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EVALUATION OF THE ANTI-INFLAMMATORY EFFECT OF ALLIUM SATIVUM.L (GARLIC) EXTRACT ON A MODEL OF BENIGN PROSTATIC HYPERTROPHY IN BALB/C MICE.

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ABSTRACT

Summary: Benign prostatic hyperplasia (BPH) is one of the most common diseases in elderly men, characterised by prostate enlargement and subvesical obstruction (SBO). The aim of this study was to determine the phytochemical composition and anti-inflammatory effect of fresh AS juice on a mouse model of BPH. **Materials and methods:** Male mice were divided into four groups: group I: control (0.2 ml/kg olive oil), group II: BPH (0.072 mg/kg TE), group III: preventive (BPH + 20 mg/kg AS + 0.072 mg/kg TE) and group IV: curative (BPH + 0.072 mg/k TE + 20 mg/kg AS). BPH was induced by intramuscular injections of testosterone enanthate (TE) and its action potentiated by the administration of b(a)p. At the end of the various treatments, the prostates were removed after the animals had been sacrificed, then weighed to determine the prostate weight index (PW). The blood sample was then used to count the number of blood components (haemogram), and the plasma levels of free TE, free PSA, CRP, IL-6 and tumour necrosis factor-α (TNF-α) were determined using enzyme-linked immunosorbent assays. Phytochemical studies were carried out to determine polyphenol and flavonoid content. Differences between the control and treated groups were analysed using the Student's t-test. **Results:** We observed a significant decrease in prostate weight index ($p<0.0024$), TE-free levels in group III ($p=0.0051$) and IV ($p=0.0253$), PSA-free levels in group III ($p=0, 0031$) and IV ($p=0.0289$), IL-6 enzymatic activity in group III ($p= 0.0073$) and TNF- α in group III and IV ($p = 0.0093$) after treatment with fresh AS juice, compared with group II. Phytochemical analysis of the fresh AS juice revealed the presence of tannins, flavonoids and reducing compounds. The highest levels of polyphenols and flavonoids were observed in the stock solution (C1=20mg/mL), i.e. 588mgEAG/100gMS in polyphenol and 8.10mgERu/100gMS in flavonoids. **Conclusion:** Fresh AS juice, administered to animals either as a preventive or curative measure, attenuates the development of BPH by regulating inflammation of the prostate.

KEYWORDS: *Allium sativum.L*, phytochemical, benign prostatic hyperplasia, inflammation.

INTRODUCTION

Benign prostatic hyperplasia (BPH) is one of the most common diseases of older men, defined by enlargement of the prostate gland and subvesical obstruction (SBO), responsible for lower urinary tract symptoms (LUTS).[1] It is caused by an excessive but non-malignant growth of the stromal and epithelial cells of the prostate.[2] The prevalence of BPH increases with age and continues to rise with the ageing of the population.[3] It is estimated that 50% of men over the age of 50 and 90% of men at the age of 80 will have BPH.[4]

The development of BPH is conditioned by high levels of androgens, imbalances in sex hormones, ageing and inflammation.[5] Clinical and experimental studies have confirmed that the excessive accumulation of reactive oxygen species (ROS) leads to tissue damage and is associated with the appearance of several inflammatory

pathologies in the elderly.[6,7] Inflammation of prostate tissue and an imbalance in oxidative stress can lead to the accumulation of inflammatory cytokines and other growth factors through which testosterone can induce BPH.[8,9] The relationship between the development of BPH and inflammation, occurs through the dissemination of pro-inflammatory cytokines which may further exaggerate pre-existing inflammation.[10]

Allium sativum.L (AS) is a medicinal plant whose pharmacological effects are attributed to several bioactive substances such as vitamins C and E, β-carotene and polyphenols, flavonoids.[11] Studies have shown that AS substances are known for their anti-tumour, anti-inflammatory and other activities.[12] The aim of our study was to determine the phytochemical composition and to investigate the anti-inflammatory effect of fresh AS juice on a mouse model of BPH induced by enanthate testosterone (TE), potentiated by benzo(a)pyren (b(a)p).

