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# Effect of *Tarantula cubensis* alcoholic extract and *Nerium oleander* distillate on cell proliferation markers in colon carcinogenesis

## Efecto del extracto alcohólico de *Tarantula cubensis* y el destilado de *Nerium oleander* sobre los marcadores de proliferación celular en la carcinogénesis de colon

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**ABSTRACT**

Colorectal Cancer (CRC) is defined as colon and rectum cancer and is among the major causes of mortality in developed Countries. *Tarantula cubensis* alcoholic extract (TCAE) and *Nerium oleander* distillate (NOD) are reported to have anticancer and antioxidative activity. In this study, it was aimed to research the impact on cell proliferation markers of TCAE and NOD given simultaneously in experimental colon cancer. A total of 24 rats, 6 in each group, were used in the study. Cancer Control (CC): Azoxymethane was administered at the beginning of the experiment at a dose of 15 miligrams (mg)· kilograms$^{-1}$ (kg), (Subcutaneous, SC) twice, with an interval of a week (wk), to induce cancer. CC+TCAE: the dosage of Azoxymethane administered was 15 mg·kg$^{-1}$ (SC) twice a wk at the beginning of the experiment, while in the case of TCAE, it was 0.2 mL·kg$^{-1}$ (SC) once a wk for 18 wk from the beginning of the experiment. Fifteen mg·kg$^{-1}$ (SC) of Azoxymethane was administered twice at one-wk intervals at the beginning of the experiment to the CC+NOD group, and NO distillate (NOD) was given with water throughout the experiment. Afterwards, animals were euthanized under appropriate conditions, paraffin blocks formed from colon tissues, histochemical AgNOR (Silver-stained nucleolar organizer regions), and immunohistochemical PCNA (proliferating cell nuclear antigen) stainings were performed. In the study, immunohistochemically, PCNA scores and AgNOR count per nucleus (AgNCI) were significantly decreased in C-TCAE and C-NOD groups ($P$<0.001). AgNOR Area index (AgNAI) ($P$<0.01), Core Area Index (CAI) ($P$<0.05), and AgNOR Area index/Core Area Index (AgNAI/CAI) ($P$<0.01) scores were significantly decreased in the C-TCAE group. As a result, it was concluded that both TCAE and NOD are effective as chemopreventive drugs and that TCAE presents a more pronounced antiproliferative effect than NOD.

**Key words:** Azoxymethane; AgNOR; immunohistochemistry; PCNA; pathology

**RESUMEN**

El cáncer colorrectal (CCR) se define como el cáncer de colon y recto, y se encuentra entre las principales causas de mortalidad en los países desarrollados. Se informa que el extracto alcohólico de *Tarántula cubensis* (TCAE) y el destilado de *Nerium oleander* (NOD) tienen actividad anticancerígena y antioxidante. En este estudio, el objetivo fue investigar los efectos de TCAE y NOD administrados simultáneamente sobre los marcadores de proliferación celular en el cáncer de colon experimental. En el estudio se utilizaron un total de 24 ratas, 6 en cada grupo. Control del Cáncer (CC). se administró azoximetano al comienzo del experimento a una dosis de 15 miligramos (mg)·kilogramos$^{-1}$ (kg) (subcutánea, SC) dos veces, con una semana (sem) de diferencia, para inducir el cáncer. CC+TCAE: la dosis de Azoximetano administrada fue de 15 mg·kg$^{-1}$ (SC) dos veces por sem al inicio del experimento, mientras que en el caso de TCAE fue de 0,2 mL·kg$^{-1}$ (SC) una vez por sem durante 18 sem desde el inicio del experimento. Se administraron 15 mg·kg$^{-1}$ (SC) de azoximetano dos veces a intervalos de una sem al comienzo del experimento al grupo CC+NOD, y se administró destilado de NO (NOD) con agua durante todo el experimento. Posteriormente, los animales fueron sacrificados en condiciones apropiadas, se realizaron bloques de parafina formados a partir de tejidos de colon, AgNOR histoquímico (regiones organizadoras nucleolares teñidas con plata) e inmunohistoquímico PCNA (antígeno nuclear de células proliferantes) se realizaron. En el estudio, inmunohistoquímicamente, las puntuaciones de PCNA y el recuento de AgNOR por núcleo (AgNCI) se redujeron significativamente en los grupos C-TCAE y C-NOD ($P$<0,001). Las puntuaciones del índice de área AgNOR (AgNAI) ($P$<0,01), el índice de área central (CAI) ($P$<0,05) y el índice de área AgNOR/índice de área central (AgNAI/CAI) ($P$<0,01) se redujeron significativamente en el C-TCAE grupo. Como resultado se concluyó que, tanto TCAE como NOD son efectivos como fármacos quimiopreventivos y que TCAE presenta un efecto antiproliferativo más pronunciado que NOD.

