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# EFFECTS OF A PROPOLIS EXTRACT ON THE VIABILITY OF AND LEVELS OF CYTOSKELETAL AND REGULATORY PROTEINS IN RAT BRAIN ASTROCYTES: AN *IN VITRO* STUDY

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A potential for the use of propolis in preventive and therapeutic purposes has been acknowledged, but little attention has been paid to estimation of possible propolis cytotoxicity with respect to astrocytes. We tried to estimate how a propolis ethanol extract (PEE) affects rat brain astrocytes *in vitro* and also to uncover crucial molecular targets of the PEE action. Primary astrocytes were exposed to PEE in doses of 10, 25, or 100 μg/ml for 24 h, and then the cell viability was monitored by MTT assay. Levels of glial fibrillary acidic protein (GFAP), transcriptional nuclear factor-κB (NF-κB), poly(ADP-ribose) polymerase (PARP), and angiostatins were measured using Western blot to identify the molecular mechanisms underlying cell responses to PEE. The PEE treatment exerted a dose-dependent cytotoxic effect on cultured astrocytes. The PEE modulated astrocyte signaling pathways through inducing the expression of NF-κB and PARP. At the same time, the PEE stimulated GFAP synthesis and fibrillogenesis, which was indicative for activation of astrocytes preceding their suppression. The PEE significantly increased the production of angiostatin isoforms by astrocytes, thus contributing to an anti-angiogenic potential of these cells. In summary, our results indicated that exposure to the PEE exerts certain cytotoxic effects on astrocytes in a dose-dependent manner; these effects are realized through modulation of cytoskeleton rearrangements and pro-apoptotic signaling pathways. A widely available, safe, and inexpensive substance, propolis, and its components and derivatives may be used in the prevention and treatment of neuronal impairments, including malignant tumors and neurodegenerative disorders associated with excessive astrocytic activation.

**Keywords: astrocytes, propolis, NF-κB, poly(ADP-ribose) polymerase (PARP), glial fibrillary acidic protein (GFAP), angiostatins.**

## INTRODUCTION

Propolis, or honeybee glue, is a hard resinous material derived by bees from plant juices and used as a cement in repairing and maintaining the hive (the word "propolis" has been derived from Greek, *pro* – before, and *polis* – city). Propolis is produced by honeybees from substances collected from different parts of plants, buds, and exudates; therefore, it contains pollen, resins, waxes, large amounts of flavonoids, and a myriad of other diverse compounds. The chemical composition of propolis depends notably on the bee species, geographic location, plant sources, climatic changes, and other factors. These differences may be the main cause of dissimilar effects of propolis reported for different areas [1]. In particular, the chemical composition of European, American, African, and Asian propolis varies dramatically, resulting in different biological activities and pharmacological properties of this means [2]. For centuries, propolis and its extracts have been widely used in the Middle East countries as food and beverage additives and also in the folk medicine (as anti-infection agents and wound healing remedies) [3].

Currently, propolis attracts significant scientific attention due to its biological and pharmacological properties. Numerous *in vitro* and *in vivo* studies have reported a plethora of biological activities of different

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worldwide propolis samples, including antibacterial, antifungal, antiviral, antiinflammatory, antioxidant, immunomodulatory, antiplatelet, hepatoprotective, antidiabetic, antiallergic, antiasthmatic, antiproliferative, and antitumor activities [4–6]. The peculiarities of biological and therapeutic effects of propolis are related to its chemical composition. The latter, in turn, depends significantly on the methods used for extraction of bioactive compounds from this substance. Biologically active propolis-derived components mostly demonstrate a low solubility in water, and the amount of phenolic compounds in propolis water extracts is an order of magnitude lower than that in ethanolic extracts. Therefore, alcoholic extracts of propolis are much more widely used [7].

Many studies have been performed to characterize and explore the diverse bioactivities of propolis and its isolated compounds, as well as to evaluate and validate its biomedical potential. Several lines of evidence suggest that, due to the presence of flavonoids, propolis may possess a noticeable therapeutic potential against neurological disorders [8]. Brazilian green propolis has been recently shown to possess neuroprotective capacities in a PC12 cell line exposed to $H_2O_2$, acting as an antioxidant against lipid peroxidation and free radical production. Furthermore, propolis was demonstrated to possess neuroprotective properties against ischemic injury, reducing occlusion-induced brain infarction in mice [9]. A macerated ethanolic extract of Indian propolis has been observed to be highly effective against β-amyloid 25-35 (Aβ25-35)-induced memory impairment in a rat Alzheimer's disease experimental model [10]. However, the exact molecular mechanisms of the above-mentioned beneficial effects of propolis in terms of signal transduction pathways remain practically unknown. It has been hypothesized that propolis-derived bioflavonoids could exert their neuroprotective effects by interacting with different receptors and modulating intracellular neuronal signaling, including protein kinase- and lipid kinase-signaling cascades. The modulation of these signaling pathways is believed to determine neurological effects governing neuronal survival, death, and/or differentiation.

Yet, there is limited information concerning the effects of propolis on astroglial cells, both in the norm and under neurodegeneration conditions. It is well known for many years that astrocytes represent the most abundant cell type in the mammal brain [11]. Astrocytes are multifunctional cells that react to insults and other damages in a special manner referred to as reactive astrogliosis. However, sustaining excessive reactive astrocytes can be deleterious to functional recovery of neurons or contribute to chronic inflammation; these units inhibit axonal regeneration and, thus, induce further neuronal dysfunction. Therefore, astrocyte activation in response to damage should be and is likely to be strictly regulated. The factors that activate astrocytes have been mostly identified. At the same time, it remains unknown: Are there factors that maintain astrocytes in a nonreactive state (or reestablish their nonreactive state) after damage?

