# The synergistic effect of the combination of *Ocimum basilicum* and *Ceratonia siliqua* on inflammation and oxidative stress

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**Abstract.** The use of basil and carob in traditional medicine to address inflammation and oxidative stress reflects a long-standing practice rooted in folk medicine. This study was conducted to demonstrate the anti-inflammatory and antioxidant properties of a rosmarinic acid-rich extract of *Ocimum basilicum* (RAE) combined with a carob aqueous extract (CAE). Paw edema in rats and vascular permeability in mice were measured to investigate the anti-inflammatory effect. The assessment of lipoprotein oxidation was carried out by measuring thiobarbituric acid reactive substances as well as determining the scavenging activity towards lipoperoxyl radicals and 2,2-diphenyl-1-picrylhydrazyl (DPPH). The combination (200 mg/kg) significantly decreased carrageenan-induced rat paw edema and vascular permeability in mice ($p$<0.001), and these effects were comparable to those of the indomethacin drug (50 mg/kg). Moreover, the combination significantly prevented plasma lipoprotein oxidation and efficiently scavenged lipoperoxyl ($IC_{50}$= 160 ± 11 µg/ml) and DPPH ($IC_{50}$= 80 ± 4 µg/ml) radicals. The present study indicates the potential nutraceutical benefits of combining Basil and Carob in the treatment of inflammatory and oxidative stress-related diseases, which is correlated with the high contents of rosmarinic acid, phenolic acids, and fibres. ***Keywords*:** *Ocimum basilicum*, rosmarinic acid, Carob, inflammation, lipid oxidation.

## 1 Introduction

The primary pathological process in numerous diseases, particularly atherosclerosis and associated cardiovascular and cerebrovascular complications, is inflammation associated with oxidative stress [1]. Atherosclerosis is an inflammatory disorder initiated by endothelial damage resulting from the generation of free radicals, which play a role in the oxidation of low-density lipoprotein (LDL) [2]. The combined impact of inflammation and oxidative stress in atherosclerosis underscores the importance of addressing both factors in therapeutic interventions [3]. Therefore, the search for a new resource of anti-inflammatory and antioxidant substances could be of great interest for preventing a number of related diseases. In this regard, polyphenols from vegetable foods, spices and medicinal plants represent promising candidates for use as anti-inflammatory and anti-lipid oxidation agents [4]. Indeed, several epidemiological studies revealed the efficiency of dietary polyphenols in preventing pathological inflammatory and oxidative complications [5]. Acknowledging the significance of plants in the quest for novel and safer therapeutic agents, the screening of medicinal plants for their medicinal activities and phytochemical compounds is a prominent and globally active research field. It is important to note that traditional practices have a long history of using herbal medicines in combination with formulas [6]. This approach returns to the very beginnings of using plants for healing purposes. The therapeutic efficacy of complex herbal mixtures is increasingly attributed to synergistic interactions between their various components [7]. *Ocimum basilicum* L. is a rich source of polyphenols and is highly diverse. The genus *Ocimum* belongs to the Lamiaceae family, and several species of *Ocimum* have been used since ancient times to treat various illnesses. Currently, there is a growing interest in the bioactive compounds found in basil (*O. basilicum*) due to its traditional use in folk medicine.

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These compounds have been found to possess antihyperlipidemic, antiplatelet aggregation and vasorelaxant properties [8, 9]. According to Prinsi, et al. [10], rosmarinic acid is the most abundant phenolic acid identified in basil. Besides, the carob tree, *Ceratonia siliqua* (Fabaceae), is frequently used in traditional medicine [11]. Its high content of dietary fiber and phenolic components is mainly responsible for its remarkable nutritional value; it has been shown to have significant potential in preventing heart disorders and reducing serum cholesterol levels [12]. The study aimed to explore the potential of combining plant extracts (RAE+CAE) as a treatment against inflammation and lipid oxidation, using both *in vivo* and *in vitro* models.

