Efficacy of hydroethanolic extract of Randia aculeata seed against the southern cattle tick Rhipicephalus (Boophilus) microplus (Acari: Ixodidae) on naturally infested cattle under field conditions

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Research Article

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Abstract

Rhipicephalus (*Boophilus*) *microplus* tick infestation is a major problem for cattle industry in tropical and subtropical regions. Chemical products are commonly applied to control it; however, their indiscriminate use has resulted in the appearance of resistant lineages. Plants have been used as an alternative to conventional acaricidal drugs. Recently, we demonstrated the higher acaricidal activity of hidroethanolyc extract of *Randia aculeata* seed (EHRA) against *R. microplus* under laboratory conditions. The utility of EHRA seed as acaricidal need to be determined under field conditions. For this reason, the aim of this study was (a) evaluate the efficacy of the EHRA against *R. microplus* by sprayed on naturally infested calves under field conditions (b) determine the effect of the EHRA seed on AChE activity in larvae *R. microplus* and (c) evaluate the stability of total phenolic compounds in EHRA after exposure to heating, UV irradiation and storage. Fourty-five male calves were divided in three groups and doused by spray G1: water, G2: EHRA 20% and G3: coumaphos 0.3%. AChE activity on *R. microplus* larvae was determined by a colorimetric assay. Total phenolic content was determined using the Folin-Ciocalteau method after to exposure of EHRA to heat, UV light and storage under sunlight and dark. Significantly fewer number of ticks were observed after 24 h on the treated group compared to control group ($p < 0.001$). EHRA significantly inhibited *in vitro* acetylcholinesterase activity in *R. microplus* larvae at all tested concentrations ($p < 0.01$). Heat, UV light and storage time under sunlight resulted in a significant decrease ($p < 0.05$) in total phenolic content. Our results contributed new data for the elucidation of the mechanisms of EHRA acaricide action and to further evaluate the use as a new alternative control agent against *R. microplus* under *in vivo* conditions

Introduction

Rhipicephalus (*Boophilus*) *microplus* tick infestation is a major problem for cattle industry in tropical and subtropical regions. This tick is an obligate hematophagous ectoparasite that can spread through hosts while their movement and transmits several pathogens such as *Babesia bovis*, *Babesia bigemina* and *Anaplasma marginale* (Silva-Lima et al. 2018). Infestation to cattle results in several economic losses due to health deterioration, weight loss, blood loss, decreases milk production and treatments (Rodriguez-Vivas et al. 2018). Traditional control methods, using chemical compounds such as organophosphates, formamidines, pyrethroids and phenylpyrazoles, have been used in tick infestation. However, the excessive and uncontrolled use of these acaricidal agents leads to the development of resistance lineages (Abbas et al. 2018). This has resulted in a search for safe alternatives as plant extracts that constitute a potential source of secondary metabolites (Ouedraogo et al. 2021). In recent years, many plants are subject to increased attention as potential agents with acaricidal activity (Ghosh et al. 2013; Adenubi et al. 2016; Rosado-Aguilar et al. 2017). Recently, we demonstrated the higher acaricidal efficacy of hidroethanolyc extract of *Randia aculeata* seed (EHRA) against larvae and engorged female of *R. microplus* under laboratory conditions (Bravo et al. 2021). This plant species, known in southern Mexico as “crucetillo” is used by population mainly against the effects of snake bites and other poisonous insects (Gallardo-Casas et al. 2012). Previous phytochemical studies carried out by Juarez-Trujillo et al.

