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# Synergistic and antagonistic effects of different bee products on coagulation mechanism: *In vitro* prothrombin time analysis using the Quick method

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## ABSTRACT

Hemostasis is a dynamic process regulated by platelets and the coagulation cascade. However, the role of bee products on coagulation parameters is not yet fully understood. We evaluated the effects of individual and combined applications of bee products on prothrombin time (PT) using the Quick method *in vitro*. The bee products studied include honey, beeswax, royal jelly, propolis, bee pollen, and bee venom, examined both individually and in various combinations (double, triple, quadruple, and quintuple). Our findings revealed that the individual bee products resulted in limited changes in PT (ΔPT: 0–3 seconds), while the combinations produced significantly greater and more variable effects. Notably, PT was significantly prolonged (with a maximum increase of 33 seconds) in double and quadruple combinations that included bee venom. Interestingly, when propolis was part of a five-component combination, the PT value returned to the control level. These results indicate that the effects of bee products do not increase linearly; instead, they display synergistic, antagonistic, and modulatory interactions that depend on the

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specific composition of each combination. In conclusion, combinational approaches should be considered for evaluating the hemostatic effects of bee products, and further studies are needed to clarify these interactions.

**Keywords:** bee products, Quick method, prothrombin time, coagulation, hemostasis

## INTRODUCTION

Hemostasis is a delicate balance mechanism vital for stopping bleeding and maintaining vascular integrity. This system is regulated by interactions among the vascular structure, platelet function, and the coagulation cascade[1]. Prothrombin time (PT), one of the key laboratory parameters used in evaluating the coagulation process, particularly reflects the functional integrity of the extrinsic and common coagulation pathways. PT measurement is sensitive to changes in factor VII, X, V, prothrombin, and fibrinogen levels. It is widely used in clinical practice across many areas, including evaluating liver function, assessing vitamin K deficiency, and monitoring anticoagulant therapy[2].

In recent years, interest in the biological effects of natural products has increased, and bee products, in particular, have attracted attention due to their functional properties. Honey, royal jelly, propolis, bee pollen, bee venom, and beeswax. Due to its rich phenolic composition, flavonoids, proteins, peptides, and lipid structures, honey exhibits antioxidant, anti-inflammatory, antimicrobial, and immunomodulatory effects[3,4]. These biological activities may have indirect or direct effects on hemostatic processes. Studies have reported that bee venom and honey play a role in blood hemostasis. It has been observed that phospholipase A2 and mellitin in bee venom prolong blood clotting time and inhibit trypsin and chymotrypsin activities[5,6]. In another study, honey inhibited platelet aggregation moderately. This suggests that honey may regulate platelet function and potentially reduce the risk of clot formation[7].

The effects of bee products on the coagulation system have been investigated in a limited number of studies, with current findings mostly focusing on platelet aggregation, fibrin formation, or general coagulation parameters[5,7]. However, studies that compare the direct effects of different bee products and their combinations on PT under the same experimental conditions are quite limited.

This study aims to investigate the effects of bee products and various combinations of these products on blood clotting time using the Quick method, and to identify possible synergistic or antagonistic interactions by revealing both the individual and combinatorial effects of bee products.

## METHODOLOGY

### Materials, chemicals, and instrumentation

The ethyl alcohol (96%), calcium chloride ($CaCl_2$), and hexane used in the study were obtained from Sigma Aldrich (USA). During the experiments, we used a precision balance (Shimadzu ATX224), a hot water bath (Nüve NB5), a vortex mixer (Velp Classic), a heater-stirrer (Scilogex MS-H280-Pro), and fine-tipped micropipettes (Eppendorf) with volumes of 10 µL and 100 µL. Lamb lung (Beykoz/Istanbul) was chosen as the tissue homogenate for the preparation of thromboplastin for the PT test. The honey, royal jelly, propolis, pollen, bee venom, and beeswax samples were obtained from La Ruche Gıda Oliva Bal (Karesi/Balıkesir). Blood plasma was obtained using a plasma pool created from the blood of at least 50 healthy individuals (male/female) from İstanbul Medipol University Mega Hospitals Complex.