## 2 Materials and methods

### 2.1 Plant material

*Ocimum basilicum* L. (Basil), which belongs to the Lamiaceae family, and *Ceratonia siliqua* (Fabaceae) were purchased from Oujda, Morocco, and were kindly subjected to authentication by Professor A. Khalil, Department of Biology, Faculty of Sciences, University Mohamed Premier. The Department of Biology has received voucher specimens with collection numbers LO-15 and CS-23 for the basil and carob, respectively.

### 2.2 Preparation of rosmarinic acid (RAE)-rich extract

*Ocimum basilicum* extract rich in rosmarinic acid (RAE) was prepared following previous methods reported by [13]. The final extraction yield was 6.47% w/w.

### 2.3 Preparation of carob aqueous extract (CAE)

The aqueous extract of carob (CAE) was prepared by infusing 100 grams of carob pod powder in 500 ml of boiling water for 30 minutes. The mixture was then centrifuged for 10 minutes at 12 000 (rotation per minute) rpm. The supernatants were collected and placed in a drying oven at 40°C to obtain the crude material. The extraction yield was 41.28%.

### 2.4 Preparation of rosmarinic acid-rich extract combined with carob aqueous extract

The two different extracts were combined in the same volume (1:1) for the combination study.

### 2.5 Estimation of total phenol content in the RAE and CAE

The total phenol content in the RAE and CAE was estimated colorimetrically by the Folin–Ciocalteu procedure with minor changes, as described previously [14]. The total phenolic content was expressed as mg rosmarinic acid/g dry extract. All measurements were performed in triplicate.

### 2.6 Estimation of flavonoid content in the RAE and CAE

The aluminium chloride reagent was used to assess flavonoids [15]. Five-millilitre samples were mixed with 2.5 ml of AlCl3. The yellow color of the samples was assessed after 10 *min*. at λmax = 430 nm *versus* a blank containing 5 ml of sample in addition to 2.5 ml of methanol. Flavonoid content was calculated using a rutin calibration curve and reported in milligrams of rutin per gram of extract. Each dose was prepared in triplicate.

### 2.7 Animal care and handling

*Wistar* rats and *albino* mice were raised in the animal house of the Faculty of Science (Oujda, Morocco). To ensure the care and handling of the animals, internationally accepted standardized guidelines for the use of laboratory animals were adopted (as approved by the local committee for the use of laboratory animals, Faculty of Medicine, with approval number: 002016).

### 2.8 Study of the anti-inflammatory activity of the combination in carrageenan-induced rat paw edema

The anti-inflammatory activity of the combination (RAE+CAE) was assessed using the carrageenan-induced rat paw edema assay, as established by Jisha, et al. [16]. Male *Wistar* rats weighing 150-200 g were divided into three groups of 6 animals each. Edema was produced by sub-plantar injection of 0.1 ml of a freshly prepared 1% solution of carrageenan in 0.9% saline solution into the right hind paws of rats in all the groups. The test group and reference group were treated 1 h before carrageenan injection with the combination (RAE+CAE at 200 mg/kg) and the standard anti-inflammatory drug indomethacin (50 mg/kg). Paw thickness was measured immediately before carrageenan injection, at 0 h and 2, 4, 6, 8, and 24 h later using a plethysmometer (Ugo Basile, Italy). The increase in paw thickness was measured as the difference between the values recorded at different times and those recorded at 0 hours. The paw edema rate and percentages of inhibition exerted by the combination and indomethacin were then calculated.

### 2.9 Study of the effect of the combination on mouse vascular permeability induced by acetic acid

This study was carried out according to the method described by Kou, et al. [17]. Three groups of five male *albino* mice weighing 26-31 g were used as the experimental model. Thus, one hour after oral administration of the combination at a dose of 200 mg/kg, animals in the first group received a single intraperitoneal injection of 0.7% acetic acid solution (10 ml/kg) to increase vascular permeability. The second group received the reference drug indomethacin (50

mg/kg) orally and the acetic acid solution intraperitoneally. The control group received oral administration of distilled water and intraperitoneal injection of saline solution at a dose of 10 ml/kg. Immediately afterward, all the groups were intravenously injected with 1% Evan's blue solution (10 ml/kg) through the tail vein. Mice were sacrificed by cervical dislocation thirty minutes later, and the abdominal cavity was rinsed with 3 ml of physiological saline, which was collected and centrifuged at 2500 rpm for 10 min. The dye intensity in the supernatant was determined spectrophotometrically at 610 nm. The following formula was used to determine the percentage inhibition of vascular permeability (1):

$$\% \text{ inhibition} = [A_{Control} - A_{test}/A_{Control}] \times 100 \quad (1)$$