MATERIALS AND METHODS

Chemicals and reagents

Standard laboratory grade chemicals and reagents were used in this study. The enanthate testosterone solution marketed under the name Androtardyl® was used. Benzo(a)pyrene was sourced from Shejiang province (China). Enzyme-linked immunosorbent assay (ELISA) kits for free testosterone, prostate-specific antigen, interleukin-6 and tumour necrosis factor-α protein C-reaction were supplied by SUNLONG BIOTECH CO. LTD, China.

Plant material and extraction of fresh AS juice

Fresh *Allium sativum.l* bulbs were obtained from markets in Brazzaville. Extraction was carried out using 500.24 g of garlic bulbs (corresponding to 12 cloves), crushed using a mortar and pestle. The crushed material was pressed and the juice filtered, then stored at +4°C. This extraction yielded 120 ml of garlic juice, giving a yield of 4.16%.

Phytochemical analysis of fresh AS juice

Chemical tube tests were used to identify the main chemical families present in the extract of fresh AS juice (alkaloids, tannins, flavonoids, reducing sugars and saponosides).

Identification of alkaloids

5 ml of AS aqueous extract was placed in a test tube. Then 1 ml of 1N hydrochloric acid and a few drops of reagents were added. The formation of a red precipitate (with Dragendorff's reagent) or a yellowish precipitate (with Mayer's reagent) indicated the presence of alkaloids.[13]

Identification of tannins

Tannins were identified by mixing 5 ml of aqueous AS decoction with 1 ml of an aqueous iron chloride solution. In the presence of tannins, a greenish or blue-blackish colour develops.[14]

Identification of flavonoids

5 ml of 5% AS decoction, 5 ml of hydrochloric acid (HCL) solution, 1 ml of iso-amyl alcohol and a few magnesium chips were mixed. A change in colour indicated the presence of the following compounds.[13]

- Orange-yellow, for flavones ;
- Purplish pink, for flavanones;
- Red for flavonols and flavanols.

Identification of reducing sugars

To 5 ml of AS decoction was added 1 ml of Fehling's liqueur. The formation of a brick-red precipitate indicated the presence of reducing sugars.[13]

Identification of saponins

5 ml of aqueous extract of AS was mixed with 5 ml of distilled water in a test tube and shaken vigorously. Stable foam formation was taken as an indication of the presence of saponins.[13]

Determination of total polyphenols (TPP)

The principle is based on determining the optical densities of the extracts in relation to those of a standard gallic acid solution of known concentration. Briefly, 0.1ml of SA extract was mixed with 0.9ml distilled water and 0.9ml Folin-Ciocalteu reagent (1N), followed by 0.2ml Na2CO3 solution (20%). This mixture was incubated at room temperature for 40 minutes, protected from light. The absorbance was then measured using a spectrophotometer at 725 nm against a methanol solution used as a blank. A calibration curve was determined using the standard solution of gallic acid under the same conditions as the samples to be analysed. The results obtained were expressed as mg gallic acid equivalent per gram of dry matter (mgEAG/gMs).[15]

Determination of total flavonoids

To 250 µl of extract and 1 ml of distilled water were added 75 µl of NaNO2 solution (5%) and then 75 µl of AlCl3 (10%), respectively, 5 minutes later. After 6 minutes, 500 µl of NaOH (1N) and 2.5 ml of distilled water were successively added to the mixture. The absorbance of the mixture obtained was measured using a UV-visible spectrophotometer at 510 nm and the results were expressed as mg rutin equivalent per 100 grams of dry matter (mgERu/100g Ms).[14]

Animals and treatment

Ten-week-old male Balb/c mice, weighing on average 18 ± 2 g and originating from the animal house of the Faculty of Health Sciences of the University Marien Ngouabi (Brazzaville, Congo) were used. They were reared under standard conditions with free access to water and food. All experiments were conducted in compliance with Directive 2010/6006/EU on the protection of laboratory animals.[16] Before the start of the experiment, the animals were acclimatised to the

laboratory conditions for 2 weeks. They were then randomly divided into four groups of 6 animals each and treated as follows:

- Group I: control (0.2 ml/kg olive oil orally),
- Group II: BPH (2 mg/kg b(a)p single dose intraperitoneally + 0.072 mg/kg TE intramuscularly),
- Group III: preventive (2 mg/kg b(a)p a single dose intraperitoneally + 20 mg/kg AS by gavage + 0.072 mg/kg TE intramuscularly),
- Group IV: curative (2 mg/kg b(a)p single dose intraperitoneally + 0.072 mg/kg TE intramuscularly + 20 mg/kg SA by gavage).