**Palabras clave:** Azoximetano; AgNOR; inmunohistoquímica; PCNA; patología

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## INTRODUCTION

Colorectal cancer (CRC) is defined as colon and rectum cancer [32]. Colon Cancer (CC), also known as CRC or bowel cancer, is used to describe neoplasia in the colon, rectum or cecum [43]. The incidence of CRC as one of the principal causes of death related to cancer around the globe, it has been increasing in recent years [27, 45]. Nowadays, one million people in the world are diagnosed with CRC, and it is foreseen that it causes the death of approximately half of them. In addition, among all the types of cancer, the death toll caused by CRC rises to 10% [16].

*Tarantula cubensis* alcoholic extract (TCAE) is frequently used in the Veterinary field as a homoeopathic product. TCAE is reported to, thanks to its effect on epithelialization, accelerate wound healing, show anti-inflammatory effects, induce the demarcation of necrotic tissues and have anti-oedematous effects [21, 24]. It has been reported that its use on tumours such as oral papillomatosis in dogs (*Canis familiaris*) and mammary tumours had successful results [21, 26].

*Nerium oleander* (NO, oleander) is cultivated as an fancy plant in parks and gardens in temperate and subtropical regions worldwide. Since all parts of the NO plant are poisonous and cause cardiotoxicity, its use for medical purposes is limited [8]. Preceding studies have pointed out that NO possesses anticancer and antidiabetic effects [7, 42]. Although NO extracts contain some toxic elements, different reports highlight the absence of toxicity in rats (*Rattus norvegicus*) in acute oral toxicity studies where NO distillate (NOD) was administered orally, which is in line with the recommendations of the Organisation for Economic Co-operation and Development (OECD). In the study performed by Bas *et al*. [7], it was expressed that after 2 weeks (wk), it did not show undesirable effects on biochemical parameters, macroscopically and microscopically. In addition, it was reported that *Nerium oleander* distillate (NOD) did not have any toxic effects after acute and subchronic administration to rats [14, 28].

Proliferating cell nuclear antigen (PCNA) is the term used to refer to a nuclear protein that plays a role in the regulation of the cell cycle as well as in the synthesis of deoxyribonucleic acid (DNA), and its expression takes place in the *nuclei* of cells in proliferation, especially in the course of the phases G1 and S [46]. PCNA begins to be synthesized in phase G1, reaching the highest point in phase S and decreasing considerably in the M and G2 phases [31]. In this context, PCNA, because of its reflection of the proliferation activity in the cell, is widely used in healthy and tumoral tissues as a marker of cell proliferation [38]. In addition, as PCNA is closely connected to the biological activity of tumour cells, it is foreseen that it plays an effective role in tumorigenesis. There is a optimistic correlation among PCNA expression and malignancy [13, 44].

Nucleolus organizer regions (NOR) are chromosomal loops of DNA which play a role in ribosomal synthesis [20]. The acrocentric chromosomes 13, 14, 15, 21, and 22 are, in their brief arms, the location place of the NORs. The argyrophilic proteins AgNORs, on the other hand, can be detected via silver nitrate staining the NORs and can be easily described as dark brown or black spots, especially in the nuclear area [5]. It is suggested that the cell activation may be deduced from the count of AgNOR points in a nucleus; thus, the activity may be assessed by this index. Being an increased AgNOR count an indicator of increased cellular activity compared to protein synthesis, it is reported to be associated with neoplastic changes [37]. Although an average of 20 black AgNOR spots are seen in a normal cell, the number of AgNORs increases in parallel with the increasing amount of DNA in dysplastic or malignant cells [5]. It has been reported that AgNOR measurements in animal skin tumours increase in malignant tumours, and AgNOR number and area indices can be considered important markers in determining malignancy [25].

PCNA and AgNOR proliferation indices are frequently used to evaluate the chemopreventive effect of certain substances in experimental models of colon cancer [3, 4]. The aim of this study aimed to determine the effects of simultaneous administration of TCAE and NOD as chemopreventive on PCNA and AgNOR proliferative indices in Azoxymethane (AOM) induced colon cancer in rats.

## MATERIAL AND METHODS

### Animal material

The material of the study was constituted of 24 rat intestinal (colon) paraffin blocks (TABLE I), which were obtained from the study named "Efficacy of *Tarantula cubensis* alcoholic extract and *Nerium oleander* distillate in experimental colon cancer" Er *et al*. [15]. The study was approved by SUVDAMEK (Decision number: 2021/113).