(2018) revealed the presence of secondary metabolites such as phenolic compounds which are potential acetylcholinesterase (AChE) inhibitors (Rollinger et al. 2004). Carbamate and organophosphate compounds have the same mechanism of action, based on the inhibition of acetylcholinesterase (AChE) in the nervous system (Tan et al. 2011). Additionally, different authors have been report that several secondary metabolites are capable to inhibited AChE activity in homogenates pools from *R. microplus* larvae (Ribeiro et al. 2012; dos Santos-Cardoso et al. 2020). On the other hand, the utility of EHRA seed as botanical acaricidal in the context of efforts to alternative control of *R. microplus* need to be determined under field conditions since that phenolic compounds concentration and their biological activity may be changed by environmental variables as well time storage. For this reason, the aim of this study was (a) evaluate the efficacy of the EHRA against *R. microplus* by sprayed on naturally infested calves under field conditions (b) determine the effect of the EHRA on AChE activity in larvae *R. microplus* and (c) evaluate the stability of total phenolic compounds in EHRA after exposure to heating, UV irradiation and storage time to contribute new data for the elucidation of the mechanisms of acaricide action and to further evaluate the use of EHRA as new alternative control products against *R. microplus* under *in vivo* conditions.

Material And Methods

Material and extract preparation

R. aculeata fruits were acquired from local markets in the municipality of Veracruz, Mexico, in November 2021. The taxonomic identification was confirmed by a member of the Herbarium of the Facultad de Ciencias Biologicas y Agropecuarias, Universidad Veracruzana, and deposited under the registration number JLBT2 (VER). Fruits were washed with distilled water and subsequently, each part of the fruit was separated to obtain the seeds. Seeds were cleaned and air dried for 7 days at room temperature and then pulverized. The extract was prepared at 1:10 (w/v) ratio by adding the hydroethanolic solution (EtOH–H_2O, 80:20) to the powdered seed. Extraction was carried out by maceration at room temperature. Each 24 h for 3 days, the contents were allowed to settle. Then, the solvent was collected and filtered. The extract was reduced under vacuum using a Buchi® Roto-Vapor at 26 °C. Finally, the concentrated extracts were lyophilized and stored.

Study area

Field study was conducted from March-April 2022 on a farm located in the municipality of Las Choapas, Veracruz, Mexico. The climate is characterized as humid tropical, with rainy in summer and a mean temperature above 28°C in all months throughout the year (INEGI, 2019).

Animals

Holstein × Zebu (3/4 Ho × 1/4 Z) male calves were selected from a local farmer who volunteered to participate in the experiment. All animals were naturally infested with *R. microplus* ticks. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. No

approval was required. Anesthesia, euthanasia, or any other kind of animal sacrifice was not a part of the study.

Acaricides

For the larval packet test (LPT) and adult immersion test (AIT), technical grade acaricides were dissolved in distilled water to prepare stock solutions of 20 mg/ml. The acaricides were as follows: amitraz 12.5 % (OP); fipronil (PYZ), flumethrin (SP), ivermectin (ML), moxidectin (ML).

Bioassays

Previously to field trial, *R. microplus* resistance status in cattle farm was determinate. The LPT and was used according to the protocol of the FAO (2004) and adapted for amitraz (Miller et al. 2002).

Larval packet test

Engorged females of *R. microplus* were collected from naturally infested cattle, washed with water and dried. Then, ticks were incubated at 27 °C and 70–80% relative humidity for 14 days to obtain egg mass. For experimental bioassays, 14-day old larval ticks were used. LPT was used for each treatment according to the FAO (2004) guidelines; three filter papers (85 mm x 75 mm) were impregnated using a 670 µl acaricide solution. After 24 h of impregnation, larvae were put inside the packets and sealed using clips; the packets were subsequently incubated in BODs. After 24 h, mortality was verified. The formula used to identify acaricide efficacy was as follows:

% mortality = [(% mortality of the treated group - % mortality of the control group) / (100 - % mortality of the control group)]

Adult immersion test

The adult immersion test (AIT) was performed followed the recommendations of Drummond et al (1973); FAO (2004) and Sharma et al. (2012). Groups of ten engorged *R. microplus* females that were weighed prior to the test and then submerged in each acaricide solution for five minutes. The ticks were then removed, dried, and placed in Petri dishes that were maintained in at 28 °C with a relative humidity of 80 % for 14 days. Subsequently, the eggs were weighed and allocated in syringes sealed with cotton and maintained in BODs until the larvae hatched for evaluation.

