JRVM
Journal of Research in Veterinary Medicine

RESEARCH ARTICLE

J Res Vet Med. 2022: 41 (2) 143-149
DOI:10.30782/jrvm.1161358

In Vitro Cytotoxicity and Genotoxicity Screening of *Cuscuta Arvensis* Beyr. and *Achillea Wilhelmsii* C. Koch

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Received 15-08-2022 Accepted 24-12-2022

Abstract

Plant-based compounds have been used for medicinal purposes since ancient times, as easily accessible and low-cost treatment options. Despite the widespread belief that plants are quite safe and devoid of side effects, scientific studies have revealed the toxicity potential of active components of plants on healthy cells. The present study was designed to investigate in vitro cytotoxicity and genotoxicity potential of Achillea wilhelmsii C. Koch and Cuscuta arvensis Beyr., which are frequently used in traditional medicine. In this context, cytotoxicity evaluation of the extracts was performed by MTT (3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Our cytotoxicity results indicate that the extract from A. wilhelmsii did not affect the viability of fibroblasts at any tested concentrations, on the contrary, significantly stimulated cell proliferation from a concentration of 25 µg/mL. On the other hand, the extract from C. arvensis significantly reduced the viability of fibroblasts at all concentrations tested. In the second part of this research, the DNA damaging potential of the extracts was investigated by in vitro comet assay at non-cytotoxic concentrations. A. wilhelmsii extract caused a significant increase in the percentage of DNA in the tail (%TDNA), which is considered as an indicator of DNA damage, only at the highest concentration, while C. arvensis extract did not significantly affect %TDNA at concentrations tested. The results of the present study indicated that the methanolic extract from A. wilhelmsii may be considered safe up to a concentration of 100 µg/mL, however, the cytotoxicity potential of C. arvensis may be a factor limiting its safe use.

Keywords: Achillea wilhelmsii C. Koch, Cuscuta arvensis Beyr., Cytotoxicity, Genotoxicity

Introduction

Medicinal plants are the major reservoir for a wide variety of pharmacologically active phytocompounds and accordingly have been used for therapeutic purposes for many years.[1,2] According to the World Health Organization (WHO), up to 80% of the world's people rely upon plant-based treatment for primary health care due to its multiple advantages over conventional therapies (such as low cost, easy accessibility, low side effects, etc.).[3] Also from the perspective of the pharmaceutical industry, the active components of medicinal plants are considered to be a valuable resource for the development of new pharmacological agents.[4] Despite the profound beneficial effects of medicinal plants, it is well known that maintaining the balance between therapeutic and toxicological effects is the most crucial factor for a plant-based therapy can be considered safe.[5] This situation has led to the necessity of investigating the toxicity potential of medicinal plants and verifying their safe use with scientific evidence-based approaches. In this context, scientific studies have revealed that the genotoxicity and carcinogenicity potentials of some bioactive components in plants are extremely important factors limiting their safe and effective use.[6,7]

The flora of Turkey is very rich in the diversity of medicinal plants as a result of the geographical location of Turkey.[8] Species belonging to the genus *Cuscuta L.* (Cuscutaceae), which is represented by 21 species in the flora of Turkey, have been frequently used in traditional medicine due

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to their pharmacological activities.[9] Among these species, Cuscuta arvensis Beyr. has been mostly used in folk medicine for the treatment of jaundice in newborns and their mothers, in the southeastern part of Turkey.[9,10] The genus Achillea L. (Asteraceae), which has a wide distribution with approximately 42 species in the flora of Turkey, has been frequently used in traditional medicine due to its beneficial properties.[11] The extracts of Achillea species have been used for food supplements and therapeutic applications as an easily accessible source of natural antioxidants.[12] Infusion and decoction of *A. wilhelmsii* C. Koch have been reported to be used in Turkish folk medicine as diuretic, anti-hemorrhoidal, and against abdominal pain.[13] The biological activities of both *C. arvensis* Beyr. and *A. wilhelmsii* C. Koch have been confirmed by *in vitro* and *in vivo* scientific studies. In this context, it has been reported that *C. arvensis* Beyr. extracts with different polarities display antioxidant, anti-inflammatory, antinociceptive, and hepatoprotective properties.[9,14] Similarly, anti-inflammatory, antinociceptive, immunomodulatory, anxiolytic, hepatoprotective and antimicrobial activities of the extracts and essential oil from *A. wilhelmsii* have been previously demonstrated.[11,15-18] Although the pharmacological activities of *C. arvensis* Beyr. and *A. wilhelmsii* C. Koch have been extensively investigated, there are quite limited reports on the toxicological properties of these plants in the scientific literature.