As a rule, astrogliosis is accompanied by overproduction of glial fibrillary acidic protein (GFAP) and intensified release of different cytokines and proinflammatory factors; GFAP is a classic marker of reactive gliosis [12]. Inflammation events caused by reactive astrogliosis augment a number of neurological disorders, such as Alzheimer's, Parkinson's, Huntington's diseases and amyotrophic lateral sclerosis. Due to the destructive effects of astrogliosis, intense attempts are currently applied for seeking new ways to treat astrogliosis [13]. This is why more research studies are needed to understand the molecular mechanisms underlying the cellular events evoked by new means (including propolis) in astroglial cells.

The overproduction of proinflammatory mediators is typically linked to inflammatory stimuli capable of causing acute and/or chronic inflammatory responses; such overproduction determines, to a significant extent, pathogenesis of many neurological diseases [14]. Anti-inflammatory effects of propolis-derived substances are well-documented as one of the important reasons for their cytoprotective activity [15]. The transcription factor NF-κB, primarily discovered in lymphocytes as a nuclear factor of kappa-chain B-cells, was later found in most cell types. NF-κB proteins represent a family of transcription factors, which targets a wide number of genes and contributes to the regulation of cell responses, including both inflammatory and apoptotic regulation [16]. NF-κB is presented as a "central mediator of the immune response," based on its ability to regulate transcription of many inflammatory factors, such as interleukins and inflammatory enzymes [17]. Other more key regulatory cellular proteins, poly(ADP-ribose) polymerases (PARPs), also known as ADP-ribosyltransferases, are members of a protein family playing important roles in cell functioning and viability [18]. Cellular functions of PARPs are diverse and include the regulations of

cell division, transcription, DNA damage repair, and mRNA stability [19]. Moreover, PARPs are involved in the cell reactivity with respect to the cytoplasmic stress response, antiviral response, and response to unfolded proteins [20].

One of the most important functions of astrocytes is their involvement in the formation, integrity, and remodeling of the blood-brain barrier (BBB). Both *in vivo* and *in vitro* studies have supported a role for astrocytes in the regulation of cerebral blood flow, which is mediated, in particular, by the control of endotheliocyte activity [21]. Recently, we have discovered that, among a variety of endothelium-regulating molecules, astrocytes produce angiostatins, the proteolytically-derived fragments of the parent protein, plasminogen [22]. Angiostatins are known as powerful endogenous suppressors of neovascularization, which underlies their involvement in inhibition of cancer growth, the metastatic process, and inflammation [23, 24]. Ruy et al. [25] demonstrated that intracerebral injection of angiostatin into rats with $A\beta_{1-42}$-induced neurotoxicity initiates a broad spectrum of effects, including reduction of the inflammatory reactivity, stabilization of vascular remodeling, and neuroprotection.

Thus, the goal of our study was to investigate effects of a propolis ethanol extract (PEE) on the astroglial response and levels of specific cytoskeletal, regulatory, and anti-angiogenic proteins in a primary culture of rat brain astrocytes.

## METHODS

**Preparation of the Propolis Extract.** Bee propolis was collected in the Bingöl region (South-Eastern Turkey) in 2016. Propolis was crushed in a ceramic mortar with thorough grinding, and 30 g of the substance was extracted in 270 g of 96% ethanol for 12 h with rotary shaking in darkness. The extract was filtered and concentrated in a rotary evaporator with controlled temperature (40°C) and reduced pressure. The concentrated ethanol extract was lyophilized to obtain a dry pellet; these samples were considered the propolis ethanol extract (PEE). The samples were protected with aluminum foil from light and stored at −80°C for long-term storage. The yield of the obtained extract (% w/w), in terms of the starting amount of propolis, was 21.7%.

**Cell Culture and Treatment Regime.** Primary rat astrocyte cell cultures were obtained from 1-day-old (newborn) rats. Briefly, the entire brain was isolated from rat pups and put in cold Hanks' solution; the blood vessels and meninges were removed. The brain tissue was minced with sterile forceps and scissors to a homogeneous state. The brain homogenate was treated with 0.25% trypsin-EDTA solution at 37°C for 5 min for cell dissociation. Digesting with trypsin was stopped by the addition of two volumes of high-glucose DMEM culture medium with 10% FBS and antibiotics (penicillin+streptomycin). The cell homogenate was filtered through a 50-μm pore sieve, washed with Hanks' solution (fivefold volume), re-suspended, and centrifuged at 1,500 rpm for 5 min. The obtained cell pellet was re-suspended in DMEM with the addition of 10% FBS, 25 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cells were seeded on cell culture flasks (75 $cm^2$) and cultured at 37°C in a humidified atmosphere with 5% $CO_2$. The medium was replaced every three days for 12 days. On the last three days, the cells were shaken for 3 h at 150 rpm with subsequent medium changing, to remove microglial cells. After 12 days, the astrocytes were deattached by trypsinization with 0.25% trypsin-EDTA solution at 37 °C for 5 min, centrifuged, dissociated in DMEM, and seeded into 7-cm Petri dishes at a $10^5$ $cm^{-2}$ density. The culture purity was determined to be no less than 95% by GFAP staining.