AS fresh juice extract was administered simultaneously to the animals four times a week for 21 days in group III (preventive) and after the establishment of BPH in group VI (curative).[17]

Induction of benign prostatic hyperplasia

BPH was induced by intramuscular injections of TE enanthate dissolved in distilled water at a dose of 0.072 mg/kg/mouse four times weekly for 21 days.[17] B(p)a, administered intraperitoneally at a dose of 2 mg/kg 24 hours before injection of TE enanthate, potentiated the development of BPH.

Animal sacrifice and sampling

At the end of the experiment, the mice were sacrificed under diethyl ether anaesthesia (Ether cooper®). Blood samples were taken from the orbital sinus in EDTA *(ethylene diamine tetra-acetic acid)* BD vacutainer dry tubes. The blood was centrifuged at 3,000 rpm for 15 minutes and the serum was aliquoted and stored at 4°C until biological analysis. The prostate glands were removed and immediately placed in phosphate-buffered saline (PBS).

Determination of the prostate index

After the animals were killed, the prostate was removed, rinsed with saline and immediately weighed. The prostate index (PW) was then calculated using the following formula:

$$\text{PW index} = \frac{\text{Prostate weight}}{\text{Body weight}} \times 100$$ [18]

Assessment of hormones

Serum concentrations of testosterone (free TE) and prostate-specific antigen (free PSA) were determined by ELISA (*enzyme-linked immunosorbent assay*), using commercial kits (free TE, free PSA *ELISA kits*) in accordance with the manufacturer's instructions.

Evaluation of haematological parameters

Haematological parameters were measured using a blood counting device *(SYSMEX Médical, XP-300)*. The blood count formula consists of red blood cells (RBC), white blood cells (WBC) and platelets (PLT). Each haematological parameter was determined using the conventional haemocytometer method, following the instructions on the machine.

Assessment of inflammatory markers

Prostates collected in 1 ml of PBS buffer (0.1 M, pH 7.4) were individually ground using an applicator. The homogenate obtained was then centrifuged at 3,000 rpm for 25 minutes. The supernatant obtained was aliquoted and used to immediately measure the levels of interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), by ELISA *(enzyme-linked immunosorbent assay)*, using commercial kits (IL-6, PSA free ELISA kits). Serum protein C-reaction (CRP) levels by immunofluorescence assay using the commercial kit *(CRP Mindrayd)*, in accordance with the device instructions.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism version 8 XML project software. Differences between the control and treated groups were analysed using Student's t-test and the significance threshold was set at $p<0.05$.

RESULTS

Phytochemical analysis

Phytochemical analysis of fresh AS juice revealed the presence of tannins, flavonoids and reducing compounds. The presence of each chemical fraction in large or small quantities was judged by the degree of coloration. The results are shown in Table I below.

Table I: Phytochemical composition of fresh AS juice.

	Chemical families				
	Alkaloids	**Flavonoids**	**Tannins**	**Saponins**	**Tri-terpenes and sterols**
Allium sativum. L	++	+++	++	-	++++

Legend: (+++): strongly present; (++): moderately present; (+): weakly present and (-): absent.

Quantitative determination of polyphenols and total flavonoids

The highest levels of polyphenols and flavonoids were observed in the stock solution (C1=20mg/mL), i.e. 588mgEAG/100gMS for polyphenols and 8.10mgERu/100gMS for flavonoids. These results are presented in Figure 1.

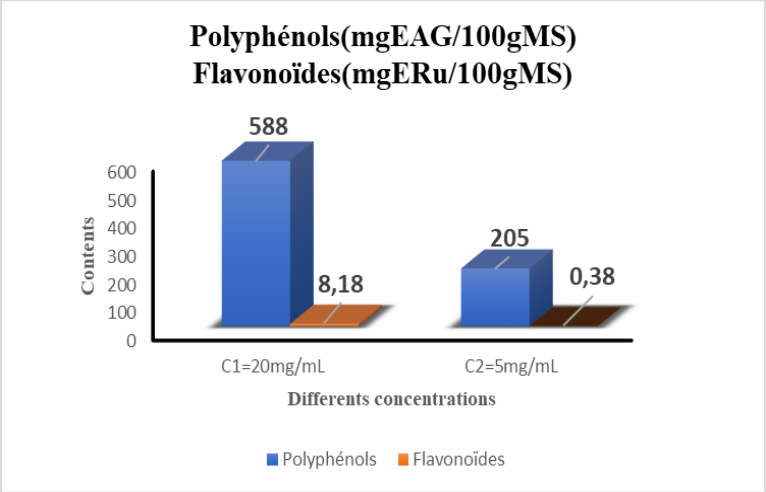

Figure 1: Total polyphenol and flavonoid content.