The current study aimed to investigate the toxicity potential of extracts from *C. arvensis* and *A. wilhelmsii* in order to confirm the safety of their therapeutic use. It is well known that the determination of genotoxicity and cytotoxicity of a compound is one of the first steps in the biological evaluation process reported in ISO- 10993-5.[19] In this direction, the methanolic extracts were investigated in terms of their genotoxic and cytotoxic activities on healthy cells under *in vitro* conditions.

Materials and methods

Plant Samples and extraction process

A. wilhelmsii C. Koch was collected from Beyşehir, Derebucak district in Turkey during Spring of 2015. An authenticated voucher specimen (GUE 3490) was maintained in the Herbarium of the Faculty of Pharmacy (Gazi University, Turkey). *A. wilhelmsii* C. Koch (100 g) was extracted with pure methanol (3× 1250 mL) for 48 hours. The extract was dried by the evaporator, then the yield of the methanolic extract was calculated as 23.34%.

C. arvensis Beyr., which was a parasitic plant found on the host lentils, was purchased from a herbalist in Mardin, Turkey. The plant material was identified compared to the specimens (KHB-78) deposited in Kilis 7 Aralik University, Department of Biology. In the extraction process, *C. arvensis* (200 g) was powdered and extracted with methanol (3 × 1500 mL) for 48 hours. The extract was dried by using the evaporator, then the yield of methanolic extract was calculated as 20.67 %.

Cell culture and stock solutions

The 3T3-Swiss albino mouse fibroblast cell line (ATCC _CCL-92) was cultured in the Dulbecco's Modified Eagle's Medium- F12 (DMEM-F12) supplemented with fetal bovine serum (FBS, 10%) and penicillin/streptomycin solution (1%) in a humidified atmosphere containing 5% CO2 at 37 °C. The culture medium was changed twice a week. When cultures reached confluence, the adherent cells were detached from the culture flask through trypsinization (Trypsin-EDTA solution, 0.25%).

For toxicological assessment, the stock solutions of the plant extracts were prepared in dimethyl sulfoxide (DMSO) followed by further required dilutions with DMEM- F12. The highest concentration of DMSO was 0.3 % in the medium and the corresponding amounts of DMSO were added to the control cells. All stock solutions were stored at (-) 20°C until use.

Cytotoxicity assessment (MTT assay)

MTT (3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was performed to assess the cytotoxicity potential of *A. wilhelmsii* and *C. arvensis* methanolic extracts. In brief, the cells were seeded into triplicate wells of a 96-well plate at a density of 1x 10^4 cells/well and incubated overnight. One hundred microliters of the medium, with or without methanolic extracts of the plants (10- 200 µg/mL), were added to each well and the cells were incubated at 37 °C. After 24 h exposure, the cell viability was evaluated using the cell proliferation kit (MTT, Roche, Germany) and the absorbance of each well was read at 595 nm. The effect of each plant extract on cell viability was determined as percent cell viability where the vehicle (DMSO)-treated cells were received as 100% viable.

Genotoxicity assessment (Comet assay)

Treatment of cells with the extracts

The determination of the highest concentration for *in vitro* genotoxicity assessment is based on the results of cytotoxicity analysis.[19,20] Accordingly, the concentration ranges that yielded a cell survival rate of approximately 70% were selected for subsequent DNA damage analysis. [19,20] 3T3-Swiss albino mouse fibroblast cells were seeded in six-well

plates at a density of 1.5 x 10^5 cells /mL and incubated for 24 h. Then, the cells were treated with non-cytotoxic concentrations of the methanolic extracts (50, 100 and 200 µg/mL for *A. wilhelmsii*; 10, 25 and 50 µg/mL for *C. arvensis*) for 3 hours. Hydrogen peroxide (300 µM) was used as the positive control for DNA damage. At the end of the treatment period, the cells were washed twice with 4 mL of cool phosphate-buffered saline (PBS, pH 7.4), suspended by trypsinization, and collected into tubes. The cells were obtained by centrifugation at 200 g for 5 min and suspended in 300 µL of cold PBS for analysis.