Efficacy trial of EHRA applied by sprayed

The experimental design was established by counting ticks between 4.5 and 8 mm (Wharton and Roulston, 1970) over the entire surface of both sides of the animals on the two days prior to experimental treatment. Ticks were recorded and used for the randomization and assignment of cattle to each treatment group to homogenize infestation levels among the experimental groups. On the day of treatment, forty-five male calves were divided into three groups with fifteen animals each: G1: untreated

control-water; G2: extract solution of EHRA seed 20%; G3: coumaphos 0.3 %. The G1 cattle were treated first to avoid contamination of water. Then, G2 were sprayed with the *R. aculeata* 20% solution and finally G3: were sprayed with the coumaphos 0.3 % solution. About 3–4 L of the EHRA solution was satisfactory to spray on one animal in each group. A 150 g bathing soap was added to each 20 L of the EHRA solution as a sticking agent. A hand-pump s was used to apply EHRA solution on the whole body of the animals while restrained in standing position. Then, calves were allowed to graze freely. The animals were observed for one hour after treatment and then at a daily basis for the presence of adverse events. Tick counts were performed on animals at beginning on the day after treatment (Day 1) and continuing through (Day 21) post-experimental treatment.

In vitro AChE activity from larvae of ticks

For experimental bioassays, 14-day old larval ticks from the same cattle farm were used. Pools of 100 mg of *R. microplus* larvae were homogenized in a volume (1:10) of cold 50 mM phosphate buffer (pH 7.0) containing 0.5% Triton-X 100. Then, homogenate was centrifuged at 2500 × *g* for 10 min at 4 °C. Subsequently, supernatants were used as the enzyme source. AChE activity was determined by slight modifications of the colorimetric assay described by Ellman et al. (1961) and Baxter et al. (1999) using acetylthiocholine iodide (1 mM) as substrate. The EHRA (final concentrations 1.5, 3 and 6 mg/mL) was incubated at 25 °C for 60 min with the enzyme source. Concentrations were decided according to previous studies by our research group (Bravo et al. 2021). Absorbance was measured at 412 nm, and AChE activity was estimated through differences in dA/min. Each sample was assayed in triplicate.

Storage and stability of total phenolic compounds in EHRA under different conditions

After to obtain the EHRA, the total phenolic content was determined using the Folin-Ciocalteau method (Zieliński and Kozłowska, 2000) with slight modifications. Absorbance was measured at 725 nm using a microplate reader (Synergy 171 H1, Biotek). Results were expressed as mg of gallic acid equivalents (GAE) per dry extract (Margraf et al. 2015). For thermal stability, hydroethanolic extract of EHRA in flask tubes with screw caps were placed in a water bath at 100 °C for 15 min. Regarding to UV stability, extract solution in glass open cuvettes, were placed under UV light (LB 301.1 BAKMED lamb, 315. nm, 2.1 mW/cm2) for 1 h. For storage stability, extract in flask tubes with screw caps were stored for 3 months under light and dark conditions at the same room temperature, to evaluate the influence of light. Illuminated samples were exposed all the time to sunlight. In the same room dark samples were stored in a closet (Bąkowska et al. 2003). After of each assay total phenolic content was measure in triplicate.

Statistical analysis

For the spray efficacy trial, data on tick number obtained from animals in the G1, G2, and G3 treatments were analyzed by the Kruskal-Wallis and Wilcoxon tests. For the AChE activity the results were expressed as median (25th/75th of percentiles) values. The pattern of distribution was assessed before statistical testing. Kruskal–Wallis followed by Dunn's multiple comparison test was used8. Student's *t*-test was used

to analyze the data obtained in the storage and stability experiments. Significance was assumed as $P < 0.05$. Statistical analysis was performed using SigmaPlot v. 12.0.

