg	3.36±0.15***	56.42±1.79
RCE	150 mg/kg	6.12±0.21***$^{\#\#\#}$	20.62±2.62$^{\#\#\#}$
RCE	250 mg/kg	4.41±0.11***$^{\#\#\#}$	42.80±2.60$^{\#\#\#}$
RCE	500 mg/kg	3.74±0.19***$^{\#}$	51.49±2.54
WSE	150 mg/kg	6.30±0.10***$^{\#\#\#}$	18.29±2.43$^{\#\#\#}$
WSE	250 mg/kg	5.18±0.33***$^{\#\#\#}$	32.81±4.94$^{\#\#\#}$
WSE	500 mg/kg	3.91±0.13***$^{\#\#}$	49.28±1.90$^{\#\#}$

Values are presented as mean±SD (n=6). Significant difference (***p<0.001) of treatments from Control group; Significant difference ($^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001) of RCE and WSE treated groups from Indomethacin. RCE: *R. communis* extract, WSE: *W. somnifera* extract.

Effect of extracts on egg albumin-induced paw edema

Egg albumin-induced paw edema model is commonly adopted to evaluate the anti-edematogenic and anti-inflammatory activities of medicinal plants [42]. This inflammation model comprises the initial phase of the upsurge of histamine, serotonin, and bradykinins, and the release of prostaglandins in the later phase [1]. The anti-inflammatory potential of RCE and WSE was observed using egg albumin as phlogistic agent-induced paw edema to further establish the anti-inflammatory activity of extracts in comparison to chlorpheniramine as a standard drug. The present study showed that treated rats with RCE and WSE

demonstrated significant (p<0.001) inhibition of paw edema from 0.5 h to 4 h. Extracts of both plants exhibited dose-dependent as well as time-dependent edema inhibitory effects and they demonstrated maximum inhibition after 4 h of study. In contrast to edema inhibition of chlorpheniramine (62.02±12.21%), maximum inhibitory effects of RCE (34.73±9.41%, 45.66±12.82%, and 46.62±8.98%) and WSE (32.25±12.48%, 39.73±14.19%, and 43.00±12.44%) were noticed at 150, 250, and 500 mg/kg dose after 4 h as mentioned in Table 2. Results are in concordance with the previous study [43] that showed the polyphenol-rich extract of *Piper guineense* extract markedly reduced paw edema by inhibiting the inflammatory agent-induced formation of prostaglandins and kinins at the level of phospholipase A_2 and cyclooxygenase which occurs during the second phase of inflammation. Edema inhibition in the first phase of inflammation after extracts administration also indicated an inhibitory effect on serotonin and histamine secretion.

Table 2. Anti-inflammatory effect of RCE and WSE on egg albumin-induced paw edema.