### 2.10 Study of the preventive effect of the combination on plasma lipoprotein oxidation

The analysis of plasma lipoprotein oxidation, considered the level of thiobarbituric acid reactive substances (TBARS), was conducted using the method outlined previously [18] with slight modifications. The combination was evaluated for its antioxidant capacity using mouse plasma rich in LDL as a substrate for the oxidative process. The lipoprotein-rich plasma was taken from animals that received 600 mg/kg Triton WR-1339 for 24h. The positive control was butylated hydroxytoluene (BHT), which is used as a standard antioxidant. The amount of malondialdehyde (MDA) was determined by an extinction coefficient equal to $1.56 \times 10^5$ M/cm, where all measurements were performed in triplicate.

### 2.11 Lipoperoxyl radical scavenging activity

Lipoperoxyl radicals created through the self-oxidation of linoleic acid kicked off the process of oxidative bleaching of β-carotene. This study employed the method described by Leouifoudi et al. [19] to assess the impact of combination (RAE+CAE) and BHT on the neutralization of lipoperoxyl radicals and the prevention of lipid oxidation. The extent of the β-carotene oxidative bleaching was calculated using the formula (3):

$$\% \text{ inhibition} = 100 - [\frac{(\text{Ablank} - \text{A sample})}{\text{Ablank}} \text{ x } 100] \quad (3)$$

The $IC_{50}$ values were derived from the logarithmic equation of the graph plotted for antioxidant activity versus sample concentration.

### 2.12 DPPH free radical scavenging activities

DPPH free radical scavenging activity assays are commonly used to assess the ability of natural metabolites to scavenge free radicals. DPPH▪ has a wide absorption band at 517 nm in its radical form, which disappears during reduction by an anti-radical compound. The anti-radical effect of the combination was screened using the DPPH assay according to the method previously described [20]. A positive control was carried out using butylated hydroxytoluene (BHT) under the same experimental conditions. All the experiments were performed in triplicate. The radical scavenging activity (RSA) was determined with the following formula (2):

$$\text{RSA } (\%) = [\frac{(\text{Ablank} - \text{A sample})}{\text{Ablank}} \text{ x } 100] \quad (2)$$

The $IC_{50}$ values were assessed using the logarithmic equation of the obtained plotted graph of the scavenging activity *versus* sample concentration.

## 3 Results

### 3.1 Phenolic acid content of the RAE and CAE

*Ocimum basilicum* extract (RAE) is a rich source of phenolic compounds, as revealed from a previously conducted study by the authors, where the total phenol content was 174.39 ± 0.92 mg rosmarinic acid equivalent/g dry extract. On the other hand, the recorded flavonoid content was 11.34 ± 0.41 mg rutin/g dry plant extract [13]. However, the total phenol content of the carob aqueous extract (CAE) was 15.79 ± 0.43 mg rosmarinic acid/g dry plant extract, and that of the flavonoids was 0.87 ± 0.001 mg rutin/g dry plant extract. Thus, the basil extract contains 11 times more total polyphenols ($p < 0.001$) and 13 times more flavonoids ($p < 0.001$) than the carob extract.

### 3.2 Anti-inflammatory effect of the combination on rat paw edema

The injection of carrageenan suspension into the hind paw resulted in local edema that progressively increased and reached its maximum after 4 h. The paw thickness of the untreated group was 58.29 ± 5.21%, which decreased at 24 h to 16.71 ± 1.48% (Table 1).

