Therapeutic effect of Arthrocnemum machrostachyum methanolic extract on Ehrlich solid tumor in mice

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Abstract

Background: The anti-cancer effect of the halophyte Arthrocnemum indicum, a member of Arthrocnemum family of salt-tolerant plants, was evaluated against colorectal cancer cell, CaCo2. However, the anti-cancer effect of another halophyte Arthrocnemum machrostachyum was not investigated yet. Herein, the anticancer effect of A. machrostachyum methanolic extract (AME) was evaluated against Ehrlich solid tumor (EST) in mice and the potential mechanism of action was also studied.

Methods: Male Swiss albino mice (n = 28) were randomly divided into 4 groups (n = 7/group). Group 1 (negative control group); group 2 (EST) injected intramuscularly by 0.2 mL Ehrlich ascitic carcinoma (2 × 10^6 cells); and groups 3 and 4 injected intratumorally with AME (180 and 360 mg/kg body weight, respectively) at D12 trice weekly for 2 weeks. Gene expression, protein expression, DNA damage, and TNFa level in tumors were determined by real-time PCR, western blot, comet assay, and Elisa, respectively.

Results: Treatment with AME induced anti-tumor effects against EST as indicated by 1) notable reduction in tumor size; 2) elevation in tissue necrosis and apoptosis, as confirmed histologically; 3) increased DNA fragmentation; 4) decreased expression of the apoptotic genes (p53, Bax and caspase 3), and increased expression of the anti-apoptotic marker Bcl2; 5) significantly upregulated cell cycle regulatory genes Cdc2 and connexin26, and; 6) decreased TNFa levels in tumor tissues. Interestingly, a high dose of AME exhibited a more potent anti-tumor effect against EST.

Conclusion: These findings indicate that AME has a potent antitumor effect against EST and could be used as an adjuvant to anticancer drugs to combat tumor, but after application of further confirmatory clinical trials.

Keywords: Ehrlich solid tumor, Arthrocnemum machrostachyum, Apoptosis, Inflammation
Cancer is the 2nd prominent cause of death on the globe preceded by cardiovascular diseases. Ehrlich is an aggressive murine mammary adenocarcinoma that can happen in almost any strain of mouse. Ehrlich tumor, particularly its solid tumor (EST) form, is easily grown and propagated in vivo. For this reason, EST was successfully used as a model to study tumor development and progression and to evaluate the therapeutic potential of new anti-cancer agents [20]. Moreover, this model was successfully used by several researchers to study the anticancer effects of natural products, synthetic chemicals, and nanoparticles [19, 21–24]. Recently, EST was used as a tumor model to investigate the anti-cancer effects of sulforaphane (an active ingredient of cruciferous vegetables such as broccoli and cabbage) [25] and Avenanthramides (a group of phenolic alkaloids found mainly in oats and white cabbage) [26]. EST is also easily induced in mice and closely similar to the in vivo breast cancer model [27]. For these advantages, EST was chosen as a tumor model in the present study.

Commercial interest is increasing in cultivating salt-resistant plants, collectively known as halophytes, for the extraction of useful phytochemicals to be used in nutrition, health, and medicine [28, 29]. Plants, other than halophytes, could be damaged by excessive release of reactive oxygen species (ROS), when they exposed to high salinities [29]. However, halophytes can tolerate high salinities through their flavonoids and phenolic phytochemicals which have potent antioxidant properties [30]. Generally, plant phytochemicals, especially phenolic ones, have potent anti-cancer effects through, at least in part, induction of cell cycle arrest, apoptosis, and inhibition of inflammation [31]. Arthrocnemum family is a large plant family that contains many perennial halophytes [32]. Of this family, A. indicum has high total polyphenol content enabling this plant to be used as a natural antioxidant and anti-cancer especially against colorectal cancer [33]. The coastal shrub A. machrostachyum (Moric.) K. Koch is another member of this family that abundantly grows in coastal zones of the Mediterranean basin, Middle East and, Asia [34]. Similar to A. indicum, A. machrostachyum also had higher phenolic contents and in vitro antioxidant properties [28, 35]. However, unlike A. indicum, the anticancer effect of A. machrostachyum has not studied yet. Moreover, as a limitation of Boulaaba, et al. (33) study, A. indicum anti-cancer effect was only evaluated in vitro on CaCo2 cells and to date, no information is available in the literature regarding the effect of these two plants on in vivo cancer model. Therefore, the present study was conducted to evaluate the antitumor activity of the A. machrostachyum against EST in mice and to illuminate the mechanism behind this effect.

