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Implication of Aqueous Extract of *Origanum majorana* and/or *Vitex agnus-castus* Mitigates Consequences of Thermal Stress on Productive and Reproductive Traits of Rabbit Does

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ABSTRACT

This study aimed to investigate the roles played by two common herbs in mitigating the adverse effects of thermal stress and enhancing the productivity of rabbit does. Thirty-two Californian rabbit does were equally allotted into four groups. Group 1 (C) served as control, given the basal diet without oral supplement; group 2 (OM) animals were given aqueous extract of *Origanum majorana*; group 3 (VAL) were given extract of *Vitex Agnus-Castus*, and group 4 (OM+VAL) were given the combination of both herbal extracts. All treated does received the extract for 11 days before insemination. Blood samples for plasma were collected at the end of the treatment from 3 does within the treatment. Results exhibited increases of estradiol, progesterone, FSH, and LH, while insulin decreased in supplemented does as compared with the control. All productive traits (i.e., kindling rate, litter size and weight at birth and weaning, survival at birth and weaning, and milk yield) expressed higher ($P<0.05$) values in treated does than in control does. Total protein, albumin, IgG, IgM, total antioxidant capacity (TAC), and HDL were higher ($P<0.05$) in treated than in control does. Contrariwise, levels of LDL, TG, TC, MDA, and ALT were lower ($P<0.05$) in the treated than in the control. The treatment did not alter total globulin levels or AST activity. In conclusion, inclusion of herbal (i.e., *Origanum majorana* and *Vitex Agnus-Castus*) extracts in the diet of rabbits living in hot climates would not only protect the animals from the adverse thermal burden, but it would also maintain and even enhance their survival and productivity.

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INTRODUCTION

The shortage of animal protein in developing countries is a well-recognized issue. Several factors contribute to this problem, including extended production cycles, inadequate nutrition and fodder, shortage of water, limited access to genetically improved breeding stock, harsh climates, and the prevalence of diseases. As a result, ruminant production, which includes cattle, sheep, and goats, has not been able to fully address the gap in protein supply **(Makkar, 2018).** The high rate of reproduction, rapid and impulsive maturity, high potential for genetic selection, efficient use of feed and land, relatively little competition for human food, and high-quality, nutritious meat are all significant factors contributing to rabbits' advantages **(Lukefahr and Cheeke, 1991).** The need to find out several natural feed additives that can be utilized in rabbits' diets to enhance their performance was a good alternative **(Ewuola *et al.*, 2011).**

However, research suggests that hormones and chemicals are considered harmful pollutants that might ultimately damage human health **(Omer *et al.*, 2013).** Several research attempts have shown that supplementing medicinal plants and herbs to the diets of sheep, cows, buffaloes, birds, and rabbits improved their productivity **(Salem and El-Mahdy, 2001).** On the other hand, the species' vulnerability to heat stress (HS) represents the most salient impediment to rabbit production in hot climates **(Ondruska *et al.*, 2011).** Due to their high susceptibility to HS, rabbits are believed to have a considerable impact on fertility **(Yagci *et al.*, 2006).** Consequently, rabbit raisers attempt to mitigate

the negative effects of the hot season by optimizing environmental elements. Heat stress is defined as a stressor that is induced by various environmental factors, resulting in physiological disturbances within an animal's body and compromising its capacity to regulate its body temperature passively **(Boni, 2019).**

Animal exposure to elevated ambient temperatures, high humidity, low wind speeds, and high levels of direct and indirect solar radiation constitutes the primary cause of this condition **(Willmer *et al.,* 2000).** Besides, the Lamiaceae family includes marjoram (Origanum majorana L.), also known as sweet marjoram. Many people have used this herb for culinary and medicinal purposes **(Ghazal *et al.,* 2022).** Its antibacterial and antioxidant properties are the basis for its numerous industrial uses **(Amor *et al.,* 2019).** Because of its spasmolytic, anti-rheumatic, diuretic, and anti-asthmatic properties, marjoram essential oil, dried leaves, and extracts have been used to treat digestive, respiratory, and urinary tract disorders **(Bina and Rahimi, 2017).**

Numerous pharmacological effects, such as antibacterial, antioxidant, cardioprotective, hepatoprotective, antiulcer, anti-inflammatory, anticoagulant, antifungal, and gastric secretory activities, have been shown by studies on O. majorana **(Ghazal *et al.,* 2022).** Moreover, abundant research has highlighted the essential oil's remarkable antifungal and antibacterial activity against Gram-positive and Gram-negative bacteria strains, in addition to specific fungi **(Amor *et al.,* 2019).** The marjoram is also abundant in flavonoids and phenolic compounds, exhibiting significant antioxidant activity. Marjoram extracts have been observed to markedly enhance the cellular antioxidant capacity in animal models **(Pimple *et al.,* 2012).** In concordance with that, marjoram essential oil reduced inflammation in rabbits and mitigated the renal damage caused by ivermectin through its levitation antioxidant capabilities **(Elmahdy and Alkalamawy, 2022).**