**Table 1.** Anti-inflammatory effect of the combination (RAE+CAE) on carrageenan-induced paw edema in rats.

| Groups | Paw edema rate (%) | | | | |
|---|---|---|---|---|---|
| | **2 h** | **4 h** | **6 h** | **8 h** | **24 h** |
| **Control** | **34.33** ±8.09 | **58.29** ±5.21 | **44.58** ±3.43 | **35.23** ±3.72 | **16.71** ±1.48 |
| **RAE+CAE** (200 mg/kg) | **18.39** ±2.10 $^{ns}$ | **26.69** ±2.40** | **27.31** ±7.5 $^{ns}$ | **7.82** ±1.69** | **1.57** ±1.82** |
| **Indomethacin** (50 mg/kg) | **27.79** ±2.21 $^{ns}$ | **7.81** ±3.43** | **4.03** ±1.65** | **1.81** ±1.05** | **0.31** ±0.76** |

The differences between the treatment and control groups were tested using ANOVA. The values are expressed as the means ± SEMs *(n = 6)*. *$p < 0.05$; **$p < 0.001$ compared to the control; ns: not significant. RAE: Rosmarinic acid-rich extract. CAE: Carob aqueous extract.

However, there was a significant ($p<0.001$) reduction in hind paw inflammation throughout the experiment when the combination was administered orally at a dose of 200 mg/kg. After 4 h, the rate of paw edema was 26.69 ±2.40%, which subsequently decreased to 1.57 ±1.82% after 24h (Table 1). On the other hand, a significant reduction in swelling was observed when the reference drug indomethacin (50 mg/kg) was administered ($p<0.001$), and the paw size observed at 4 h was 7.81 ±3.43%, which showed a significant decrease at the end of 24 h, that is, 0.31 ±0.076% ($p<0.001$). This effect was relatively comparable to that exerted by our tested combination.

### 3.3 Effect of the combination on acetic acid-induced vascular permeability in mice

The combination's anti-inflammatory activity was further investigated by assessing its ability to inhibit vascular permeability, serving as a marker for peritoneal inflammation. The results obtained indicated that the combination at a dosage of 200 mg/kg reduced the intensity of peritoneal inflammation by 50.65% ($p<0.001$) compared to 60.56% for the indomethacin drug ($p=0.001$) (Figure 1).

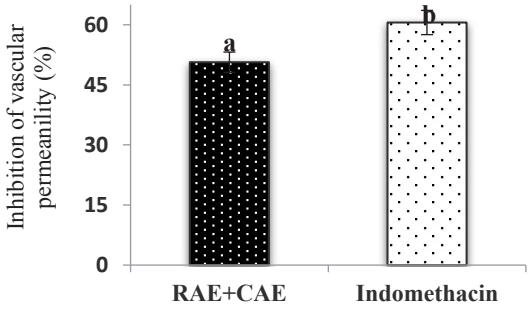


**Fig. 1.** Effect of the combination (RAE+CAE) and indomethacin on acetic acid-induced vascular permeability in mice. The values are expressed as the means ± SEMs ($n = 5$). [a]$p<0.001$, [b]$p=0.001$. RAE: Rosmarinic acid-rich extract. CAE: Carob aqueous extract.

### 3.4 Effect on mice plasma lipoprotein oxidation

The plasma lipoprotein oxidation produced by copper with or without treatment with the combination (RAE+CAE) and BHT was determined by measuring the MDA content at 532 nm. At 37°C, copper significantly increased plasma lipoprotein oxidation relative to that in controls ($p < 0.001$). Results illustrated in Figure 2 showed that treatment of copper-added lipoprotein-rich plasma with different concentrations of the combination (RAE+CAE) exerted significant and dose-dependent decreases in MDA levels.
Oxidation of plasma lipoprotein was decreased by 34% ($p < 0.001$), 80% ($p < 0.001$) and 86.3% ($p < 0.001$) at doses of 40, 100 and 400 µg/ml, respectively.

