ORIGINAL ARTICLE

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Effect of Propolis on Precocious Puberty in Female Rats

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What is already known on this topic?

Nutrition and exposure to various chemicals, including environmental pollution, insecticides, and plant phytoestrogens (having oestrogen-like effects), are environmental factors that affect puberty onset.

What this study adds?

This study investigated the effects of propolis on precocious puberty and the reproductive system in prepubertal female rats. Although propolis has estrogenic effects, to the best of our knowledge, no previous study has evaluated the relationship between propolis and puberty onset. This study provides comprehensive information about the stimulant effect of propolis on puberty.

Abstract

Objective: Nutrition and exposure to various chemicals, including environmental pollution, insecticides, and plant phytoestrogens (having oestrogen-like effects), are environmental factors that affect puberty onset. The aim of this study was to identify the effects of propolis, which has been reported to have oestrogenic effects, on precocious puberty and the reproductive system in prepubertal female rats (ovary, endometrium, breast).

Methods: Thirty-four 25-day-old, prepubertal, female Sprague-Dawley rats were included. Rats were randomly divided into the propolis (n = 17) and control groups (n = 17). The primary endpoint was the number of rats that developed vaginal opening, a sign of puberty, at 12-day follow-up. In addition, the effect of propolis on ovary, uterus and breast tissue was evaluated histologically.

Results: Vaginal patency occurred earlier (about 7.5 days sooner) in the propolis group and all animals in the propolis group had vaginal patency by day 12. The number of ovarian follicles (in all follicles), endometrial thickness, and mammary gland secretory gland area were significantly higher in the propolis group than in the control group (all $p < 0.001$). In addition, Ki-67 activity in the endometrium, breast tissue and ovary was more intense in the propolis group compared to the control group (all $p < 0.001$).

Conclusion: Propolis triggers precocious puberty in female rats, possibly by interacting with the oestrogen receptor. The mechanism of action of propolis should be considered before prescribing it. In addition, further studies are needed to explore the mechanism of action of propolis and to determine the component of propolis that triggers puberty.

Keywords: Phytoestrogens, propolis, precocious puberty, rat

Introduction

Adolescence is the transition period from childhood to adulthood and involves the development of secondary sexual characteristics and reproductive ability, and sexual maturation (1). Even under similar living conditions, the timing of puberty varies significantly among individuals, suggesting that many factors affect the onset of puberty, such as genetic and environmental factors, socioeconomic status, stress, metabolic rate, bone maturation, and body fat ratio (2,3,4). In addition, nutrition and exposure to various chemicals, including environmental pollution, insecticides, and plant phytoestrogens, which have oestrogen-like effects, are environmental factors that affect puberty onset (2,5,6,7,8). Propolis is a product from *Apis mellifera* (honey bee) hives, containing plant resins, beeswax, and minor

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constituents, including pollen and minerals (9). Propolis is very heterogeneous and the composition is dependent upon plant sources and/or types of bees (10). Although propolis has estrogenic effects (11,12,13), to the best of our knowledge, no previous study has evaluated the relationship between propolis and pubertal onset. The aim of this study was to investigate the effects of propolis on precocious puberty and the reproductive system in prepubertal female rats.

Methods

This study was approved by the Animal Experiments Local Ethics Committee, Sakarya University, Turkey (date: 01.07.2020, decision no: 34). Thirty-four 25-day-old, prepubertal, female, Sprague-Dawley rats were included. The number of rats was determined using G Power analysis (95% confidence interval, 80% power). Rats were randomly divided into the propolis (n = 17) and control groups (n = 17). The weight of the rats was recorded before the experiments. The rats were sedated using anaesthetic doses of ketamine and xylazine; blood was obtained from the rats to measure the levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), oestradiol, and testosterone. Water-soluble propolis (1 cc at a dose of 200 mg/kg; based on other similar studies) (11,14) was administered to the propolis group by gavage for 12 days (equivalent to approximately 1 human year, comparing relative expected life-spans). Water-soluble propolis contained 10% pure propolis and was prepared using water and glycol solution for gavage. The content of the propolis used is presented in Supplementary Table 1. The control group was administered 1 cc of water by gavage. The animals were provided food and water *ad libitum*. To determine the time of puberty onset, vaginal openness was measured, and estrous cycle status was observed with vaginal smear at baseline and then daily. Vaginal openness was measured at baseline and then daily to determine the time of puberty onset. The number of rats attaining puberty after 12 days of treatment was recorded. After 12 days of treatment, the rats were weighed. Then the rats were sedated with the appropriate anaesthetic dose, blood was obtained to measure hormone levels, and the rats were sacrificed. Uterine, ovarian, and breast tissues were obtained for histopathological and immunohistochemical evaluation. This study was not designed as part of a translational medicine study.

