OTJHS
Online Turkish Journal of Health Sciences

e-ISSN: 2459-1467

OTSBD
Online Türk Sağlık Bilimleri Dergisi

Online Turkish Journal of Health Sciences 2025;10(2):193-199 *Online Türk Sağlık Bilimleri Dergisi 2025;10(2):193-199*

Apoptotic and Antiproliferative Effects of *Ajuga reptans* L. Ethanol Extract via Inhibition of NF-κB and eNOS Activity in HCT116 Colon Cancer Cells

Ajuga reptans L. Etanol Özütünün HCT116 Kolon Kanseri Hücrelerinde NF-κB ve eNOS Aktivitesinin Inhibisyonu Yoluyla Apoptotik ve Antiproliferatif Etkileri

[1]Ilker KILICCIOGLU, [1]Gorkem DULGER, [2]Basaran DULGER

[1]Department of Medical Biology, Faculty of Medicine, Duzce University, 81620, Konuralp/Duzce, Türkiye
[2]Department of Biology, Faculty of Science and Arts, Duzce University, 81620, Konuralp/Duzce, Türkiye

Ilker Kiliccioglu: https://orcid.org/0000-0003-3367-9665
Gorkem Dulger: https://orcid.org/0000-0002-1506-1549
Basaran Dulger: https://orcid.org/0000-0002-3184-2652

ABSTRACT

Objective: This study aimed to investigate the ethanol extract of *Ajuga reptans* L. (Lamiaceae) on cell proliferation, apoptotic response, expression levels of Caspase-3, NF-κB and eNOS proteins on HCT116 human colorectal carcinoma cells.

Materials and Methods: *A. reptans* was extracted using a Soxhlet apparatus with ethanol, and the obtained extract was applied to HCT116 and HUVEC cells (as healthy controls) in increasing concentrations (0–800 µg/mL). Cell viability was analysed by the WST-1 assay at 24 h and 48 h. Morphological changes were observed via inverted microscopy. ELISA was performed to quantify Caspase-3, NF-κB, and eNOS protein levels.

Results: *A. reptans* ethanol extract showed concentration and duration-dependent cytotoxic effects on HCT116 cells, with IC_{50} values of 206.9 µg/mL (24 h) and 147.5 µg/mL (48 h). Minimal cytotoxicity was observed in HUVEC cells. Microscopy confirmed morphological signs of apoptosis in HCT116 cells. ELISA results demonstrated increased Caspase-3 and decreased NF-κB and eNOS levels, indicating induction of apoptosis and suppression of pro-survival pathways.

Conclusions: The ethanol extract of *Ajuga reptans* selectively inhibited the proliferation of colorectal cancer cells by apoptotic induction and modulation of NF-κB and eNOS signaling, suggesting its potential as a plant-based adjunctive agent in colorectal cancer therapy.

Keywords: *Ajuga reptans*, caspase-3, colon cancer, eNOS, NF-κB

ÖZ

Amaç: Bu çalışmanın amacı, *Ajuga reptans* L. (Lamiaceae) etanol ekstraktının, HCT116 insan kolorektal karsinom hücrelerinde hücre proliferasyonu, apoptotik yanıt, Kaspaz-3, NF-κB ve eNOS proteinlerinin ifade düzeyleri üzerine etkilerini araştırmaktır.

Materyal ve Metot: *A. reptans* bitkisi Soxhlet cihazı kullanılarak etanol ile ekstrakte edilmiştir. Elde edilen ekstrakt, HCT116 ve HUVEC (sağlıklı kontrol) hücrelerine artan konsantrasyonlarda (0–800 µg/mL) uygulanmıştır. Hücre canlılığı, WST-1 testi ile 24 ve 48 saatlik sürelerde değerlendirilmiştir. Morfolojik değişiklikler inverted mikroskopi ile incelenmiştir. Kaspaz-3, NF-κB ve eNOS protein düzeylerinin kantifikasyonu için ELISA analizi uygulanmıştır.