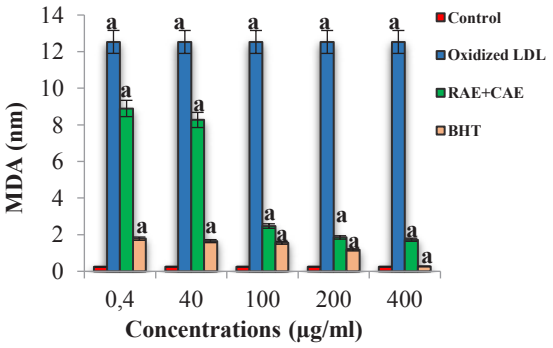


**Fig. 2.** Effect of combination and BHT on plasma lipoprotein oxidation; RAE: rosmarinic acid-rich extract from basil, CAE: carob aqueous extract, combination RAE+CAE, BHT: butylated hydroxytoluene; [a]p < 0.001, (oxidized LDL *vs* control; combination RAE+CAE and BHT *vs* oxidized LDL).

### 3.5 Lipoperoxyl radical scavenging activity

In this study, this combination was compared to the widely recognized synthetic antioxidant BHT. Our results indicated that the combination (RAE+CAE) exhibited notable dose-dependent antioxidant activity. The results illustrated in Table 2 revealed that the combination effectively prohibited the oxidation of the linoleic acid β-carotene-system in a good manner, which was statistically lower than that of BHT. In addition, the mean total antioxidant activity of BHT was the highest ($IC_{50}$= 4.19 ± 0.12 µg/ml), followed by that of the combination ($IC_{50}$= 160 ± 11 µg/ml).

**Table 2.** Antioxidant activity of the combination (RAE + CAE) and BHT using DPPH and lipoperoxyl radical scavenging activity assays expressed as $IC_{50}$ values (µg/ml).

| Samples | DPPH | *β*-carotene |
|---|---|---|
| RAE+CAE | 80.00 ± 4.00 | 160.00 ± 11.00 |
| BHT | 15.91 ± 0.71 | 4.19 ± 0.12 |

### 3.6 DPPH radical scavenging activity

The combination (RAE+CAE) exhibited significant antioxidant potential against DPPH. As shown in Table 2, RAE+CAE scavenged DPPH radicals in a dose-dependent manner, and the radical scavenging activity was estimated based on the $IC_{50}$ value.
The results illustrated in Table 2 revealed that the combination exhibited pronounced antioxidant potential ($IC_{50}$ = 80 ±4 µg/ml, $p$ <0.001) compared to the standard antioxidant BHT ($IC_{50}$= 15.91 ±0.71 µg/ml, $p$ <0.01).

## 4 Discussion

Inflammation is a serious problem in the field of medicine, and various treatments are needed to address it. Swelling, known as edema, is a primary outcome of inflammation and serves as a defensive response in living organisms. However, sometimes, swelling can also be the main illness itself. The current study aimed to assess the anti-inflammatory and anti-lipoprotein oxidation effects of our tested combination abundant in rosmarinic acid and fibers (RAE+CAE). The findings showed that this combination can reduce swelling in the hind paws of rats caused by an injection of carrageenan, demonstrating its effectiveness in alleviating acute inflammation. The combination also reduced the intensity of inflammation in the abdomen of mice caused by acetic acid. Moreover, the combination showed dose-dependent activity, effectively preventing the oxidation of plasma lipoproteins and scavenging free radicals. Rat paw edema induced by carrageenan serves as a well-established model for studying acute inflammation and is commonly used to evaluate the beneficial effects of natural products [21]. Based on the percentage inhibition of paw edema volume, the anti-inflammatory activity of the combination (RAE+CAE) at 200 mg/kg was assessed in this experimental model at different time points (2 h, 4 h, 6 h, 8 h, and 24 h) and

compared to that of the control and reference drug (indomethacin).

The findings indicated that the edema reached its highest point four hours after the carrageenan injection, and then it decreased after 24 hours. The combination treatment had a very effective anti-inflammatory effect, reducing the paw thickness of the rats by 54%. Indomethacin, which decreased the increase in paw volume by 87%, had a stronger effect on this inflammation model. Phlogistic agents induce biphasic edema, and it seems that the early phase commences with the production of acute mediators such as histamine, serotonin, bradykinin, and cyclooxygenase products. Conversely, the second phase is characterized by prostaglandin overproduction within 2-3 hours [16]. This suggests that cyclooxygenase inhibition or antioxidant activity may explain the delayed phase. As indicated by previous works, phenolic acids can inhibit cyclooxygenase and the release of its products [22, 23].

