Research Article

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INVESTIGATION OF VIBURNUM OPULUS L. EXTRACT'S ANTIOXIDANT EFFECTS IN DIFFERENT SOLVENTS AND CYTOTOXIC EFFECTS ON COLON CANCER CELL LINES

Keywords

Antioxidant effects, Colon cancer, Cytototoxic effect, Viburnum opulus L.

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ABSTRACT

Cancer is a disease that develops as a result of the uncontrolled proliferation of cells and can be fatal if left untreated. Side effects of cancer treatment reduce the cure rate of this disease. For this reason, studies in the field of health have shifted to new treatment methods in cancer. As a result of these studies, natural products have become important targets for the development of new anticancer agents. There are many plants belonging to Viburnum opulus L. species. These plant species have economic importance in many sectors due to essential and aromatic oils and secondary metabolites. These plant species are commonly used for the treatment of coughs, stomach aches, and infectious diseases. In this study, the antioxidant and antiproliferative properties of the endemic species Viburnum opulus L. extract were investigated in colon cancer cell lines. In this way, the aim is to develop an effective therapeutic agent for the treatment of cancer.

INTRODUCTION

Colorectal cancer (CRC) is a serious cancer with high incidence and mortality rates in developed countries. Colorectal cancer (CRC) is the third most common cancer diagnosed in both men and women in the United States. Colon cancer and rectal cancer are often grouped together because they have many common features. The precursors of colon cancer are polyps that develop into cancerous cells over time. Colonoscopy is the most widely accepted standard for the detection of these polyps and colon cancer screening[1].

The treatment method in colon cancer varies from person to person depending on the size, distribution and stage of the tumour. The main treatment modalities are surgery, chemotherapy and radiotherapy[2].

Natural therapeutics are very important because they support treatment in all stages of cancer, are less toxic or non-toxic compared to chemotherapeutic agents, are easily accessible, easy to use and generally show synergistic effects with drugs[3].

Recent research has shown that many compounds derived from seeds, fruits, bark, roots and leaves of plants have anti-carcinogenic properties. They can regress colon cancer growth in many ways, such as increasing the level of

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superoxide dismutase; reducing DNA damage mediated by oxidative stress; inducing cell cycle checkpoint arrest in G1 phase, G1/S phase, S phase and G2/M phase to increase apoptosis; reducing anti-apoptotic protein levels such as BCL2 (B-cell lymphoma 2) and BCL-XL (B-cell lymphoma-extra large); reducing the expression of PI3K (phosphoinositide 3-kinase), AKT (Ak strain transformation) and MMP (matrix metalloproteinase) levels; induces the expression of various cell cycle inhibitors such as p53, p21 and p27 and apoptotic markers such as BCL2-related cell death agonist, BCL2-related X protein (BAX), Caspase3, Caspase7, Caspase8 and Caspase9 protein[4].

The use of natural sources such as strawberries, grapes, plums, pomegranates, green tea, cruciferous vegetables, vegetables, soya beans, tomatoes, garlic, turmeric, ginger, olives, whole grains and mushrooms, garlic and pomegranate can inhibit development and colon carcinogenesis by promoting apoptosis and cell cycle arrest. About 35,000 herbal bioactive compounds are extracted from plants, seas and other sources, which minimise the negative effects of using modern technology to treat cancer, such as chemotherapy and radiological therapy. Medicinal plants are the most reliable source of bioactive compounds for natural medicines that improve medicines to alternative systems as a green approach in CRC treatment. Terpenoids, saponins, essential oils, flavonoids, phenolics, quinones and alkaloids have a strong cytotoxic effect against CRC cells with lower risk and fewer side effects[4].

Viburnum opulus L. (VO) is a plant belonging to the genus Viburnum L. of the family Adoxaceae, sometimes included in the monotypic family Viburnaceae, formerly in the family Caprifoliaceae. In Turkey it is known as viburnum rose, European viburnum, cranberry bush, water elder, rose elder, rose marbling, cherry tree, krampbark, viburnum tree and gilaburu. VO is a valuable decorative, medicinal and food plant. In the countries of Russia and Ukraine, red VO berries, despite their astringent-bitter-sour taste, are used in traditional cuisine, for example, as a component of marmalades, jams, liqueurs and liqueurs and "Kalinnikov" pies, as well as herbal teas. VO is widely used for medicinal purposes. Gilaburu juice is traditionally used to treat coughs, colds, tuberculosis, rheumatic pains, ulcers, liver disease, diabetes and hypertension, as well as to prevent certain stomach and kidney problems. VO bark (Cortex Viburni) is used in the treatment of gastric or uterine bleeding and haemorrhoids. The results of published in vitro studies show that it has antimicrobial, antidiabetic, anti-obesity, anti-inflammatory and anti-cancer effect. The properties of different morphological parts of VO have been shown in animal studies to have a beneficial effect on the urinary system, anti-inflammatory and vasorelaxant activities of VO. The health benefits of VO are due to the presence of bioactive components such as phenolic compounds, vitamin C, carotenoids, iridoids and essential oils, among others[5]. In this study, we aimed to investigate the antioxidant and anti-cancer activity of Viburnum opulus L. in colon cancer cell line.