Effect of AS fresh juice extract on prostate weight index.

Table II shows the weight and prostate index of the animals after treatment with the different products. Analysis of these results shows a significant reduction in the prostate weight index of animals in groups III and IV ($p<0.0024$) treated with AS fresh juice extract, compared with animals in group II (BPH).

Table II: Effects of AS juice extract administration on body weight and prostate weight index in animals.

Groups	Body weight (g)	Prostate weight	Prostate weight index
Group I (CTRL)	22,9 ±1,060	0,022±0,001	0,103±0,010
Group II (HBP)	29,4±1,020	0,058±0,002 [a]	0,230±0,008 [a]
Group III (preventive)	27,5±0,883	0,034±0,005 [b]	0,136±0,021 [b]
Group VI (curative)	27,7±0,748	0,035±0,008 [b]	0,141±0,032 [b]

Results are expressed as mean ± standard error. [(a)]: p= 0.0001 group I vs group II. [(b)]: p= 0.0024 group III and IV vs group II. n=06 animals per group.

Effects of extract of fresh AS juice on hormone levels

Effects on free testosterone levels

Administration of AS juice extract caused a significant decrease in circulating free TE levels in animals in groups III (p=0.0051) and IV (p=0.0253), compared with animals in group II (BPH). These results are shown in Figure 2.

Effects on free PSA levels

These results show that the extract of fresh AS juice administered to the animals caused a significant decrease in the free PSA levels of animals in groups III (p=0.0031) and IV (p=0.0289), compared with animals in group II (BPH). These results are shown in Figure 3 below.

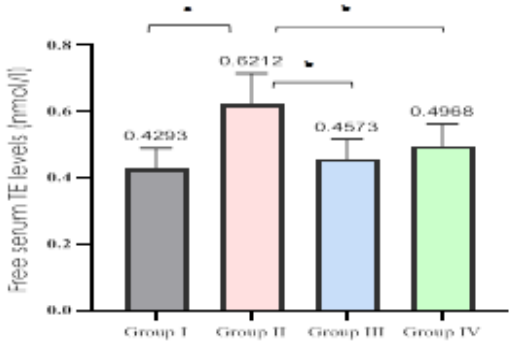

Figure 2: Effects of AS juice extract administration on free testosterone levels in animals.

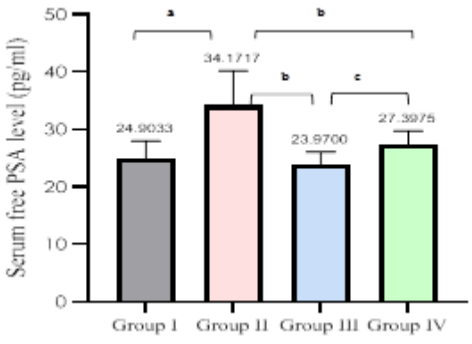

Figure 3: Effects of AS juice extract administration on free PSA levels in animals.

Results are expressed as mean ± standard error. ([a]): p=0.0020 group I vs group II. ([b]): group II vs group III (p=0.0051) and group IV (p=0.0253). n=06 animals per group.

Results are expressed as mean ± standard error. ([a]): p=0.0075 group I vs group II; ([b]): group II vs group III (p=0.0031) and IV (p=0.0289); ([c]): p= 0.0241 group III vs group IV, n=06 animals per group.

Effect of fresh AS juice extract on haematological parameters

Table III shows the effects of fresh AS juice on haematological parameters in animals. These results show a significant increase (p=0.0073) in the number of leukocytes compared with group I (control). However, after treatment, SA juice as a preventive or curative measure by did not improve the leucocyte count.

Table III: Effects of fresh AS juice on haematological parameters in animals.