**Methods**

**Plant collection and extract preparation**

*Arthrocnemum machrostachyum* plant was gathered from Siwa, Matruh, Egypt by Dr. Mohamed Abd El-Maboud, Desert Research Center, Egypt, and was identified by Dr. Omran Ghaly, Plant Taxonomy Department, Herbarium of Desert Research Center with Identification Code Number: CAIH-4-6-2015. The whole plant was washed, and shade dried for a week and was milled to the fine powder. The entire plant was cleaned and dried for 7 days and then was milled to get the powder form. The extract was obtained from the powder (20 g) using 80% methanol (200 ml). The obtained methanolic extracts were left for 2 days at 4 °C, filtered (using Whatman n°4 filter paper), evaporated, and dissolved in DMSO to get 2.5% stock concentration.

**Animals**

Male Swiss albino mice (20–25 g weight) purchased from National Cancer Institute, Cairo University were housed in plastic cages (7/cage) and maintained on laboratory standard experimental conditions (temperature 25–27 °C, relative humidity 55–60% with dark/light cycle 12/12 h, and free access to balanced diet and water). Mice were acclimatized to laboratory conditions for 1 w before experimentation. Animal’s procedures agreed with the international rules for the management and use of laboratory animals and followed Helsinki declaration of animal ethics which approved by Animal Ethical Committee of King Abdul Aziz University.

**Median lethal dose (LD50) determination**

The LD50 was determined as previously described [36]. Briefly, different concentrations of AME (300, 900, 1500, 2100, 2700, and 3300 mg/kg) were obtained by dissolving different weights of AME in 2% DMSO and phosphate buffer saline (PBS) followed by vortexing for 7 min until the solution became clear. A volume of 0.2 ml of each concentration was intramuscularly injected into 6 groups of mice (n = 5 mice/group), then mortality was recorded after 24 h.

**Experimental design**

The animals (n = 28) were randomly divided into four groups (n = 7/group). Group 1 was considered as a negative control group. In group 2 [Ehrlich ascites carcinoma (EAC) solid tumor (EST) group] mice were intramuscularly injected with a single dose of 0.2 mL EAC (2 × 10⁶ cells) in the femoral region at day zero (D0) [37] and by vehicle (DMSO-PBS) at D12 (the day at which the tumor was developed). In group 3 (low dose-treated group) mice were intratumorally injected with *A. machrostachyum* methanolic extract (AME, at a dose equals 1/10 LD50, 180 mg/kg body weight) at D12 trice.
weekly for 2 weeks. In group 4 (high dose-treated group) mice were treated as in group 3 but with a dose equals to 1/5 LD_{50}; 360 mg/kg body weight.

At the end of the experiment (D25), mice were euthanized (by decapitation under ether anesthesia) and the tumors were weighed and divided into the following 3 parts: the first part was preserved in 10% formalin (for histopathology), the second part was kept in −80°C (for real-time PCR and western blot), and the third part was frozen at −20°C (for Elisa and comet assay).

The tumor inhibition rate
The tumor inhibition rate was expressed according to the following formula: tumor inhibition rate (%) = (The mean tumor weight of control group – the mean tumor weight of treated group)/ (the mean tumor weight of control group) × 100.

Histopathological examination
The fixed tumors were embedded in paraffin and cut in 4 μm sections. The sections were stained with Hematoxylin and Eosin and then examined under a light microscope.