Histopathological and Immunohistochemical Evaluation

The tissue samples were fixed with 10% formalin solution for 48 hours and dehydrated with 60%, 70%, 80%, 96%, and 100% alcohol. Then the samples were passed through a xylol series to render the tissues transparent. The tissues were embedded in paraffin and cut using a microtome. The sections were stained with hematoxylin-eosin to observe the histological changes in the ovary, endometrium, and mammary gland tissues. Photographs were acquired under a light microscope (Olympus CX31-Japan). Ten sections (10 µm each) were obtained from each ovary to determine the effects of propolis on the number of follicles. Only follicles with oocyte nuclei were counted to determine the follicle count. Follicles were classified into five stages: primordial, primary, secondary, antral, and atretic follicles (15). The automated image analysis software, Image J®, was used to measure endometrial thickness (in µm). All slides were examined under the microscope at 100× magnification (14). Mammary gland tissues were examined using a Nikon eclipse inverted microscope (Nikon Corp., Tokyo, Japan), and the area (μm^2) of secretory epithelium and fat cells and the area of stroma were calculated using the NIS-element imaging system from the same manufacturer. The ratio of

Table 1. Comparison of the laboratory data of rats in the propolis and control groups

	Control group (n = 17) Median values (min-max)	Propolis group (n = 17) Median values (min-max)	p
Starting weight (g)	50 (30.1-61.9)	46.6 (38.2-52.3)	0.540
Final weight (g)	92 (77.8-104.0)	93.4 (76.6-104.8)	0.812
Starting LH	1.12 (0.57-2.13)	1.40 (0.70-2.97)	0.345
Final LH	1.39 (0.61-2.88)	1.52 (0.77-3.83)	0.563
Starting FSH	2.35 (1.88-3.13)	2.70 (1.43-4.17)	0.160
Final FSH	2.48 (1.61-3.55)	2.63 (2.35-3.52)	0.170
Starting oestradiol	86.48 (63.2-114.3)	86.48 (54.9-119.1)	0.683
Final oestradiol	62.83 (48.8-83.0)	77.78 (60.3-108.9)	0.020*
Starting testosterone	209.30 (160.3-272.2)	200.30 (98.6-302.3)	0.540
Final testosterone	146.10 (93.1-278.4)	276.50 (200.7-338.5)	< 0.001*

Mann-Whitney used for continuous variables. *Statistical significance ($p < 0.05$).
FSH: follicle stimulating hormone, LH: luteinizing hormone, min-max: minimum-maximum

the area of the secretory epithelium and fat cells to the area of the stroma was then calculated (16). Ki-67 staining was used to demonstrate tissue stimulation and proliferation in the endometrium, mammary glands, and ovaries. Four micron thick tissue samples were cut from paraffin-embedded blocks and deparaffinized using a decreasing alcohol series. Citrate buffer was heated in the microwave for 20 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2. The primary antibody used was anti-Ki-67 (1/400 dilution, GeneTex; Cat. No: GTX16667; USA). The secondary antibody [Ultra Vision Large Volume Detection System Anti-rabbit by LabVision, conjugated with horse radish peroxidase (HRP)] was used in accordance with the manufacturer's instructions. DAB (3,3'-diaminobenzidine) was used for immunohistochemical staining of HRP-conjugated secondary antibody-labeled proteins in tissues. Mayer's hematoxylin was used as the counterstain. The prepared slides were covered in mounting medium (Aqueous Mounting Medium by ScyTek). Proliferative activity, as assessed by Ki-67 staining, was semi-quantitatively analysed (h-score) by selecting 10 random fields, and 100 epithelial cells were photographed in each area. The Ki-67 index was calculated as the percentage of positively stained cells among the total cells assessed (17,18). In the immunohistochemical analysis, Ki-67 staining and cell division rates in the mammary glands, ovary, and endometrium were compared between the control and propolis groups.