### Preparation of thromboplastin solution (Quick method)

To prepare the thromboplastin solution used in the Quick method for PT analysis, lamb lung tissue was cleaned of unwanted parts, such as membranes, fat, bronchi, and pleura, using scissors and a scalpel. The cleaned tissue was separated into small pieces and homogenized in a mixer. 140 g of lung homogenate, weighed on a precision balance, was mixed with 140 g of physiological saline (SF) using a glass stirring rod. The mixture was filtered through an 8-layer gauze pad pre-moistened with SF, transferred to an Erlenmeyer flask, and refrigerated for 8 hours, stirring every 30 minutes[8]. After an overnight incubation, the tissue homogenate was stored in 2 mL Eppendorf tubes at -80°C until use; it was dissolved in a 37°C water bath during the experiment[6].

### Preparation of $CaCl_2$ solutions

A 0.2 M stock $CaCl_2$ solution was prepared to initiate coagulation in PT determination. For this purpose, 2.22 g of $CaCl_2$ was weighed, dissolved in a small amount of distilled water, and brought to a volume of 100 mL in a volumetric flask. At the time of the experiment, 1 mL of this stock solution was taken, and 9 mL of distilled water was added to obtain a fresh 0.02 M dilute working solution.

### Preparation of bee product solutions

All bee products were prepared as stock solutions at 1 mg/mL. 10 mg of each honey, bee venom, royal jelly, and pollen sample were weighed and dissolved in 10 mL of saline solution[9-11]. Since beeswax requires an organic solvent due

to its ester structure, 10 mg of beeswax was dissolved in a mixture of 4 mL of hexane and 6 mL of distilled water[9,12]. Since propolis is soluble in ethyl alcohol, we used a mixture of 10 mg of propolis, 3 mL of 96% ethyl alcohol, and 7 mL of distilled water. The bee products were stored at +4°C until the experiment[13].

### *In vitro* PT measurement with Quick method

In this study, the PT test was performed manually *in vitro* using the Quick method to evaluate the common and extrinsic coagulation pathways[8,14]. Based on the plasma-to-anticoagulant ratio of 9:1, bee products were included in the system as anticoagulants. We performed measurements in two stages: a control group (plasma only) and an experimental group (plasma + bee product combinations). Coagulation times were recorded in seconds (s) with a stopwatch in triplicate and expressed as mean (s) ± standard deviation (SD).

### PT measurement in the control group (plasma)

100 µL of thromboplastin was placed in a test tube and incubated in a 37°C water bath for 1 minute. 100 µL of control plasma, obtained by mixing 90 µL of plasma with 10 µL of distilled water, was added and vortexed. Next, 100 µL of 0.02 M $CaCl_2$ solution was added to the mixture, and the time to fibrin formation (clotting) was recorded with a stopwatch[6].

### PT measurement with bee products

Similarly, 100 µL of thromboplastin was incubated at 37°C for 1 minute, then 90 µL of plasma and 10 µL of bee product (or a combination of bee products) were added to the tube and homogenized by vortexing. Finally, PT determination was performed by starting the stopwatch at the moment 100 µL of 0.02 M $CaCl_2$ was added to the mixture. In the experiments, we integrated bee products into the system in combinations of one (10 µL at 0.1 mg/mL concentration), two (5 µL of each product at 0.2 mg/mL concentration), three (3.3 µL of each product at 0.3 mg/mL concentration), four (2.5 µL of each product at 0.4 mg/mL concentration), and five (2 µL of each product at 0.5 mg/mL concentration) to test their effects on coagulation[6]. All measurements were performed in triplicate, and all PT values are reported as mean (s) ± SD calculated from three independent measurements. Differences from the control group were calculated as ΔPT (s), and percentage changes were determined accordingly. Data analysis and graphical visualization were performed in Microsoft Excel.