Results

Rhipicephalus microplus resistance test

For acaricides evaluated using the AIT and LPT *R. microplus* strain tested are susceptible to all acaricide classes used in the assay (Table 1).

Efficacy trial of EHRA applied by sprayed

Regarding to assay, no adverse effects or signs of systemic intoxication were observed in calves after experimentally administration of treatment. EHRA solution 20 % concentration gave a reduction in tick counts after application. Figure 1 showed the efficacy on reduction in tick load infestation on cattle through day 21. The observed tick reductions were found to be statistically significant when compared to control group ($p<0.001$). Further, there was a significant reduction in tick loads on animals within 24 h after treatment.

In vitro AChE activity from larvae of ticks

The effect of *EHRA* on AChE activity is showed in Figure 2. The results demonstrated that this extract reduced AChE activity. The inhibition was significant at 1.5, 3 and 6 mg/mL to larvae *R. microplus* when compared to control group (*p<0.01).*

Storage and stability of total phenolic compounds in EHRA under different conditions

In the present study, the analysis of EHRA quantified high levels of total phenolic content (154.89 mg GAE/g extract). On the other hand, the results showed that heat, UV light and storage time under sunlight resulted in a significant decrease ($p<0.05$) in total phenolic content, except in storage in a dark room (Fig.3).

Discussion

In Mexico, the *R. microplus* resistance pattern has been previously described to several acaricidal classes (Miller et al., 1999; Rodriguez-Vivas et al., 2006, 2007; Li et al., 2007; Rosado-Aguilar et al., 2008; Perez-Cogollo et al., 2010; Miller et al., 2013; Higa et al. 2020). On the other hand, natural products have been widely used in the world showing that it is a good alternative even together with chemical acaricides helping to control ticks. Several plants species have been described with acaricidal properties based on laboratory assess but few of these have been validated using environmental conditions (Klafke et al. 2021; Lee at al. 2022). Regarding this, we tested EHRA in a natural setting and were able to confirm the higher acaricidal *in vivo* activity. This effect may be related to the synergism among the different compounds in the EHRA (Rosado-Aguilar et al. 2017; Bravo et al. 2021). On the other hand, phenolic

compounds are the major phytochemicals responsible of biological properties in plants (Derakhshan et al. 2018). In this study, the total phenolic content of the EHRA was determined, however, the phenolic composition of the extracts was not analyzed as it was not within the scope of present investigation. Further work in this area is currently in progress. Currents studies have been reported that the main phenolic compounds in *Randia* genus is acid chlorogenic acid, rutin, 4-coumaric acid, and caffeic acid. Other phenolic compounds, such as ferulic acid, kaempferol, vanillic acid, quercetin, (-)-epicatechin, 4-hydroxybenzoic acid, quercetin, vanillin, 2,4-dimethoxy-6-methylbenzoic acid and scopoletin were found in lower concentrations (Juarez-Trujillo et al. 2018). For this reason, EHRA can be used as a good alternative in field for integrated management of ticks impacting livestock health and production. However, several environmental variables such as heat, UV light or storage stability can degrade secondary metabolites present in EHRA, thereby decreasing the acaricidal activity. In this study, EHRA were subjected to heat treatment at 100°C (15 min), which resulted in a significant decrease of total phenolic content. We infer that he decreases in total phenolic content in EHRA might be due to the formation of novel compounds having prooxidant activity upon heat processing (Javanmardi et al. 2003). Regarding to influence of storage time, the sunlight had a degradation influence on total phenolic content in EHRA after 3 months. These results are suported by the results of Kapoor (2018) who reported that in samples kept in the presence of light the concentration of total phenolic content decreases more strongly than in samples kept in the dark. After 1 h UV irradiation, a decreased in total phenolic content were observed in EHRA; is well known that UV irradiation induce changes in gene expression enhancing synthesis of UV-absorbing pigments (Csepregi et al.2017), and other changes that modify the metabolic profile of plant tissues (Hectors et al. 2014). On the other hand, our data suggest that EHRA acts as an AChE inhibitor. The AChE inhibition in the larvae of *R. microplus* was significant at all concentrations tested (1.5, 3 and 6 mg/mL). We hypothesized that the extract toxicity was associated with the presence of scopoletin that have been reported as a potential acetylcholinesterase (AChE) inhibitor (Rollinger et al. 2004) or the synergism among the different compounds. To the best of our knowledge, we herein report the first findings on cholinesterase inhibitory activity of *Randia acueleata.* We can suppose that this effect may be related to its tick's toxicity. Moreover, isolation and determination of EHRA secondary metabolites is not exhausted at this point and it is important to find out what or which molecules are responsible for inhibitory AChE properties. Cattle could be sprayed with EHRA and other plant extracts as part of a rotational schedule involving several acaricidal agents with different modes of action (Klafke et al. 2021). This approach would help manage the grand challenge of resistance to multiple classes of acaricides among populations of several tick's species in some parts of the world (Rodriguez-Vivas et al., 2018). Finally, in the present study we can concluded that if spraying of EHRA is performed correctly as recommended, this method remains an alternative for *R. microplus* control. Additionally, is needed continued research of EHRA related to storage stability, action mechanism at molecular level and find best application mode comparing pour on and spray performance to contribute development of novel and safer natural acaricide.