Cyclooxygenases are widely recognized for their ability to convert arachidonic acid into prostaglandins and other inflammatory mediators. By inhibiting cyclooxygenase, phenolic acids can decrease the release of these inflammatory products, leading to a reduction in the inflammatory response. The anti-inflammatory effects of rosmarinic acid result from its ability to inhibit lipoxygenase and cyclooxygenases, interfere with the complement cascade, inhibit the expression of inflammatory cytokines and inactivate the inflammatory pathway [24,25]. The combination was observed to exert a potent effect during the late phase. Therefore, the edema volume decreased by 78% after 8 hours of carrageenan injection in comparison to that of the standard drug (95%). This effect implies that the inhibitory effect of the combination would be more pronounced on cyclooxygenases, which are responsible for prostaglandin synthesis. To confirm the anti-inflammatory effect of the combination *in vivo*, we studied its possible impact on vascular permeability using acetic acid-induced mice. This model of inflammation is distinguished by an increase in the peritoneal content of prostaglandins (PGE2α and PGF2α), serotonin, and histamine caused by acetic acid [26]. This leads to the dilation of arterioles and venules, as well as an increase in vascular permeability [27]. A significant reduction (50.65%) in peritoneal vascular permeability was observed following oral administration of the combination (RAE + CAE), suggesting that it suppresses the vascular response in acute inflammatory processes and modulates the amplitude of inflammation. Notably, numerous studies have shown that the production of free radicals from various biological and environmental sources results from an imbalance in natural antioxidants, contributing to various diseases associated with inflammation, particularly atherosclerosis and cardiovascular events [28]. In this context, we assessed the antioxidant activity of the combination (RAE + CAE). It is necessary to highlight that the results from this study indicate that the combination has phytochemical constituents that might donate hydrogen to a free radical, thereby scavenging potential damage. In addition, the combination demonstrated a potent preventive effect against plasma lipoprotein oxidation, which is recognized as the initiating factor in the atherosclerosis process [29]. Indeed, the combination demonstrated the neutralization of lipoperoxyl radicals, which could significantly contribute to the inhibition of lipoprotein oxidation. Hence, given the significant associations between oxidative stress, inflammation, and the development of atherosclerosis, this combination has emerged as a valuable source of natural compounds with preventive potential against cardiovascular diseases [30]. Metabolic profiling of the rosmarinic acid-rich extract from *Ocimum basilicum* (RAE) using HPLC indicated the presence of four major phenolic acids, namely, rosmarinic, caftaric (2,9%), caffeic (4,3%), and chicoric (5,5%) acids [13]. Moreover, quantitative analysis revealed that rosmarinic acid was the predominant phenolic compound in the extract, representing 87,3% of the total identified phenolic acids [13]. In addition, phytochemical analysis indicated that the aqueous extract of carob (CAE) was abundant in polyphenols, flavonoids and alkaloids, with high levels of gallic acid and catechin [31], and gallic acid was identified as the main component. A study conducted by Torun, et al. [32] confirmed the presence of gallic acid along with five other phenolic acids, namely, protocatechuic acid, gentisic acid, syringic acid, p-coumaric acid, and synaptic acid, which contribute to the potent antioxidant properties exhibited by carob pods. Therefore, the rosmarinic acid from the RAE and gallic acid from the CAE may be the primary compounds responsible for the anti-inflammatory and antioxidant properties of the combination (RAE+CAE).

## 5 Conclusion

In conclusion, these results suggest that basil and carob, which are usually consumed as spices during food seasoning, could be considered promising natural alternative treatments for inflammatory diseases and oxidative stress, leading to the incidence and progression of atherosclerosis and cardiovascular complications. Based on our results, this combination appears to be a promising candidate for treating inflammatory diseases.

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