After 2 or 3 days, when the confluence reached 70-80%, the cells were incubated overnight in a medium without FBS and then treated with the PEE for 24 h. A dried PEE pellet was diluted in pure sterile DMSO and then mixed with the medium for 10 mg/ml PEE and 10% DMSO final concentrations. Specific dilutions were prepared from this stock solution for each assay, to achieve different propolis concentrations, 10, 25, or 100 μg/ml. The highest amount of DMSO (0.1%), corresponding to the DMSO amount in the 100 μg/ml PEE sample, was dissolved in the medium and added to control cells.

**Cell Viability Test.** The level of cell viability was measured by monitoring the capability of cells of reducing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) [26]. The PEE effect on the primary astroglial cell viability was studied with different PEE concentrations for 24 h. Cells were harvested from confluenced plates, and $10^5$ cells/ml samples were suspended in DMEM complemented with 10 % FBS. The cells were seeded into 96-well plates at $2 \cdot 10^4$ cells per well and incubated overnight at 37°C in a humidified atmosphere with 5%

$CO_2$. After the medium was changed, the cells were exposed to various concentrations of freeze-dried PEE (10, 25, and 100 μg/ml) dissolved in DMSO. Untreated (control) cells were exposed to 0.1% DMSO considering that the final DMSO concentration in each well did not exceed this value. Concentrations of the tested substances were chosen based on the published data [27].

Each PEE concentration for exposures was represented in four wells and replicated twice. The medium was removed after 24-h-long incubation, and the wells were rinsed with PBS. The washed cells were incubated with 180 μl PBS and 20 μl of the MTT reagent (5 mg/ml) for 4 h at 37°C in a humidified atmosphere with 5% $CO_2$. After 4 h, the MTT reagent solution was removed, and DMSO was added in a 180 μl per well volume for 10 min. The absorbance was measured at a 570 nm wavelength in the presence of 20 μl of Sorensen's buffer. The data are presented as percentages of the control value.

**Protein Sample Preparation and Western Blot.** The cells were washed thrice with cold PBS after a 24-h-long exposure and collected with scratching without trypsinization. The astrocytes were lysed in the RIPA buffer [28] containing a proteinase and phosphatase inhibitor cocktail, and proteins were extracted for 60 min at 4°C. The whole-cell lysates were centrifuged for 20 min at 40,000g. The protein content in the supernatants was measured by the Bradford method [29] using BSA as the standard. The supernatant of each lysate was diluted (1:1) in Laemmli sample buffer containing 0.1 M dithiothreitol and boiled for 5 min (for angiostatin detection, samples were prepared with the use of the nonreducing buffer). Protein samples were frozen and stored at –20°C, no longer than two weeks until analysis.

Proteins were separated by PAGE in 10% acrylamide; then they were transferred on a PVDF membrane by electroblot. After blocking in 5% BSA-PBS, the membrane was probed with rabbit anti-GFAP (Santa Cruz, sc-9065, USA), anti-NF-κB (Abcam, ab16502), anti-PARP (Abcam, ab6079), and anti-actin as loading control (Abcam, ab1801; all USA) antibodies. Anti-angiostatin antibodies were produced in rabbits, as was described elsewhere [28]. Secondary anti-rabbit IgG antibodies conjugated with horseradish peroxidase (Abcam, ab6721, USA) were used to visualize the corresponding polypeptide bands. Immunostaining was developed by an enhanced chemiluminiscence method with the use of X-ray films (Konica Minolta, Japan).

Densitometric analysis of the immunostaining intensities was performed with the use of TotalLab TL120 software (USA). The intensity value in each band was normalized with respect to the intensity of the corresponding actin band. Every track on the blotogram was corrected for the background level by subtracting a tracing of the nonreactive area on the X-ray film.

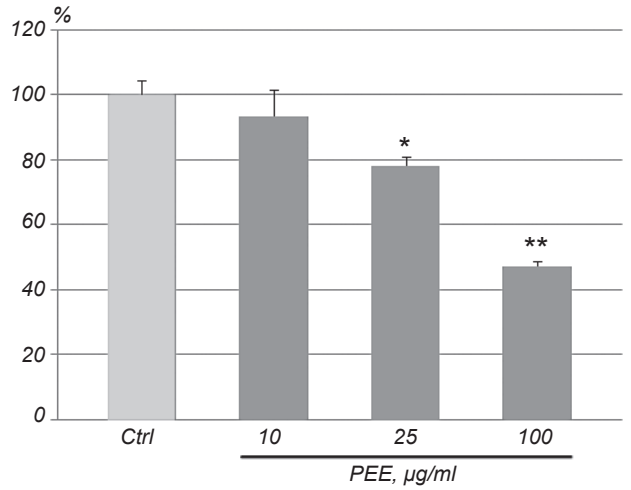
**Statistical Analysis.** Statistical comparisons of the data were made using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. Numerical data are presented below as means ± ± s.e.m. obtained in at least three independent measurements. Differences with $P$ values less than 0.05 were considered statistically significant.

## RESULTS

The patterns observed in this study reflect the astrocyte response on the action of compounds present in the alcoholic extract of propolis obtained from a definite geographic region (South-Eastern Turkey). In this case, cellular responses of astrocytes are realized through multiple ways, which involve alterations of the astrocytic proteome on the levels of signaling proteins, including the pro-apoptotic pathway regulators and inhibitors of angiogenesis.