Bulgular: *A. reptans* ekstraktı, HCT116 hücrelerinde konsantrasyona ve süreye bağımlı sitotoksik etki göstermiştir; IC_{50} değerleri sırasıyla 24 saatte 206.9 µg/mL, 48 saatte 147.5 µg/mL olarak hesaplanmıştır. HUVEC hücrelerinde minimum düzeyde sitotoksisite gözlenmiştir. Mikroskobik incelemeler, HCT116 hücrelerinde apoptozu düşündüren morfolojik değişiklikleri doğrulamıştır. ELISA analizleri, Caspase-3 düzeylerinde artış ve NF-κB ile eNOS düzeylerinde azalma göstermiştir.

Sonuç: *Ajuga reptans*'ın etanolik ekstraktı, kolorektal kanser hücrelerinde seçici proliferasyon baskılanması, apoptoz indüksiyonu ve NF-κB ile eNOS sinyallerinin modülasyonu yoluyla antikanser etki göstermiştir. Bu bulgular, ekstraktın kolorektal kanser tedavisinde bitki kaynaklı tamamlayıcı bir ajan olarak potansiyelini ortaya koymaktadır.

Anahtar Kelimeler: Ajuga reptans, eNOS, kaspaz-3, kolon kanseri, NF-κB

Sorumlu Yazar / Corresponding Author:
Ilker Kiliccioglu
Department of Medical Biology, Faculty of Medicine, Duzce University, Konuralp Campus, 81620 Duzce - Türkiye
Tel.: 05376518429
E-mail: ilkerkiliccioglu@duzce.edu.tr

Yayın Bilgisi / Article Info:
Gönderi Tarihi/ Received: 28/04/2025
Kabul Tarihi/ Accepted: 16/06/2025
Online Yayın Tarihi/ Published: 30/06/2025

Atıf / Cited: Kiliccioglu İ and et al. Apoptotic and Antiproliferative Effects of Ajuga reptans L. Ethanol Extract via Inhibition of NF-κB and eNOS Activity in HCT116 Colon Cancer Cells. *Online Türk Sağlık Bilimleri Dergisi* 2025;10(2):193-199. doi: 10.26453/otjhs.1685503

INTRODUCTION

Phytochemicals have been shown to exhibit anticancer effects by modulating molecular pathways involved in cell cycle modulation, apoptosis, inflammation, and angiogenesis.[1,2] *Ajuga reptans* L. is a perennial plant belonging to the Lamiaceae family, and is broadly spread across Europe and Asia. Traditionally used for its anti-inflammatory, antioxidant, hepatoprotective, and wound-healing properties, recent studies have begun to explore its potential anticancer activities.[3,4]

Phytochemical analyses have identified several secondary metabolites in *A. reptans*, including important phenolic compounds, which are known for their biological activities.[5,6] These constituents are hypothesized to regulate various cellular targets and pathways associated with cancer development and progression.

Among the key molecular players implicated in colon cancer pathogenesis are nuclear factor kappa B (NF-κB) and endothelial nitric oxide synthase (eNOS). NF-κB is an important transcription factor that modulates proteins involved in inflammatory and apoptotic pathways. Its abnormal activation has been commonly detected in colon cancer and is associated with chemoresistance and poor prognosis.[7] Similarly, eNOS, which catalyzes the production of nitric oxide (NO), plays a dual role in cancer, but its overexpression has been linked to enhanced tumor angiogenesis and metastatic potential in colorectal tumors.[8]

In this study, we aimed to observe the anticancer potential of ethanol extract of *A. reptans* on HCT116 human colorectal carcinoma cells. Specifically, we assessed its effects on cell proliferation, apoptotic response, and modulation of NF-κB and eNOS expression levels. To our knowledge, this is the first study to systematically investigate the cellular processes underlying the effects of *A. reptans* on colon cancer cells, highlighting its potential as a novel phytotherapeutic agent.