## RESULTS and DISCUSSION

The PT test, which evaluates the common and extrinsic pathways of coagulation, was performed *in vitro* using the manual Quick method. In the experiment, PT

measurements were first taken using only plasma, serving as the control group. The plasma PT times measured in the control group were 17, 18, and 19 seconds, respectively, and the plasma reference value was set at an average of 18 seconds. To determine the effects of bee products on PT, single, double, triple, quadruple, and quintuple combinations were prepared, and each measurement was repeated three times, with the average values recorded in seconds (s).

### Effects of bee products and their combinations on PT

In this study, the effects of individual and combined use of bee products on PT were evaluated *in vitro* using the Quick method, and it was found that the effects varied with the number of components in the combinations. The findings indicate that while bee products alone exerted limited effects, their combinations resulted in significantly greater and non-linear responses.

The PT values of bee products tested individually are presented in Table 1. PT ranged from 18 to 21 seconds across individual bee products. While beeswax and honey did not change the coagulation time and remained equal to the reference value (18 seconds), bee venom (19 seconds), pollen (20 seconds), royal jelly (21 seconds), and propolis (21 seconds) prolonged the coagulation time. Compared to the control group, the highest increase was observed in the propolis and royal jelly groups (ΔPT + 3 sec, 16.7%).

**Table 1.** Effects of individual bee products on PT

| Bee Product | Mean PT (s) ± SD | ΔPT (s) | Change (%) |
|---|---|---|---|
| Honey | 18 ± 1 | 0 | 0.0 |
| Beeswax | 18 ± 1 | 0 | 0.0 |
| Bee venom | 19 ± 1 | +1 | 5.6 |
| Bee pollen | 20 ± 1 | +2 | 11.1 |
| Propolis | 21 ± 1 | +3 | 16.7 |
| Royal jelly | 21 ± 1 | +3 | 16.7 |

PT values are expressed as mean (s) ± SD of three independent measurements. ΔPT values show the difference of each combination group compared to the control group (Control PT value: 18 ± 1 s). Percentage change was calculated using the formula [(PT – 18) / 18] × 100.

Single bee products showed PT times of 18 to 21 seconds, with no significant anticoagulant effect. In contrast, with the inclusion of bee venom in combinations, significant increases in PT time were observed, especially in binary combinations, with values reaching 30–32 seconds (Table 2). In combination with two bee products, PT ranged from 18 to 32 seconds, compared with the control group (18 seconds). The highest increase was observed in honey + bee venom and beeswax + bee venom combinations, with a ΔPT increase of +14 seconds and 77.8% in both groups. The fact that bee venom (and binary/quadruple combinations containing bee venom) showed the strongest anticoagulant effect by extending the PT to 32-33 seconds. These findings are supported by studies showing that phospholipase A2 (PLA2) obtained from bee venom exhibits antiplatelet and anticoagulant properties, and it may be a potential antithrombotic agent[15]. A study showed that bee venom significantly prolonged blood clotting time PT and activated partial thromboplastin time - aPTT) in a dose-dependent manner. It was noted that it exerts an anticoagulant effect by inhibiting the activity of coagulation factors, serine proteases involved in the intrinsic and extrinsic coagulation pathways[5]. Bioactive peptides such as PLA2 and melittin, found in bee venom, are key anticoagulation factors that prolong blood clotting time[6]. This is consistent with the prolongation of clotting time observed in the bee venom-containing groups in our study. Furthermore, although bee venom inhibits clotting factors, it does not impair Thrombin Time (TT) or normal fibrinolytic activity. Because it does not directly inhibit the conversion of fibrinogen to fibrin, it provides an anticoagulant effect without causing abnormal bleeding times (bleeding disorders)[5]. However, the limited effect of bee venom alone in our findings suggests that this effect occurs in combination with other bee products.