Hormonal Assessment

The rats were sacrificed and blood samples were collected. When the specimens had completely clotted, they were centrifuged at 1500 *g* for 10 minutes. Serum fractions were collected and frozen at -40 °C until further use. LH, FSH, testosterone, and oestradiol levels were determined using a double antibody enzyme-linked immunosorbent assay [YLBiont brand Sandwich enzyme linked immunosorbent assay (ELISA); Shanghai YL Biotech Co., Ltd., Shanghai, China] containing hormone-specific monoclonal antibody coated wells. 40 µL of rat serum and 10 µL of antibody were added to the wells. 50 µL of streptavidin HRP conjugate was added to all wells except the blank well (standard and sample well) and the wells were incubated at 37 °C for 60 min. After incubation, the wells were washed to remove unbound antibody. The specimens were incubated with chromogen at 37 °C for 10 min to develop a blue colour. Stop solution was added to terminate the reaction, reflected by a change in the colour of the solution from blue to yellow. The intensity of the yellow colour was directly proportional to the analyte concentration. The colorimetric readings were performed using the inappropriate wavelength for the micro ELISA reader. A standard curve was generated to calculate the sample concentrations. The results and the measurement range were specified as rat LH 0.1-38 mIU/mL, rat FSH 0.2-60 mIU/mL, rat testosterone 10-3000 ng/L, rat oestradiol 3-900 ng/L respectively. The within-run and between-run CV% of the assays were given as $<10\%$.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences, version 20.0 software (IBM Inc., Chicago, IL, USA). Numerical variables were summarized using median values (minimum-maximum) as appropriate. Normality of the numerical variables was assessed with the Kolmogorov-Smirnov test. To compare independent groups, the number of rats in the groups was low and therefore nonparametric tests were used, including the Mann-Whitney U test. A p-value less than 0.05 was considered statistically significant.

Results

Laboratory, histopathological, and immunohistochemical data are presented in Tables 1 and 2. Histopathological and immunohistochemical images are shown in Figures 1 and 2, respectively. The control and propolis groups had similar initial ($p=0.535$) and final weights ($p=0.809$) and baseline levels of LH ($p=0.241$), FSH ($p=0.158$), testosterone ($p=0.524$), and oestradiol ($p=0.667$). On day 12, the oestradiol and testosterone levels were higher in the propolis than control group ($p=0.021$ and $p<0.001$, respectively). The testosterone level decreased from baseline to day 12 in the control group, whereas it increased in the propolis group. Although the oestradiol level decreased in both groups, the decrease was smaller in the propolis compared with the control group. Vaginal openness was observed in only two rats (both on day 12) during the 12-day follow-up in the control group, whereas it was observed in all rats in the propolis group. Furthermore, in the propolis group nine (52.9%) rats exhibited vaginal openness on day 4 and eight (47.1%) rats on day 5. The number of ovarian follicles, endometrial thickness, and mammary gland secretory area were significantly higher in the propolis than control group ($p<0.001$, $p<0.001$, and $p<0.001$, respectively). In addition, Ki-67 activity in the endometrium, breast, and ovarian tissues was greater in the propolis than control group ($p<0.001$, $p<0.001$, and $p<0.001$, respectively).

Discussion

Many factors affect the age of puberty onset, including nutrition and exposure to environmental pollution, insecticides, and plant phytoestrogens, which have

oestrogen-like effects (2, 5-8). Propolis consists of many chemicals that vary depending on the type of plant the bees have collected the pollen and nectar from. Several studies have reported that some of these chemicals, such as flavonoids, coumaric acids, and caffeic acids, have oestrogen-like activity (11,12). Okamoto et al. (11) showed that propolis increased the uterine wet weight and endometrial thickness in ovariectomized rats and stimulated ductal cell proliferation in the mammary glands via the oestrogen receptor. In the present study, propolis increased the endometrial thickness and secretory area of adipose tissue in the mammary glands. Additionally, it increased the number of follicles in the ovaries and Ki-67 staining in the ovary, uterus, and breast tissues, suggesting increased cell proliferation.