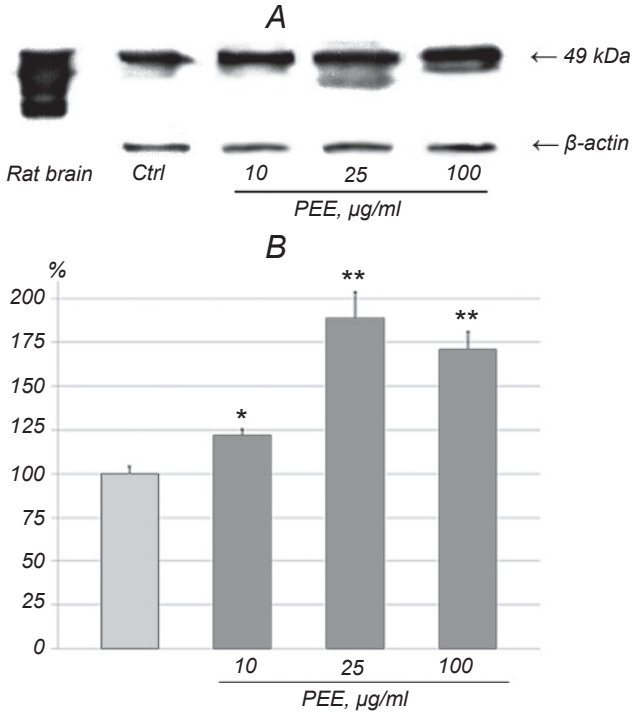
**Effects of the PEE on the Viability of Primary Astrocytes.** To test whether exposure to the PEE interfered with the astrocyte viability, primary astrocytes treated by 10 to 100 μg/ml PEE were assayed with the MTT test. As is seen in Fig. 1, the PEE in doses of 25 and 100 μg/ml manifested a significant cytotoxicity with respect to astrocytes. The MTT assay revealed that the astrocyte viability was reduced with increasing PEE doses. The lowest PEE dose applied did not exert a statistically significant effect on the cell viability (while showing some trend), though 25 and 100 μg/ml expositions caused marked decreases in the survival rate (22 and 53% decrements, respectively; $P<0.05$). These data demonstrate that treatment with PEE overdoses may induce the noticeable cytotoxic effect on astrocytes.

**Effects of the PEE on GFAP Expression.** Results of immunochemical detection of GFAP, a principal astroglial marker and stress-related protein, suggest that astrocytes treated with the PEE acquired a reactive phenotype prior to triggering the cell death. However, a non-monotonic dose-response relationship between the PEE exposure and changes in the GFAP



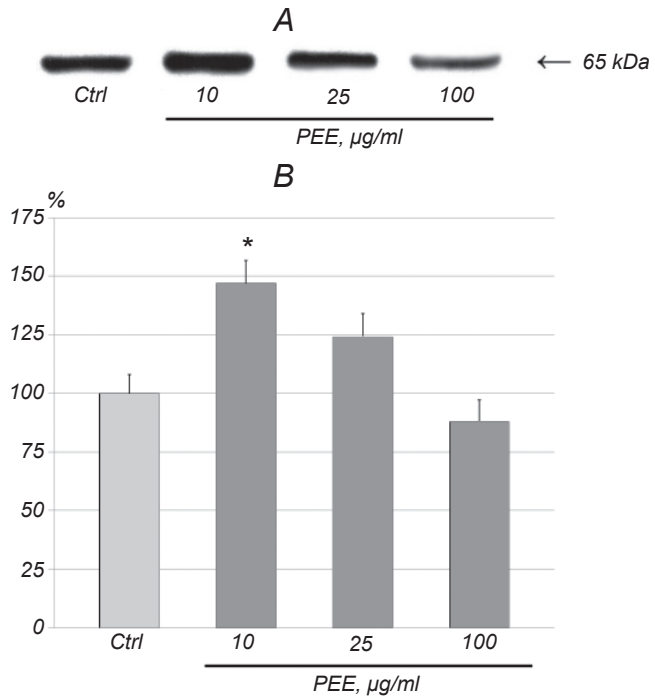

**F i g. 1.** Effects of the propolis ethanol extract (PEE) on the viability of primary cultured astrocytes (results of MTT assay); *$P < 0.05$, **$P < 0.01$ vs. control.

**Р и с. 1.** Вплив спиртового екстракту прополісу (СЕП) на життєздатність астроцитів у первинній культурі (результати МТТ-тесту); *$P < 0.05$, **$P < 0.01$ у порівнянні з контролем.




**F i g. 2.** Effects of the propolis ethanol extract (PEE) on the level of glial fibrillary acidic protein (GFAP) in primary cultured astrocytes: A) Western blot of control and PEE-exposed astrocytes; B) relative content of GFAP in control and PEE-exposed astrocytes. The rat brain protein extract was loaded as a positive control; *$P < 0.05$, **$P < 0.01$ vs. control.

**Р и с. 2.** Вплив спиртового екстракту прополісу на рівень гліального фібрилярного кислого протеїну в культивованих астроцитах (первинна культура).