MATERIALS AND METHODS

Ethics Committee Approval: This study was conducted using commercial cell culture. Approval from an ethics committee is not necessary.

Ethanol Extraction of Ajuga reptans: The aerial parts of the plant were collected in April 2024 from the campus area of Duzce University, Türkiye (Voucher number: GD 06-04-24; 40º54'25"N, 31º11'5"E) alt. 248 m). For the extraction procedure, 20 g of the powdered plant material was macerated in 200 mL of ethanol (Merck) in a Soxhlet extractor (Termal Lab, Istanbul, Türkiye) for 12 h. After the extracts were passed through Whatman filter No. 1, the ethanol was removed with a rotary evaporator at 55°C. The dried extracts obtained were dissolved in dimethyl sulfoxide to a final concentration of 100 mg/mL.

Cell Culture: HCT116 colon cancer cells and HUVEC (human umbilical vein endothelial cells) were used in this study. HCT116 is a human colorectal carcinoma cell line derived from the colon tissue of a male patient. These cells exhibit microsatellite instability and carry a mutant KRAS allele while retaining the wild-type p53 gene. HCT116 cells are adherent and grow as an epithelial monolayer with relatively high proliferation rates. Due to their sensitivity to various chemotherapeutic agents, HCT116 cells serve as an important model for evaluating cytotoxicity, apoptosis, cell cycle, and signaling pathways in colorectal cancer studies. Cells were cultured in RPMI-1640 and DMEM mediums (Capricorn Scientific, Germany) supplemented with heat inactivated 10% fetal bovine serum, 200 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Capricorn Scientific, Germany) in a humidified incubator (Nuve, Ankara, Türkiye) at 37ºC with 5% CO_2.

Cell Viability Assay: The effect of *A. reptans* ethanol extract on cell viability was analyzed using the WST-1 cell proliferation assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. HCT116 and HUVEC cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 24 h to allow cell attachment. Cells were then treated with various concentrations of the extract (0, 100, 200, 400, 600, and 800 µg/mL) for 24 and 48 h. After treatment, 10 µL WST-1 reagent was added to each well and incubated for 2 hours. Absorbance was measured at 450 nm using a microplate reader (Allsheng Instruments, Hangzhou, China). Each experiment was performed in three independent replicates.

Morphological Evaluation: To evaluate morphological changes, both HCT116 and HUVEC cells were seeded in 6-well plates at 3×10^5 cells/well and treated with the ethanol extract at 100, 200 and 400 µg/mL concentrations. After 24 h of treatment, morphological alterations were visualised with an inverted microscope (Euromex, Arnhem, Netherlands) equipped with a CMEX-5 Pro camera at 20x magnification (Olympus CKX53).

Protein Isolation and ELISA Assay: Following treatment with the extract, total protein was isolated from HCT116 cells using RIPA lysis buffer (A.B.T, Ankara, Türkiye). Protein concentrations were quantified using the bicinchoninic acid (BCA) assay (ABP Biosciences, USA). The expression levels of Caspase-3, eNOS and NF-κB were measured using human-specific ELISA kits (BT LAB, Shanghai, China and ELK Biotechnology CO., Ltd, Denver,

USA), following the manufacturers' protocols. Optical densities were read at 450 nm on an ELISA reader (Allsheng Instruments, Hangzhou, China). Each experiment was performed in three independent replicates.

***Statistical Analysis**:* GraphPad Prism 9.0 was used to perform the statistical analysis. Statistical significance was determined using one-way ANOVA and two-way ANOVA, followed by Dunnett's post-hoc test. A significance level of $p<0.05$ was accepted as significant.

