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# ANTICARCINOGENIC AND ANTIPLATELET EFFECTS OF CARVACROL

*S. Karkabounas[1, *], O. K Kostoula[1, 5], T. Daskalou[1], P. Veltsistas[2], M. Karamouzis[4], I. Zelovitis[1], A. Metsios[1], P. Lekkas[1], A. M. Evangelou[1], N. Kotsis[1], I. Skoufos[3]*

[1]*Laboratory of Physiology, Faculty of Medicine, University of Ioannina, Ioannina, Greece*
[2]*Laboratory of Analytical Chemistry, Department of Chemistry, University of Ioannina, Ioannina, Greece*
[3]*Laboratory of Infectious Diseases and Hygiene of Animals, Department of Animal Production, Technological Education Institute of Epirus, Arta, Greece*
[4]*Laboratory of Biological Chemistry, Faculty of Medicine, University of Thessalonica, Greece*
[5]*Department of Biological Applications and Technology, University of Ioannina, Ioannina, Greece*

***Aim:*** **To investigate the effect of carvacrol on chemical carcinogenesis, cancer cell proliferation and platelet aggregation, and to find possible correlation between all these processes and the antioxidant properties of carvacrol.** ***Materials and Methods*****: 3,4-benzopyrene-induced carcinogenesis model using Wistar rats was used. Leiomyosarcoma cells from Wistar rats were used to study carvacrol antiproliferative activity** ***in vitro*****. The carvacrol antiplatelet properties were investigated with platelet aggregation assay and flow cytometry technique. The production of thromboxane B2, final metabolite of platelet aggregation, was evaluated by radioimmunoassay.** ***Results*****: Our study revealed significant anticarcinogenic properties of carvacrol. We observed 30% decrease of 3,4 benzopyrene carcinogenic activity** ***in vivo*****. Antiproliferative activity of carvacrol ($IC_{50}$) was 90 μM and 67 μM for 24 h and 48 h of incubation of cells, respectively. Carvacrol possessed also mild antiplatelet effect, inducing the decrease of thromboxane A2 production in platelets and as a result — restrictive expression of the GPIIb/IIIa platelet receptor.** ***Conclusion*****: Our data demonstrated that carvacrol possesses anticarcinogenic, antiproliferative and antiplatelet properties.**

***Key Words:*** **carvacrol,** ***Origanum vulgaris*****, experimental carcinogenesis, leiomyosarcoma cells, platelet aggregation, GPIIb/IIIa platelet receptors.**

Carvacrol [isopropyl-0-cresol, $C_6H_3(OH)(C_3H_7)$] is one of main substances of essential oil from the herb *Origanum vulgaris subs Hirtum* possessing antiseptic, antibacterial, antiviral and antifungal properties [1–3]. It was reported that carvacrol in low concentrations (0.15 mg/ml), inhibits growth of microbes, human, animal or plant pathogens, such as bacteria *Camby-lobacter jejuni, Escherichia coli, Salmonella. Enterica*, methicillin-resistant staphylococci and several fungi. Its antimicrobial effect is more potent than other *Origanum* compounds, such as sineol and camphora [4–7].

It is also well known that essential oils, which are rich in carvacrol, possess strong antioxidant properties equivalent to those of ascorbic acid, butyl hydroxyl toluene (BHT) and vitamin E [8, 9]. Since many antioxidants exert antiplatelet [10–12] and anticarcinogenic effects [13–16], it is possible that carvacrol functions in a similar way. Basing on this hypothesis and reported data, in this study we examined carvacrol anticarcinogenic effect *in vivo* and its antiproliferative property *in vitro*. Also we investigated of platelet aggregation, induced by carvacrol, and tried to find correlation between mentioned processes.

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*Correspondence: Fax: +30 651097850
E-mail: ametsios@cc.uoi.gr

*Abbreviation used*: ADP – adenosine diphosphate; ARA – arachidonate; B[a]P – 3,4-benzopyrene; BHT – butyl hydroxyl toluene; COX1 – cycloxygenase 1; COX2 – cycloxygenase 2; CP – creatine phosphate; $CP_{B[a]P}$ – carcinogenic potency of B[a]P; CPK – creatin phoshokinase; DMEM – Dulbecco's Modified Eagle Medium; GpIIb/IIIa – glycoprotein IIb/IIIa; 5-LOX – 5-lipoxygenase; MST – mean survival time; Mabs – monoclonal antibodies; NSAD – non steroid anti-inflammatory drug; PAF – platelet activating factor; PPP – platelet poor plasma; PRP – platelet rich plasma; $TXA_2$ – thromboxane $A_2$.