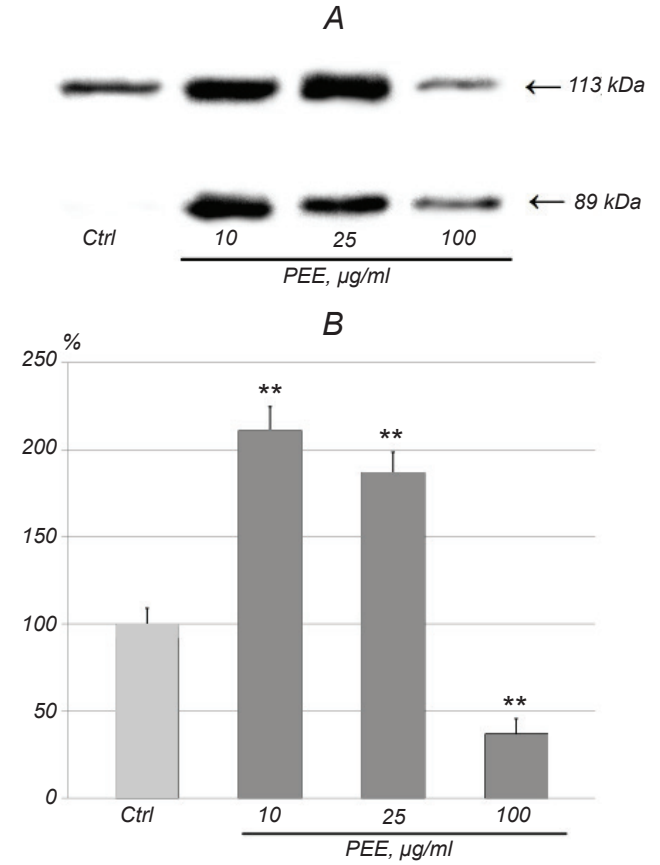

**F i g. 3.** Effects of the propolis ethanol extract (PEE) on the NF-κB level in primary cultured astrocytes: A) Western blot of control and PEE-exposed astrocytes; B) relative content of NF-κB in control and PEE-exposed astrocytes; *$P < 0.05$ vs. control.

**Р и с. 3.** Вплив спиртового екстракту прополісу на рівень NF-κB у культивованих астроцитах.

levels was observed, as is shown in Fig. 2. It should be noted that signs of astrocytosis (a 22% elevation of the GFAP level; $P < 0.05$ vs. control) were observed even after treatment of the culture with the lowest PEE dose used (10 μg/ml). The exposure of astrocytes to 25 and 100 μg/ml PEE caused statistically significant dramatic elevations of GFAP content (by 89 and 71% vs. control, respectively). These results indicate that, prior to the viability loss, astrocytes became reactive and began to synthesize an abundant amount of GFAP. It is possible that cellular protective mechanisms are exhausted after PEE exposure, and astrocytes begin to swell.

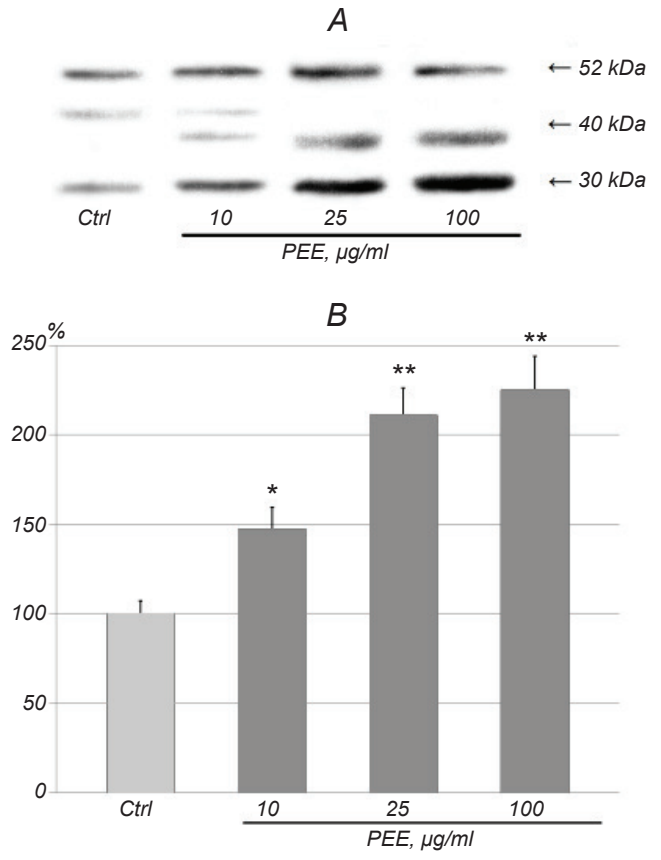
**Effects of the PEE on NF-kB Expression.** Results of Western blot analysis (Fig. 3A) showed that PEE exposure significantly affects NF-kB expression in primary astrocytes.

Intriguingly, the most prominent effect on the NF-κB level was exerted by 10 μg/ml PEE (45% increase, as compared to the control, $P < 0.05$). A higher PEE concentration (25 μg/ml) caused a 24 % elevation in the NF-κB expression (statistically insignificant,




**F i g. 4.** Effects of the propolis ethanol extract (PEE) on the poly(ADP-ribose) polymerase (PARP) level in primary cultured astrocytes: A) Western blot of control and PEE-exposed astrocytes; B) relative content of PARP in control and PEE-exposed astrocytes; $^{**}P < 0.01$ vs. control.

**Р и с. 4.** Вплив спиртового екстракту прополісу на рівень полі(АДФ-рибозо)полімерази в культивованих астроцитах.




**F i g. 5.** Effects of the propolis ethanol extract (PEE) on angiostatin production by primary cultured astrocytes: A) Western blot of control and PEE-exposed astrocytes; B) relative content of angiostatins in control and PEE-exposed astrocytes; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

**Р и с. 5.** Вплив спиртового екстракту прополісу на продукцію ангіостатинів культивованими астроцитами.

$P > 0.05$ vs. control). Treatment with the highest PEE dose (100 μg/ml) led to a tendency toward a decrease in NF-κB expression (by 12%, on average, as compared to the control); however, this change did not reach the level of statistical significance (Fig. 3B). These results suggest that effects of the low-dose PEE treatment may be mediated through modulation of NF-κB signaling.