Additionally, *Origanum majorana* essential oil has enhancing roles on the lipid fractions by increasing HDL and decreasing LDL in monogastric animals **(Maral *et al.,* 2021).** *Vitex agnus-castus,* also known as the chaste tree or Vitex, is a diminutive shrub or tree that climbs up to six feet in height. It is native to western Asia and the Mediterranean, even though it is particularly prevalent throughout Egypt, where it is cultivated in a variety of gardens. *Vitex agnus-castus L.* is a remarkable traditional herbal medicine plant belonging to the *Verbenaceae* family **(Kamal *et al.,* 2022).** The plant is employed in the treatment of a variety of reproductive system conditions, including premenstrual syndrome (PMS), abnormal menstrual cycles, amenorrhea, mastodynia, hyperprolactinemia, premenstrual dysphoric disorder, and lactation difficulties **(Hoberg *et al.,* 2001; Niroumand *et al.,* 2018)**. Vitex chemical composition is dominated by iridoid glycosides, flavonoids, diterpenes, and volatile oil. The plant's chemical composition has been found to include a combination of iridoids and flavonoids, which have been observed to exhibit structural similarities to sex hormones **(AbdAlmajeed, 2007).** Similarly, **Hoberg *et al.,* (2000)** referred to the fact that rich phytochemicals found in *Vitex agnus-castus* include flavonoids, alkaloids, diterpenoids, agnuside, p-hydroxybenzoic acid, and steroidal hormone precursors**.** It is hypothesized that these substances exert a normalizing or balancing effect on reproductive hormone production **(Schellenberg, 2001).** Therefore, this study aimed to explore the roles played by *Origanum majorana* and *Vitex Agnus-Castus* in mitigating the thermal stress on the productivity and reproductive performance of rabbit does.

MATERIALS AND METHODS

Area of study

Farm work was conducted at a private farm in Qalyubia governorate, north of Egypt. The laboratory duties were done at Al-Sabahia station, East Alexandria, Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt.

Meteorological parameters

The overall means of ambient temperature (AT), relative humidity (RH), and temperature-humidity index (THI) during the whole experimental period (July-August) were 30.91±0.14◦C °C, 72.01±0.64%, and 29.47±0.11, respectively. These summer months are considered the hottest months of the year in the country of Egypt **(Table 1).**

Table 1: The mean values of ambient temperature (AT), relative humidity (RH), and temperature–humidity index (THI) during the trial duration (July-August).

Parameter	July	August	Overall
AT (◦C)	31.09±0.15[a]	30.73±0.21[a]	30.91±0.14
RH (%)	72.10±0.88[a]	71.91±1.07[a]	72.01±0.64
THI	29.65±0.11[a]	29.31±0.16[a]	29.47±0.11

[a,b,c] Values with different superscript within the same row, significantly differ (P<0.05).

Herbal extracts preparation

The *Origanum majorana* and *Vitex* leaves were rinsed with tap water, dried at room temperature in the dark for 5 days, then ground to a powder using an electric blender. Two hundred and fifty grams of each herb was placed in 1000 ml of boiling distilled water for four to five days in a glass flask (1L) with continuous stirring. Afterward, the mixture was filtered through filter paper (Whatman No.1). The aqueous filtrate was concentrated by evaporation, and the yield of *Origanum majorana* and Vitex powder was stored in a refrigerator at 4°C until used **(Méabed *et al.,* 2018).** The main active constituents in both herbs are listed in **Table 2**.

Table 2: Bioactive components of *Vitex agnus-castus* and *Origanum majorana* L extracts.

V. agnus-castus flower leaves (Ethyl acetate extract)		
Total Phenolics (µg GAE/mg)	Total Flavonoids (µg QE/mg)	Total Flavonols (µg QE/mg)
203.00± 1.86	47.36± 0.76	37.20± 1.80
Origanum majorana (aqueous extract)		
Total Phenolic in Gallic acid equivalent (mg of GAE/g dry weight of the extract	Total Flavonoid in (mg Quercetin /g of the extract)	DPPH, 2.2-Diphenyl-1-picrylhydrazylscavenging activity (EC50 in mg/ml)
9.2 ± 0.3	57.3 ± 0.2	12.34±0.1

Méabed *et al.,* 2018; Berrani *et al.,* 2021.

Animal housing, management and experimental design

The experiment was conducted during July-August 2023, and ethical approval was obtained from the Animal Care and Use Committee of the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Egypt. Thirty-two does (7 months old) of the Californian rabbits were used in this study for three consecutive parities. The rabbits were divided into four groups. All experimental animals were healthy and were kept under the same managerial, hygienic, and environmental conditions throughout the experimental period. They were individually housed in galvanized wire batteries provided with feeders and automatic stainless-steel nipples for supplying free access to clean and fresh water. The doe's batteries were also provided with a parturition and rearing nest box measuring 50×35×35 cm (L×W×H). All batteries were located in an open area rabbitry. The animals were exposed to the ambient environmental temperature, a photoperiod of 16h light and 8h dark, and ventilation by windows and ceiling electric fans. All rabbits were fed on basal diets, and G2, G3, and G4 were orally supplemented with the designed herb extract (OM, VAL, and OM+VAL, respectively) for 11 days before insemination, following the recommendation of **Al-Kushi (2014).** Control (C) animals were given the basal diet alone. Then, each doe that exhibited the signs of receptivity was artificially inseminated by a pool of semen from untreated bucks. All inseminated does were palpated 14 days post-insemination to determine pregnancy. In case of pregnancy failure, artificial insemination was repeated, keeping in mind that the herbal supplementation wasn't repeated. Five days before kindling, does were provided with access to nest boxes attached to each cage and supplied with fine rice straw to provide comfortable and warm bedding for kindling and rearing bunnies.