MATERIALS AND METHODS

Preparation of Extracts

The collected Viburnum opulus L. were washed with tap water, then distilled water and dried in the laboratory for 2 weeks. The dried samples were ground and 100 gram samples were extracted twice in 1000 mL of different solvents (water, ethanol, n-hexane and ethyl acetate solvents) at 45°C for 45 min in an ultrasonic bath. The extracts were filtered through Whatman No.1 filter paper and concentrated in vacuo to obtain the extracts. The extracts obtained were stored at -20°C for use in experimental procedures.

Determination of Antioxidant Properties

Determination of Radical Scavenging Activity (DPPH)

DPPH determination was performed according to the method developed by Cuendet et al. DPPH- radical (2,2-diphenyl-1-picrylhydrazyl) is a commercially available radical and was dissolved in 100 µM ethanolic solution of this radical. According to this method, the sample was treated with DPPH- radical and kept in the dark for 50 min and then absorbance was measured at 517 nm in a spectrophotometer. (Ascorbic acid was used as standard).

Determination of Iron (Fe+3) Reducing Power in Extracts

The presence of reducing agents such as antioxidants causes the reduction of the Fe+3-ferricyanide complex to Fe+2. In this method, the colour of the test solution changes from yellow to green depending on the reducing power of the sample under test. The resulting green colour gives maximum absorbance at 700 nm and increasing absorbance indicates increasing reducing power [100]. According to this method, trolox was used as standard antioxidant compound.

The experiment was modified and instead of spectrophotometric cuvettes, the first six steps of pipetting in Table 1 were performed in 1.5 ml ependorfs and the subsequent steps were performed in 96-well microplane [101].

Table 1. *Determination of iron (Fe+3) reducing power of Viburnum opulus L. extract*

	Blank	Sample	Standart
Solvent	40 µl	-	-
Extract	-	40 µl	-
Standart	-	-	40 µl
0.2 M pH:6.Phosphate Buffer	100 µl	100 µl	100 µl
1%-$K_3Fe(CN)_6$	100 µl	100 µl	100 µl
Incubated at 50°C for 20 minutes and cooled.			
10% TCA	100 µl	100 µl	100 µl
Centrifuged at 3000 g for 10 minutes.			
100 µl of the upper phases were taken and transferred to a 96-well micropleyt.			
Distilled water	100 µl	100 µl	100 µl
0.1% $FeCl_3$	20 µl	20 µl	20 µl
Incubated at room temperature for 5 minutes in the dark.			
At 700 nm, absorbance was measured on a micropleyt reader.			

Total Flavonoid Content Determination

Total flavonoid content of the extracts was determined by aluminium chloride colorimetric method. The principle of the method is based on the fact that AlCl3 forms acid stable complexes with C-4 keto group and C-3 or C-5 hydroxyl groups of flavones and flavonols. In addition, AlCl3 forms complexes with ortho-dihydroxyl groups of A- or B- rings of flavonoids [98]. According to this method, quercetin was used as a standard [99].

Table 2. *Determination of filavonoid content of Viburnum opulus L. extract*

	Blank	Sample	Standart
Solvent	20 µl	-	-
Extract	-	20 µl	-
Standart	-	-	20 µl
80% Ethanol	172 µl	172 µl	172 µl
10% Al $(NO_3)_3$	4 µl	4 µl	4 µl
1 M KCH_3COO	4 µl	4 µl	4 µl
Incubated at room temperature for 40 minutes in the dark.			
At 415 nm, absorbance was measured on a micropleyt reader.			

Cell Culture

Cells

In this study, colon cancer (HT-29-HTB-38™) cell lines were grown in DMEM medium containing 10% (v/v) fetalbovine serum (FBS), 1% penicillin-stretomycin and healthy mouse fibroblast cells (L-929) were grown in RPMI-1640 medium containing 10% (v/v) fetalbovine serum (FBS), 1% penicillin-streptomycin in an incubator at 5% CO2, 95% humidity and 37°C. Mouse fibroblast cell line L929 was used as a healthy cell line.

Cytotoxicity Tests

The cytotoxic effects of Viburnum opulus L. extract, which was found to be the most effective antioxidant, on colon and healthy cells were performed using MTT method. Firstly, cells were seeded in plates at 5 × 104 cells/well, respectively. All cells were treated with different concentrations of the extracts and incubated for 24 hours. After incubation, MTT solution was added and incubated for 4 hours. After the plate was poured, DMSO was added and formazone crystals were formed. Absorbance values were obtained by reading on an ELISA reader at 545 nm.