Groupes	Haematological Parameters		
	WBC (x 10^3/ µL)	RBC (× 10^6/µL)	PLAT (x 10^3/ µL)
Groupe I (CTRL)	2,73±0,310 [a]	9,64±0,423 [ns]	595±147 [ns]
Groupe II (HBP)	8,08±1,56 [a]	9,13±0,405 [ns]	517±263 [ns]
Groupe III (préventif)	9,56±1,38	9,85±0,268	700±185
Groupe VI (curatif)	7,79±1,81	10,7± 0,412	785±198

Legende: WBC: white blood cells , RBC: red blood cells, PLAT: platelets

Results are expressed as mean ± standard error. ([a]): p= 0.0073 group I vs group II, ns: not significant, n= 06 animals per group.

Effect of fresh AS juice extract on inflammatory markers

Effect on interleukin-6 and C-reactive protein

The development of BPH in the animals caused a significant increase in the levels of markers of inflammation, interleukin-6 (p= 0.0001) and C-reactin protein (p= 0.0035) in group II (HBP) compared with group I (control) animals. Fresh AS juice extract administered either as a preventive or curative measure improved interleukin-6 levels in groups III (p= 0.0354) and IV (p= 0.0064) compared with animals in group II (BPH), with no significant difference between the two groups. These results are presented in Table IV below.

Table IV: Effect of fresh AS juice extract on inflammatory markers.

Groupes	Inflammatory markers	
	l'interleukin-6 (IL-6),	C-reaction protein (CRP)
Groupe I (CTRL)	7,725 ± 0,2283 [a]	2,387± 0,4791 [c]
Groupe II (HBP)	12,08 ± 0,6810 [a,b]	7,897± 1,367 [c]
Groupe III (préventif)	9,243 ± 0,4674 [b]	7,638± 1,099
Groupe VI (curatif)	10,11 ± 0,4350 [b]	8,890± 0,9036

Results are expressed as mean ± standard error. ([a]): group I vs II (p= 0.0004). ([b]): group II vs group III (p= 0.0354), group IV (p= 0.0064). ([c]) : group I vs group II (p= 0,0035). n=6 animals per group.

Effect on tumour necrosis factor (TNF- α)

Figure 4 shows the effects of AS juice on TNF- α enzymatic activity in animals. Analysis of these results shows a significant decrease in TNF- α in the prostate tissues of animals in groups III (p= 0.0093) and IV (p= 0.0272) that received fresh AS juice, compared with group II (BPH).

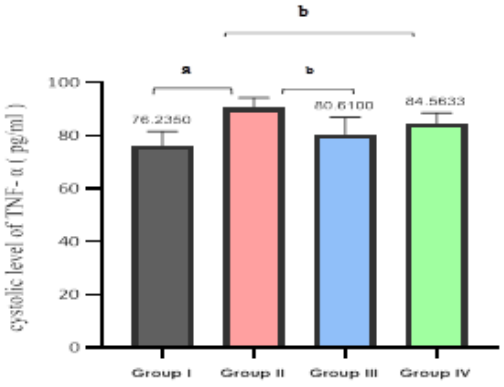

Figure 4: Effects of fresh AS juice administration on cystolic TNF- α levels in animals.

Values are expressed as mean ± standard error, with ([a]): p= 0.0004 vs II. ([b]): group II vs group III (p= 0.0093) and IV (p=0.0272), n= 06 animals per group.

DISCUSSION

Allium sativum.L (garlic) is a plant of the alliaceae family containing several chemical compounds with antioxidant properties.[19] Our objective was to determine the phytochemical composition and to evaluate the anti-inflammatory effect of fresh SA juice on the BPH model in vivo. BPH is a chronic andrological disease characterised by extensive testosterone-induced inflammation in epithelial cells and hyperplasia.[20] Clinically, BPH is characterised by heavy urination and lower urinary tract symptoms, affecting patients' quality of life. Numerous complications of BPH have been described, including urinary tract infections, acute urinary retention, urinary stones and renal failure.[21] Several aetiological factors, including hormonal alterations, inflammation of the prostate and growth factors, have been implicated in its development.[22]

Chemical screening revealed the presence of alkaloids, tannins, flavonoids and reducing sugars. However, we did not find any saponosides. Quantification of phenolic compounds (polyphenols and total flavonoids) in the extract was carried out using the aluminium trichloride (AlCl3) method. The results obtained show that the highest levels of polyphenols and flavonoids were

observed in the solution (C1=20mg/mL), with a highly positive level of flavonoids recovered of 8.18 compared to 0.38 in the solution (C2=5mg/mL). These observations are in line with those made by other authors.[23, 24]