Basal diet: A balanced pelleted ration was used *ad libitum* to meet the reproductive demand of animals. Chemical analysis of the diet **(Table 3)** was determined according to the **AOAC (2005)**.

Experimental groups

1- **Group 1 (**C, n=8**):** does were fed *ad libitum* with basal diet serving as a control group.
2- **Group 2 (**OM, n=8**):** does were fed basal diet plus 3ml OM/doe daily*.
3- **Group 3 (**VAL, n=8**):** does were fed basal diet plus 3ml VAL/doe daily.
4-**Group 4 (**OM+VAL, n=8**):** does were fed basal diet plus 3ml of the combination OM +VAL/doe daily.
*Groups G2, G3, and G4 animals were orally given the designed aqueous extract once daily for 11 days until the day of insemination.

Table 3: The composition and chemical analysis of the basal experimental diet.

Ingredient	%	Calculated analysis			
Yellow corn	6.22	Crude protein, %		18.8	
Soybean meal, 44%	22.33	Crude fiber, %		13.0	
Wheat bran	23.33	Ether extract, %		3.0	
Barley	15.00	Digestible energy (kcal/kg diet)		2680	
Alfalfa hay	30.12	n-6 poly unsaturated FAs%		0.3	
Ground limestone	1.00	n-3 poly unsaturated FAs%		1.03	
Dicalcium Phosphate	1.20	Determined analysis (g/kg)			
Common salt	0.50	Dry matter	897.1	Crude fiber	138.5
Vitamin premix[a]	0.30	Organic matter	801.4	Ether extract	26.2
Total	100.00	Crude protein	169.8	Nitrogen-free extract	575.0
				Ash	87.9

[a]Each 3 kg of premix contains: Vit.A: 12, 000,000 IU; Vit.D3: 3, 000,000 IU; Vit.E: 10.0 mg; Vit.K3: 3.0 mg; Vit.B1: 200 mg: Vit.B2: 5.0 mg Vit.B6: 3.0 mg: Vit.B12: 15.0 mg; Biotin: 50.0 mg; Folic acid: 1.0 mg; Nicotinic acid: 35.0 mg: Pantothenic acid: 10.0 mg; Mn: 80 g; Cu: 8.8 g; Zn: 70 g; Fe: 35 g; I: 1 g; Co: 0.15g and Se: 0.3g.

Artificial insemination of does

Semen ejaculates were individually evaluated microscopically, and only those with active progressive motility percentages greater than 70% were pooled and extended in TRIS extender. The final concentration of motile sperm was 80-100 million sperm per ml. Does were inseminated using a multiple-diluted heterospermic pool (1 part semen: 3 parts extender) collected from 16 fertile bucks. Artificial insemination (AI) was performed by depositing 0.5 ml of fresh diluted semen deeply into the vaginal region using a sterile catheter. Following insemination, 0.8 µg of buserelin (0.2 ml Receptal, a GnRH analog, Hoechst, Frankfurt, Germany) was administered intramuscularly to induce ovulation **(Lopez and Alvariño, 2000).**

Reproductive traits

The reproductive traits tested include: kindling rate, litter size, viability, and litter weight at birth and weaning in all groups.

Kindling rate

Kindling rate was defined by dividing the number of does that gave birth by the total number of bred does ×100 **(El-Speiy *et al.,* 2024).**

Litter size and litter weight at birth and weaning

The litter number and weight of pups at birth and weaning were determined by counting and weighing pups after kindling and at weaning, respectively. The survival rate was defined by dividing the number of pups born and weaned alive at 28 days of age on the total number of pups born **(El-Speiy *et al.,* 2024).**

Blood collection and biochemical attributes determinations

At the end of the female treatment for 11 days before insemination, blood samples were taken from the marginal ear vein in 5 ml heparinized test tubes at 9.00 h from three does within each group and centrifuged at 3000 rpm for 20 minutes. Plasma samples were harvested and stored frozen at -20ºC until analyses. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using the method established earlier by **Reitman and Frankel (1957).** Total protein (TP) levels were determined following the protocol outlined by Song et al. in 2023. Albumin (Alb) levels were quantified based on the method developed by **Doumas and Watson (1971)**; however, globulin was estimated by the difference. Total lipids and triglyceride (TG) levels were assessed using the method described by **Fossati and Prencipe (1982)**. The determinations of total cholesterol (TC), high-density lipoprotein (HDL), and low-density lipoprotein (LDL**)** were calculated using the formula LDL (mg/dl) = Total cholesterol - {HDL + (TG/5)}, which was explained by **Bachorik (1989)**. All biochemical analyses were performed using commercial kits (Human, Liquicolor, Germany).