**Effects of the PEE on PARP Expression.** Next, there was the question whether the PEE is capable of affecting the expression of PARP as in the case of NF-κB. As is shown in Fig. 4A, a single PARP polypeptide (113 kDa) was visualized in the control samples, while two PARP immunoreactive bands, corresponding to intact and truncated (89 kDa) polypeptides, were detected by Western blot in lysates of astrocytes exposed to various PEE concentrations.

The effects of PEE treatment on the PARP level demonstrated a bidirectional pattern. When smaller PEE concentrations (10 and 25 μg/ml) were used, the PARP level in primary cultured astrocytes dramatically (more than two times) increased. At the same time, when PEE was applied in the high concentration (100 μg/ml), the above-mentioned index demonstrated a significant (nearly threefold) drop; it corresponded only to about 37% of the control level (Fig. 4 B).

**Effects of the PEE on Angiostatin Production.** Western blot analysis of primary astrocyte lysates revealed at least three major immunoreactive angiostatin bands with the apparent molecular masses of 50, 38, and 30 kDa (Fig. 5A).

As an important proof of PEE effects on astrocytic angiogenesis-regulating molecules, we found that

PEE treatment promotes angiostatin generation in a dose-dependent manner. Thus, 10, 25, or 100 μg/ml PEE induced 47, 111, and 125% elevations in the angiostatin level, respectively, and each of these alterations was statistically significant (Fig. 5B). These data indicate that the conversion of plasminogen into angiostatins was strongly enhanced by PEE treatment of primary rat brain astrocytes.

## DISCUSSION

Propolis demonstrates a wide spectrum of biological and pharmacological properties, which have been extensively studied in cell and animal experiments; thus, this substance should be considered as a drug. More than 300 chemical compounds were identified in propolis samples collected in different regions. The main chemical classes and the most bioactive compounds found in propolis are phenolic compounds (including phenolic acids), flavonoids, and their derivatives [2, 30]. Due to the fact that the propolis composition is highly variable and depends on the season, region and altitude of the biotope, collecting procedures, food availability, and activity developed during propolis exploitation, propolis can exert a variety of biological effects, from antioxidative and cytoprotective to pro-oxidative and cytotoxic [4, 31-33]. Through *in vitro* assays, it is possible to define different mechanisms of actions of propolis constituents, but it should be taken into account that this greatly depends on the type of cell culture studied. Many studies emphasize proapoptotic effects of propolis-derived caffeic acid phenethyl ester and chrysin (5,7-dihydroxyflavone) on normal and transformed cells [15, 34]. Earlier, an attempt to clarify effects of the propolis-extracted compounds in the human astrocytic cell line SVGp12 has been made [35]. The authors observed pronounced inhibition of cell growth, mitochondrial membrane damage, and induction of an intrinsic apoptosis pathway.

To our knowledge, the present study is the first performed to identify PEE-induced effects with respect to brain astrocytes in a primary culture. Such a type of experiments is regarded to more closely mimic the physiology of cells *in vivo*. Furthermore, we expand our understanding of the propolis action on astrocytes reporting that PEE-caused cytotoxicity is accompanied by the cytoskeleton rearrangement, modulation of signaling protein expression, and alteration of the angiogenesis regulatory levels.

The up-regulation of GFAP expression is one of the most prominent hallmarks of the astrocytic reactivity; it reflects the general synthetic capability of astrocytes [12, 13]. However, results of our study, in which elevation of the GFAP levels in astrocytes was suppressed by PEE treatment, seem to be contradictory with respect to the loss of cell survival. Nevertheless, the respective observations may be related to the overactivity of cells, when intensified GFAP synthesis (an early response) was followed by a metabolic collapse due to exhausting of energy reserves in PEE-exposed astrocytes (resulting in a decrease in the index of viability). There are some facts supporting this idea; several research groups [27, 35, 36] have mentioned that cell shrinkage, condensation of chromatin, nuclear fragmentation, appearance of cytoplasmic membrane blisters, and formation of apoptotic bodies due to increased metabolism and enhanced requirements for energy are provoked by propolis treatment. Based on the data of GFAP Western blots, we can assume that the shift from a hyperactive state toward depressed metabolic conditions induced by exposition of primary astrocytes to the PEE is developed via intense cytoskeletal reconstruction.

Several biochemical events have been proposed to explain the cytotoxic effects of propolis and its polyphenolic compounds; these are inhibition of signal molecules, modulation of the activity of oncogenes encouraging the processes of apoptosis/necrosis, change in the redox state of treated cells, etc. [4, 7]. Although a large body of evidence suggests that phenolic acids and their analogs display potent antioxidant capacities, other authors [37] report that drupanin, baccharin, and cinnamic acid derivatives are able to inhibit the growth of several cell lines by inducing intense apoptosis. There are several conditions where excessive production of mitochondria-produced reactive oxygen species (ROSs) can be associated with cell apoptosis [38]. Propolis can induce ROS formation in yeasts (*S. cerevisiae*), as well as in some types of mammalian cells [39]. Moreover, despite the high cytotoxicity displayed by most of the propolis samples against tumor cell lines, some types of propolis also demonstrated obvious toxicity with respect to nontumor (normal) cells, e.g., to those in liver primary culture (PLP2) or human normal epithelial embryonic kidney culture (Hek-293) [40]. Da Silva et al. [41] have recently demonstrated that propolis induced apoptosis and cell cycle arrest in G2/M phase Hep-2 cells, while pre-incubation of the cells with an antioxidant compound, NAC, exerted significant

protective effects against propolis-induced cell death. These results indicate that oxidative stress can be a possible mechanism responsible for intensification of apoptosis and necrosis events induced by propolis. It is obvious that differences between the responses of various cell types to propolis treatment could be explained by the specific propolis composition, cells phenotype, and experimental conditions. Therefore, future studies are needed to check whether ROS-mediated cell death in primary astrocytes is induced by alcohol extracts of propolis.