### Study design

In the study, 4 groups of rats were created. While 8 rats formed the control group, the other 30 rats were divided into 3 groups equally. Cancer Control (CC): in order to induce cancer, Azoxymethane was administered at the beginning of the experiment at a dose of 15 mg·kg$^{-1}$ subcutaneous (SC) twice, with an interval of a wk. CC+TCAE: the dosage of AOM administered was 15 mg·kg$^{-1}$ (SC) twice a wk at the beginning of the experiment, while in the case of TCAE, it was 0.2 mL·kg$^{-1}$ (SC) once a wk for 18 wk from the beginning of the experiment. Fifteen mg·kg$^{-1}$ (SC) of AOM was administered twice at one-wk intervals at the beginning of the experiment to the CC+NOD group, and NOD was given with water throughout the study. At the end of the 18th wk, anaesthesia was implemented to the rats in a dose of 95 mg·kg$^{-1}$ (SC) ketamine + 5 mg·kg$^{-1}$ (SC), xylazine and euthanasia was performed by the cervical dislocation method [15]. A method described in the literature was used to obtain NOD [7]. In this study, the paraffin blocks of intestinal samples (colon tissue of 24 rats in total, 6 in each group) taken from the study, the design of which was described above, were used (TABLE I).

***TABLE I***
**Number of rats used in the study**

| Groups | C | CC | C-TCAE | C-NOD |
|---|---|---|---|---|
| Number of rats (n) | 6 | 6 | 6 | 6 |

C: Control, CC; Cancer Control, C-TCAE: Cancer+TCAE, C-NOD: Cancer+NOD

### PCNA Immunohistochemistry staining

After 4-5 milimicras (μm) sections from those paraffin blocks that best represented the tumour were taken to adhesive slides, they were kept in an incubator (Binder ED 56, Darmstadt, Germany) at 60°C for 20 minutes (min). Afterwards, paraffin extraction and rehydration processes were performed on the sections. Immunohistochemistry (IHC) staining was performed with the UltraVision Detection System Anti-Polyvalent, HRP (Horseradish Peroksidaz) (Ready-To-Use, TP-060-HL, Lab Vision, USA) IHC kit following the manufacturer's

recommendations. The antigen was recovered by treating the tissues in a microwave oven (UTD-1420, Utest, Turkey) with Citrate buffer (pH 6) solution for 20 min at 750 watts. Mouse monoclonal Anti-PCNA antibodies (Dako, clone PC10, Cat No; M0879, 1-hour (h) incubation were used as primer and 3,3 diaminobenzidine (DAB) as chromogen. The incubation of sections with phosphate buffered saline (PBS) rather than primary antibodies was used to obtain the negative controls. After counterstaining with Mayers Hematoxylin, it was passed through alcohol and xylene series, covered with a coverslip and examined under a light microscope (Olympus BX51, Tokyo, Japan). The Allred scoring method was used for the extent and intensity of staining in the IHC scoring of the sections [23]. Staining intensity score (0; absent, 1; weak, 2; moderate, 3; strong) and staining extent (0: absent; 1: >0-1/100; 2: > 1/100-1/10; 3:; >1/10-1/3; 4:1/3-2/3 and 5: >2/3-1) total scores were obtained.

### AgNOR staining and quantification

From the paraffin blocks that best represent the tumour, slides were cut with 4-5 µm thickness and stained for AgNOR, according to Hatipoglu *et al*. [25]. Tissues were passed, in order, through xylol, alcohol and distilled water series. Afterwards, mixing 1 unit of 2% gelatin with 1% aqueous formic acid solution and 2 units of 50% aqueous AgNO3 solution resulted in a mixture which was filtered through a 0.2 µm filter in the dark. Ensuing, the tissues were incubated in the existing mixture at 37°C for 25 min. Then bidistilled water was used to treat the tissues, and they were kept for 5 min in 5% sodium thiosulfate. Afterwards, the tissues were passed through alcohol, xylene and bidistilled water and adhered with synthetic adhesive. The examination of the dysplastic crypts was performed under a light microscope using an immersion objective (1000X magnification). Photos that would later be transferred to a computer were taken from the areas with dysplastic crypts with a digital imaging system (Olympus, DP12-BSW, version 01.03, Olympus, Tokyo, Japan). The evaluation was performed on 25 dysplastic crypt epithelium using the image analysis program (Digital Life Science Imaging, analySIS® LS Starter, 2.2, Build 1110, An Olympus Company, Munster, Germany). The following indices were calculated from each case; AgNOR Area Index (AgNAI)(Total NOR area/25), Core Area Index (CAI)(Total Core Area/25), AgNOR Count Index (AgNCI)(Total NOR Point Count/25), AgNAI/CAI index (AgNOR Area Index/Core Area Index).

### Statistical analysis

IHC and AgNOR data obtained in the study were evaluated with One-way ANOVA and Duncan test as Post hoc test (SPSS,Inc., Chicago, IL, USA 25.0). A value of $P<0.05$ was considered statistically significant.

## RESULTS AND DISCUSSION

Statistical scores of PCNA and AgNOR analysis in control and experimental animals are shown in TABLE II. Regarding PCNA staining, the highest scores were found in the experimental groups. Especially in the experimental CC induced by AOM, the PCNA expression was increased by the intense nuclear staining (FIGS. 1-2, $P<0.001$). PCNA stainings showed an intense expression mostly in dysplastic crypts (FIG. 2). A significant decrease in PCNA nuclear expression was detected in the C-TCAE and C-NOD groups in comparison with the CC group (FIGS. 1-2, $P<0.001$).