Declarations

Declaration of Competing Interest

The authors report no declarations of interest

CRediT authorship contribution statement

Jose L. Bravo-Ramos: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Maria G. Sanchez-Otero:** Conceptualization, Methodology, Investigation. **Sokani Sanchez- Montes:** Writing - original draft, Supervision. **Angelica Olivares-Muñoz:** Methodology, Investigation. **Dora Romero-Salas:** Writing - original draft, Supervision, Project administration, Funding acquisition.

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Tables

Table 1. Larval packet test and adult immersion test efficacy using *Rhipicephalus microplus* strain from the southern region of Veracruz, Mexico

Acaricide efficacy %					
Bioassay	Amitraz 12.5 % (OP)	Fipronil (PYZ)	Flumethrin (SP)	Ivermectin (ML)	Moxidectin (ML)
LPT	92.1 %	93.2%	88.2%	86.1 %	83.5 %
AIT	88.4 %	91.2	84.3 %	80.5 %	78.4 %
LPT: larval packet test; AIM: adult immersion test					

Figures

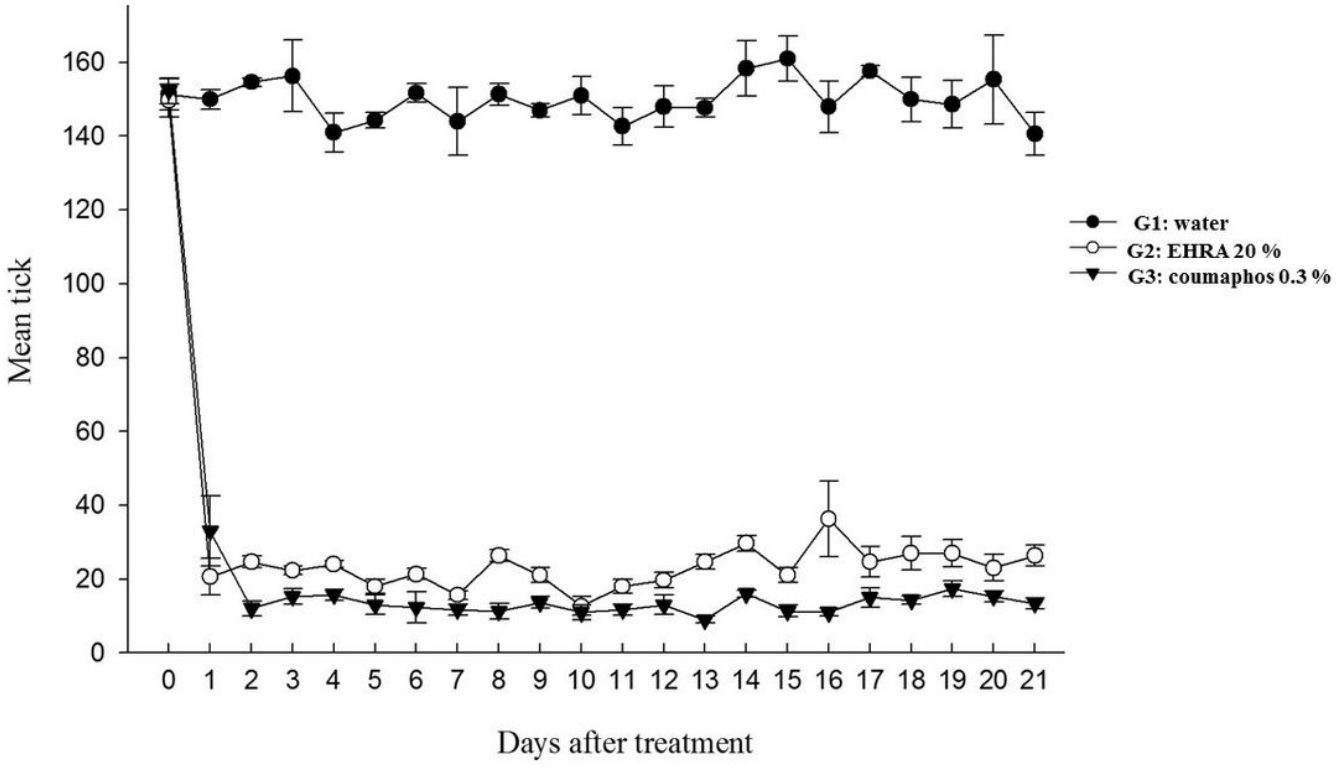

Figure 1

Mean number of *R. microplus* ticks on cattle after sprayed treatment with water, EHRA and coumaphos 0.3 %.