In female rats, vaginal opening, the first external sign of ovarian activity, is considered a sign of puberty and occurs at approximately postnatal 35-37 days. The estrous cycle may start immediately after vaginal opening or within a week (19,20). Our study showed that the vaginal opening developed significantly earlier in the propolis compared with the control group. In the control group vaginal openness was observed in only two rats (both on day 12) during the 12-day follow-up, whereas it was observed in all rats in the propolis group. Furthermore, in the propolis group nine rats exhibited vaginal openness on day 4 and eight rats on day 5. In addition, while none of the rats in the control group could enter the estrous cycle (in vaginal smear), all the rats in the propolis group were in the estrous cycle.

Although genistein is predominantly found in soy, it is also one of the main components of propolis and estrogenic effect has been shown in previous studies (21,22,23,24). Chrysin, the flavone group found in propolis, was found to inhibit the aromatase enzyme in most *in vitro* studies, leading to reduced oestrogen production (25). A human study found no increase in urine testosterone level after chrysin administration, suggesting that aromatase was not inhibited (26). In the present study, the testosterone level was higher in the propolis than control group, suggesting that the propolis used in this experiment inhibits aromatase. However, the oestradiol level was also significantly higher in the propolis group at the day 12 timepoint.

This study also demonstrated a significant stimulating effect of propolis on the ovary, endometrium, and breast. However, the unexpected low estrogen level detected at the end of the study was interpreted as the increase in the cumulative estrogenic effect due to the estrogen-like effect of propolis rather than a direct estrogen increase. These changes may be due to a large intra-cycle oestradiol change due to the high number of rats in the estrous cycle in the propolis group. For this reason, estradiol values can be very different according to the period of the rats in the propolis group (especially in the proestrus period). In fact, it is inaccurate to compare estradiol between groups because we do not know exactly what stage of the estrous cycle the rats in the propolis group were in at sacrifice. At the end of the study, no difference was found in the gonadotropin level between the propolis and control groups. Presumably, propolis triggers precocious puberty by interacting with the oestrogen receptor and oestradiol/testosterone ratio, rather than increasing the gonadotropin or oestradiol level. In addition, the steroid/oestrogen-like side chain rings of some flavonoids/phenolics, which are abundant in propolis, may induce changes in the steroid pathway and trigger precocious puberty due to interaction with the oestrogen receptor. This condition can only be determined by conducting studies at the estrogen receptor level. Contrary to our study, some

Table 2. Comparison of the immunohistochemical findings in the propolis and control groups

	Control group (n = 17) Median values (min-max)	Propolis group (n = 17) Median values (min-max)	p values
Number of primordial follicles	52 (37-59)	61 (54-65)	**< 0.001***
Number of primary follicles	24 (20-29)	32 (25-36)	**< 0.001***
Number of secondary follicles	4 (2-6)	12 (9-15)	**< 0.001***
Number of antral follicles	3 (1-4)	5 (4-6)	**< 0.001***
Number of atretic follicles	2 (0-2)	4 (3-5)	**< 0.001***
Endometrial thickness (μm)	178 (156-189)	193 (185-198)	**< 0.001***
Mammary gland entire area (μm^2)	44.18 (39.1-49.3)	77.84 (69.5-95.7)	**< 0.001***
Mammary gland secretory area (μm^2)	6.64 (4.1-45.1)	36.08 (32.1-39.5)	**< 0.001***
Ovarian Ki-67 (%)	15 (9-22)	33 (25-44)	**< 0.001***
Endometrium Ki-67 (%)	13 (7-16)	40 (35-46)	**< 0.001***
Mammary gland Ki-67 (%)	17 (10-21)	43 (39-46)	**< 0.001***

Mann-Whitney used for continuous variables. *Statistical significance ($p < 0.05$).
Min-max: minimum-maximum

studies have shown that polyphenols in green tea prevent prepubertal puberty (27,28). This opposite effect of green tea compared to propolis may be due to the different ratios of polyphenols in the food used as the proportion of catechin was high in green tea, while the proportion of chrysin and caffeic acid phenethyl ester was higher in the propolis used in the present study.