Regarding AgNOR staining, black-brown NORs were observed in all control and experimental groups (FIG. 3). It was determined that all the indices in the experimental groups were increased in comparison with the Control group (C). When the AgNOR Area Index (AgNAI) was evaluated, the highest scores were found similar in the C-NOD group and the CC group. In the C-TCAE group, the scores decreased compared to the CC group and were similar to the Control group (C)($P<0.01$). In the Core Area Index (CAI), the highest score was again in the C-NOD group, being statistically similar to the CC group, and the C-TCAE group had a lower score ($P<0.05$) than the C-NOD group, although C and CC group were similar. In the evaluation of the AgNOR Count Index (AgNCI), the scores in the CC group were found to be significantly higher ($P<0.001$) in comparison with the other groups. The scores of the C-TCAE and C-NOD groups decreased, and the C-TCAE group was found closer to the C group. Although the effect of AgNAI/CAI chemopreventives was statistically similar, it was observed that the C-TCAE group was similar to the C group and was statistically significant ($P<0.01$).

CRC is, considering its mortality and morbidity, a very important disease in developed Countries. Although classical treatment methods are used in the treatment of CRC today, different treatment options are sought because of the many side effects in individuals [2, 45]. Experimental CRC models induced with chemicals are clinically and pathologically similar to human CRC [35]. In this context, AOM-induced experimental colonic carcinogenesis is known as a reliable model that is frequently used today to evaluate the chemopreventive effect of many drugs [4, 17]. In the present study, the effects of simultaneous TCAE and NOD administration on PCNA and AgNOR proliferative indices were determined in AOM-induced colon cancer in rats.

PCNA is a protein whose IHC determination is frequently used to examine the proliferation activity in the process of carcinogenesis. It is especially used as an intermediate marker in chemoprevention studies of colon cancer carcinogenesis, and its expression levels

***TABLE II***
**Distribution of AgNOR indices and PCNA indices into groups (Mean ± SD)**

| | C (n = 6) | CC (n = 6) | C-TCAE (n = 6) | C-NOD (n = 6) | *P*-value |
|---|---|---|---|---|---|
| AgNAI | 1.82 ± 0.23$^{b}$ | 3.89 ± 0.64$^{a}$ | 2.78 ± 0.82$^{b}$ | 3.92 ± 1.39$^{a}$ | <0.01 |
| CAI | 18.23 ± 3.34$^{c}$ | 23.57 ± 3.14$^{ab}$ | 20.96 ± 3.46$^{bc}$ | 26.06 ± 4.67$^{a}$ | <0.05 |
| AgNCI | 1.82 ± 0.28$^{c}$ | 3.44 ± 0.33$^{a}$ | 2.03 ± 0.18$^{c}$ | 2.42 ± 0.19$^{b}$ | <0.001 |
| AgNAI/CAI | 0.10 ± 0.02$^{c}$ | 0.17 ± 0,03$^{a}$ | 0.13 ± 0.02$^{bc}$ | 0.15 ± 0.03$^{ab}$ | <0.01 |
| PCNA | 3,83 ± 1.17$^{c}$ | 7,50 ± 0,84$^{a}$ | 5,83 ± 0,41$^{b}$ | 5,83 ± 0,75$^{b}$ | <0.001 |

$^{a,b,c}$: Indicates statistical significance between groups (C, CC, C-TCAE, C-NOD). (C: Control, CC: Cancer Control, C-TCAE: Cancer+TCAE, C-NOD: Cancer+NOD, AgNAI: AgNOR Area Index, CAI: Core Area Index: AgNCI; AgNOR Count Index, AgNAI/CAI: AgNOR Area Index/Core Area Index, PCNA: Proliferating cell nuclear antigen, AgNOR: Silver-stained nucleolar organizer regions)