Comet assay

The comet assay was performed under alkaline conditions (pH >13) using the method of Singh et al.[21] A 25 µL aliquot of the cell suspension was mixed with 75 µL of low melting point agarose in PBS at 37 °C (0.65%). This mixture was rapidly placed on microscope slides previously covered with normal melting point agarose (0.5%). Coverslips were added to each slide and the slides were maintained at 4 °C for 10 min to solidify. After removing the coverslips, the slides were left in a cold freshly prepared lysing solution (2.5M NaCl, 100mM Na-EDTA, 10mM Tris, pH: 10.0, DMSO (10%) and Triton X-100 (1%) mixed prior to use) at least 1 h, at 4°C. The slides were removed from the lysing solution and immediately incubated in a horizontal electrophoresis tank filled with a freshly prepared electrophoresis buffer (0.3 M NaOH, 1mM EDTA, pH >13), for 20 min. Subsequently, electrophoresis was performed at 25 V/300mA, for 30 minutes. The slides were neutralized with 0.4 M Tris buffer solution (pH: 7.5), fixed with ethanol for 2 minutes, and dried at room temperature. All slides were kept in a closed container until image analysis. All steps of comet assay were performed in the dark to avoid additional DNA damage.

Staining and image analysis

Before image analysis, each slide was stained with 50 µL of ethidium bromide solution (20 µg/mL). Two slides were prepared from each sample and the analysis was performed on randomly 50 cells per slide, using an Olympus BX51fluorescence microscope (Olympus Optical® Co. Ltd, Japan). Image analysis was performed by using image analysis software (Bs 200 Pro Software®, BAB Imaging System in Turkey). The percentage of DNA in the tail (TDNA %) was considered the indicator of DNA damage.

Statistical analysis

Data were expressed as the mean of triplicates ± standard deviation (SD). Statistical analysis of the results was performed by one-way ANOVA followed by Dunnett's multiple comparisons test by using Graphpad Prism® (version 9.1.0, Graphpad Software® Inc., CA, USA). All the results were considered significant at $p < 0.05$. The concentrations of extracts needed for 50% inhibition of cell viability (IC50) were determined by using the Biosoft CalcuSyn software (Biosoft, UK).

Results

Figure 1 represents the percentage of cell viability after treated with various concentrations (10-200 µg/ mL) of methanolic extracts from *A. wilhelmsii* and *C. arvensis*. As shown in the figure, *A. wilhelmsii* extract did not reduce the viability of fibroblasts at any concentrations tested, as well as significantly stimulated fibroblast proliferation from a concentration of 25 µg/ mL ($p<0.001$). The IC50 value could not be detected for *A. wilhelmsii* extract at the range of concentrations used, since it did not reduce the viability of fibroblasts. In contrast, *C. arvensis* extract reduced fibroblast viability by 27.4% to 60.4% compared to untreated cells (IC50: 176.07 µg/ mL).

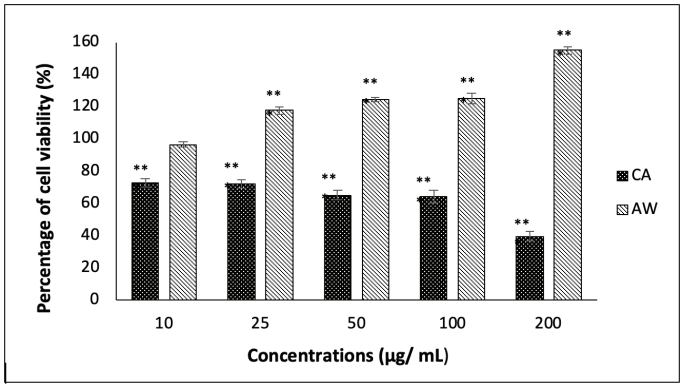

Figure 1. Cytotoxicity of the methanolic extracts from A. wilhelmsii and C. arvensis on Swiss-3T3 albino mouse fibroblasts. ***p<0.001, versus negative control. CA: Cuscuta arvensis Beyr.; AW- Achillea wilhelmsii C. Koch.