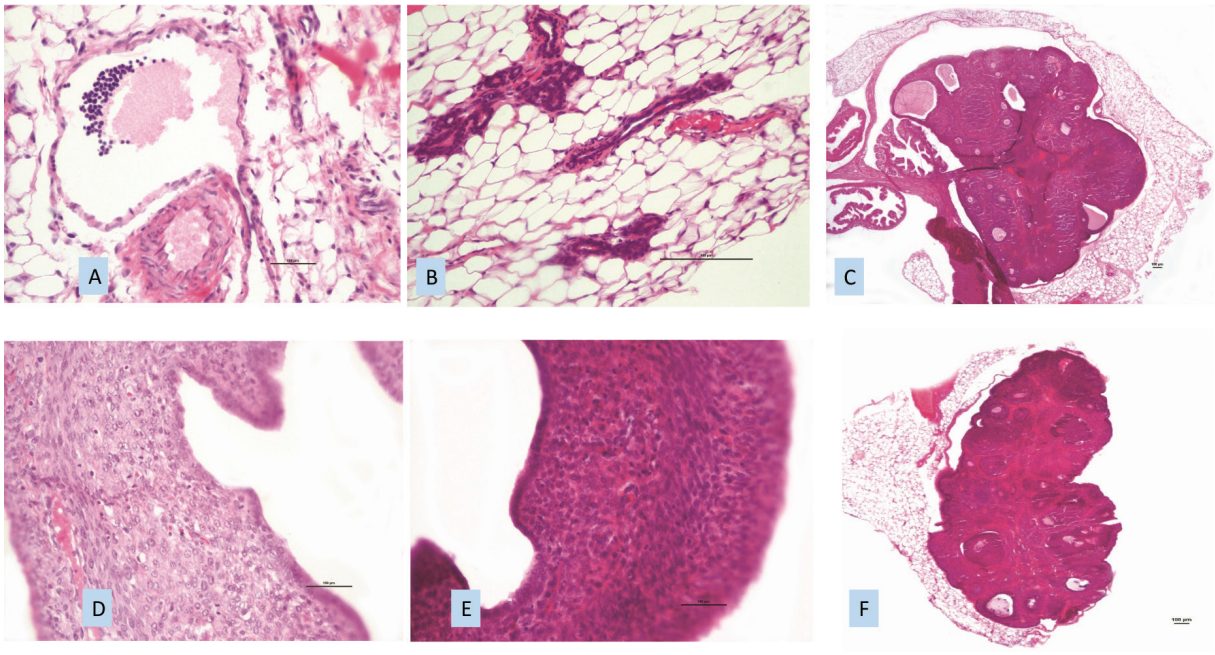

Figure 1. Larger secretory areas (active state) were observed in the adipose tissue of the mammary glands in the propolis group (A) than control group (B). In ovarian tissue, there were more secondary, antral, and corpus luteum follicles in the propolis group (C) than control group (F). The endometrial layer was thicker in the propolis group (E) than control group (D). hematoxylin-eosin pictures. 40x lens, 100 µm scale bar