Hormone assays

All samples were analyzed in one assay for each hormone; thus, no inter-assay CVs were estimated.

Progesterone (P4) in plasma was determined by **Simersky *et al.,* (2007)** using a commercial ELISA kit (Diagnostics Test Canada, Inc., Ontario, Canada). The intra-assay coefficient of variation (CV) was 6.5%. Plasma estradiol-17ß (E2) was analyzed by the method of **Ratcliff *et al.,* (1988)** using an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostics Test Canada, Inc., Ontario, Canada), having an intra-assay CV of 5.2%. Plasma FSH was determined by a commercial ELISA kit (MyBioSource, San Diego, CA, USA) with an intra-assay CV of 8.6%. Plasma LH values were determined by a commercial ELISA kit (My BioSource, San Diego, CA, USA), having an intra-assay CV of 6.5%. Plasma insulin samples were determined according to **Frier *et al.,* (1981)** by rabbit insulin ELISA kit (DRG Diagnostics, Germany). Intra-assay coefficient of variation was 2.2%.

Determination of antioxidant activity

The total antioxidant capacity (TAC) and levels of malondialdehyde (MDA) in plasma were measured using commercial kits with a spectrophotometer (GNW-Model: SM-721), following the methods described by **Gawel *et al.,* (2004).**

Immunoglobulin determination

Rabbit immunoglobulins G (IgG) and M (IgM) levels were assessed using commercial ELISA kits (Abcam Limited, Cambridge, UK). The sensitivity of the IgG assay was 0.23 ng/mL and an intra-assay coefficient of variation (CV) of 6.5%, while the IgM assay had a sensitivity of 0.19 ng/mL and an intra-assay CV of 7.2%.

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Milk yield estimation

Milk yield for each doe was recorded from parturition until 28 days of lactation. For doing this, bunnies were separated from their dams at 8.00 pm; thereafter, the bunnies were allowed to suckle at 8.00 am the next day. Milk production was calculated as the average difference between the weight of each doe and their bunnies before and after suckling (weight-suckling-weight method) as described previously **(Davies *et al.,* 1964).**

Statistical analysis

The statistical model used was: Data were analyzed using general linear model procedure of SAS software **(SAS, 2002).** Difference among treatment means were tested for significance using Duncan's Multiple Range Test **(Duncan, 1955).**

$Yijk = \mu + Ti + eijk$

Where: Yijk = the observation, μ = Overall mean, Ti = Treatment effect, eijk = Experimental error, associated with i, j and k observations assumed to be randomly distributed.

RESULTS

Reproductive hormones and insulin profiles

As shown in **Table 4**, all treatments significantly ($P < 0.05$) elevated E2, P4, FSH, and LH but diminished insulin levels. The improvement of the E2 secretion in does that were given herbal extracts ranges between 24.91% and 58.39% over the control values. Moreover, the increase of progesterone ranges between 85.45% and 87.32% over control, with the highest value resulting from OM+VAL extract. Likewise, both FSH and LH were elevated in the three herbal groups. The level of increase ranges between 15-19% for FSH and 8-13% for LH. However, insulin decreased between 22.55% and 43.89% in treated groups compared with the control.

Table 4: Effect of aqueous extracts of *Origanum majorana* and *Vitex* on plasma hormones of Californian rabbit does raised under thermal stress (n=3/G).

Item	Treatment					
	C	OM	VAL	OM+ VAL	SE	P-value
E2 (pg/ mL)	37.85^{d}	47.28^{c}	57.42^{b}	59.95^{a}	0.72	≤0.0001
P_4 (ng/mL)	1.42^{b}	2.52^{a}	2.68^{a}	2.66^{a}	0.07	≤0.0001
FSH (mIU/mL)	4.44^{b}	5.11^{a}	5.24^{a}	5.30^{a}	0.14	≤0.0001
LH (mIU/mL)	6.83^{c}	7.41^{b}	7.71^{a}	7.74^{a}	0.10	≤0.0001
Insulin (μIU/mL)	7.45^{a}	5.77^{b}	4.58^{c}	4.18^{c}	0.11	≤0.0001

a,b,c,d Values with different superscripts in the same row differ significantly ($P<0.05$). C: control; OM: *Origanum majorana*; VAL: Vitex; OM+VAL: *Origanum majorana* + *Vitex*. E2: Estradiol17β; P4: Progesterone; LH: luteinizing hormone; FSH: follicle-stimulating hormone.