Determination of apoptosis-regulatory genes expression
Total RNA was isolated from tumor tissues using GeneJET RNA Purification Kit (Thermo Scientific, # K0731, USA) and CDNA was synthesized using Revert Aid H Minus Reverse Transcriptase (Thermo Scientific, # EP0451, USA) according to the manufacturer’s protocol. The primers sequences were as follows: p53 forward (F): 5′-CCCAGG TCCAGATGAAG-3′, and reverse (R): 5′-CAGACGG GAAACCGTAGC-3′; Bax forward (F): 5′-GGCTGAC ACCTGGACTTCT-3′, and reverse (R): 5′-GGTGGAG GACTCCAGGCCACCA-3′; Bcl2 F: 5′ CATGCCAAGAGG AAACACCGAGG AAACAACCGAA 3′ and R: 5′ GTGCTTTTGGATTCT TGGA TGAGGG-3′; caspase3 F: 5′ TCCATTATTCCAGGC TGGCACGG 3′ and R: 5′ TTCTGACAGCCCATGTCA TCTCTCA 3′; Cdc2 (Cdk1) 5′ AAGTGGGCCAGAA GTCGAG3′ and 5′ TGCTCCAGTTCTGTACGTG 3′; connexin26 (gap junction protein beta 2, Gjb2) 5′ CTCGAGGGTGTCACAAACA 3′ and 5′ CACGAG GATCATGATGCGGA-3′ and β-actin (internal reference) F: 5′ CTGTCCTCCTATGCTCCTG 3′ and R: 5′ ATGTCA CGCAGCATTTCC3′. Quantitative real-time PCR (qPCR) was performed using QuantiTect SYBR Green qPCR Master Mix in a StepOnePlus real-time PCR system and the melting curve condition and fold change calculation were done as previously described [38].

Western blot
Western blot was used to determine changes in the expression of p53, Bcl2, and caspase 3 proteins following different treatments using a method as detailed by El-Magd, et al. [39]. In brief, after lysis of the tumor (using RIPA buffer), the obtained proteins were separated using SDS-PAGE gels. Following plotting on polyvinylidene fluoride membrane, the primary antibodies p53 (1:150; Bioworld), cleaved caspase 3 (1:100; Abcam), and Bcl2 (1:100, Santa Cruz Biotechnology) were incubated with the secondary antibody (1: 5000; Santa Cruz Biotechnology.) for 1 h at room temperature before color development using tetramethylbenzidine (Sigma, St. Louis, MO, USA). The band density was normalized by β-actin and was quantified using Image J software.

Single cell gel-electrophoresis (comet assay)
Comet assay was performed to detect the effect of different treatments on DNA fragmentation of tumor tissues and was done as previously described [40, 41]. The DNA samples were stained with GelRed stain which gave red color under a fluorescent microscope. DNA damage parameters including tail length of the comet (degraded DNA), percentage of degraded DNA, and tail moment were evaluated using Komet 5 image analysis software (Kinetic Imaging, Liverpool, UK). Approximately, 50 to 100 cells were analyzed per sample.

Determination of TNFα level by Elisa
Collected tumor samples were homogenized and then centrifuged at 7000 rpm for 10 min at 4°C. The supernatant was used for the determination of tumor necrosis factor alpha (TNFα) using the mice TNFα ELISA kit (ab100747; Abcam) according to the manufacturer’s instructions. The detection limit was 1 pg/mL and intra-assay and inter-assay precision were 7 and 9%, respectively.

Statistical analysis
One-way ANOVA using GraphPad Prism 7 was used to determine the difference between the groups. Comparison of means was carried out with Tukey’s Honestly Significant Difference test. Data were presented as a mean ± standard error of the mean (SEM) and significance was declared at P < 0.05.

Results
Effect of A. machrostachyum on tumor growth
As detailed in Table S1, the calculated intramuscular LD_{50} value of AME was 1800 mg/kg. Intratumoral injection of AME into EST-bearing mice significantly reduced tumor weight in a dose-dependent manner with a size of 2.85 ± 0.06 g and tumor inhibition rate of 22% in low dose [180 mg/kg, body weight (bw)]-treated group and 1.96 ± 0.05 g and tumor inhibition rate of 46% in high dose (360 mg/kg bw)-treated group as compared to the untreated EST group (3.68 ± 0.17 g, P ≤ 0.05, Table 1).
Effect of *A. machrostachyum* on tumor cell histology

The histological examination of tumor masses in EST group showed anaplastic cells with mitotic figures (yellow arrow) with no evidence of cell necrosis or apoptosis (yellow arrow, Fig. 1a, b). However, treated groups showed clear features of necrosis (including pyknotic and karyolitic nuclei, and homogenous acidophilic cytoplasm) (dashed line in Fig. 1c) and apoptosis with the appearance of a large amount of subcutaneous adipose tissue (yellow arrow, Fig. 1d).