## MATERIALS AND METHODS

***Reagents***. 3,4 benzopurene (B[a]P) was purchased from Fluca (Germany). Adenosine diphosphate (ADP), platelet activating factor (PAF), creatine phoshate (CP) creatin phoshokinase (CPK), arachidonic acid (ARA), acetylsalicylic acid, carvacrol, trypan blue, gingkolide A, gingolide B and tricapryline were purchased from Sigma (Germany). Thromboxane $A_2$ ($TXA_2$) RIA kit was provided by Isotop, Institute of Isotopes Co Ltd, Platelet GpIIb/IIIa Occupancy kit was provided by American Diagnostics Inc (USA). Dulbecco's Modified Eagle Medium (DMEM) for the cell cultures was purchased from Fluka and Sigma (Germany).

***Chemically induced carcinogenesis.*** 40 male Wistar rats 2 months old (mean weight of 130 ± 15 g) were divided into two groups (20 animals per group): control group and experimental. Animals were kept in plastic cages in room with constant temperature (21 ± 1 °C), using alternate 12 h period of light and dark, and were fed with standard rat chow. Animals were weighted once a week, till the end of experiments. 20 ml of carvacrol ($\varepsilon$ = 976 mg/ml) were mixed with 200 mg of B[a]P in glass tube and incubated at room temperature during 24 h under continuous steering. 200 mg of B[a]P were dissolved in 20 ml of tricapryline in other glass tube and incubated at room temperature under continuous steering. Animals of control group were then anesthetized with diethyl ether and 1 ml of B[a]P — tricapryline solution, containing 10 mg of B[a]P, was injected in dorsal area of each animal, while each animal of experimental group was injected in dorsal area with 1 ml of solution B[a]P — carvacrol, containing 10 mg B[a]P in 976 mg of carvacrol.

The survived animals of both group were sacrificed on 350th day after B[a]P or B[a]P – carvacrol injection. All developed tumors were carefully excised, weighted, fixed in 8% formaldehyde solution and submitted to histological examination. All animal procedures were performed under strict rules of recommendations of Ethic committee.

***Measurement of B[a]P carcinogenic potency.*** The evaluation of B[a]P carcinogenic potency ($CP_{B[a]P}$) was carried out on established rat model, using the following mathematic formula [13]:

$CP_{B[a]P}$ = [Percentage (%) of tumor induction/Mean survival time of the rats]X100

The inhibition of chemical carcinogenesis in Wistar rats by carvacrol we estimated by calculating the difference between $CP_{B[a]P}$ of the control group and $CP_{B[a]P}$ of the experimental group:

B[a]P carcinogenesis inhibition = $CP_{B[a]P}$ (control group) — $CP_{B[a]P}$ (experimental group) X100

***Cell cultures***. Malignant cells (leiomyosarcoma cells from Wistar rats) were used for *in vitro* experiments. Cells were cultured in DMEM at 37 °C, 5% $CO_2$ and were plated in density of $10^4$ cells per plate. Two plates were used as control ones, and to other plates carvacrol was added at different concentrations (2 plates per each concentration): **10 μM, 25 μM,** 50 μM, **75 μM, 100 μM, 150 μM, 300 μM, 600 μM,** 1000 μM, **1500 μM and 4000 μM.** Cell growth rate and cytotoxicity of carvacrol were estimated after 24 h and 48 h of incubation, using Newbauer cytometer and staining cells with Trypan Blue.

***Platelet aggregation assay.*** Venous blood from 10 healthy, non smoking, male volunteers (24 ± 1.5 years old) was collected in 25 ml tubes, containing citrate as anticoagulant (1 ml of citrate per 9 ml of blood). Blood samples were then centrifuged at 900 rpm for 10 min and platelet reach plasma (PRP) was isolated as supernatant. For the calibration of Ca-500 aggregometer (Chronolog Co, USA), the rest of blood samples were centrifuged again at 3100 rpm for 15 min and platelet poor plasma (PPP) was collected as supernatant.