The results of the comet assay were shown in Figure 2 and the representative images were presented in Figure 3. The highest DNA damage was detected in cells treated with H2O2 (%TDNA 83.29 ± 1.72). Our findings demonstrated that *A. wilhelmsii* extract did not cause a significant increase in the %TDNA at concentrations of 50 and 100 µg/mL ($p>0.05$). Treatment of *A. wilhelmsii* extract induced DNA damage merely at the highest concentration, as shown by a %14 increase in %TDNA compared to control. (43.34 ± 1.57 versus 37.9 ± 1.63; $p < 0.05$). On the other hand, no significant increase was determined in %TDNA of cells treated with *C. arvensis* extract, indicating that this extract did not induce significant DNA damage at low concentrations ($p > 0.05$).

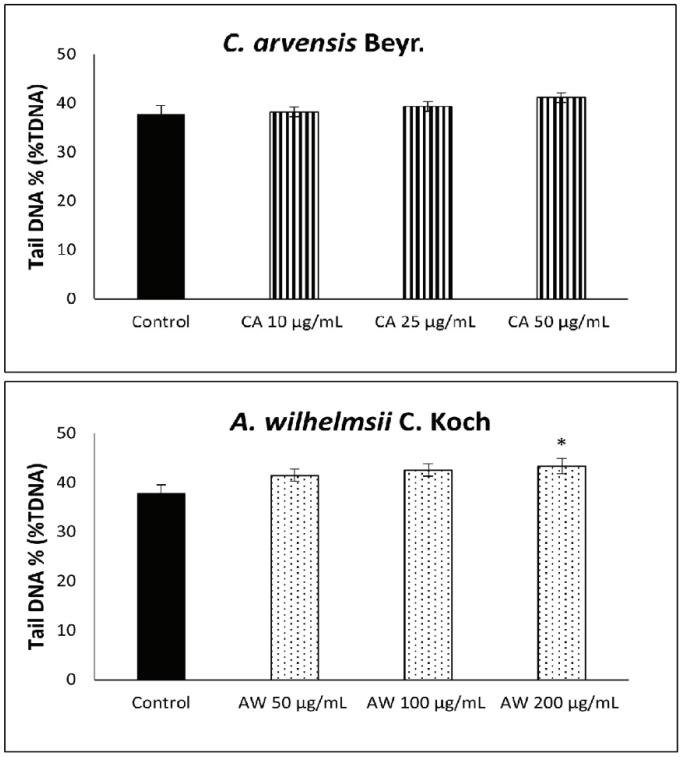

Figure 2. Percentage of tail DNA in cells after treatment with C. arvensis and A. wilhelmsii methanolic extracts. *p<0.05 versus control. CA- Cuscuta arvensis Beyr., AW- Achillea wilhelmsii C. Koch.

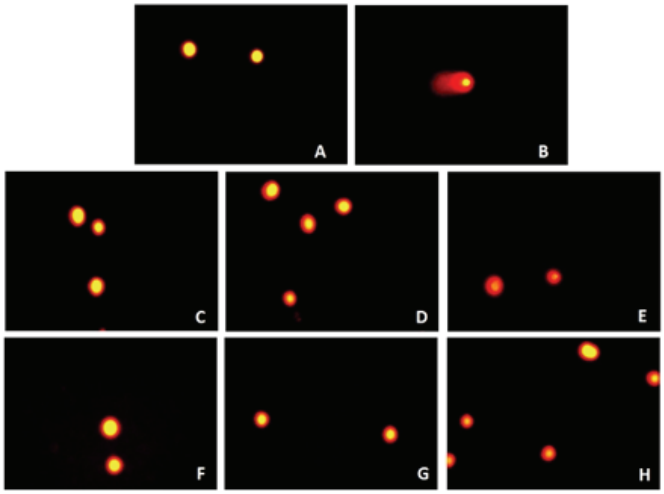

Figure 3. Comet images in fibroblasts treated with the methanolic extracts from A. wilhelmsii and C. arvensis (100x magnification). A) Control, B) Positive control- H2O2 (300 µM), C) A. wilhelmsii extract (50 µg/ mL), D) A. wilhelmsii extract (100 µg/ mL), E) A. wilhelmsii extract (200 µg/ mL), F) C. arvensis extract (10 µg/ mL), G) C. arvensis extract (25 µg/ mL), H) C. arvensis extract (50 µg/ mL). H2O2- Hydrogen peroxide.