In our study, additional evidence of activation of the ROS-mediated pathway in primary astrocytes exposed to the PEE has been obtained. NF-κB is widely used by eukaryotic cells as a regulator of genes, which controls cell proliferation and cell survival [16]. Oxidative stress, inflammation, and reactive astrogliosis are closely interconnected in the CNS, and the pro-inflammatory nuclear factor NF-kB pathway is activated in astrocytes [14]. It is of interest that an increased NF-kB level was first identified in rat reactive astrocytes after kainic acid-induced seizure [42]. Recent studies have also shown that cultured astrocytes exposed to pro-inflammatory compounds (a mixture of gangliosides) demonstrate reactive astrocytosis, overexpression of GFAP, and intensification of NF-kB production. Moreover, NF-kB regulates expression of the *GFAP* gene, and activation of NF-kB contributes to increased GFAP expression and the development of reactive astrocytosis [43]. These observations are completely consistent with our Western blot data indicating that there is a parallel elevation in both GFAP and NF-kB levels induced by 10 μg/ml PEE treatment. NF-κB controls many genes involved in inflammation; this peptide was found to be chronically active in many inflammatory diseases, including neurodegenerative conditions. There are experimental data indicating activation of the NF-κB pathway, which is responsible for axonal loss, gliosis, demyelination, deterioration of the white matter integrity, and memory impairment [44]. Among all known inducers of the NF-κB expression, ROSs are of particular interest. Our data are partly in line with the results of Conti et al. [45] who showed that propolis activates the NF-κB production in human dendritic cells. Although we found that PEE treatment exerted a relatively mild effect on NF-κB expression in astrocytes, the question whether propolis can affect NF-κB-mediated physiological processes *in vivo* (such as learning and memory) remains open and should be clarified.

In contrast to NF-κB, the PARP levels appeared to be more susceptible to PEE treatment. It is known that PARP is an enzyme/enzyme complex that utilizes $NAD^+$ to form poly(ADP-ribose) polymers on specific acceptor proteins. PARP was identified as an enzyme performing a central role in the repair of damaged DNA. Many additional functions of PARP isoforms in biochemical and molecular signaling have now been demonstrated. Apart from its role in repairing DNA damage, PARP-1 also plays important roles in transcription, cardiac remodeling, vasoconstriction, regulation of astrocyte and microglial functions, long-term memory, and aging [46]. At the same time, excessive PARP activation promotes cell death under conditions that cause extensive DNA damage, including ROS-induced breakage of DNA strands. As is believed, the main reason for PARP-induced cell death is depletion of $NAD^+$ and ATP pools [28]. At present, it is generally accepted that PARP cleavage catalyzed by "suicidal" proteases (such as caspases, calpains, cathepsins, granzymes, and MMPs) can be considered the sign of cell death [47]. Therefore, PARP fragmentation, yielding proteolytic fragments and observed in our study as an effect of the PEE on primary astrocytes, may be indicative of the fact that PEE-induced initiation of critical events in the cell survival program has begun. Based on our findings, it can be supposed that excessive ROS production induced by PEE treatment can result in DNA damage, PARP overexpression and cleavage, and subsequent poly(ADP-ribosyl)ation by PARP, which can trigger the cell death programs. Decreases in the NF-κB, PARP, and GFAP levels (though having different kinetics), which are observed in astrocytes exposed to the highest PEE dose (100 μg/ml), can be related to the failure of total protein synthesis due to metabolic depletion.

Astrocytes are involved in many processes in the CNS, including synaptic formation and plasticity, recycling of neurotransmitters, maintenance of the blood-brain barrier, trophic and metabolic support of neurons, injury reparation, etc. [11, 13]. Among all astrocytic functions, regulation of angiogenesis (formation of new blood vessels in addition to the pre-existing ones) is of particular importance [48]. It is generally accepted that angiogenesis is normally kept at an adequate level by a tightly balanced production of pro- and anti-angiogenic factors. Angiogenesis is implicated in the growth of all solid malignant tumors, providing sufficient supply of oxygen and nutrients to the processes of growth and development

of metastases [49]. It should be mentioned that brain tumors, in particular, are highly angiogenic, and astrocytes are involved directly in the angiogenesis in these neoplasms [50]. It has been observed that brain tumor cells noticeably activate their glial nontransformed surrounding. For example, glioma cells have been found to produce factors promoting the proliferation and motility of stromal astrocytes, thus accounting for the high mortality and morbidity of this disease [51]. Reactive astrocytes have been found to contribute to the brain metastatic capacity of melanoma cells through production of proteinases that degrade the extracellular matrix. Since reactive astrocytes are the main source of MMP-2, glioma cells effectively influence astrocytes and increase their own invasiveness via stimulation of the uPA-plasmin cascade [52].

Angiostatins, a family of proteolytically derived fragments of a 92-kDa plasmin proenzyme, plasminogen, consist of various numbers of kringle domains of the latter. Angiostatins are among the most powerful endogenous inhibitors of the vessel growth; these agents are the main antagonists of pro-angiogenic factors. They can be formed predominantly on the surface of various cells due to plasminogen fragmentation performed by matrix metalloproteinases (MMPs), neutrophil elastase, and cathepsins or to the development of autoproteolysis of plasmin. The resultant multiple fragments of the kringle domains of plasminogen have been shown to specifically inhibit endothelial cell proliferation and migration, as well as to induce apoptosis of endotheliocytes. Therefore, the antitumor effects of angiostatins are to a considerable extent based on their capacity to effectively restrict tumor-induced neovascularization [23, 24].