**Table 2.** Effects of two-component bee product combinations on PT

| Combination | Mean PT (s) ± SD | ΔPT (s) | Change (%) |
|---|---|---|---|
| Beeswax + royal jelly | 18 ± 1 | 0 | 0.0 |
| Honey + royal jelly | 19 ± 1 | +1 | 5.6 |
| Honey + propolis | 19 ± 1 | +1 | 5.6 |
| Beeswax + bee pollen | 20 ± 1 | +2 | 11.1 |
| Royal jelly + bee pollen | 20 ± 1 | +2 | 11.1 |
| Beeswax + propolis | 22 ± 1 | +4 | 22.2 |
| Royal jelly + propolis | 22 ± 1 | +4 | 22.2 |
| Honey + bee pollen | 24 ± 1 | +6 | 33.3 |
| Honey + beeswax | 25 ± 1 | +7 | 38.9 |
| Bee pollen + propolis | 25 ± 1 | +7 | 38.9 |
| Bee pollen + bee venom | 25 ± 1 | +7 | 38.9 |
| Propolis + bee venom | 27 ± 1 | +9 | 50.0 |
| Royal jelly + bee venom | 30 ± 1 | +12 | 66.7 |
| Honey + bee venom | 32 ± 1 | +14 | 77.8 |
| Beeswax +bee venom | 32 ± 1 | +14 | 77.8 |

PT values are expressed as mean (s) ± SD of three independent measurements. ΔPT values show the difference of each combination group compared to the control group (Control PT value: 18 ± 1 s). Percentage change was calculated using the formula [(PT – 18) / 18] × 100.

In this study, honey did not prolong plasma reference PT alone. A study evaluating the effects of honey on hemostasis *in vitro* found that honey inhibited platelet aggregation and significantly prolonged clotting times, such as aPTT, PT, and TT, in a dose-dependent manner (3.5%, 7.5%, and 15% v/v). Honey also reduced fibrinogen levels, thus demonstrating multifaceted inhibitory effects on hemostasis and revealing its anticoagulant potential[7]. In our study, a

low honey concentration (0.1 mg/mL) alone may not have provided a sufficient inhibitory effect to prolong PT time. However, when combined with protease inhibitors such as bee venom (32 seconds), it acted as a very potent synergistic agent blocking the coagulation cascade.

Propolis is a lipophilic resinous substance composed of beeswax, essential oils, pollen, and other compounds. Besides its high flavonoid, phenolic acid, and ester content, it contains approximately 300 different organic compounds identified to date[16]. Propolis, owing to its diverse bioactive constituents, has been reported to exert anticoagulant and antithrombotic effects and to regulate hemostasis. These effects are thought to be mediated by inhibiting excessive platelet activation, enhancing fibrinolytic activity, and maintaining hemostatic balance. In addition, its antioxidant and anti-inflammatory properties may indirectly reduce thrombotic risk by attenuating oxidative stress and systemic inflammation[17]. Caffeic acid phenethyl ester (CAPE), one of the main bioactive components of propolis, has demonstrated dose-dependent fibrinolytic activity in human whole blood clots. These effects are achieved by enhancing fibrinolysis, the breakdown of existing blood clots[18].

Royal jelly is a natural, yellowish, creamy mixture with a slightly acidic taste, secreted by nurse bees. It is notable for containing proteins and sugars, as well as unique fatty acid compounds specific to royal jelly, such as (E)-10-hydroxy-2-decenoic acid (10-HDA) and its precursor 10-hydroxydecanoic acid (10-HDAA)[10,19]. Royal jelly is often recommended as a dietary supplement due to its high nutritional value and antioxidant, anti-inflammatory, and immunomodulatory effects[20]. Royal jelly may have clinically relevant effects on hemostasis, especially when co-administered with anticoagulant therapy. In a reported case, INR levels increased to 7.29 after royal jelly supplementation in a patient on anticoagulant treatment, leading to severe bleeding complications, including hematuria[21]. This interaction is thought to be due to the synergistic effect of royal jelly on clotting factors (Factors II, VII, IX, and X), increasing the risk of bleeding[22].