FINDINGS

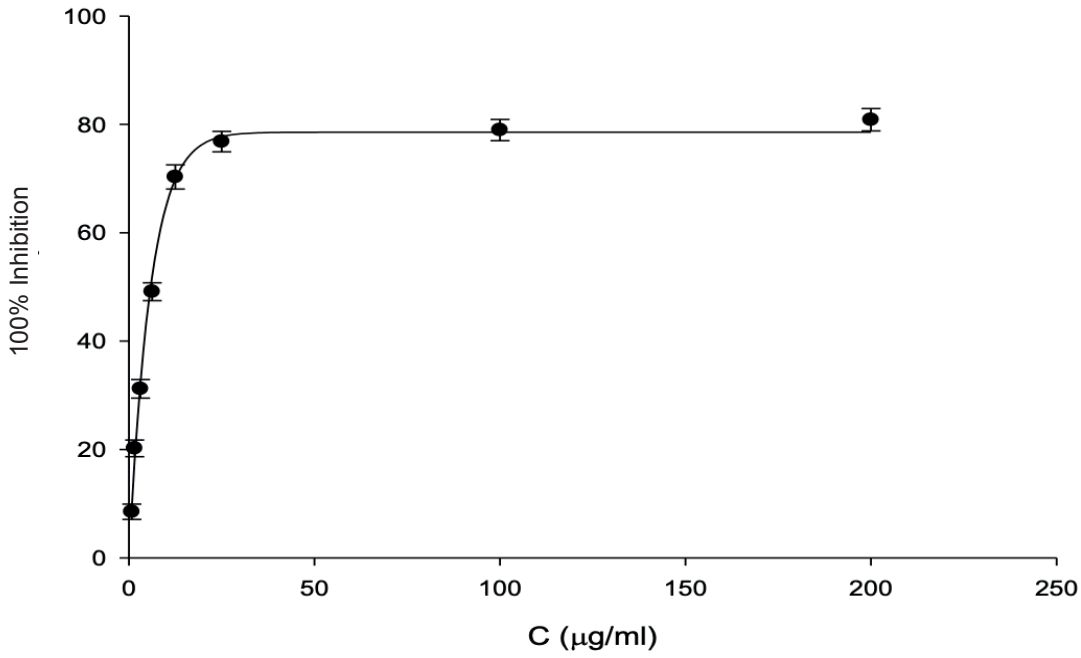
Antioxidant Parameters

DPPH is a dark purple radical and in the presence of antioxidant, it takes a proton and turns into a colourless compound DPPH reduced molecule. Measurement of the absorbance at 517 nm of the reaction of DPPH with antioxidant is one of the most widely used decolarisation analysis methods [103].DPPH (1,1-Diphenyl-2-picrylhydrazyl) is used for the determination of free radical scavenging activity. DPPH radical was determined in the extracts of Viburnum opulus L. obtained from ethanol, water, ethyl acetate and n-hexane. DPPH is purple in colour and as the plant shows antioxidant effect, the DPPH radical is scavenged and the purple colour lightens. The colour change is determined by absorbance change with UV-Vis spectrophotometer. Ascorbic acid was used as standard. DPPH scavenging activity of Viburnum opulus L. extract in the study is given in the table below.

DPPH radical scavenging activity was determined for different concentrations of Viburnum opulus L. extract and ascorbic acid (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.562 µg/mL). For this purpose, % inhibition values at each concentration were calculated from the absorption values obtained and plotted against the concentration. The following formula was used for calculation [104].

Figure 1. *Ascorbic acid standard graph*

$$I\,(\%) = \left[\frac{A_{blank} - A_{sample}}{A_{blank}}\right] \times 100$$



IC50 value of Viburnum opulus L. extract was 62.5±1.23 µg/mL water, 53±2.76 µg/mL ethanol, 65±2.34 µg/mL ethyl acetate, 44±1.98 µg/mL n-hexane. The IC50 value of ascorbic acid was found to be 4.67±0.09 µg/mL (Table 3).

Table 3. *DPPH values*

	Water	Ethanol	Ethyl acetate	n-hexane	Ascorbic Acid
µg/mL	62.5±1.23	53±2h.76	65±2.34	44±1.98	4.67±0.09

Total Flavonoid Content Determination

Viburnum opulus L. extract was analysed according to the method of Chang et al. The amount of total phenolic matter was calculated as quercetin equivalent. The concentrations of total flavanoid compounds in Viburnum opulus L. extracts (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.562 µg/mL) were calculated as quercetin equivalent from the equation obtained from the quercetin standard graph calibration curve given in Figure 2. The obtained graph equation was found as y = 0.0032x+0.0768 (R2=0.9997). The results of the total flavonoid content of Viburnum opulus L. extracts of water, ethanol, ethyl acetate and n-hexane solutions are given in Table 4.