Discussion

Evaluation of cytotoxic activity is a very important parameter in revealing the safety profiles of plant-based compounds.[22] Furthermore, since detected DNA damage can be a secondary effect of cytotoxicity, performing a cytotoxicity analysis is essential to avoid false interpretations of genotoxic activity.[22,23] In the present study, screening of cell viability was performed both to evaluate the cytotoxic activities of the extracts and to determine the concentrations to be used in studies on the DNA-damaging potentials of plant extracts.

Our results showed that methanolic extract from *A. wilhelmsii* did not adversely affect fibroblast viability even at high concentrations. Moreover, according to our results, this extract significantly stimulated fibroblast proliferation from a concentration of 25 µg/ mL ($p<0.001$). Previous studies on the biological activities of Achillea species confirm that phytocompounds contained in various extracts of these species can stimulate fibroblast proliferation. Similar to the result of our study, Ghobadian et al.[24] have suggested that the hydroalcoholic extract of A. millefolium stimulates proliferation of human skin fibroblasts at concentrations below 20 mg/mL. In another study investigating the effects of different Achillea species on the treatment of skin wounds, the stimulating effects of A. kotschyi extract on fibroblast proliferation have been reported (at the concentration of 2.5-20 µg/mL).[25] Contrary to the results of these studies, there are also reports indicating the cytotoxicity potential of Achillea species, especially on cancer cell lines. In this context, Sargazi et al.[26] demonstrated the significant antiproliferative activity of hydroalcoholic extract from *A. wilhelmsii* on HeLa cervical cancer cells. This can be explained by the difference in the sensitivity of cancer cells and healthy cells to the extracts or the differences in the phytochemical composition of various Achillea extracts.

According to International Standard Organisation (ISO)-10993-5, cell viability below 70% compared to untreated cells is considered a sign of cytotoxic effect.[19] In the present study, the viability of cells treated with *A. wilhelmsii* extract was above the standard level of cytotoxicity at all tested concentrations, indicating the non-cytotoxic behavior of this extract on mouse fibroblasts. On the other hand, methanolic extract of *C. arvensis* can be reported as cytotoxic in mouse fibroblasts according to the ISO 10993-5 standard, as it reduces cell viability below 70%, at concentrations higher than 25 µg/mL. Despite the widespread use and high therapeutic value of *C. arvensis*, no study was found in the scientific literature evaluating the toxicity potential of the extracts obtained from this plant. On the other hand, a limited number of studies evaluating the toxicity profile of other species from this genus have revealed the cytotoxic profile of Cuscuta extracts, similar to our findings. The findings of the study conducted by Abedini et al.[27] showed that ethanolic extract of C. epithymum exhibited

concentration-dependent cytotoxic activity on fibroblasts from a concentration of 312 µg/mL. In our study, the cytotoxic effect of *C. arvensis* extract was detected at lower concentrations (10-200 µg/mL) than the concentration reported by Abeni et al.[27] It is known that multiple factors such as the type of extraction solvent or extraction technique can affect the quantity of isolated bioactive compounds in plant extracts.[28] In our study, the observed effect on fibroblast viability even at low concentrations can be explained by the possibility that the methanolic extract may contain higher amounts of cytotoxic components. Similarly, it has been reported that chloroform extract of C. reflexa reduced the proliferation of RAW 264.7 murine macrophages and HEK 293 (human embryonic kidney) cells.[29] The antiproliferative activity of C. reflexa has been attributed to the presence of four compounds purified from the extract; scoparone, stigmasta-3,5-diene, p-coumaric acid, and 1-O-p-hydroxy cinnamoyl glucose.[30] On the other hand, in the study performed by Koca- Caliskan et al.[9], kaempferol-3-O-rhamnoside, a flavonol glycoside with antiproliferative activity, has been reported as the major component of *C. arvensis* methanolic extract.[31] In the present study, although no evaluation was carried out on the phytochemical composition of *C. arvensis*, it is possible that the cytotoxic effect on fibroblasts may be related to the phytocompounds with antiproliferative activity previously reported in this species.