Bee pollen is an important food source collected by honeybees from flowers and used within the hive, especially for feeding larvae. In addition to essential nutrients (such as amino acids, fats, sugars, vitamins, and minerals), it also contains various flavonoids and phenolic acids[4,9]. The complex chemical composition of bee pollen can lead to variability in clinical response, particularly in terms of its effects on blood viscosity and clotting time. Indeed, the literature reports that bee pollen inhibits platelet aggregation and exhibits fibrinolytic activity. These effects are thought to occur via α-linolenic

acid (ALA), a precursor of prostaglandin-3, which plays a role in platelet aggregation[23,24]. However, the limited changes observed in PT duration in the groups containing bee pollen in our study suggest that the effect of this product is primarily through platelet function (primary hemostasis) and its effect on the coagulation cascade (secondary hemostasis) may be more limited.

Beeswax is a yellowish-brown, natural lipid mixture produced by honeybees for building their hives and it is widely used in cosmetics today[4,9]. In our study, beeswax alone did not alter PT. The most important component in beeswax that plays a role in coagulation is a mixture called policosanol, which consists of long-chain aliphatic alcohols. In one study, a policosanol extract from rice bran did not affect PT or aPTT values; however, it reduced platelet aggregation by modulating eicosanoid pathways[25]. This suggests that beeswax exerts an anticoagulant effect without affecting the coagulation cascade.

In the triple bee product combinations, PT varied between 20 and 29 seconds compared to the control group (18 seconds) (Table 3). The highest increase was observed in the "honey + royal jelly + bee venom" and "honey + pollen + bee venom" combinations, with a ΔPT increase of +11 seconds and 61.1% in both groups. The lowest increase was observed in the "honey + beeswax + bee venom" combination (ΔPT increase of +2 seconds and 11.1%).

ΔPT values in individual bee products remain in the 0–3 sec range, suggesting that these products alone have a limited effect on the coagulation system. This supports the idea that the biological effects of bee products may depend primarily on complex component interactions. In contrast, the significant increases in ΔPT observed in binary combinations suggest that bioactive components in different bee products may produce a stronger effect when combined. The association of higher ΔPT values with combinations containing bee venom, in particular, indicates that this component may play a dominant role in the coagulation process. The more heterogeneous distribution of ΔPT values in triple combinations compared to binary combinations suggests that synergistic and antagonistic interactions may occur between the components.

**Table 3.** Effects of three-component bee product combinations on PT

| Combination | Mean PT (s) ± SD | ΔPT (s) | Change (%) |
|---|---|---|---|
| Honey + beeswax + bee venom | 20 ± 1 | +2 | 11.1 |
| Honey + beeswax + royal jelly | 21 ± 1 | +3 | 16.7 |
| Beeswax + royal jelly + bee pollen | 23 ± 1 | +5 | 27.8 |
| Beeswax + bee pollen + propolis | 23 ± 1 | +5 | 27.8 |
| Beeswax + royal jelly + propolis | 24 ± 1 | +6 | 33.3 |
| Beeswax + royal jelly + bee venom | 24 ± 1 | +6 | 33.3 |
| Beeswax + propolis + bee venom | 25 ± 1 | +7 | 38.9 |
| Honey + royal jelly + bee venom | 29 ± 1 | +11 | 61.1 |
| Honey + bee pollen + bee venom | 29 ± 1 | +11 | 61.1 |

PT values are expressed as mean (s) ± SD of three independent measurements. ΔPT values show the difference of each combination group compared to the control group (Control PT value: 18 ± 1 s). Percentage change was calculated using the formula [(PT – 18) / 18] × 100.

In the quadruple bee product combination, PT was 33 seconds, resulting in a ΔPT of 15 seconds and 83.3% compared to the control group. In the five-component combination group, PT was found to be completely similar to the control group (18 seconds), and no change was detected (ΔPT = 0 seconds, 0.0%) (Table 4).