RESULTS

Figure 1A shows that the ethanol extract of *A. reptans* exhibited a dose-dependent antiproliferative effect with an IC_{50} value of 206.9 µg/mL for 24 h. Cell proliferation decreased significantly as the concentration of the extract increased, indicating strong cytotoxic activity in the applied dose range. The IC_{50} value was 147.5 µg/mL for 48 h (Figure 1B). This result indicated that the anticancer activity of the extract increased with the incubation time.

As shown in Figure 2, *A. reptans* ethanol extract significantly reduced cell viability in a dose and time -dependent profile. Cell proliferation decreased by up to 22% for increasing concentrations at 24 h. This antiproliferative effect was more pronounced at 48 h. Above 200 µg/mL, the proliferation inhibition of HCT116 cells exceeded 50%. These data highlighted that the inhibitory effect of *A. reptans* on the proliferation of HCT116 colon cancer cells was concentration and exposure time-dependent.

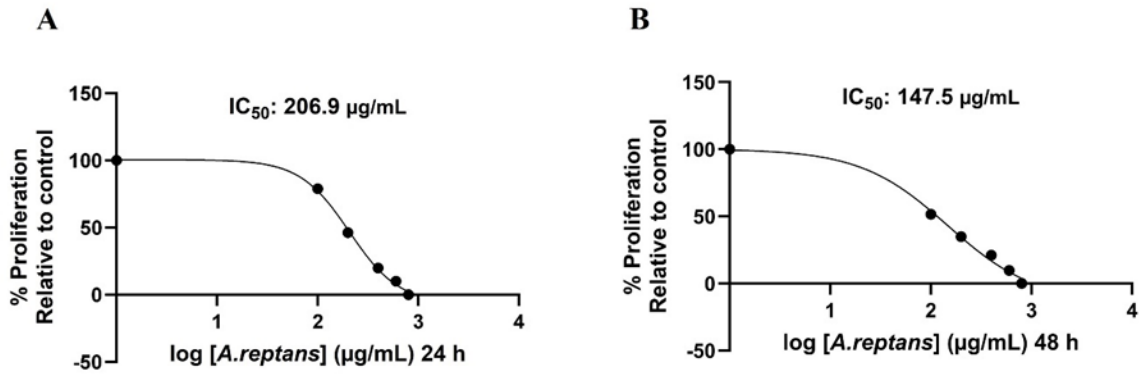

Figure 1. Antiproliferative effect curves of *A. reptans* ethanol extract on HCT116 colon cancer cells. A: Antiproliferative effect for 24 h; B: Antiproliferative effect for 48 h.

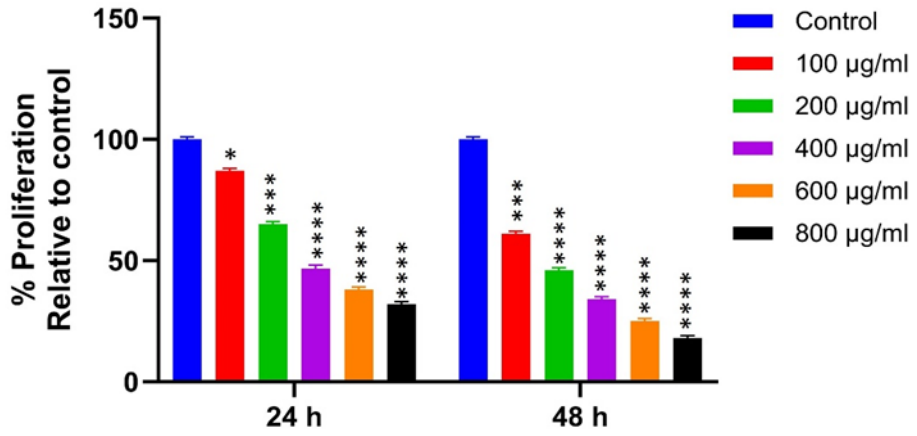

Figure 2. The antiproliferative effect of *A. reptans* ethanol extract on HCT116 colon cancer cells for 24 h and 48 h. *: $p<0.05$; ***: $p<0.001$; ****: $p<0.0001$.