Platelet aggregation was performed into the aggregometer's cuvettes using 450 μl of PRP, pretreated with the specific two of the three pathways inhibitors of the platelet aggregation, as follows: 1) addition of adequate doses of CP/CPK and aspirin for complete blocking of ADP and arachidonic acid pathways respectively. After this treatment, only the PAF pathway remains active, 2) addition of adequate doses of aspirin and ginkgolides A and B for blocking the arachidonic acid and PAF pathways. By this treatment, only ADP pathway remains activated, 3) CP/CPK and Ginkgolides A and B were addited in PRP for blocking the ADP and PAF pathways respectively and only arachidonic acid pathway remains active.

The concentrations of the specific inhibitors of ADP, PAF and ARA pathways in platelets were respectively: [CP] = 28 mM in PRP, [CPK] = 0.0118 units/ml in PRP, [acetylsalicylic acid] = $1.79 \times 10^{-4}$ M in PRP, [ginkgolide A] = [ginkgolide B] = $2 \times 10^{-4}$ M in PRP. In this manner, we can determine possible selective inhibition of carvacrol (or other substances) in platelet aggregation pathways [17, 18]. Carvacrol was added in aggregometer cuvettes with 450 μl of PRP in increasing doses: 0.488 mg, 0.976 mg, 1.46 mg, 1.952 mg, 2.44 mg, 2.928 mg, 3.904 mg, 4.88 mg, 9.76 mg, 14.64 mg and 19.52 mg. Each dose of carvacrol was added 2–3 min before the addition of the selected agonist and then PRP and carvacrol were mixed well by continuous stirring. ADP, arachidonic acid and PAF at concentrations of 12 μM, 0.7 μM and 15 μM, respectively, were used as agonists of platelet aggregation. Results were expressed as percentage of inhibition of maximum platelet aggregation by carvacrol *via* each aggregation pathway. Carvacrol concentration inducing 50% inhibition of platelet aggregation ($IC_{50}$), was calculated.

***Thromboxane $A_2$ production by platelets.*** Reagents were provided by Isotop (Institute of Isotopes Co Ltd). Levels of $TXA_2$ were estimated by radioimmunoassay as described [19, 20]. Samples were divided on three groups: a) 450 μl of PRP treated with 1.25 mg of indomethacin (resting platelets control); b) 450 μl of PRP pretreated with agonists and 7 min later treated with 1.25 mg of indomethacin (agonist control); c) 450 μl of PRP pretreated with different concentrations of carvacrol that induces complete inhibition of aggregation and then treated with agonists. Seven minutes after the addition of agonist, 1.25 mg of indomethacin were also added to the samples. Indomethacin was added in order to stop the production of $TXA_2$ after the end of the platelet aggregation experiments, by blocking platelet cycloxygenase 1 (COX1). All samples prepared as described before were kept for 10 days at –80 °C.

For estimation of $TXA_2$ concentration in the samples, each sample after thawing was centrifuged at 8000 rpm for 5 min and 400 μl of the supernatant were used for lipoids extraction in ethyl acetate on activated minicolumn [21]. Samples with extracted lipoids in ethyl acetate were evaporated under nitrogen stream and were treated with the radiolabeled specific antibody against $TXB_2$, final stable metabolite of $TXA_2$. The radioactivity of each sample was detected using γ-counter (Nucleus Model 1600) and the results were expressed as pg $TXA_2$/ml of PRP.

***Expression of the GpIIb/IIIa platelet receptor.*** The expression of platelet membrane receptor GpIIb/IIIa was studied by the flow cytometry assay, using ADIAflo Platelet occupancy kit (American Diagnostics, USA) and system Epics XL-MCL (Beckman-Coulter Co, USA) [22, 23]. Monoclonal antibodies (Mabs) were used and the content of GpIIb/IIIa platelet receptor was evaluated, using recorder fluorescence intensity.

For estimation of platelet receptor content, PRP was prepared as described above and 450 **μl of** preparation were used per assay. Before each assay, platelets were tested in aggregometer to confirm their functional activity. Carvacrol was added in PRP samples at the concentration, which inhibited platelet aggregation totally. The samples without carvacrol were used as control. Then GpIIb/IIIa receptors in samples were labeled with fluorescent substance, ac-

cording to instructions, and fluorescence intensity was analyzed. A standard curve was initially constructed as described in the kit. The number of receptors per platelet was extrapolated by standard curve.