Reproductive performance of does

Table 5 summarizes data of reproductive traits in control and treated rabbit does. Percentage of doe kindling increased in treated females by 9.7-12.7% over control. Litter size at birth and weaning significantly (P<0.01) increased in treated does compared with control does. The percentage of the increase in litter size at birth due to treatment ranges between 40.6% and 46.9%, with the highest live pups' outcome in does that are given the two-herb combination. Based on the fact that weaning was achieved at 28 days of age, survival at weaning increased (P<0.01) by about 49% in offspring produced by does that supplemented with herb extracts compared with control counterparts. Total litter body weight at weaning of treated does exceeds (P<0.01) that derived from control does by 41.4-82.9%, with the OM+VAL combination extract producing the heaviest weight at weaning. Moreover, milk yield and milk conversion ratio improved significantly (P<0.01) at weaning in treated does.

Table 5: Effect of *Origanum majorana* and *Vitex* aqueous extracts on reproductive traits of Californian rabbit does exposed to heat stress (n=8/G).

Item	Treatment					
	C	OM	VAL	OM+VAL	SE	P-value
Kindling %	77.83^{c}	85.37^{b}	85.87^{b}	87.73^{a}	0.513	≤0.0001
Litter size at birth (n)	6.19^{b}	8.70^{a}	8.85^{a}	9.09^{a}	0.225	≤0.0001
Litter size at weaning (n)	5.77^{c}	7.79^{b}	8.42^{a}	8.62^{a}	0.11	≤0.0001
Litter weight at birth (g)	357.22^{d}	415.15^{c}	448.94^{b}	514.89^{a}	4.84	≤0.0001
Kid weight at birth (g)	61.75^{a}	53.37^{c}	50.91^{d}	57.10^{b}	0.71	≤0.0001
Litter weight at weaning (g)	1859.85^{c}	2630.43^{b}	3311.58^{a}	3401.39^{a}	48.72	≤0.0001
Kid weight at weaning (g)	322.33^{b}	337.66^{b}	393.30^{a}	394.59^{a}	4.56	≤0.0001
Milk yield at d 28 (g)	3750.10^{c}	4521.28^{b}	4564.51^{b}	5213.76^{a}	64.47	≤0.0001
Milk conversion ratio*	2.49^{a}	2.06^{b}	1.62^{d}	1.81^{c}	0.045	≤0.0001

a, b, c, d Values with different superscripts in the same row differ significantly (P<0.05).C: control; OM: *Origanum majorana*; VAL: *Vitex* extracts; OM+VAL: *Origanum majorana* + *Vitex* extracts; *Milk conversion ratio = g milk/g weight gain.

Biochemical attributes

Table 6 demonstrates notable (P<0.01) increases in total protein (TP), these increases range between 18.3-20.4% over control. Similarly, albumin (Alb) levels increased (P<0.01) over control by 29-31%. However, no differences (P>0.05) were detected in globulins among groups. In contrast, ALT levels significantly (P<0.05) decreased in does that administered herbal extracts with a range of reduction between 19.5-23.5%. While, AST activity was relatively similar among groups.

Table 6: Effect of aqueous extracts of *Origanum majorana* and *Vitex* on the blood Biochemical compounds of Californian rabbit doe's exposed to heat stress (n=3/G).

Item	Treatment					
	C	OM	VAL	OM+VAL	SE	P-value
Total protein (g/dL)	5.53^{b}	6.57^{a}	6.54^{a}	6.66^{a}	0.11	≤0.0001
Albumin (g/dL)	2.89^{b}	3.73^{a}	3.75^{a}	3.79^{a}	0.09	≤0.0001
Globulin (g/dL)	2.64	2.84	2.79	2.87	0.13	≤0.068
ALT(U/L)	33.48^{a}	26.96^{b}	25.69^{b}	25.61^{b}	0.46	≤0.0001
AST (U/L)	13.50	13.53	13.72	13.85	0.20	≤0.089

a,b Values with different superscripts in the same row differ significantly (P<0.05). C: control; OM: *Origanum majorana*; VAL: *Vitex*; OM+VAL: *Origanum majorana* + *Vitex*.

Lipid profile

Table 7 illustrates data on lipid fractions. All treatments improved lipid profiles by reducing total cholesterol, triglycerides, and LDL, while elevating HDL. The reduction rates in treated rabbits for bad lipid fractions amounted to 13.7, 26.6, and 51.3% for total cholesterol, triglycerides, and LDL, respectively. Whereas, the increase of the HDL in the herbal supplemented does approached 49% over the control.

Table 7: Effect of aqueous extracts of *Origanum majorana* and *Vitex* on the blood plasma lipid profiles of Californian rabbit does exposed to heat stress (n=3/G).