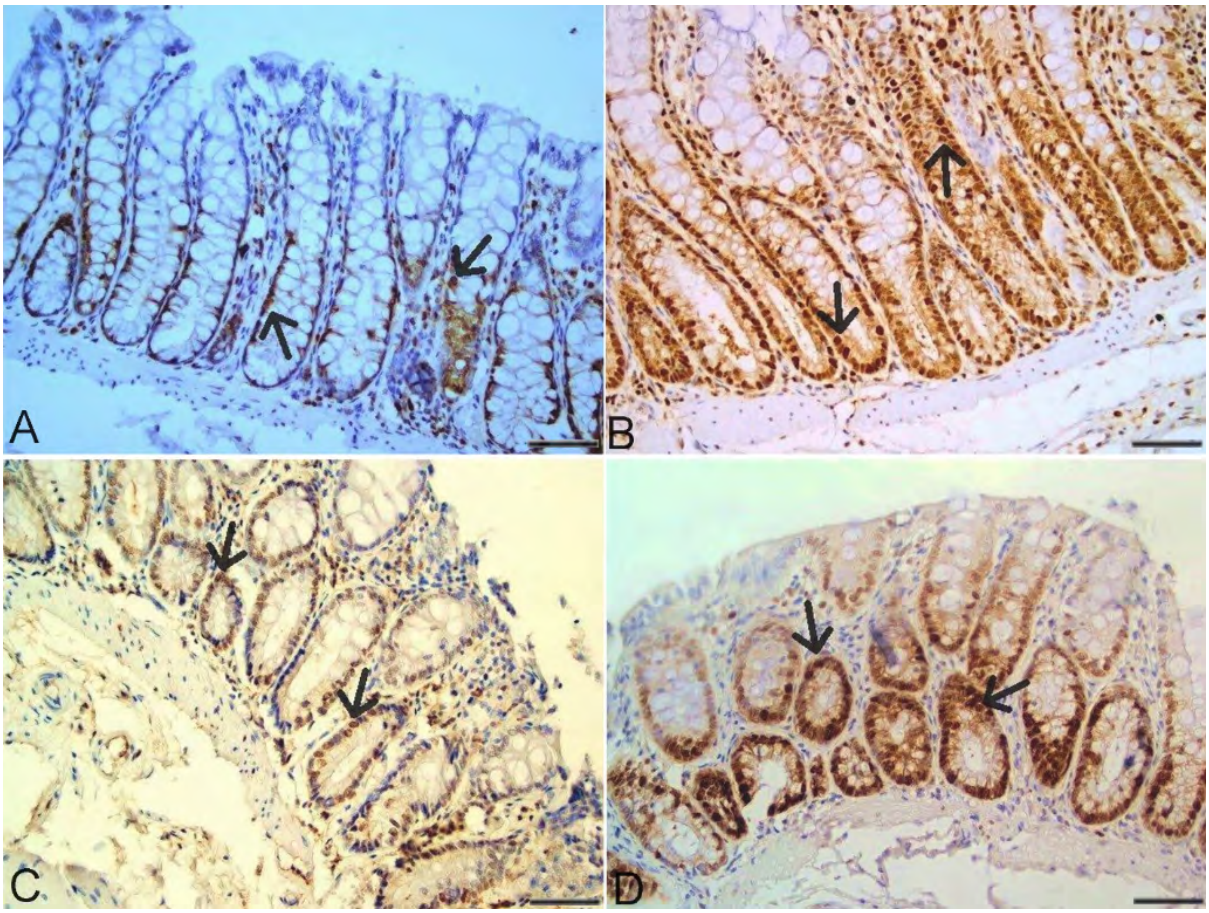


**FIGURE 1. Effect of TCAE and NOD on PCNA IHC (DAB) in AOM-induced Colon cancer, 400X. A. Normal level of nuclear PCNA expression in crypts in the C group (arrows), B. Severe nuclear PCNA expression in crypts in the AOM-induced (CC) group, C. Decreased nuclear PCNA expression in crypts in the C-TCAE group, D. Decreased nuclear PCNA expression in crypts in the C-NOD group PCNA expression. Bar: 50 µm. (C: Control, CC: Cancer Control, C-TCAE: Cancer+TCAE, C-NOD: Cancer+NOD, PCNA: proliferating cell nuclear antigen, NOD: *Nerium oleander* distillate)**

are examined in many studies [3, 4]. In this study, it was determined that, in according to the control group, the PCNA expression in the experimental groups was increased. In the C-TCAE and C-NOD groups, the scores were found to be significantly reduced compared to the CC group (TABLE II, $P<0.001$). In the study, the severe increase in PCNA immunoreactivity in hyperplastic and dysplastic crypts in experimental groups, especially in the CC group, can be interpreted as a correlation between proliferative activity and hyperplasia and dysplasia. The findings of this study show parallelism with the findings of chemical-induced experimental colon cancer or colorectal cancer studies in the literature [3, 4, 33].

Growth factors can regulate the stabilization of gene and protein expressions of PCNA. Changes in the concentrations of growth factors and receptor levels, or their combinations, can affect PCNA expressions in some hyperplastic polyps [10]. Especially Nuclear EGFR (Epidermal Growth Factor Receptor) can induce or stabilize DNA-PK (DNA-dependent protein kinase) and PCNA to increase DNA repair and replication [9]. In addition, it has been reported that the induction of PCNA tyrosine phosphorylation by EGFR inhibits MMR (DNA mismatch repair) so that mismatches occur during DNA synthesis [29]. In a study by Fichera *et al.* [17], it was announced that EGFR signals increased in an experimental study induced by AOM. In the present study, the high scores of PCNA expression in the cancer model induced by AOM, the CC group, can be interpreted as a result of increased DNA replication or the formation of DNA mutations. In addition, the decrease of the PCNA scores in C-TCAE and C-NOD groups leads to the possibility of a positive effect on EGFR signalling.