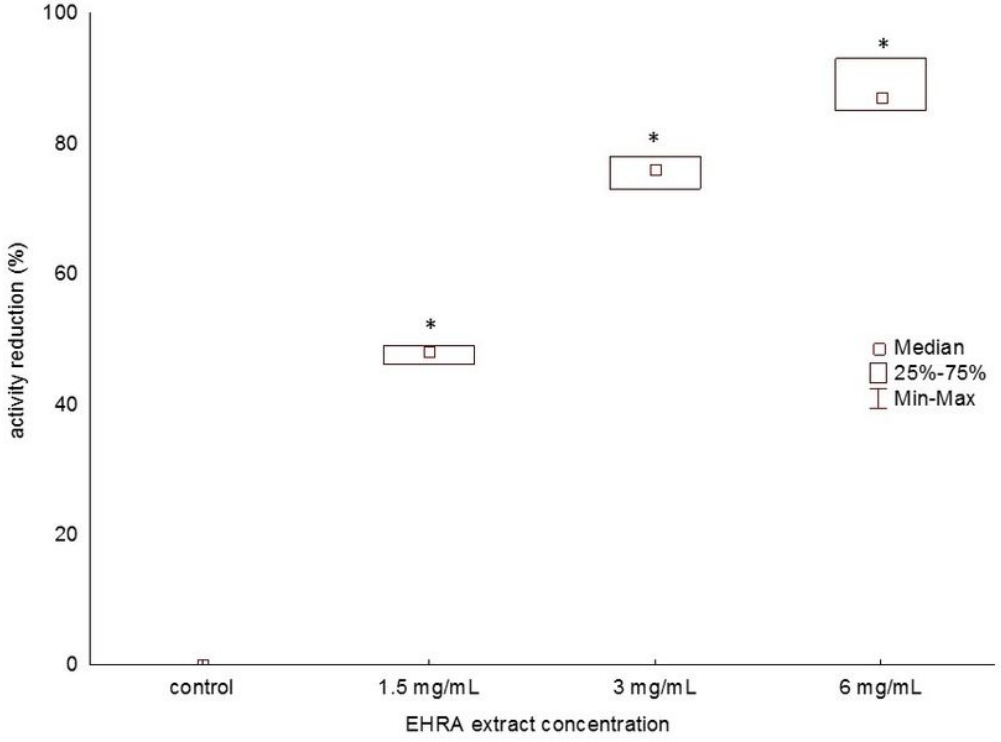

Figure 2

Effect of *EHRA*(1.5, 3 and 6 mg/mL) on AChE activity reduction in the larvae of *R. microplus*. AChE activity reduction (as percentage of the control group) after 60 min of incubation. Values significantly different from control group; *$P < 0.001$