Item	Treatment					
	C	OM	VAL	OM+VAL	SE	P-value
TC (mg/dL)	92.27^{a}	78.59^{b}	78.06^{b}	79.02^{b}	0.198	≤0.0001
TG (mg/dL)	98.63^{a}	72.88^{b}	72.03^{b}	71.99^{b}	0.493	≤0.0001
HDL (mg/dL)	28.69^{b}	41.80^{a}	42.10^{a}	42.87^{a}	0.560	≤0.0001
LDL (mg/dL)	44.27^{a}	22.39^{b}	21.55^{b}	21.75^{b}	0.905	≤0.0001

^{a,b}Values with different superscripts in the same row differ significantly (P<0.05). C: control; OM: *Origanum majorana*; VAL: *Vitex*; OM+VAL: *Origanum majorana* + *Vitex*. TC: Total cholesterol, TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

Immunoglobulins and antioxidants

Table 8 presents data on immunoglobulins and antioxidant bioindicators. All herbal extracts enhanced (P<0.05) immunoglobulin fractions. The IgG improvement surpassed the control by a range of about 74-88%. However, the IgM levels improved by about 22.4-29% above the control. Moreover, TAC as an indicator of the anti-oxidation biomarker remarkably (P<0.05) increased due to treatments. The improvement in TAC ranges between 33.07% and 33.07%-37.01% above control values. Contrariwise, levels of malondialdehyde (MDA) were significantly (P<0.01) reduced by about 35% below the control.

Table 8: Effect of aqueous extracts of *Origanum majorana* and *Vitex* on the blood immune-globulins and antioxidant indicators of Californian rabbit does exposed to heat stress (n=3/G).

Item	Treatment					
	C	OM	VAL	OM+VAL	SE	P-value
IgG (ng/mL)	38.44^{c}	66.87^{b}	67.40^{b}	72.30^{a}	0.558	≤0.0001
IgM (ng/mL)	72.69^{c}	88.98^{b}	89.05^{b}	93.74^{a}	0.523	≤0.0001
TAC (µmol/ml)	1.27^{b}	1.69^{a}	1.68^{a}	1.74^{a}	0.023	≤0.0001
MDA (nmol/mL)	0.48^{a}	0.34^{b}	0.33^{b}	0.31^{b}	0.015	≤0.0001

^{a,b}Values with different superscripts in the same column differ significantly (P<0.05). C: control; OM: *Origanum majorana*; VAL: *Vitex*; OM+VAL: *Origanum majorana* + *Vitex*. IgG: Immunoglobulin G, IgM: Immunoglobulin M; TAC: Total antioxidant capacity; MDA: Malondialdehyde.

DISCUSSION

The THI obtained in the present study indicates that the doe rabbits were suffering from severe HS, according to **Marai *et al.,* (2002)** who stated that exposing female rabbits to a temperature-humidity index (THI) below 30 impairs their ability to reproduce and lessens their vulnerability to illness. The percentages of conception, embryonic development, litter size, litter weight, and milk production were all found to be lower in female rabbits exposed to HS. Additionally, it has been demonstrated that HS delays the age of puberty and increases pre- and post-weaning kit mortalities **(Marai *et al.,* 2002).** Moreover, HS impaired rabbit immunity via increased oxidation parameters; in addition, it impaired the reproductive traits, fertility, and physiological characteristics of female rabbits **(Liang *et al.,* 2022).** Likewise, **Marai *et al.,* (2004)** found that female rabbits raised in a hot climate in Egypt had lower milk yields on day 7 of suckling and lower milk consumption per kit at days 7 and 14 postpartum. Elevated temperatures also negatively deteriorate oogenesis, particularly during meiotic telophase and metaphase II, which impacts the quality of oocytes **(Liang *et al.,* 2022).** The detrimental effects of HS on animals are particularly tough to ameliorate in tropical and subtropical settings,

particularly when high humidity and high temperatures combine. Recently, **Fayed *et al.,* (2025)** reported the necessity for supplementing dairy cows with phytogenic sources to restore reproductive and lactational potential to the thermoneutral level. Moreover, in a study on broiler chicks, **Fayed *et al.,* (2024)** attributed the enhancement of immunity, gut health, and welfare of birds to the feed additive of *Terminalia bellirica* and *Andrographis paniculata*. To shield the rabbits from the negative effects of HS, breeders may need to use a variety of tactics, such as changing housing arrangements, modifying diets, and choosing animals that can tolerate high temperatures. Whether employed alone or in conjunction with other tactics, nutritional interventions are successful and profitable among the methods tried to lessen the detrimental effects of HS on animals **(Marai *et al.,* 2002).**

Bioactive components include medicinal herbs that have positive biological effects on animal health **(Lamson *et al.,* 2020).** It has been shown that the active ingredients in medicinal herbs, such as flavonoids and flavones, play vital antioxidant roles, leading to maintaining normal rectal temperature in rabbits exposed to a hot climate **(El-Ratel *et al.,* 2021).** Additionally, **Elspeiy *et al.,* (2020)** stated that utilizing *Vitex agnus* extract in the rabbits' diets would be a valuable tool for summer rabbit farming to maximize immunological response, physiological status, and growth performance.