In the study performed by Ghasemi-Dizgah *et al.* [19], it was announced that TCAE reduces free oxygen radicals and can activate apoptosis by inducing the caspase-3 pathway *in vitro*. In addition, in a study conducted on different cancer cell lines *in vitro*, it was reported that TCAE is an apoptosis-inducing agent [39]. In a different study, it was reported that after treatment with TCAE in canine mammary tumours, Bcl-2 (B cell lymphoma-2) and Ki-67 expressions were IHC reduced [21]. Guzińska-Ustymowicz *et al.* [22] reported that there is a positive correlation between PCNA and Bcl-2 in CRC in humans. Also, studies have reported a negative correlation between PCNA expressions and p53 [6, 41]. In particular, Umesalma *et al.* [41] attributed decreased PCNA expression to increased p53 expression in their study. In addition, in a different study induced by AOM/DSS, it was reported that messenger Ribonucleic acid (mRNA) expression levels of p53 and Bax decreased while Bcl-2 expression increased [34]. In

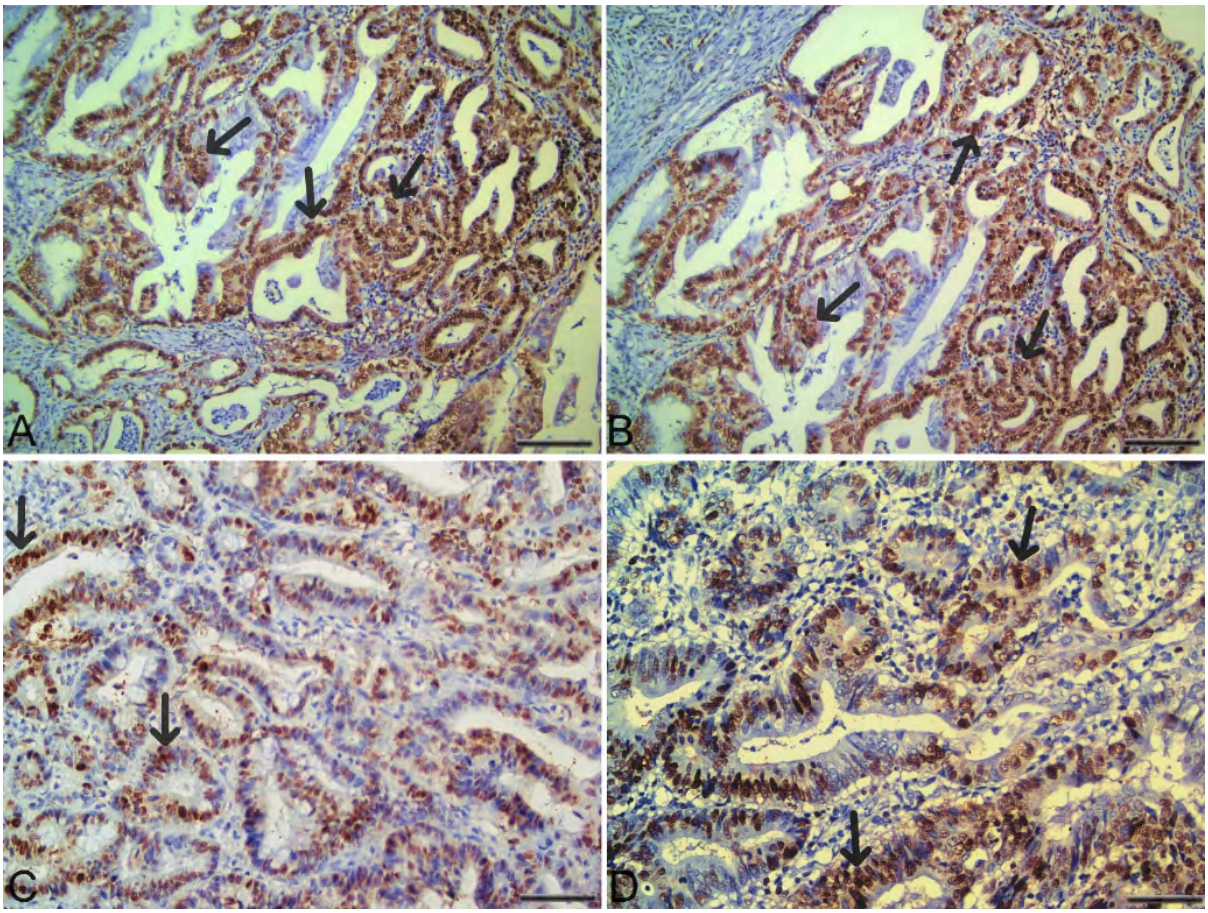


**FIGURE 2. The effect of TCAE and NOD on PCNA as immunohistochemically (DAB) in dysplastic crypts in AOM-induced colon cancer, 400X. A-B. Severe nuclear PCNA expression (arrows) in dysplastic crypts in the AOM-induced (CC) group (arrows) C. Decreased nuclear PCNA expression in dysplastic crypts in the C-TCAE group compared to the CC group (arrows), D. Decreased nuclear PCNA in dysplastic crypts in the C-NOD group expression (arrows). Bar: 50 µm. (C: Control, CC: Cancer Control, C-TCAE: Cancer+TCAE, C-NOD: Cancer+NOD, PCNA: proliferating cell nuclear antigen, NOD: *Nerium oleander* distillate)**