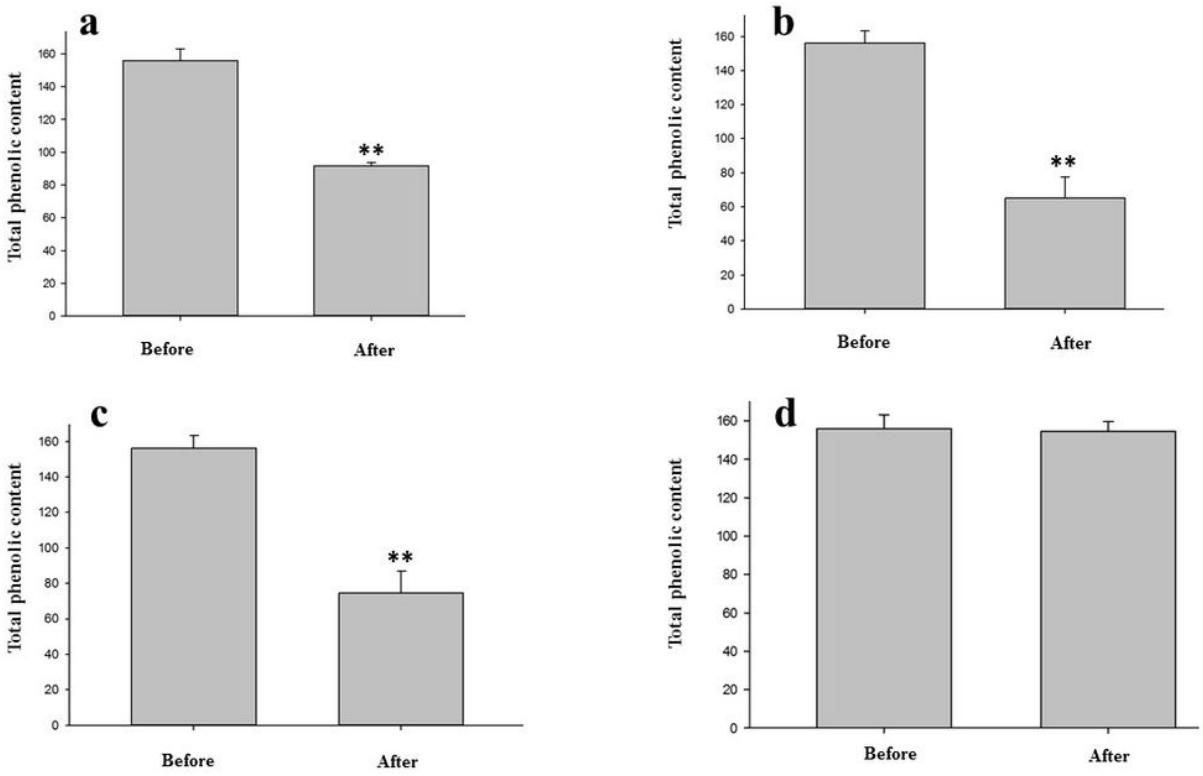

Figure 3

Total phenolic content before and after exposure to **a)** heat (100°C); **b)** UV light and stored 3 months **c)** under sunlight; **d)** dark room. Values statically significantly $*P < 0.001$