To evaluate the selectivity of the extract towards cancer cells, concentrations were applied to HUVEC cells. As shown in Figure 3, *A. reptans* showed relatively low cytotoxicity in HUVEC cells. Only slight decreases in proliferation were observed at higher concentrations (600 and 800 µg/mL) at 48 h. These results indicate that the toxicity of *A. reptans* extract towards healthy cells is quite low.

Microscopic examination revealed distinct morphological changes in HCT116 cells after treatment with 100, 200, and 400 µg/mL of *A. reptans* ethanol extract for 24 h (Figure 4). Treated cancer cells appeared shrunken with condensed cytoplasm and membrane blebbing, which are hallmarks of apoptotic cell death. In contrast, HUVEC cells largely retained their normal spindle-like morphology, and minimal morphological changes were observed even at higher extract concentrations, further supporting selective toxicity against cancer cells.

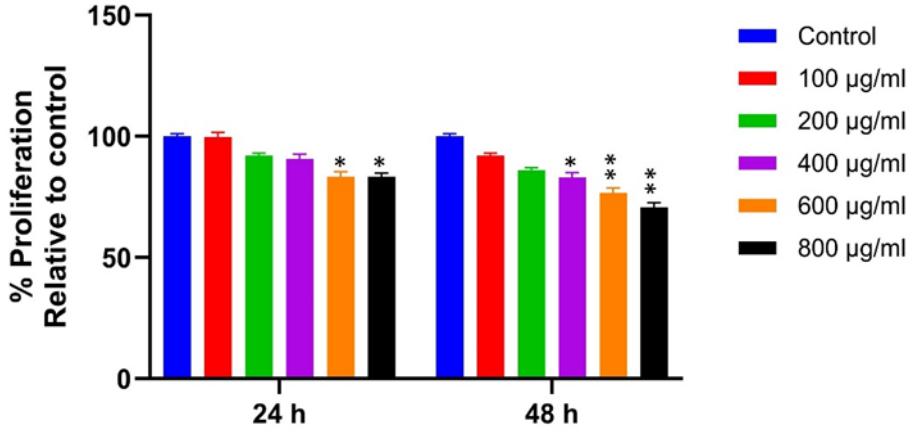

Figure 3. The minimal cytotoxicity level of *A. reptans* ethanol extract on HUVEC cells. *: p<0.05; **: p<0.01.

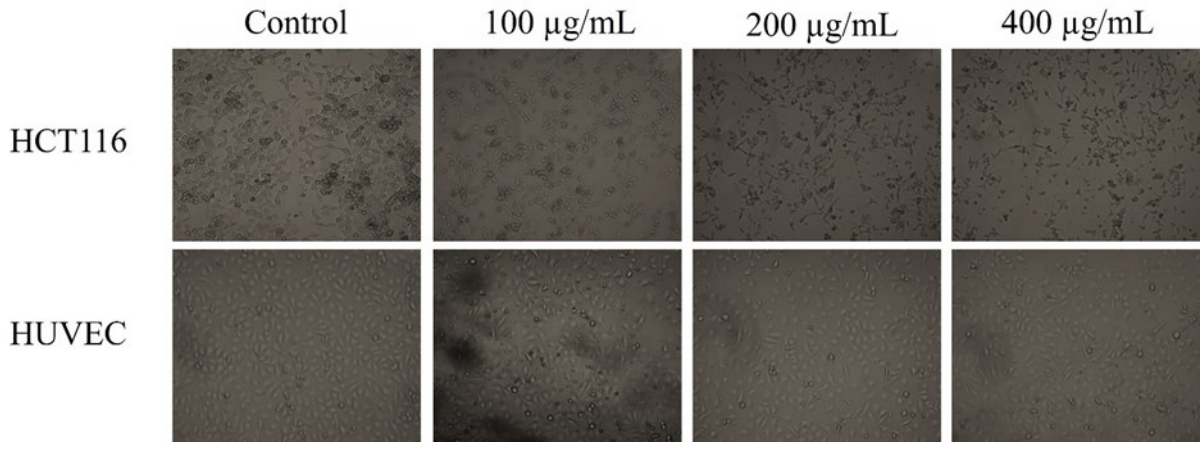

Figure 4. Inverted microscope examination of morphological changes in HCT116 and HUVEC cells upon *A. reptans* treatment.