Effect of *A. machrostachyum* on cell cycle regulatory and apoptotic-related genes expression

In order to determine the expression level of apoptotic markers in treated tissues, the mRNA levels of *p53*, *Bax*, *caspase 3*, and *Bcl2* were evaluated in tumor tissues by qPCR after treatment with AME. *P53* expression was up-regulated in tumor tissues, with 3.21-fold in the low dose-treated group and 7.92-fold in the high dose-treated group as compared to the untreated EST and negative control groups (Table 2). The expression of the other two apoptotic genes was also up-regulated (2.43-fold and 3.85-fold for *Bax* and 1.92-fold and 2.8-fold for *caspase 3*) following treatment with AME at low and high doses, respectively. In contrast, *Bcl2* expression was markedly down-regulated in the tumor after AME administration at a low dose (0.26-fold) and high dose (0.10-fold) relative to untreated EST and negative control groups (Table 2). Additionally, treatment with 180 mg/kg and 360 mg/kg doses of AME significantly increased *Cdc2* (1.83-fold and 2.08-fold) and *connxin26* (1.94-fold and 2.87-fold) genes expression, respectively as compared to the other two groups (Table 2).

Effect of *A. machrostachyum* on apoptosis-related proteins

Western blot data revealed that treatment with AME at the two doses significantly increased *p53* and *caspase 3* protein expression and significantly decreased *Bcl2* protein expression relative to the other two groups (Fig. 2, Fig. S1). In all cases, high dose-treated mice showed significantly higher *p53* and *caspase 3* expression than low dose-treated animals. However, there was no significant difference in *Bcl2* expression between low and high doses.

### Table 1 Effect of *A. machrostachyum* extract on tumor size

| Group                        | Tumor size (g) | Inhibition % |
|------------------------------|----------------|--------------|
| EST                          | 3.68 ± 0.17 a  |              |
| *A. machrostachyum* (180 mg/kg) | 2.85 ± 0.06 b  | 22%          |
| *A. machrostachyum* (360 mg/kg) | 1.96 ± 0.05 c  | 46%          |

Data are expressed as mean ± SEM (*n* = 7). Values with different superscript letters [a (the highest value) – c (the lowest value)] in the same column are significantly different at *p* < 0.05. All groups were compared to each other.

**Fig. 1** Light micrographs of the tumor tissues sections stained with H&E in (a and b) the EST group, (c) low dose AME-treated group (180 mg/kg b.w.), and (d) high dose AME-treated group (360 mg/kg b.w.). X400 in A, B, D and X200 in C. Yellow arrow in (a) refers to anaplastic cells with mitotic figures, while dashed line in (c) outlines the necrotic area, and yellow arrow in (d) refers to subcutaneous adipose tissue.
Effect of *A. machrostachyum* on DNA fragmentation

AME induced considerable DNA fragmentation in tumor tissues as indicated by increased tailed DNA %, untailed DNA %, tail DNA%, tail length and tail moment as compared to untreated EST group and negative control (muscular tissues) group (Table 3 and Fig. 3). Again, the high dose-treated group showed a higher level of DNA damage. However, no significant difference was noticed between negative control and untreated EST groups.

Effect of *A. machrostachyum* on the level of TNFa in tumor tissues

The level of TNFa in tumor of animals treated with AME was significantly diminished, with lowest level in the high dose-treated group, as compared to the untreated EST group (Table 4). However, this reduced level remained higher than that in the muscular tissue of the negative control mice.

Discussion

This study aimed to assess the potential anticarcinogenic effects of the Egyptian medicinal plant *A. machrostachyum* against Ehrlich solid tumor (EST) in mice. To the best of our knowledge, this may be the first study to report an anti-cancer effect for *A. machrostachyum* methanolic extract (AME) against EST. This overall conclusion was based on the findings that treatment with *A. machrostachyum* resulted in: 1) decrease in tumor volume; 2) increase in tissue necrosis and apoptosis (as confirmed histologically); 3) down-regulation of Bcl2 gene and protein; 4) up-regulation of p53 Bax, and caspase3 markers; 5) upregulation of Cdc2 and connexin26 genes; 6) increase in DNA damage; and 7) decrease in TNFa level in tumor tissues.