**Table 4.** Effects of four- and five-component bee product combinations on PT

| Combination | Mean PT (s) ± SD | ΔPT (s) | Change (%) |
|---|---|---|---|
| Honey + beeswax + royal jelly + bee venom | 33 ± 1 | +15 | 83.3 |
| Honey + beeswax + royal jelly + bee venom + propolis | 18 ± 1 | 0 | 0.0 |

PT values are expressed as mean (s) ± SD of three independent measurements. ΔPT values show the difference of each combination group compared to the control group (Control PT value: 18 ± 1 s). Percentage change was calculated using the formula [(PT – 18) / 18] × 100.

The effects of individual and combination bee products on PT are presented in Figure 1 as ΔPT (s). Higher ΔPT values indicate prolonged clotting time. In Figure 1, ΔPT values varied widely among all groups. In individual bee products, ΔPT values remained limited to 0–3 seconds; honey and beeswax showed the same value as the control group (ΔPT = 0 seconds), while increases of +1, +2, and +3 seconds were observed in the bee venom, pollen, propolis, and royal jelly groups, respectively. In contrast, ΔPT values increased significantly in the combination groups. High ΔPT values were obtained, especially in binary combinations containing bee venom, with the highest values of ΔPT +14 seconds observed in honey + bee venom and beeswax + bee venom combinations. In triple combinations, ΔPT values generally ranged between +2 and +11 seconds; The highest values were determined in the honey + royal jelly + bee venom and honey + pollen + bee venom combinations (ΔPT +11 sec). In the four-component combination, the largest increase was observed in the ΔPT +15 sec group. In contrast, in the five-component combination group, the ΔPT value was found to be 0 seconds, and the PT was the same as that of the control group.

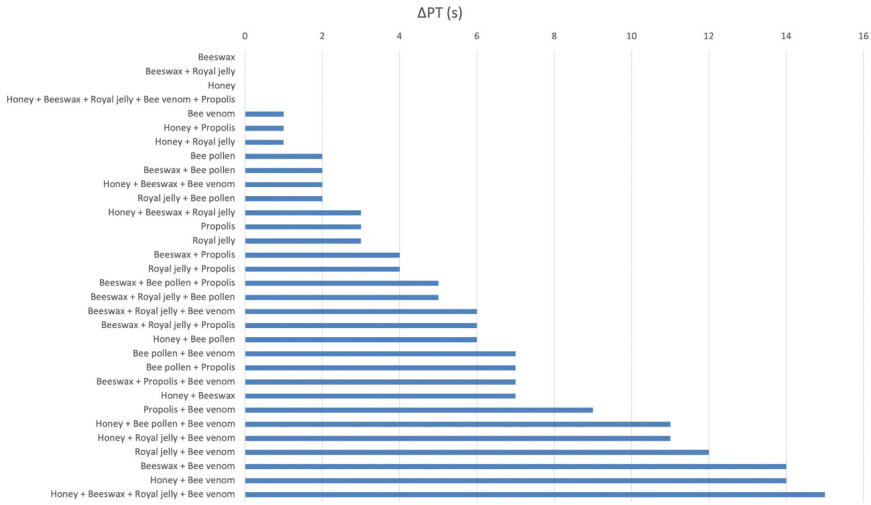


**Figure 1.** ΔPT (s) values of bee product combinations compared to the control group (control PT value: 18 ± 1 s).

Figure 2 presents the trend (ΔPT) as a function of the number of components. A non-linear association was observed: mean ΔPT values increased from individual conditions to binary combinations, then increased more moderately in three-component combinations. The highest mean ΔPT was observed in the four-component combination, whereas the five-component combination

showed a sharp decline, approaching the control level. These findings indicate that the effects of bee product combinations do not follow a linear pattern but instead exhibit a variable response profile depending on the number of components.