A. reptans ethanol extract significantly increased the expression of Caspase-3 at 200 and 400 µg/mL, indicating induction of apoptosis. In addition, NF-κB and eNOS levels were significantly down-regulated compared to control ($p < 0.01$ to $p < 0.001$), indicating inhibition of pro-survival and inflammatory pathways (Figure 5). These results suggest that A. reptans induces apoptosis in colorectal cancer cells via caspase activation and regulation of NF-κB and eNOS signaling.

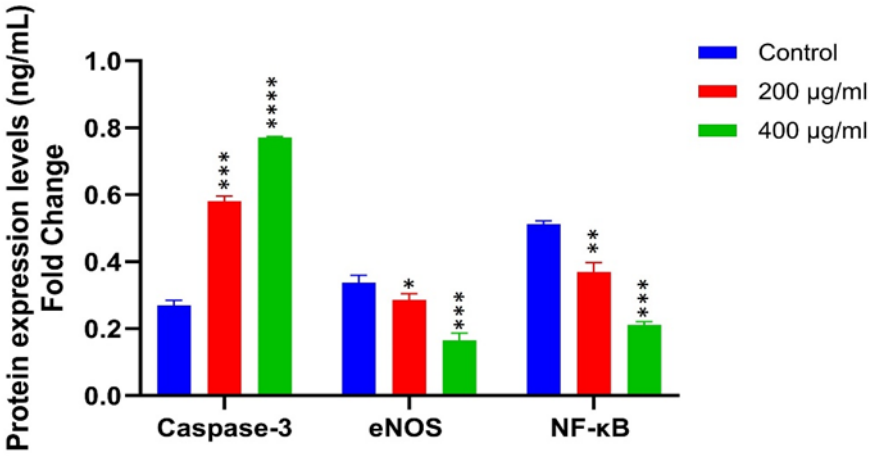

Figure 5. Effect of *A. reptans* ethanol extract on the expression levels of Caspase-3, eNOS and NF-κB proteins in HCT116 colon cancer cells. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$.

DISCUSSION AND CONCLUSION

The present study provides novel insights into the anticancer potential of *Ajuga reptans* ethanol extract on HCT116 human colorectal carcinoma cells, highlighting its selective cytotoxicity, time and dose-dependent antiproliferative activity, and its ability to regulate apoptotic and inflammatory pathways. These findings provide important evidence to support the therapeutic potential of plant-derived compounds in the treatment of colorectal cancer.[9,10]

Our dose-response analyses revealed that *A. reptans* extract significantly inhibited the proliferation of HCT116 cells, with an IC_{50} value of 206.9 µg/mL at 24 h, which decreased to 147.5 µg/mL at 48 h. The reduction in IC_{50} over time reflects an enhanced cytotoxic efficacy with prolonged exposure. These results are in agreement with previous studies reporting the cytotoxic potential of *A. reptans* and other *Ajuga* species on various cancer cell lines. *A. reptans* extracts showed cytotoxic effects on prostate cancer cell lines by inhibiting cell proliferation and inducing apoptosis at concentrations of 300 µM and 500 µM, respectively. Similarly, methanolic and water extracts of *A. orientalis* showed moderate cytotoxic activity against various cancer cell lines. These findings suggest a broad-spectrum anticancer potential in the *Ajuga* genus.[11]

Importantly, when tested on HUVEC cells, *A. reptans* ethanol extract showed minimal cytotoxicity, even at higher concentrations (600–800 µg/mL). This selectivity toward cancer cells while sparing normal cells is crucial for the development of safer chemotherapeutic agents. Morphological analysis under inverted microscopy further supported the cytotoxic findings. These observations support that *A. reptans* induces cell death via apoptotic mechanisms rather than necrosis, minimizing the inflammatory response.