***Statistics.*** Results were statistically evaluated by Student's *t*-test, and value of $P < 0.05$ was considered statistically significant.

## RESULTS

***Inhibition of chemically induced carcinogenesis by carvacrol.*** Animals (n = 20), treated with B[a]P in presence of carvacrol, developed malignant tumors in 70% of cases at the site of injection, while all animals from control group (n = 20) developed malignant tumors (100%). The mean survival time (MST) of the animals from control group was 182.4 ± 20.4 days (min ST 145 and max ST 212 days – all animals died before 350th day), while MST in experimental group was 231.6 ± 92.0 days (min ST — 130 days, max ST — 350 days), exhibiting statistically significant life prolongation, compared with the control group ($P < 0.05$) (Fig.1).

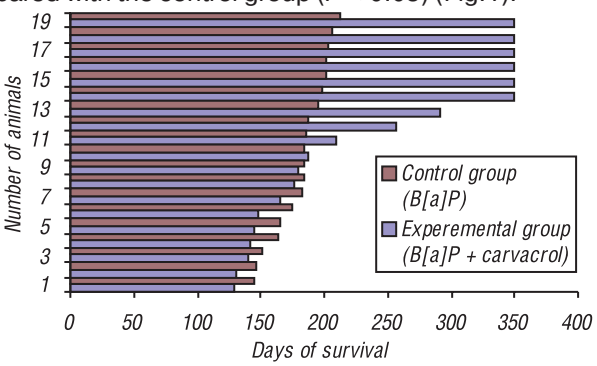


**Fig. 1.** Survival rate of animals in the control) and experimental group

Carcinogenic potency (Cp) of B[a]P in the control group was 54.82 units *versus* 30.22 units in experimental group, what represents decrease of Cp by 44.87%. Histological examination of tumors revealed that in control group 18 leimyosarcomas (90%) and 2 fibrosarcomas (10%) developed, while in animals from experimental group, 10 from 14 tumors were leiomyosarcomas (71.4%) and 4 — fibrosarcomas (28.6%). Mean weight of tumors in control and experimental group was 59.4 ± 15.0 g and 55.2 ± 17.3 g, respectively (difference between this groups was not statistically significant).

***Inhibition of leiomyosarcoma cell growth by carvacrol.*** Carvacrol was added at various concentrations (10 μM–4000 μM) in leiomyosarcoma cell cultures and inhibited the cell growth significantly at 24 h and 48 h of incubation (Fig. 2, 3). $IC_{50}$ indices of carvacrol were 90 μM for 24 h of incubation and 67 μM for 48 h of incubation.

***Platelet antiaggregating effects of carvacrol. Inhibition of platelet aggregation.*** Carvacrol in a dose dependent manner inhibited maximal irreversible aggregation of platelets, induced by each of three physiological agonists (ADP, arachidonate and PAF): $IC_{50}$ for PAF was 34.7 mM, for ADP — 39.2 mM and for arachidonate — 33.3 mM (Table).

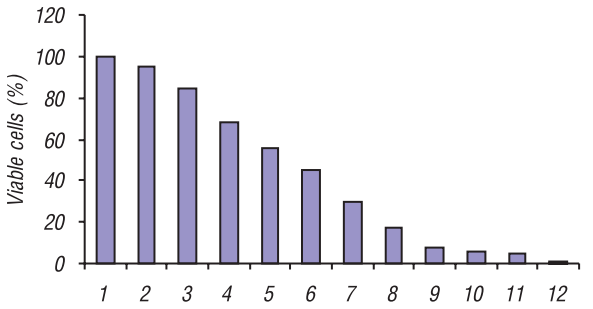


**Fig. 2.** Dose-dependent effect of carvacrol on leiomyosarcoma cell viability (24 h of incubation).
Concentrations of carvacrol: 1: 0 μM, 2: 10 μM, 3: 25 μM, 4: 50 μM, 5: 75 μM, 6: 100 μM, 7: 150 μM, 8: 300 μM, 9: 600 μM, 10: 1000 μM, 11: 1500 μM, 12: 4000 μM