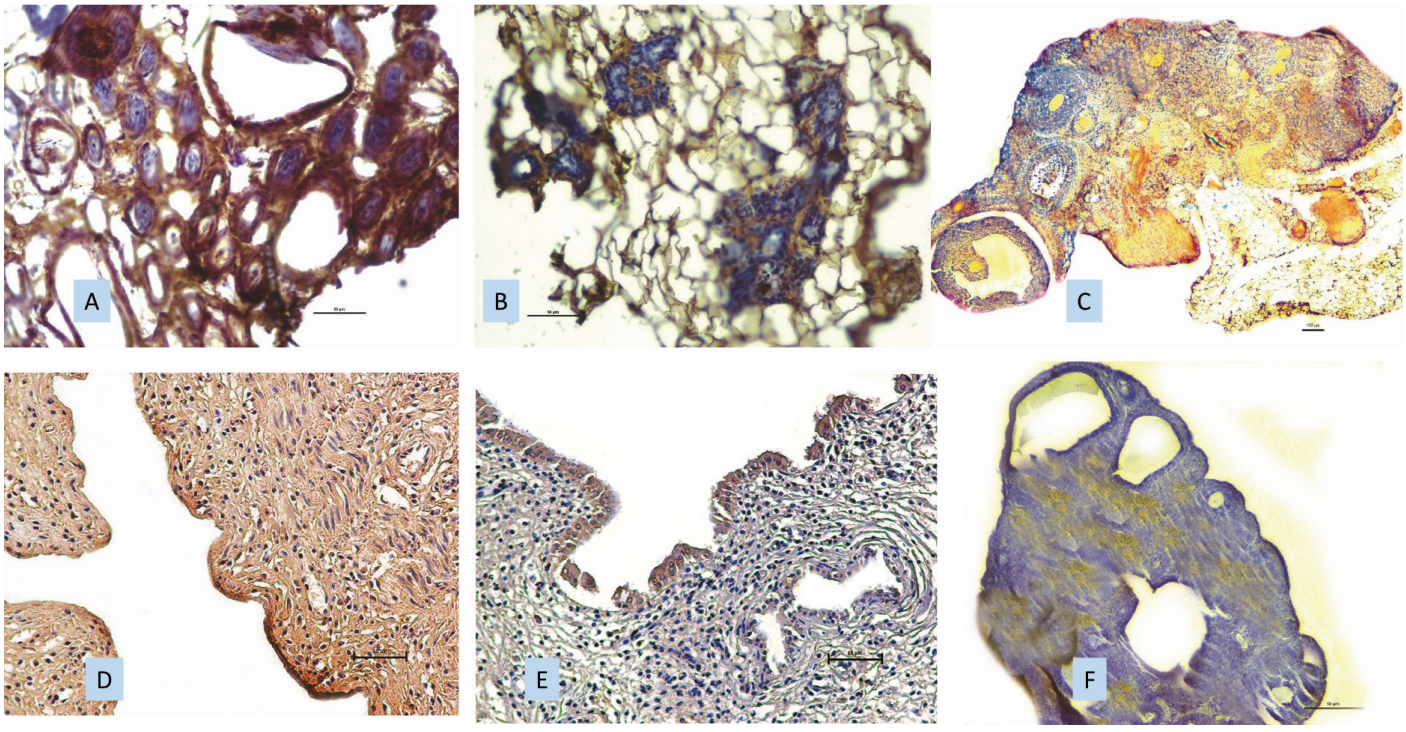

Figure 2. Due to cell development and proliferation, Ki-67 staining intensity was greater in the mammary glands, ovary, and endometrium (due to thickening) in the propolis group (A, C, and D, respectively) compared with the control group (B, E, and F, respectively). Ki-67 immunoreactivity preparations. 200x lens, 100 µm scale bar

Study Limitations

The small sample size limits our results. To account for this, conservative statistical methods, including nonparametric tests, were employed to mitigate the risk of type 1 error. Clinical findings and histological findings were supported by immunohistochemical staining.

Conclusion

This was the first study to evaluate the relationship between propolis and puberty, to the best of our knowledge. Propolis triggered precocious puberty in female rats, possibly by interacting with the oestrogen receptor. This is an interesting finding which should be investigated further. However, this finding does not yet have a direct impact on clinical practice. Composition and effective dose of propolis varies from product to product. Moreover, duration of use, differences in individual receptor sensitivity, and exposure to other chemicals with anti-estrogenic/androgenic effects (cumulative effect) might differ for each individual. The mechanism of action of propolis should be considered before prescribing it. In addition, further studies are needed to explore the mechanism of action of propolis and to determine the component of propolis that triggers puberty.

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The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/yFiRRO

Ethics

Ethics Committee Approval: This study was approved by the Animal Experiments Local Ethics Committee. Sakarya University, Turkey (date: 01.07.2020, decision no: 34).

Informed Consent: Animal experiments.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Recep Polat, Fatıma Betül Tuncer, Design: Recep Polat, Erdem Çokluk, Özcan Budak, Data Collection or Processing: Recep Polat, Erdem Çokluk, Özcan Budak, Fatıma Betül Tuncer, Analysis or Interpretation: Recep Polat, Erdem Çokluk, Özcan Budak, Fatıma Betül Tuncer, Literature Search: Recep Polat, Erdem Çokluk, Fatıma Betül Tuncer, Writing: Recep Polat, Erdem Çokluk, Özcan Budak.

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