Treatment	Paw edema (mm)/(% Inhibition)					
	Pre-treated	Post-treated				
		0.5 h	1 h	2 h	3 h	4 h
Control	5.30±0.21	8.19±0.10	9.21±0.23	9.01±0.17	8.79±0.26	8.36±0.29
CPM (60 mg/kg)	5.40±0.16	6.84±0.29*** (50.13±11.32)	7.24±0.27*** (52.78±8.21)	7.11±0.30*** (53.59±10.14)	6.92±0.35*** (56.42±13.17)	6.56±0.28*** (62.02±12.21)
RCE (150 mg/kg)	5.38±0.11	7.84±0.37$^{###}$ (15.57±12.86$^{###}$)	8.21±0.27***$^{###}$ (27.62±7.20$^{##}$)	8.04±0.29***$^{###}$ (28.41±6.46$^{##}$)	7.72±0.26***$^{###}$ (32.95±10.73$^{##}$)	7.37±0.20***$^{###}$ (34.73±9.41$^{###}$)
RCE (250 mg/kg)	5.35±0.19	7.36±0.20***$^{#}$ (30.52±7.47$^{#}$)	7.91±0.24***$^{##}$ (34.07±11.23)	7.67±0.23***$^{#}$ (36.93±10.25)	7.34±0.29*** (42.85±11.60)	7.00±0.26*** (45.66±12.82)
RCE (500 mg/kg)	5.30±0.21	7.16±0.21*** (35.98±3.98)	7.69±0.46*** (38.87±10.31)	7.50±0.44*** (41.09±8.79)	7.28±0.44*** (43.70±10.66)	6.95±0.43*** (46.62±8.98)
WSE (150 mg/kg)	5.33±0.19	7.94±0.42$^{###}$ (10.32±13.72$^{###}$)	8.17±0.43***$^{###}$ (27.81±7.96$^{##}$)	8.02±0.42***$^{###}$ (27.99±7.79$^{##}$)	7.75±0.40***$^{###}$ (30.73±12.49$^{##}$)	7.41±0.40***$^{###}$ (32.25±12.48$^{###}$)
WSE (250 mg/kg)	5.40±0.16	7.53±0.23**$^{##}$ (26.19±7.01$^{##}$)	8.04±0.42***$^{###}$ (31.89±11.76$^{#}$)	7.81±0.44***$^{##}$ (34.33±13.94$^{#}$)	7.42±0.44*** (41.64±14.60)	7.23±0.23***$^{##}$ (39.73±14.19$^{##}$)
WSE (500 mg/kg)	5.37±0.23	7.32±0.19*** (31.51±9.51)	7.82±0.26***$^{#}$ (36.01±12.78)	7.60±0.25*** (38.79±10.41)	7.39±0.22*** (41.07±11.56)	7.08±0.25*** (43.00±12.44$^{#}$)

Values are presented as mean±SD (n=6). Significant difference (**p<0.01, ***p<0.001) of treatments from Control group; Significant difference ($^{#}p$<0.05, $^{##}p$<0.01, $^{###}p$<0.001) of RCE and WSE treated groups from Chlorpheniramine-treated group. CPM: Chlorpheniramine, RCE: *R. communis* extract, WSE: *W. somnifera* extract.

Effect of extracts on carrageenan-induced paw edema

The carrageenan-mediated biphasic inflammatory response is arbitrated by an upsurge of histamine, kinins, and serotonin in the initial phase and release of acute inflammation associating prostaglandins [44]. RCE and WSE were assessed for their anti-inflammatory activities using carrageenan-induced paw edema. Rats treated with different doses of extracts significantly (p<0.001) inhibited paw edema from 1 h to 6 h of the study showing maximum percentages of inhibition after 4 h. The highest dose of each plant extract showed maximum edema inhibition that was comparative to the diclofenac. RCE and WSE may produce an anti-inflammatory effect by blocking the synthesis of prostaglandins as well as inhibit the cyclooxygenase pathway similar to the action of diclofenac. Results mentioned in Table 3 indicating that pretreatment with different doses of extracts significantly prevented an increase in paw edema in comparison to the control group. Percentages of edema inhibition of RCE (36.43±21.09%, 61.27±23.88%, and 72.88±13.79%) and WSE (33.15±30.32%, 50.04±20.27%, and 57.81±17.43%) at the dose of 150, 250 and 500 mg/kg, p.o., were observed after 6 h of carrageenan injection, meanwhile, diclofenac showed 89.93±18.53% inhibition. The anti-inflammatory activity of both extracts corroborates the previous study [45] which reported the inhibitory effect on pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and mast cell proliferation, as well as a reduction in oxidative stress-related biomarkers in carrageenan, injected localized tissue pretreated with antioxidants containing grape seeds extract.

Table 3. Anti-inflammatory effect of RCE and WSE on carrageenan-induced paw edema.