Inhibition of apoptosis is considered as an important initial step in tumorigenesis. This is crucial for cancer cells to maintain their uncontrollable proliferation. Therefore, triggering apoptosis is a main mode of action for most of the anti-cancer agents [16, 17, 19, 42–45]. In the present study, *A. machrostachyum* extract reduces EST size through induction of apoptosis as evidenced histologically (presence of apoptotic cells), and molecularly (up-regulation of the apoptotic genes p53, Bax, and caspase 3, and downregulation of the anti-apoptotic gene Bcl2 as well as increase DNA damage). This effect was confirmed at protein levels of p53, Bcl2, and caspase 3 via western blot and the obtained results were consistent with that of the qPCR. In agreement, it was reported that *A. indicum* had an anti-cancer effect against CaCo2 cells and this effect was attributed to the high total polyphenol content [33]. Similarly, extracts and active compounds from other medicinal plants also induced apoptosis in HepG2 cells via a similar molecular pathway (downregulation of Bcl2 and upregulation of p53 and caspase 3) [46, 47]. Shivaprakash, et al. (4) also reported that *Caesalpinia bonducella* plant reduced Bcl2 expression and increased Bax expression in Ehrlich ascites tumor cells. *Atriplex halimus* was also reported to induce the upregulation of p53 and Bax and the downregulation of Bcl2 genes and proteins [46].

A large body of studies focused on the essential role played by p53 in the balance between apoptosis and proliferation [48]. The p53 gene controls the cell cycle through inhibition of the G2/M transition to prevent any possible deleterious effect post that point. Upon damage to DNA in cells, p53 causes cell cycle arrest and triggers DNA damage repair through its exonuclease activity and activation of DNA repair genes to ensure genomic stability [49]. If DNA damage is not remedied, p53 would trigger the transcription of the apoptotic genes [50]. In the mitochondria-dependent apoptotic pathway, induction of Bax stimulates the release of cytochrome c to the cytoplasm where it activates caspase 3 cleavage to produced cleaved caspase 3 which is considered as the end results of apoptosis [51]. This apoptotic effect can be hindered by the release of anti-apoptosis markers (Bcl-xL, Bcl2, survivin) [52] through stabilization of the mitochondrial membrane [53].

The obtained results showed that *A. machrostachyum* extract induces cell cycle deregulation as revealed by the upregulation of the cell cycle regulatory genes, Cdc2 and connexin26. In consistence, *A. indicum* causes cell cycle arrest of cancer cells on the G2/M phase [33]. Cdc2 (Cdk1) controls the G2/M

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**Table 2** Effect of treatment with *A. machrostachyum* at two doses on cell cycle regulatory and apoptosis-related genes as assessed by qPCR

| Group                  | P53            | Bax            | Caspase3   | Bcl2       | Cdc2       | Connexin26 |
|------------------------|----------------|----------------|------------|------------|------------|------------|
| Negative control       | 1 ± 0.38 c     | 1 ± 0.25 c     | 1 ± 0.29 a  | 1 ± 0.20 a  | 1 ± 0.24 a  | 1 ± 0.24 a  |
| EST 180 mg/kg          | 2.31 ± 0.41 a  | 2.31 ± 0.41 a  | 1.92 ± 0.12 b | 0.26 ± 0.03 b | 1.83 ± 0.10 a | 1.94 ± 0.11 b |
| EST 360 mg/kg          | 7.92 ± 0.35 a  | 3.85 ± 0.15 a  | 2.8 ± 0.15 a | 0.10 ± 0.01 c | 2.08 ± 0.09 a | 2.87 ± 0.14 a |

Data are expressed as fold change mean ± SEM (n = 7). Values with different superscript letters (a (the highest value) – c (the lowest value)) in the same column are significantly different at p < 0.05. All groups were compared to each other for each gene.
transition of the cell cycle through participating in nuclear envelope disintegration, and the formation of the mitotic spindle [54, 55]. Some traditional Chinese plant extracts were reported to upregulate the expression of Cdc2, p53, and caspase 3 in human nasopharyngeal carcinoma cells [50]. However, some other
natural products such as Safranal, a main component of saffron, mediated HepG2 cycle arrest at the G2/M phase through, at least in part, inhibition of Cdc2 expression [55]. Following translation, the putative tumor suppressor connexin26 gene produces a gap junction protein. Overexpression of connexin26 resulted in apoptosis (as indicated by the downregulation of Bcl2) and G2/M cell cycle arrest of prostate cancer cells [56]. Connexins, including connexin26, mediate the passage of apoptotic markers intracellularly and extracellularly through both gap junction and hemichannel in the cell membranes of cells (reviewed in [57]).