Caspase-3 is a key executioner protease in the apoptotic cascade, responsible for cleavage of structural and regulatory cellular proteins.[12] Its activation is widely regarded as a hallmark of apoptosis, and its induction by *A. reptans* suggests that the extract facilitates intrinsic apoptotic signaling. In addition, NF-κB levels were significantly reduced following extract treatment. NF-κB is a transcription factor that regulates genes involved in cell survival, proliferation, and inflammation. Its constitutive activation has been linked to tumorigenesis, metastasis, and chemoresistance in colorectal cancer.[13] Inhibiting NF-κB can sensitize cancer cells to apoptosis and disrupt their proliferative advantage.[14] Therefore, the downregulation of NF-κB by *A. reptans* may partly explain the enhanced apoptosis and reduced proliferation observed.

Similarly, the downregulation of endothelial nitric oxide synthase (eNOS) further supports the anti-tumor effects of the extract. eNOS can support tu-

mor growth by promoting angiogenesis and tumor blood supply under pathological conditions.[15] Reduced eNOS expression may contribute to an anti-angiogenic effect, thereby limiting tumor progression. These results suggest that *A. reptans* interferes not only with tumor cell survival and proliferation but also with their microenvironmental support systems.

Phytochemical constituents potentially responsible for these effects include phytoecdysteroids, iridoid glycosides, flavonoids, and diterpenes, all of which have been reported in the *Ajuga* genus.[6,16] For instance, 20-hydroxyecdysone, a major ecdysteroid in *A. reptans*, has been shown to modulate signaling pathways associated with apoptosis and inflammation.[17-19] However, future studies involving chromatographic isolation and compound-specific testing are necessary to identify and characterize the bioactive principles responsible for the observed effects.

In conclusion, these findings suggest that *A. reptans* ethanol extract exerts its anticancer effects through a multi-faceted mechanism involving the induction of apoptosis, suppression of inflammatory signaling, and selective cytotoxicity. Our results showed that this plant has significant potential as a complement to conventional treatments. Further studies, including in vivo validation, pharmacokinetic profiling, and target identification, are warranted to advance *A. reptans* as a candidate for colorectal cancer therapy.

Ethics Committee Approval: This study was conducted using commercial cell culture. Approval from an ethics committee is not necessary.

Conflict of Interest: No conflict of interest was declared by the authors.

Author Contributions: Concept – IK, GD; Supervision – BD; Materials – IK, GD; Data Collection and/or Processing – BD; Analysis and/or Interpretation – IK; Writing –IK, GD.

Peer-review: Externally peer-reviewed.