Treatment	Paw edema (mm)/(% Inhibition)					
	Pre-treated	Post-treated				
		1 h	2 h	3 h	4 h	6 h
Control	5.11±0.18	7.77±0.01	7.82±0.18	7.57±0.24	7.29±0.23	6.49±0.10
Diclofenac (50 mg/kg)	5.05±0.22	5.99±0.41*** (61.86±22.44)	5.82±0.32*** (70.69±16.26)	5.69±0.18*** (73.18±13.11)	5.47±0.19*** (80.52±13.43)	5.18±0.16*** (89.93±18.53)
RCE (150 mg/kg)	5.20±0.35	6.76±0.19***### (38.37±20.52#)	6.64±0.18***### (45.39±15.53#)	6.62±0.23***### (40.50±15.77##)	6.35±0.30***### (45.59±18.22##)	6.09±0.32### (36.43±21.09###)
RCE (250 mg/kg)	5.44±0.22	6.59±0.21***## (54.30±20.66)	6.36±0.19***## (65.68±14.23)	6.21±0.28***# (67.23±21.41)	6.09±0.17***## (68.71±18.43#)	5.96±0.21*### (61.27±23.88#)
RCE (500 mg/kg)	5.37±0.11	6.32±0.35*** (62.84±17.76)	6.27±0.36*** (67.22±8.61)	6.15±0.35*** (68.51±10.42)	6.01±0.32***# (71.13±11.47#)	5.74±0.23***## (72.88±13.79#)
WSE (150 mg/kg)	5.30±0.32	6.86±0.22***### (38.79±16.72#)	6.70±0.19***### (46.83±13.30#)	6.68±0.23***### (42.36±14.43##)	6.42±0.18***### (46.78±17.13##)	6.18±0.27### (33.15±30.32###)
WSE (250 mg/kg)	5.24±0.32	6.48±0.19***# (51.14±18.51)	6.39±0.21***## (55.89±19.34)	6.35±0.28***### (52.72±23.90#)	6.19±0.26***### (53.88±23.09#)	6.04±0.23### (50.04±20.27##)
WSE (500 mg/kg)	5.43±0.31	6.43±0.25*** (62.26±7.03)	6.29±0.27***# (68.23±9.57)	6.20±0.32***# (69.35±10.22)	6.18±0.33***### (66.28±9.31#)	6.02±0.32*### (57.81±17.43#)

Values are presented as mean±SD (n=6). Significance (*p<0.05, ***p<0.001) of treatments from Control group; Significant difference (#p<0.05, ##p<0.01, ###p<0.001) of RCE and WSE treated groups from Diclofenac-treated group. RCE: *R. communis* extract, WSE: *W. somnifera* extract.

CONCLUSION

The search for plant-based sources may help to bring new natural products into the food production, cosmetic and pharmaceutical industries. The present study findings suggest that *R. communis* leaves and *W. somnifera* roots contain considerable bioactive compounds responsible for significant free radicals scavenging activities in the *in vitro* assays and contribute to potent anti-inflammatory effects in chemical-induced inflammation in experimental models. Conclusively, this study demonstrated that *R. communis* leaves and *W. somnifera* roots could be explored as a valuable source of new and potent phytocompounds with anti-inflammatory properties. In addition to conventional medicines, both plants may provide safe and effective therapeutic options for a variety of inflammatory disorders.

Acknowledgments: This study was partially supported by the Higher Education Commission (HEC), Pakistan-funded project (7510/Sindh/NRPU/R&D/HEC/2017) approved to BA. The authors are grateful to Dr. Sajjad-ur-Rahman, Institute of Microbiology, University of Agriculture, Faisalabad, for providing animal house facility and Dr. Muhammad Shahid, Department of Biochemistry, University of Agriculture, Faisalabad for guiding in *in vitro* analysis.
Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