Assessment of the potential genotoxicity of plant extracts is a very crucial issue as DNA damage can lead to critical mutations and thus increase the risk of cancer.[6] In the present study, we determined the genotoxicity potential of *A. wilhelmsii* and *C. arvensis* by comet assay, which is a highly sensitive method for assessing cell-specific DNA damage.[23] The increase in % TDNA value was considered to be correlated to the intensity of DNA damage occurring in the fibroblasts. Previous studies have reported that DNA damage can occur as a result of direct interaction of genotoxic compounds with DNA, or it can be an indirect result of oxidative stress induction or cytotoxicity.[32] In order to eliminate conflicting results related to cytotoxicity, we used non-cytotoxic concentrations of the extracts for the comet assay. Our results indicate that while *A. wilhelmsii* extract did not cause DNA damage at concentrations of 50 and 100 µg/mL, it induced DNA damage only at the highest concentration (200 µg/mL). The fact that *A. wilhelmsii* extract stimulated DNA damage (at a concentration of 200 µg/mL) without causing a concomitant increase in cytotoxicity may indicate that high concentrations of the extract may contain direct DNA-reactive compounds.

During the literature review, no study was found that evaluated the genotoxic activities of *A. wilhelmsii* extracts on healthy cells. On the other hand, the potential of *A. wilhelmsii* to cause DNA damage has been evaluated in only cancer cell lines. According to a previous report evaluating the genotoxicity potential of *A. wilhelmsii* in cancer cells, hydroalcoholic extract of *A. wilhelmsii* can cause DNA damage in HeLa cervical cancer cells (at 100 µg/mL concentration), by increasing the phosphorylation of H2AX as a very sensitive marker of unrepaired DNA damage.[26] Considering that *A. wilhelmsii* extract did not cause significant DNA damage up to 200 µg/mL concentration in our study, it can be concluded that this extract may be safe in healthy cells at concentrations reported to cause DNA damage in cancer cells. This can be interpreted as *A. wilhelmsii* extracts can be considered safe on healthy cells at the specified concentrations and can be a potential source for raw material in the pharmaceutical industry. On the other hand, to the best of our knowledge, no reports are available regarding the genotoxicity potential of the extracts from *C. arvensis*. In our study, the genotoxic potential of the methanolic extract from *C. arvensis* was examined in a very low concentration range (10-50 µg/mL), in order to avoid false positive results associated with cytotoxicity. Whereas, in a pharmacological activity study conducted with *C. arvensis*, it has been reported that the methanolic extract exhibited protective activity against acetaminophen-induced hepatotoxicity in the range of 125-250 mg/kg.[9] It is noteworthy that in our study, the non-genotoxic profile of *C. arvensis* extract was revealed at a lower concentration range than the concentrations at which it exhibited pharmacological activity. Upon also considering our cytotoxicity findings, it could be speculated that *C. arvensis* extracts may have toxicity potential on healthy cells, especially at higher concentrations. This view can be supported by the observation of Dokuparthi et al.[33] who reported the mutagenic activity of C. reflexa methanolic extract by Ames test, only at high concentrations (5000 µg/plate and 10000 µg/plate).

Conclusion

Natural products including medicinal plants are very important reservoirs for therapeutic applications and play a crucial role in the designing of new drugs. Despite a general perception that plant-based compounds are quite safe and devoid of side effects, the cytotoxicity/genotoxicity potentials of plant constituents are major factors limiting their use. The present study revealed the *in vitro* toxicological profile of methanolic extracts from *A. wilhelmsii* and *C. arvensis*. Our results demonstrated the cytotoxicity potential of *C. arvensis* extract and the safety profile of *A. wilhelmsii* extract (up to the concentration of 100 µg/mL) on mouse fibroblasts. Although the genotoxicity potential of *A. wilhelmsii* needs to be confirmed by different genotox-

icity assays especially at higher concentrations, this plant can be considered a safe therapeutic alternative at indicated concentrations.

Acknowledgments

This work was supported by the Department of Scientific Research Projects, Istanbul University-Cerrahpasa, Turkey (Grant numbers: BYP-2017-25470 and BYP-2017-27877).

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