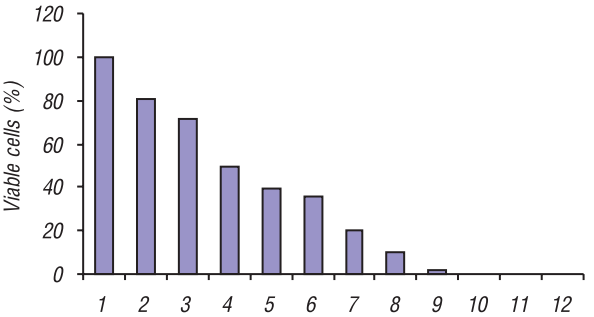


**Fig. 3.** Dose–dependent effect of carvacrol on leiomyosarcoma cell viability (48 h of incubation)

**Table.** Inhibition (%) of ADP, arachidonate and PAF-induced platelet aggregation by carvacrol

| Agonist | Carvacrol (mM) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6.5 | 13 | 19 | 25 | 32 | 39 | 52 | 65 | 130 | 195 | 260 |
| PAF | 7 | 12 | 18 | 32 | 46 | 57 | 81 | 95 | 100 | 100 | 100 |
| ADP | 5 | 9 | 14 | 28 | 41 | 52 | 79 | 97 | 100 | 100 | 100 |
| ARA | 8 | 14 | 21 | 35 | 48 | 58 | 86 | 98 | 100 | 100 | 100 |

***Inhibiting effects of carvacrol on $TXA_2$ production by platelets.*** Carvacrol at high concentration (130 mM) seems to inhibit dramatically $TXA_2$ production by platelets, when ADP, arachidonate and PAF are used as agonists. Fig. 4 reflects process of $TXA_2$ production in resting and activated platelets. In resting PRP, basal $TXA_2$ level was 125 ± 54 pg/ml. $TXA_2$ levels in ADP activated PRP reached 800 pg/ml, followed $TXA_2$ level in ARA activated PRP - 1100 pg/ml and the highest $TXA_2$ production in PAF activated PRP - 1800 pg/ml. Addition of carvacrol into PRP activated with ADP, PAF and arachidonate caused 79.2% ($P < 0.001$), 92.0% ($P < 0.001$) and 85.7% ($P < 0.001$) decrease of $TXA_2$ concentration, respectively, compared with the control (Fig. 3).

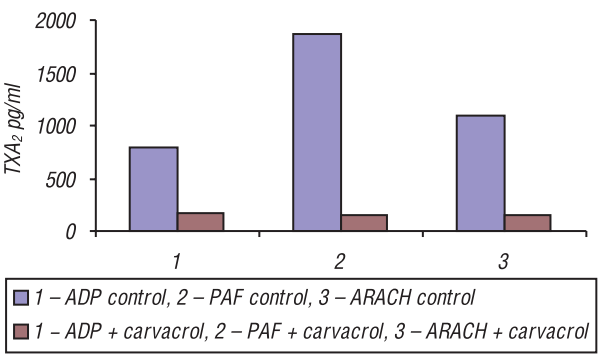


**Fig. 4.** Inhibition of $THA_2$ production $A_2$ by platelets

*Effects of carvacrol on expression of GP IIb/IIIa platelet receptor.* Carvacrol, at the concentration of 130 mM (when platelet aggregation was inhibited completely), induced decrease of Gp IIb/IIIa receptor expression per platelet up to 97% (Data not shown).

## DISCUSSION

The results of present study indicate that carvacrol exerts *in vivo* remarkable anticarcinogenic effects. This is supported by: a) 30% lower tumor incidence in Wistar rats when the carcinogen was incubated in carvacrol in comparison with the tumor incidence by B[a]P not incubated in carvacrol; b) the significant prolongation of survival time of animals treated with B[a]P in the presence of carvacrol compared with the control group; c) the significant decrease of B[a]P carcinogenic potency (44%), and d) the induction of tumors of lower malignancy in the experimental group compared with the control group.

We have previously reported that substances possessing antioxidant properties, such as ascorbic acid, α-tocopherol and β-carotene, reveal anticarcinogenic effects on B[a]P – induced tumors in Wistar rats and that anticarcinogenic effects of water-soluble antioxidant molecules, such as ascorbic acid, are enhanced, when these compounds are combined with oil soluble antioxidants, such as α–tocopherol and β-carotene [13, 14]. It is already known from published data that carvacrol, which is oil soluble cresol, possess stronger antioxidant properties than ascorbic acid and tocopherols [3, 9, 24]. Taking into account its antioxidant properties, carvacrol could scavenge reactive oxygen species and B[a]P — diol — epoxides which are produced during the metabolic activation of B[a]P [25]. These B[a]P metabolites reveal mutagenic effects and function as complete carcinogens (tumor initiators and promoters). Another hypothesis suggests that carvacrol, incubated with B[a]P, may induce B[a]P chemical neutralization, reducing the double bonds, which are responsible for its carcinogenic properties [24], *via* its reaction with π — electrons on K and L molecular regions of B[a]P.