this context, although in this study, no findings of the expressions of Bcl-2 and p53 proteins were detected, in the light of the literature, it can be interpreted that the cells are directed to apoptosis as a result of decreased PCNA expressions by means of increased p53 and decreased Bcl-2 expressions. In the present study and in the light of the literature, it is possible to think that the increase in p53 expression and the decrease in Bcl-2 expression caused by TCAE may be behind the PCNA scores decrease in the C-TCAE group.

Known as a tumor suppressor gene, p21 has functions as a cell cycle inhibitor and antiproliferative effector in normal cells. These functions are based on its function as cyclin-dependent kinase (CDK)-cyclin complexes and PCNA inhibitor as a mediator of p53 tumour suppressor activity [1]. In one study, it was reported that spider *Macrothele raveni* venom increased p21 mRNA and protein expressions in human hepatocellular carcinoma *in vitro* HepG2 cell line, and HepG2 cells were directed to apoptosis. They also attributed this to the G2/M phase cell cycle arrest [18]. Due to the phylogenetic susceptibility of *Tarantula cubensis* and *Macrothele revani*, the cytotoxic functions and mechanisms of their venoms may be similar. In another study, an *in vitro* CRC study with Odoroside, an active ingredient obtained from NO leaves, reported that Odoroside A treatment stopped the cell cycle in CRC in the G2/M phase due to increased p21 expression levels. It has also been reported that it is directed to apoptosis in a p53-dependent manner, which is associated with the activation of the p21 protein [11]. In a different *in vitro* CRC study, it has been reported that NO inhibits cell proliferation by stopping cells in the G2/M phase. It is also stated to induce apoptosis in cancer cells [30]. In this context, the decrease in PCNA expression levels in K-TCAE and K-NOD groups may also be caused by its activity on p21. More molecular studies are needed in the future to reveal the reason for the decreased PCNA expressions in the K-TCAE and K-NOD groups in this study.

AgNOR proteins present various morphological changes in the number and size that are eligible for being used as a marker of cell proliferation. In this context, it is considered a predictive prognostic marker for cancer proliferation [33]. In addition, AgNOR image analysis is a useful method in the evaluation of the cell proliferation rate [5]. Previous studies have announced that the increase in the number of AgNORs is significantly higher in malignant cells than in normal cells, and it is interpreted as related to ribosomal RNA transcription rate, cell proliferation and DNA ploidy. Additionally, it has been reported that