The improvement in steroid and gonadotrophin hormones in the current study is in agreement with previous data reported by **Hussein and Alzubaidi (2022)**, who found that rats given OM revealed lower-than-control levels of prolactin accompanied by higher levels of FSH, LH, and progesterone. Also, **Rababah et al., (2015)** stated that giving female rats an alcoholic extract of OM increases the synthesis of steroid hormones in females, leading to higher levels of progesterone and estradiol. The main polyphenols in marjoram and Vitex are quercetin and caffeic acid, which have diverse health activities such as activating the cellular enzymes cytochrome P450 and 17 α-hydroxylase and the regulatory protein involved in steroidogenesis **(Hasegawa *et al.,* 2013).** Furthermore, **Haj-Husein *et al.,* (2016)** found that marjoram tea significantly reduced blood insulin. Besides, giving Marjoram **(Elfiky *et al.,* 2025)** or Vitex **(Saul, 2017)** extract to polycystic ovarian syndrome (PCOS) rats with hormonal irregularities resulted in the restoration of the normal levels of FSH and LH**.** Also, using Vitex tablets (Vitagnus®) was effective in reducing the hyperprolactinemic effects in women by increasing their blood levels of progesterone and estradiol. Moreover, **Soleymanzadeh *et al.,* (2020)** indicated that diabetic rats receiving Vitex fruit extract revealed higher levels of serum LH, FSH, estrogen, and progesterone compared with the control group.

Also, **Haerifar *et al.,* (2020)** reported that hyperprolactinemic women treated with Vitagnus significantly increased blood estradiol and progesterone. Surprisingly, Vitex produces large amounts of androgen, which turns into estrogen **(Ahangarpour *et al.,* 2017). Liu *et al.,* (2004)** also noted that polyphenols in Vitex can activate certain estrogen-inducible genes by binding to estrogen receptors.

Regarding rabbit does's productivity, **Lobna *et al.,* (2016)** observed that does that were supplemented with marjoram produced significantly larger litter sizes and weights at weaning (28 days of age) compared to the control does. Further, **Seleem *et al.,* (2007)** demonstrated that incorporating *Origanum majorana* into rabbit diets significantly increased litter size at birth, total litter weight, and milk yield per doe compared to the control does. Interestingly, medicinal plants exhibit lactogenic properties that effectively boost serum prolactin levels **(Gaya *et al.,* 2009).** In parallel, **Abd-El Ghany *et al.,* (2017)** found that incorporating Vitex significantly enhanced conception rates, litter size, and litter weight at birth and weaning. Furthermore, it reduced pre-weaning mortality and increased milk yield across three parities. Notably, it also boosts milk intake among kids during most lactation periods, resulting in higher individual bunny weights at weaning. Additionally, **Loch *et al.,* (2000)** stated that Vitex can aid in balancing hormones essential for milk production. As a result, this herb is thought to stimulate lactation by helping to regulate the progesterone-to-estrogen ratio. Likewise, **Basyony and Abdel-Khalek (2021)** found that when rabbit females were orally administered an extract of *Vitex agnus-castus* leaves, there was a notable increase in milk production (g/doe), as well as improvements in both litter size at birth and litter weight at weaning, in comparison to the control does.

Concerning liver functions, the present data agree with the findings of **Ahmed and Abdel-Ghany (2015),** who mentioned that feeding rabbits on *an Origanum majorana* diet increased cytochrome P450, 17 α-hydroxylase, and the regulatory protein involved in steroidogenesis; in addition, it increased albumin, globulins, and total protein, while AST activity didn't change. Also, **EL Bushuty and Shanshan (2012)** disclosed that rats treated with marjoram revealed lower ($P<0.05$) serum activities of ALT and AST compared with controls. Similarly, **Elspeiy *et al.,* (2020)** reported that administration of Vitex extracts for nine weeks improved total protein and globulins in growing male California rabbits. However, this effect was not

observed in mature female rabbits even after one month of supplementation **(Abd-El Ghany *et al.,* 2017).** Furthermore, **EL-Ashmawy *et al.,* (2005)** proposed that the presence of isoflavones, polyphenols, and other antioxidants in herbal plants may be responsible for the decrease in liver transaminases (ALT & AST) activity. Additionally, **Rodriguez-Meizoso *et al.,* (2006)** revealed remarkable reductions in the activity of serum aminotransaminases upon administration of marjoram in various forms (i.e., volatile oil, alcoholic, and aqueous extracts). However, broilers fed a marjoram diet showed non-significant increases in ALT and AST activities **(Osman *et al.,* 2010).**

The decline in ALT activity among the growing rabbits that received Vitex aqueous extract in the present study aligns with the findings in male California rabbits **(Elspeiy *et al.,* 2020)** but was denied by the lack of effect seen in mature female rabbits **(Abd-El Ghany *et al.,* 2017).** In the common health status of mammals, the normal AST/ALT ratio must not exceed 1.15, but the increase of this ratio is an indication of liver cell damage **(Lala *et al.,* 2023).** Apparently, in the control rabbits in the current study, the AST/ALT ratio approached 0.40; however, in the rabbits supplemented with herbal extracts, the ratio was 0.50, 0.53, and 0.54 for OM, VAL, and OM+VAL, respectively. In a rabbit clinical pathology study conducted previously, it has been reported that ALT is not the critical indicator in rabbit liver damage, like in other herbivores, keeping in mind that slightly elevated ALT is a common case in healthy rabbits **(Melillo, 2007).**