In this study, we modelled BPH in mice by repeated administration of testosterone. The short-term effect of testosterone was potentiated by a single injection of b(a)p, as reported by Konishi N et al (1995).[25] The establishment and progression of hyperplasia was confirmed by increases in the prostate weight index (PW), PSA levels and inflammatory markers, as described in the literature.[26,27] In fact, repeated administration of testosterone has been shown to induce prostate hyperplasia, resulting in an increase in the weight of this organ (and therefore in the prostate weight index) and an increase in inflammatory cytokines.

In experimental models, exogenous testosterone is known to induce cell proliferation associated with inhibition of apoptosis.[28] This action was potentiated by b(a)p in the present study and resulted in a significant increase in the prostate index. However, treatment with 20 ml/kg of fresh AS juice resulted in a significant decrease ($p<0.05$) in the prostate weight index (Table II). This effect could be explained by an inhibition of the abnormal regulation of apoptosis caused by testosterone.[29] The (figure 2,3), showed us a remarkable increase in the levels of (free TE and free PSA) in the treated groups. In fact, the injection of exogenous TE increases the level of intra-prostatic TE which produces a higher activity of the 5 α -reductase enzyme, leading to an accumulation of dihydrotestosterone (DHT). This accumulation in prostate tissue is sufficient to produce an increase in androgen-dependent expression of growth factors, stimulating NF-κB/p65 signalling pathways, creating expression of inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin (IL)-1β, IL-6, IL-8 involved in inflammation and immune responses in addition to cell development and growth.[30,31] However, fresh AS juice extract administered to animals significantly ($p<0, 05$) reduced (TE-free, PSA-free) levels. These effects could be due to the bioactive substances in AS, which increase the degradation of testosterone, necessary for prostate cancer growth, and reduce the enzymatic activity of specific antigens.[32] The development of inflammation in prostate tissue can lead to the accumulation of inflammatory cytokines and other growth factors.[8] This is consistent with our study where induction of benign prostatic hypertrophy resulted in increased leukocyte counts associated with the frequent presence of inflammatory prostate cells, overexpression of inflammatory cytokines including interleukin-6 (IL-6), tumour necrosis factor (TNF- α) and increased levels of C-reactive protein (CRP) compared with control animals. However, the administration of AS juice extract, as a preventive or curative measure, improved the activity of inflammatory cytokines, resulting in a significant decrease in IL-6 and the cystolic activity of tumour necrosis factor (TNF- α).

Thus, the biological effects observed with fresh AS juice would be supported by the chemical composition of this extract, rich in flavonoids and phenolic compounds known for their anti-tumour, anti-inflammatory and other activities.[19] Indeed, the proliferation of stromal and epithelial cells during BPH may be promoted by IL-6 and IL-8 through fibroblast-myofibroblast differentiation and also by indirect stimulation of fibroblast growth factor secretions. This may have a stimulating effect on prostate growth.[33] In addition, TNF-α has various important functions in inflammation, cell differentiation, proliferation and cell death.[34] Furthermore, extract of fresh AS juice administered as a preventive or curative measure improved the activity of interleukin-6 (IL-6) and tumour necrosis factor (TNF- α). Diallyl trisulphide (DATS) has a suppressive effect on the NF-κB pathway.[35] Allyl sulphides suppress proliferation by blocking G2/M phase cells and by inducing apoptosis.[36] This led to the ameliorative effect of the extract of fresh AS juice administered preventively or curatively in this study.

CONCLUSION

The present study has shown that fresh AS juice possesses anti-inflammatory properties as a preventive or curative measure for TE-induced BPH in mice. These effects are reflected in a decrease in the prostate weight index, testosterone and prostate-specific antigen levels, and a decrease in the inflammatory cytokines interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α). The biological activity of fresh AS juice is attributed to the presence of certain chemical compounds such as flavonoids and polyphenols. Further studies could clarify the mechanism of this antiproliferative activity of AS and support the development of new therapeutic strategies based on this plant.

AUTHORS' CONTRIBUTIONS

All the authors contributed to this work. The authors of this article have read and approved its contents.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest in relation to this work.

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