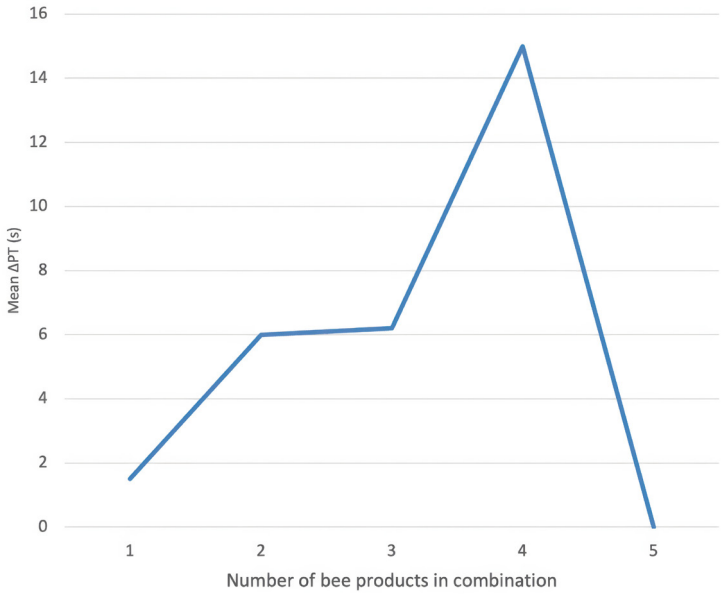


**Figure 2.** Changes in (ΔPT) by number of components

One of the most striking findings in our study is that PT was prolonged to 33 seconds in the quadruple combination of honey, beeswax, royal jelly, and bee venom. However, the addition of propolis to this system (quintuple combination) abruptly returned the PT value to the reference level of 18 seconds. The observation of the highest PT value in the quadruple combination indicates a synergistic anticoagulant effect among these components. In contrast, the return of the PT time to baseline levels with the addition of propolis suggests that propolis may have played an antagonistic or modulatory role in this combination. It is reported that although it exhibits strong antiplatelet properties, propolis may not significantly alter classical coagulation parameters such as PT and aPTT at certain concentrations. This suggests that the effect of propolis largely occurs through platelet activation and aggregation, rather than directly inhibiting factors in the coagulation cascade. Indeed, it has been reported that some propolis extracts (e.g., AF-08 of Brazilian origin) exhibit this selective effect[18,26]. Furthermore, the strong antagonistic or modulatory effect of propolis can also be explained by the chemical activity of its high levels

of polyphenols and flavonoids. This can be interpreted as phenolic compounds in propolis interacting with protein/enzyme-containing components (such as PLA2 or melittin) in bee venom and royal jelly, thereby weakening the coagulation-inhibitory effect and limiting the anticoagulant effect[9,16].

In conclusion, these findings suggest that the biological effects observed in combinations of bee products are shaped not only by the individual properties of their components but also by synergistic, antagonistic, and modulatory interactions among them. This highlights the capacity of multi-component natural products to produce complex and, at times, unpredictable effects in biological systems. Accordingly, evaluating the effects of bee products requires approaches that go beyond individual components and incorporate their combinational interactions.

This study has some limitations. Firstly, the evaluations were conducted *in vitro*, and the generalizability of the findings to the clinical setting is limited. Secondly, PT reflects only the extrinsic coagulation pathway, and a holistic assessment of hemostasis requires examination of aPTT, platelet function, and other relevant parameters. Future studies should standardize and evaluate the chemical composition of bee products using total phenolic content or specific biomarkers, thereby enabling more accurate interpretation of findings and improving comparability.

## STATEMENT OF ETHICS

Ethical approval was obtained from the Ethics Committee of İstanbul Medipol University (Approval No: 861, Date: 23.10.2019).

## CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

## AUTHOR CONTRIBUTIONS

Conceptualization: OEE and GZO; methodology: OEE; investigation: OEE; formal analysis: MP and OEE; data curation: MP, OEE, and GZO; visualization: MP; original draft writing: MP, OEE, and GZO; review and editing: MP, OEE, and GZO.

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