REFERENCES

1. Cragg GM, Newman DJ. Natural products: a continuing source of novel drug leads. Biochim Biophys Acta. 2013;1830(6):3670-95. doi:10.1016/j.bbagen.2013.02.008
2. Afrin S, Giampieri F, Gasparrini M, et al. Dietary phytochemicals in colorectal cancer prevention and treatment: A focus on the molecular mechanisms involved. Biotechnol Adv. 2020:38:107322. doi:10.1016/j.biotechadv.2018.11.011
3. Rana S, Narinderpal K, Poonam DA. Review on pharmacognostic profile of Ajuga reptans. J. Pharmacogn. 2020;7:9-18.
4. Nagarkoti K, Kanyal J, Prakash O, Kumar R, Rawat DS, Pant,AK. Ajuga L.: A systematic review on chemical composition, phytopharmacological and biological potential. Curr Bioact Compd. 2021;17(9),11-37. doi:10.2174/1573407216999210101230234
5. Maliuvanchuk S, Grytsyk A, Popadynets O, Kotyk T, Raal A, Koshovyi O. Ajuga reptans L. Herb Extracts: Phytochemical composition and pharmacological activity screening. Plants. 2025;14(2),219. doi:10.3390/plants14020219
6. Dziki A, Malinowska MA, Szopa A, Sikora E. Comparative study of the phytochemical profile and biological activity of Ajuga reptans L. leaf and root extracts. Appl Sci. 2024; 14(12),5105. doi:10.3390/app14125105
7. Karin M. Nuclear factor-κB in cancer development and progression. Nature. 2006;441 (7092):431-6. doi:10.1038/nature04870
8. Gao Y, Zhou S, Xu Y, Sheng S, Qian SY, Huo X. Nitric oxide synthase inhibitors 1400W and L -NIO inhibit angiogenesis pathway of colorectal cancer. Nitric Oxide. 2019;1:83:33-39. doi:10.1016/j.niox.2018.12.008
9. Newman DJ, Cragg GM. Natural products as sources of new drugs over the nearly four decades from 1981 to 2019. J Nat Prod. 2020;83 (3):770-803. doi: 10.1021/acs.jnatprod.9b01285
10. Mukkavilli V, Ramakrishnan G, Gujjula KR, Balachandran S, Chamarthy S, Mekala JR. Molecular understanding and pharmacological potency of plant-derived compounds in colorectal cancer (CRC): A Critical Analysis and Future Perspectives.
    Cell Biochem Biophys. 2024;82(3):1777-1795. doi:10.1007/s12013-024-01370-1
11. Caskurlu A, Karavus SN, Biltekin SN, Helvaci EZ, Karadag AE. In vitro cytotoxicity evaluation and phytochemical analysis of Ajuga reptans L. extracts. J Res Pharm. 2023;27(5):2115-2123. doi:10.29228/jrp.490
12. Hussar, P. Apoptosis regulators bcl-2 and caspase-3. Encyclopedia. 2022;2(4),1624-1636. doi:10.3390/encyclopedia2040111
13. Martin M, Sun M, Motolan A, Lu T. The pivotal player: components of NF-κB pathway as promising biomarkers in colorectal cancer. Int J Mol Sci. 2021;22(14):7429. doi:10.3390/ijms22147429
14. Xie Y, Liu F, Wu Y, et al. Inflammation in cancer: therapeutic opportunities from new insights. Mol Cancer. 2025;24(1):51. doi:10.1186/s12943-025-02243-8
15. Wang H, Wang L, Xie Z, et al. Nitric oxide (NO) and NO synthases (NOS)-based targeted therapy for colon cancer. Cancers (Basel). 2020;12 (7):1881. doi:10.3390/cancers12071881
16. Luan F, Han K, Li M, et al. Ethnomedicinal uses, phytochemistry, pharmacology, and toxicology

of species from the genus Ajuga L.: a systematic review. Am J Chin Med. 2019;47(5):959-1003. doi:10.1142/S0192415X19500502

17. Jin Z, Wang B, Ren L, et al. 20-Hydroxyecdysone inhibits inflammation via SIRT6-mediated NF-κB signaling in endothelial cells. Biochim Biophys Acta Mol Cell Res. 2023;1870(5):119460. doi:10.1016/j.bbamcr.2023.119460
18. Romaniuk-Drapała A, Lisiak N, Totoń E, et al. Proapoptotic and proautophagic activity of 20-hydroxyecdysone in breast cancer cells in vitro. Chem Biol Interact. 2021:342:109479. doi:10.1016/j.cbi.2021.109479
19. Arif Y, Singh P, Bajguz A, Hayat S. Phytoecdysteroids: Distribution, structural diversity, biosynthesis, activity, and crosstalk with phytohormones. Int J Mol Sci. 2022;23(15):8664. doi:10.3390/ijms23158664