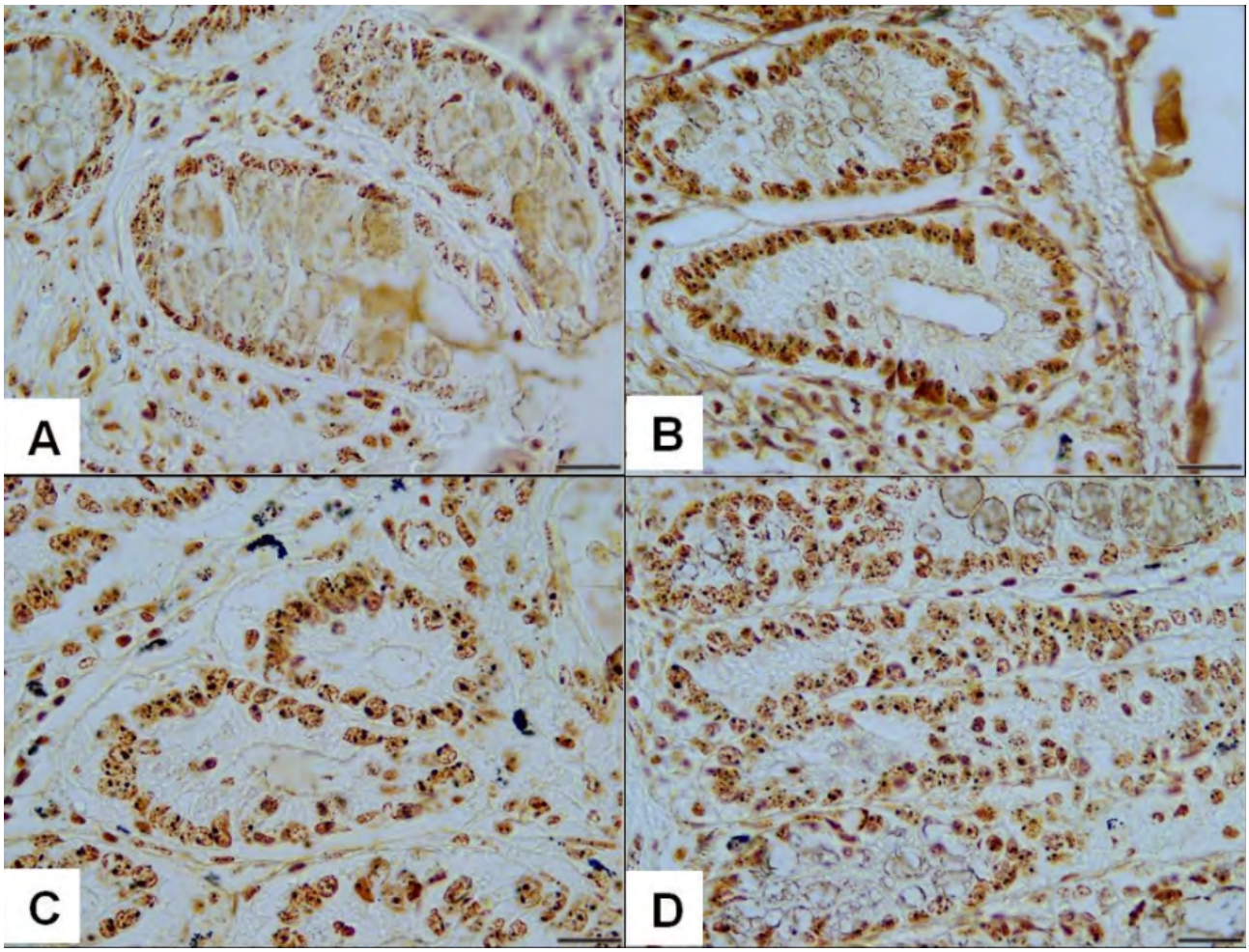


**FIGURE 3. AgNOR staining in groups, 1000X. A. AgNOR staining in crypts in the control (C) group, B. Increase in AgNOR number and size in dysplasic crypts in the CC group, C. C-TCAE group, D. C-NOD group. Bar: 20 µm. (C: Control, CC: Cancer Control, C-TCAE: Cancer+TCAE, C-NOD: Cancer+NOD, AgNOR: Silver-stained nucleolar organizer regions, NOD: *Nerium oleander* distillate)**

AgNOR sizes in tumour cells with high malignancy are smaller than in benign and less malignant tumour cells [12, 25]. When the findings of this study were evaluated, it was determined that, in according to the Control group (C), there was an increase in the NOR area (AgNAI) ($P$<0.01), NOR count (AgNCI) ($P$<0.001), Core area index (CAI) ($P$<0.05) and AgNOR area index/core area index (AGNAI/CAI) ($P$<0.01) in the experimental groups. This increase in the experimental groups can be attributed to the increased cellular activation and mutations in the DNA as a result of some biochemical changes in the nucleophilic region of the DNA of the control groups induced by AOM [36]. The obtained finding regarding the NOR count (AgNCI) were consistent with the experimental studies of colon cancer induced by chemical agents in the literature [3, 4].

It was determined that the AgNOR area (AgNAI) and count (AgNCI) indices decreased significantly in the C-TCAE group, being similar to the control group. In the C-NOD group, it was determined that there was no difference with the CC in terms of AgNOR area (AgNAI); in contrast, it was closer to the control group due to the decrease in the count (AgNCI) (TABLE II). Many factors such as temperature, time, pH and fixation solutions may affect the results in AgNOR staining. The prolongation of the incubation period in the staining process leads to the emergence of a giant-sized structure as a result of staining all of the nucleoli. It also gathers in one region to form a single, round structure [13, 40]. The fact that the AgNOR area (AgNAI) in the C-NOD group was statistically similar to the CC group might be justified by merging more than one NOR body that formed AgNOR clusters that were considered a single point. In the chemoprevention studies of the literature, AgNOR counts (AgNCI) for the antiproliferative effect take centre stage and are evaluated [3, 4]. In this context, the present experimental model created with AOM clearly shows that TCAE and NOD administration inhibits abnormal cell proliferation by inducing a decrease in the number of AgNORs. In the evaluation of CAI and AgNAI/CAI, it was determined that the K-TCAE group was statistically similar to the control group (TABLE II). The findings of this study show that, although the application of TCAE and NOD has effects on AgNOR (AgNCI) counts, TCAE is more effective in other indices, and this may be due to the better efficacy of TCAE on Aberrant Crypt Foci (ACF) and dysplastic crypts compared to NOD [15].

## CONCLUSION

In conclusion, the antiproliferative effects as chemopreventive of TCAE and NOD were evaluated in experimental colon cancer induced by AOM, and it was found that both TCAE and NOD had antiproliferative effects on PCNA and AgNOR indices. The results of this study suggest that both TCAE and NOD were effective as chemopreventive agents and that, compared to NOD, TCAE is one step ahead as an antiproliferative agent in colon cancer. In addition, AgNAI, AgNCI and PCNA can be used safely in colon cancer studies in the follow-up of carcinogenesis and in determining the prognosis.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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