1. Newton K, Vishva MD. Signaling in innate immunity and inflammation. Cold. Spring Harb. Perspect. Biol. 2012;4:a006049.
2. Nathan C. Points of control in inflammation. Nature. 2002;420:846-52.
3. Tasneem S, Saleem M, Saeed SA. Nonsteroidal anti-inflammatory drugs as potential ecto-nucleotide phosphodiesterase inhibitors. Braz. J. Pharm. Sci. 2020;56:e18271.
4. Hao D, Pei-gen X. Pharmaceutical resource discovery from traditional medicinal plants: Pharmacophylogeny and pharmacophylogenomics. Chin. Herb. Med. 2020;12(2):104-17.
5. Bahadur S, Khan MS, Shah M, Shuaib M, Ahmad M, Zafar M, Begum N, Gul S, Ashfaq S, Mujahid I, Hussain F. Traditional usage of medicinal plants among the local communities of Peshawar valley, Pakistan. Acta Eco. Sinica. 2020; 40:1-29.
6. Jim´enez-Estrada M, Vel´azquez-Contreras C, Garibay-Escobar A, Sierras-Canchola D, Lapizco-Vazquez R, Oritz-Sandoval C, Robles-Zepeda RE. *In vitro* antioxidant and antiproliferative activities of plants of the ethnopharmacopeia from northwest of Mexico. BMC Complement. Altern. Med. 2013; 13:12.
7. Jing W, Zhong J, Ping LJ, Yan LH. Relationship between oxidative stress and inflammation in peripheral and cerebral system of oxonate-induced hyperuricemic rats. Braz. J. Pharm. Sci. 2017; 53(4):e00229.
8. Gallego R, Bueno M, Herrero M. Sub- and supercritical fluid extraction of bioactive compounds from plants, food-by-products, seaweeds and microalgae–an update. TrAC-Trends Anal. Chem. 2019; 116:198-13.