Astrocytes are able to synthesize plasminogen and also components of the plasminogen activation cascade (tPA, uPA) and several types of MMPs, which are involved in converting plasminogen into angiostatins. Thus, astrocytes can be considerably responsible for producing angiostatin in the CNS [52, 53]. Indeed, we have shown in our recent study that the production of angiostatins by quiescent primary astrocytes is a common event [22]. To extend these findings, we investigated here whether exposition to the PEE has an impact on the astrocytic ability to generate angiostatins *in vitro*. The enhanced level of angiostatin formation induced by increasing PEE doses can be explained by the fact that dying cells are characterized by elevated expression of plasminogen receptors. Our finding that increasing angiostatin levels correlate with PEE-induced cytotoxicity corroborates the results of O'Mullane et al. [54]. These authors clarified the role of the plasminogen activation system in a degradation phase of cellular apoptosis. Whether angiostatins that are side-products of plasminogen/plasmin degradation can be responsible for the regulation of angiogenesis in the CNS or not, remains to be elucidated. The above hypothesis obtained some support; intracranial injections of angiostatin into animals with experimental Aβ-induced Alzheimer's disease exerted a neuroprotective effect, inhibited inflammation, and stabilized vascular remodeling [25]. It is possible that treatment directed toward inhibition of astrocyte activity may improve the formation of angiostatin, which in turn would suppress tumorigenic vessel growth.

Like other functions of astrocytes, the control of angiogenesis is related to the abilities of these gliocytes to uptake and release certain substances. Reactive astrocytes are known to secrete vascular endothelial growth factor (VEGF) in response to ischemia or other injury, and this factor helps to restore the blood supply. At the same time, excessive VEGF release can result in disruption of the endothelial barrier [55]. A few studies demonstrated that propolis possesses significant anti-angiogenic capacities via inhibiting VEGF secretion and inducing apoptosis of endotheliocytes [27, 56]. In the case of astrocytes, it is possible that the presence of angiostatins counteracts the expression of pro-angiogenic growth factors (such as VEGF), and this may have plausible physiological and pathophysiological significance. Thus, considering current findings, it is possible to propose an alternative mechanism for the PEE-mediated anti-angiogenic effects based on activation of angiostatin formation by astrocytes.

In conclusion, results of our study for the first time provide certain evidence that the alcoholic extract prepared from Turkish propolis displays the noticeable cytotoxicity in cultured primary astrocytes. This effect is related to the modulation of expression of the proteins responsible for cytoskeletal, signaling, and angiogenic regulations. These findings can open some novel prospects for the use of propolis as a complementary medication that can be implemented in the prevention and treatment of CNS disorders associated with excessive glial activation.

All procedures with animals were performed in compliance with the Guidelines for Care and Use of Laboratory Animals by the National Institute of Health (NIH) and were approved by the Animal Experimentation Ethics Committee of the Bingöl University (Bingöl, Turkey; protocol No. 201602).

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## ВПЛИВИ ЕКСТРАКТУ ПРОПОЛІСУ НА ЖИТТЄЗДАТНІСТЬ АСТРОЦИТІВ ГОЛОВНОГО МОЗКУ ЩУРІВ ТА ВМІСТ ЦИТОСКЕЛЕТНОГО ТА РЕГУЛЯТОРНИХ ПРОТЕЇНІВ У ЦИХ КЛІТИНАХ: ДОСЛІДЖЕННЯ *IN VITRO*

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Р е з ю м е

Перспективність використання прополісу як профілактичного та лікувального засобу є загальновизнаною, проте його цитотоксичні ефекти щодо астроцитів вивчені недостатньо. Ми досліджували впливи спиртового екстракту прополісу (СЕП) на астроцити головного мозку щура в умовах первинної культури, визначаючи ключові молекулярні мішені для дії такого екстракту. Первинні астроцити інкубували з додаванням СЕП у дозах 10, 25 або 100 мкг/мл протягом 24 год, після чого життєздатність клітин оцінювали за допомогою МТТ-тесту. Для встановлення молекулярних механізмів, що лежать в основі клітинної відповіді на дію СЕП, аналізували вміст гліального фібрилярного кислого протеїну (ГФКП), транскрипційного ядерного фактора NF-κB, полі(АДФ-рибозо)полімерази (ПАРП) та ангіостатинів, використовуючи Вестерн-блотинг. СЕП зумовлював цитотоксичний вплив, і відповідні ефекти були дозозалежними. Під дією СЕП відбувалася модуляція сигнальних шляхів в астроцитах через впливи на експресію NF-κB та ПАРП. У той же час СЕП посилював синтез ГФКП та процес фібрилогенезу; це призводило до активації астроцитів, що передувало пригніченню їх життєздатності. СЕП спричиняв істотне посилення продукції астроцитами ізоформ ангіостатинів; це може забезпечувати набуття антиангіогенних властивостей зазначеними клітинами. Отримані дані свідчать про те, що дозозалежний цитотоксичний вплив СЕП на астроцити опосередковується модуляцією реконструкції астроцитарного цитоскелета та функціонування проапоптотичних сигнальних шляхів. Прополіс, його компоненти та похідні є широкодоступними, безпечними та недорогими агентами, котрі можуть бути використані як ефективні засоби для профілактики та терапії низки патологій нервової системи, в тому числі злоякісних новоутворень та нейродегенеративних станів, асоційованих із надмірною активацією клітин астроглії.

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