In the present study, *A. machrostachyum* extract reduced the pro-inflammatory cytokine TNFa in the tumor tissues in a dose-dependent manner. The survival of cancer cells is accomplished by the maintenance of inflammatory conditions [58, 59]. Cancer cells can directly cause inflammation by producing pro-inflammatory cytokines, or indirectly by causing physical tumor damage in ordinary tissue leading to hypoxia [60].

Herein, I only studied the effect of the whole extract of *A. machrostachyum* on EST as an in vivo cancer model, which was a limitation to our research. Many cancer cell types, a wider range of models, and active

### Table 3 Effect of *A. machrostachyum* extract on DNA fragmentation as revealed by comet assay

| Group                     | Tailed DNA (%) | Untailed DNA (%) | Tails length (μm) | Tail DNA (%) | Tail moment |
|---------------------------|----------------|------------------|-------------------|--------------|-------------|
| Negative control          | 2              | 98               | 1.73 ± 0.09 c     | 1.72         | 2.98        |
| EST                       | 3              | 97               | 2.19 ± 0.11 c     | 1.9          | 4.16        |
| *A. machrostachyum* (180 mg) | 7             | 93               | 3.64 ± 0.23 b     | 2.93         | 10.67       |
| *A. machrostachyum* (360 mg) | 15            | 85               | 6.34 ± 0.35 a     | 4.42         | 28.02       |

Tail length data are expressed as mean ± SEM (n = 7). Different superscript letters [a (the highest value) – c (the lowest value)] in the same column of tail length showed significance difference at *P* < 0.05. All groups were compared to each other for tail length.
principles of the plant could be investigated in further studies.

Conclusions
To the best of our knowledge, this may be the first study to report an anti-cancer effect for *A. machrostachyum* methanolic extract against Ehrlich solid tumor. This effect could be mediated through induction of apoptosis (as revealed by upregulation of p53, Bax, caspase3, Cdc2 and connexin 26, and reduction of Bcl2 gene/protein expression), and inhibition of inflammation (as evidenced by the reduction of TNFα levels). In this study, empirical evidence is provided supporting the use of *A. machrostachyum* extract in the treatment of cancer after the application of further confirmatory clinical trials.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12906-020-02947-y.

Additional file 1: Table S1. Median lethal dose (LD50) determination for *Arthrocnemum machrostachyum* methanolic extract. Figure S1. Full blots of proteins.

Abbreviations
A. indicum: Arthrocnemum indicum; A. machrostachyum: Arthrocnemum machrostachyum; AME: A. machrostachyum methanolic extract; ANOVA: Analysis of variance; Bcl2: B-cell lymphoma 2; Cdc2: Cell division control protein 2; DMSO: Dimethyl sulfoxide; EAC: Ehrlich ascites carcinoma; EST: Ehrlich solid tumor; LD50: lethal dose 50; PBS: phosphate buffer saline; SDS-PAGE: Sodium dodecyl sulfate-Polyacrylamide Gel Electrophoresis; SEM: Standard Error of the mean; TNFa: Tumor necrosis factor alpha.

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Author’s contributions
I am the sole author for this manuscript. The author(s) read and approved the final manuscript.

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

Table 4 Effect of *A. machrostachyum* extract on the level of tumor TNFa of tumor bearing mice

| Group                          | TNF-α (pg/g tumor) |
|--------------------------------|-------------------|
| Negative control               | 33.97 ± 4.15 d    |
| Untreated control EST          | 120.95 ± 5.71 a   |
| *A. machrostachyum* (180 mg)  | 88.31 ± 4.56 b    |
| *A. machrostachyum* (360 mg)  | 58.79 ± 3.82 c    |

Results are expressed as mean ± SEM (n = 7). Values with different superscript letters [a (the highest value) – d (the lowest value)] in the same column are significantly different at p < 0.05. All groups were compared to each other.

Ethics approval and consent to participate
The protocol for the mouse studies was approved by the Ethics Committee for Animal Care and Use, King Abdul Aziz University, Saudi Arabia. All animal procedures were performed in compliance with institutional guidelines.

Consent for publication
Not applicable since the manuscript does not involve human subjects.

Competing interests
The authors report no conflicts of interest in this study.

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