9. Abdul WM, Hajrah NH, Sabir JS, Al-Garni SM, Sabir MJ, Kabli SA, Saini KS, Bora RS. Therapeutic role of *Ricinus communis* L. and its bioactive compounds in disease prevention and treatment. Asian Pac. J. Trop. Med. 2018;11:177-85.
10. Kumar M. A review on phytochemical constituents and pharmacological activities of *Ricinus communis* L. plant. Int. J. Pharmacogn. Phytochem. Res. 2017;9(4):466-72.
11. Shokeen P, Anand P, Murali YK, Tandon V. Antidiabetic activity of 50% ethanolic extract of *Ricinus communis* and its purified fractions. Food Chem. Toxicol. 2008;46(11):3458-66.
12. Zarai Z, Chobba IB, Mansour RB, B´ekir A, Gharsallah N, Kadri A. Essential oil of the leaves of *Ricinus communis* L.: *in vitro* cytotoxicity and antimicrobial properties. Lipids Health Dis. 2012; 11:102.
13. Hussain A, Aslam B, Muhammad F, Faisal MN, Kousar S, Mustaq A, Bari MU. Anti-arthritic activity of *Ricinus communis* L. and *Withania somnifera* L. extracts in adjuvant-induced arthritic rats via modulating inflammatory mediators and subsiding oxidative stress. Iran. J. Basic Med. Sci. 2021; 24(7):951-61.
14. Tripathi N, Shrivastava D, Mir BA, Kumar S, Govil S, Vahedi M, Bisen PS. Metabolomic and biotechnological approaches to determine therapeutic potential of *Withania somnifera* (L.) Dunal: A review. Phytomedicine. 2018;50:127-36.
15. Andulla B, Radhika B. Hypoglycemic, diuretic and hypocholesterolemic effect of winter cherry (*Withania somnifera*) root. Indian J. Exp. Biol. 2010;38(6):607-9.
16. Udayakumar R, Sampath K, Ayyappan V, Thankaraj SM, Jessudass JSR, Chang WC, Andy G, Sei CK. Antioxidant effect of dietary supplement *Withania somnifera* L. reduce blood glucose levels in alloxan induced diabetic rats. Plant Foods Hum. Nutr. 2010;65:91-8.
17. Süntar I. Importance of ethnopharmacological studies in drug discovery: Role of medicinal plants. Phytochem. Rev. 2020;19:1199-1209.
18. Sultana B, Anwar F, Ashraf M. Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. Molecules. 2009;14(6):2167-80.
19. Ilavarasan R, Mallika M, Venkataraman S. Anti-inflammatory and free radical scavenging activity of *Ricinus communis* root extract. J. Ethnopharmacol.2006; 103(3):478-80.
20. Srikanth G, Babu SM, Kavitha CHN, Rao MB, Vijaykumar N, Pradeep CH. Studies on *in vitro* antioxidant activities of *Carica papaya* aqueous leaf extract. Res. J. Pharm. Biol. Chem. Sci. 2010;1(2):59-65.
21. Beauchamp C, Fridovich I. Superoxide dismutase: Improved assays and an Assay applicable to acrylamide gels. Anal. Biochem. 1971;44(1):276-87.
22. Oyaizu M. Antioxidant activity of browning products of glucosamine fractionated by organic solvent and thin-layer chromatography. J. Jpn. Soc. Food Sci. 1988;35(11):771-5.
23. Hosseinzadeh H, Ramezani M, Salmani GA. Antinociceptive, anti-inflammatory and acute toxicity effects of *Zataria multiflora* Boiss extracts in mice and rats. J. Ethnopharmacol. 2000;73(3):379-85.
24. Akindele AJ, Oladimeji-Salami JA, Usuwah BA. Antinociceptive and anti-inflammatory activities of *Telfairia occidentalis* hydroethanolic leaf extract (Cucurbitaceae). J. Med. Food. 2015;18(10):1157-63.
25. Mattioli R, Francioso A, d'Erme M, Trovato M, Mancini P, Piacentini L, Casale AM, Wessjohann L, Gazzino R, Costantino P, Mosca L. Anti-inflammatory activity of a polyphenolic extract from *Arabidopsis thaliana* in *in vitro* and *in vivo* models of Alzheimer's disease. Int. J. Mol. Sci. 2019;20(3):708.
26. Ren M, Tang Q, Chen F, Xing X, Huang Y, Tan X. Mahuang Fuzi Xixin decoction attenuates Th1 and Th2 responses in the treatment of ovalbumin-induced allergic inflammation in a rat model of allergic rhinitis. J. Immunol. Res. 2017;1-12.
27. Sobhani M, Farzaei MH, Kiani S, Khodarahmi R. Immunomodulatory; anti-inflammatory/antioxidant effects of polyphenols: A comparative review on the parental compounds and their metabolites. Food Rev. Int. 2020;1-53.
28. Magrone T, Magrone M, Russo MA, Jirillo E. Recent advances on the anti-inflammatory and antioxidant properties of red grape polyphenols: *In vitro* and *in vivo* studies. Antioxidants. 2020;9(1):35.
29. Mechchate H, Es-Safi I, Amaghnouje A, Boukhira S, Alotaibi A, Al-Zharani M, Nasr F, Noman OM, Conte R, Amal EHEY, Bekkari H. Antioxidant, anti-inflammatory and antidiabetic proprieties of LC-MS/MS identified polyphenols from coriander seeds. Molecules. 2021;26(2):487.
30. Ramzan T, Aslam B, Muhammad F, Faisal MN, Hussain A. Influence of *Ocimum sanctum* (L.) extract on the activity of gliclazide in alloxan-induced diabetes in rats. Rev. Chim. 2020;71(10):101-10.
31. Hussain Z, Khan JA, Arshad MI, Muhammad F, Abbas RZ. Protective effects of cinnamon, cinnamaldehyde and kaempferol against acetaminophen-induced acute liver injury and apoptosis in mouse model. Pak. Vet. J. 2021;41(1):25-32.
32. Mushtaq A, Aslam B, Muhammad F, Khan JA. Hepatoprotective activity of *Nigella sativa* and *Piper nigrum* against concanavalin a-induced acute liver injury in mouse model. Pak. Vet. J. 2021;41(1):78-84.