We have shown that *in vitro* carvacrol in a concentration dependent manner exhibited antiproliferative effects; concerning platelet aggregation, it function as mild nonselective inhibitor of platelet physiological reactions (production of final metabolite $TXA_2$ and expression of GP IIb/IIIa receptor). Small differences in $IC_{50}$ indices of carvacrol for representative platelet agonists (ADP, ARA, PAF) are not significant. It is already known that $TXA_2$ was produced *via* arachidonic metabolic pathway, where cycloxygenase 1 (COX1) plays most important role. The inhibition of this enzyme with aspirin or other nonsteroid anti-inflammatory drugs (NSAD), stops $TXA_2$ synthesis [26, 27]. The results of our study indicated that carvacrol could function as anti-inflammatory factor.

It is well known that when cancer cells enter blood stream, platelets interact with them and form small thrombus, consisted of outer layer of agglutinated platelets and core of tumor cells [28]. This is due to $TXA_2$ and $PGE_2$ production in tumor cells and these arachidonic metabolites aggregate platelets [28]. Our results on platelet aggregation reveal that leiomyosarcomatic cells produced high concentrations of prostanoids ($TXA_2$ — 10000 pg/ml and $PGE_2$ — 7000 pg/ml) and induce platelet aggregation (unpublished data). According to our results, antiaggregating property of carvacrol seems to be related to its antioxidant properties, since antioxidants are functioning as inhibitors of platelet activation and aggregation, inhibiting platelet cycloxygenase [10, 11]. Antiaggregating activity of different antioxidants is due to the neutralization of free radicals, produced in cycloxygenase pathway, resulting in low production of $TXA_2$ and lower accessibility of GpIIb/IIIa platelet receptors to fibrogen molecules [29]. So, carvacrol may function in similar way with other antioxidants.

In conclusion, carvacrol, exerting anticarcinogenic effects *in vivo*, *in vitro* and *ex vivo* antiplatelet effects, could be important anticarcinogenic and antiplatelet agent that has to be further investigated.

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# АНТИКАНЦЕРОГЕННЫЙ И АНТИТРОМБОЦИТАРНЫЙ ЭФФЕКТ КАРВАКРОЛА

***Цель:*** **изучить влияние карвакрола на химический канцерогенез, пролиферацию опухолевых клеток и на процесс аггрегации тромбоцитов, а также выявить возможные взаимосвязи между этими процессами и антиоксидантными свойствами карвакрола.** ***Материалы и методы*****: применена модель химического канцерогенеза с использованием крыс линии Вистар и 3,4-бензо[а]пирена как химического индуктора канцерогенеза. Клетки лейомиосаркомы от крыс линии Вистар были использованы для изучения антипролиферативной активности карвакрола *in vitro*. Антитромбоцитарные свойства карвакрола исследовали при помощи методов аггрегации тромбоцитов и проточной цитометрии. Продукцию тромбоксана В2, конечного продукта процесса аггрегации тромбоцитов, оценивали радиоиммунологическим методом.** ***Результаты:*** **выявлено антиканцерогенное действие карвакрола: снижение канцерогенной активности 3,4-бензо[а]пирена составило 30% в системе *in vivo*. Антипролиферативная активность карвакрола ($IC_{50}$) составила 90 и 67 μM при 24 и 48 ч инкубации клеток с агентом соответственно. Карвакрол также обладал слабым антитромбоцитарным эффектом, индуцируя снижение синтеза тромбоксана А2 в тромбоцитах и как результат — ограниченную экспрессию тромбоцитарных рецепторов GPIIb/IIIa.** ***Выводы:*** **наши данные продемонстрировали, что карвакрол обладает антиканцерогенными, антипролиферативными и антитромбоцитарными свойствами.**

***Ключевые слова:*** **карвакрол, *Origanum vulgaris*, экспериментальный канцерогенез, клетки лейомиосаркомы, реакция аггрегации тромбоцитов, GPIIb/IIIa рецепторы тромбоцитов.**