The data of lipid fractions are aligned with the results of **Mossa *et al.,* (2013)**, who found that incorporating *Origanum majorana* into the diets of growing rabbits reduced liver enzyme activity and improved plasma lipid profiles (i.e. TC, TG, HDL and LDL), suggesting that *Origanum majorana* possesses hypolipidemic properties that enhance tissue functionality. Also, **EL Bushuty and Shanshan (2012)** found that male rats given marjoram exhibited significant reductions in serum cholesterol, triglycerides, and low-density lipoprotein, while the levels of high-density lipoprotein increased when compared to the control. Additionally, **Nagm (2002)** stated that the administration of marjoram extract resulted in a notable decrease in triglyceride levels in treated rats compared to the control. Earlier, **Rang and Dale (1991)** suggested that the cholesterol-lowering effect of marjoram may be due to the presence of isoflavones, which inhibit cholesterol absorption in the intestine by competing for absorption sites. Recently, **Zeidabadi *et al.,* (2022)** tested the supplementation of Vitex to postmenopausal women and reported reductions in cholesterol, LDL, and TG, along with HDL increase. Similar findings using sage extract in type II- diabetic patients were stated by **Kianbakht *et al.,* (2016).** This reduction of lipid fractions may be attributed to the extract's potent ability to inhibit free radicals **(Kianbakht *et al.,* 2011).** The phytoestrogenic properties of flavonoids in these herbs mimic the natural estrogen hormone, which mainly reduces blood cholesterol **(Myasoedova *et al.,* 2016).** Furthermore, a study indicated that polypeptides, steroids, and flavonoids in herbs may have lipid-lowering effects **(Sedighi *et al.,* 2017).**

A proposed mechanism for the lipid-lowering effects of flavonoids includes the reduction of enzyme activity involved in cholesterol acyltransferase in hepatic cells, which is responsible for cholesterol esterification and storage **(Matralis and Kourounakis, 2014).** Likewise, **Elspeiy *et al.,* (2020)** found that supplementing weaned rabbits with Vitex led to significant decreases in blood TG, TC, and LDL. The increase of MDA is a strong indicator of the accumulation of reactive oxygen species, which is reduced in the present study by herbal extracts. Besides, **Liang *et al.,* (2022)** concluded that heat stress negatively affects immune function, mainly through the hypothalamic-pituitary-adrenal axis via stimulating the release of adrenal glucocorticoids, leading to the suppression of the immune system. Increased glucocorticoid levels have detrimental consequences on the cellular and humoral immune systems. Consequently, heat stress results in substantial reductions in rabbit production, since weakened immune function makes them more susceptible to pathogens **(Marai *et al.,* 2002).** The current study reveals that incorporating *Origanum majorana* into the diets of rabbit does results in higher levels of IgG and IgM, the indicators of cellular immunity, compared to the control animals.

Additionally, **Marai *et al.,* (2002)** indicated that the involvement of *Origanum majorana* in rabbit diets expressed significant enhancements in humoral immunity, as demonstrated by increased lysosomal activity and elevated amyloid A levels. Further, **Eklund *et al.,* (2012)** suggested that improved lysozyme activity could facilitate pathogen elimination due to its enzymatic degradation properties. Amyloid A also plays vital immunological roles, functioning as a chemotactic factor for mast cells and neutrophils while promoting cytokine production. **Aziz *et al.,* (2018)** highlighted that the beneficial effects of *Origanum majorana* on immune function can be attributed to its varied biological activities, which encompass anti-allergic, anti-inflammatory, antiviral, and antimicrobial effects. Supportive of our findings, previous studies indicated that rabbit diets supplemented with *Origanum majorana* revealed improved antioxidant ability with reduced oxidative stress and inflammatory indicators

(Farag *et al.*, 2023), in addition to increases of antioxidant enzymes such as GSH, with reductions of MDA and DNA oxidation **(Bina and Rahimi, 2017).** The possible mechanism of the phenolic compounds in these herbs is attributed to their ability to donate a hydrogen atom from the phenolic hydroxyl group, leading to scavenging excess free radicals under heat stress **(Mossa and Nawar, 2011).** Moreover, other active ingredients such as flavonoids, castein, orientin, and isovitexin were identified in *Vitex agnus-castus,* showing remarkable antioxidant and free radical scavenging properties **(Sarikurkcu *et al.*, 2009).** In agreement with this context, **Chen *et al.*, (2020)** concluded that supplementing Vitex extract to mice not only enhances the immune system but also improves animal health and well-being.

CONCLUSION

Under stressful circumstances in general, and heat stress in specific, it is advisable to supplement the animal diets with these herbs' extracts. Further studies are needed to widely interpret these phenomena by testing the roles of each individual ingredient in these herbs to confirm its precise mechanism(s) to alleviate the adverse surrounding elements in the field of animal production. Also, monitoring such a phenomenon in small ruminants would be helpful to interpret the adverse consequences on herbivores.

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Conflicts of interest

The authors declare that they have no competing interests.

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Data Availability

The data obtained in this study are available from the corresponding author upon a reasonable request.

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