33. Chand T, Anil B, Bhupendra KK, Pawank B, Sanjay S, Rajesh V. *In vitro* antioxidant activity of alcoholic extract of seed of *Cucumis callosus* (Rottl.) cogn. Am. J. Pharmtech. Res. 2012;2(3):2249-87.
34. Pan Y, Wang K, Huang S, Wang H, Mu X., He C, Ji X, Zhang J, Huang F. Antioxidant activity of microwave-assisted extract of longan (*Dimocarpus longum* Lour.) peel. Food Chem. 2008;106(3):1264-70.
35. Taşkın T, Gezmiş T, Çam ME, Taşkın D, Çelik BÖ, Şenkardeş İ, Süzgeç-Selçuk S. The *in vitro* and *in vivo* investigation of biological activities and phenolic analysis of *Helichrysum plicatum* subsp. plicatum. Braz. J. Pharm. Sci. 2020;56:e18345.
36. Manian R, Nagarajan A, Perumal S, Sellamuthu M. The antioxidant activity and free radical scavenging potential of two different solvent extracts of *Camellia sinesis* (L.) O. Kuntz, *Ficus bengalensis* L. and *Ficus racemosa* L. Food Chem. 2008;3(1):1000-7.
37. Zulfikar AHM, Saha MR, Sarwar S, Nahar L, Hamid K, Rana MS. Hypoglycemic and *in vitro* antioxidant activity of ethanolic extracts of *Ficus racemosa* Linn. fruits. Am. J. Sci. Ind. Res. 2011;2(3):391-400.
38. Dorman HJD, Peltoketo A, Hiltunen R, Tikkanen MJ. Characterisation of the antioxidant properties of de-odourised aqueous extracts from selected Lamiaceae herbs. Food Chem. 2003;83(2):255-6.
39. Yildirim A, Mavi A, Oktay M, Kara AA, Algur OF, Bilaloglu V. Comparison of antioxidant and antimicrobial activities of tilia (*Tilia arentea* Desf ex DC), sage (*Salvia triloba* L.) and black tea (*Camellia sinensis* L.) extracts. J. Agr. Food Chem. 2000;48(10):5030-4.
40. Chatzigeorgiou A, Antoios H, Konstantinos K, Elli K. The use of animal models in the study of diabetes mellitus. In Vivo. 2009;23(2):245-58.
41. Singsai K, Charoongchit P, Chaikaew W, Boonma N, Fhanjaksai P, Chaisatan K. Antilipoxygenase and anti-inflammatory activities of *Streblus asper* leaf extract on xylene-induced ear edema in mice. Adv. Pharmacol. Pharm. Sci. 2020;3176391.
42. Hira A, Shubhra KD, Md. Sariful IH, Arif A, Hemayet H, Ismet AJ. Anti-inflammatory and antioxidant activities of ethanolic extract of aerial parts of *Vernonia patula* (Dryand.) Merr. Asian Pac. J. Trop. Biomed. 2013;3(10):798-5.
43. Akinloye OA, Alagbe OA, Ugbaja RN, Omotainse SO. Evaluation of the modulatory effects of *Piper guineense* leaves and seeds on egg albumin-induced inflammation in experimental rat models. J. Ethnopharmacol. 2020;255:112762.
44. Uko MS, Usman A, Toma I, Okhale SE, Magili ST, Adzu B. Evaluation of active phytochemical constituents linked to the analgesic and anti-inflammatory property of *Cassia singueana* Del. root bark. J. Med. Plants Res. 2019;13(12):288-95.
45. Abd-Allah AA, El-Deen NA, Mohamed WA, Naguib FM. Mast cells and pro-inflammatory cytokines roles in assessment of grape seeds extract anti-inflammatory activity in rat model of carrageenan-induced paw edema. Iran. J. Basic Med. Sci. 2018;21(1):97.