Figure 2. *Standard graph of quercetin*

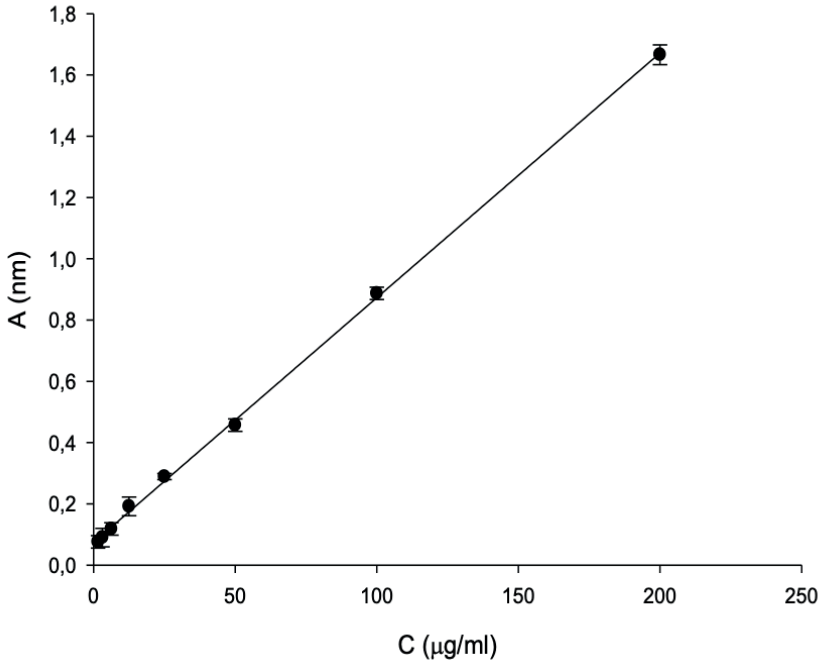


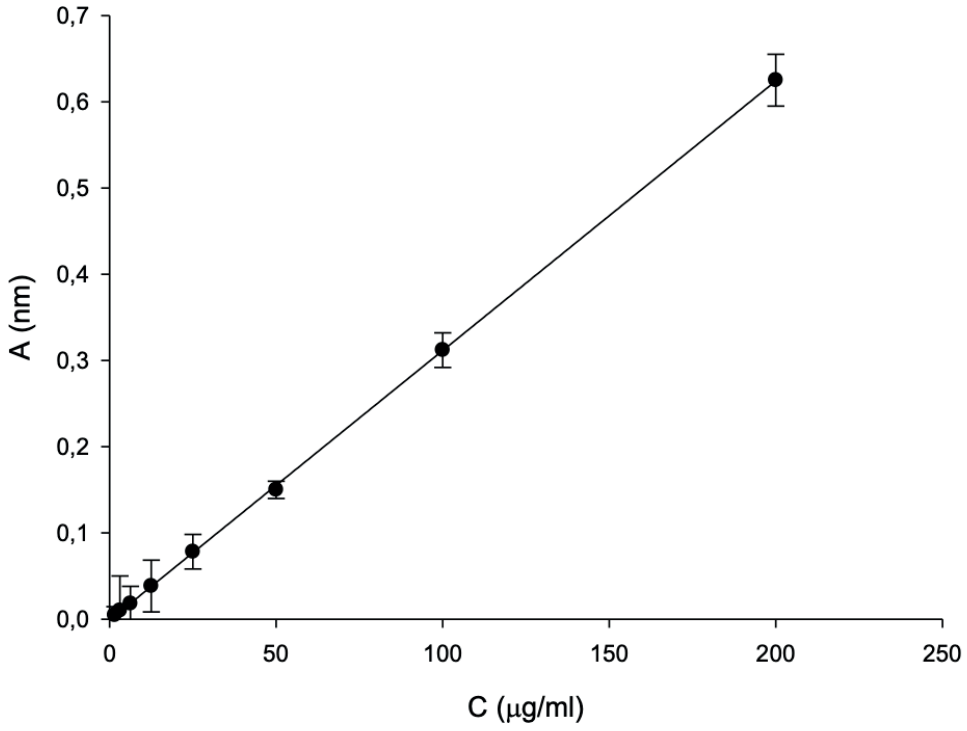
Table 4. *Flavonoid values*

	Water	Ethanol	Ethyl acetate	n-hexane
µg/mL	28.67	29.87	25.98	27.75

FRAP Antioxidant Power Determination Results

In the analysis of Viburnum opulus L. extracts according to Benzie and Strain method, the amounts of FRAP antioxidant power were calculated as trolox equivalents. The concentrations of iron reducing compounds (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.562 µg/mL) in *Viburnum opulus L.* extract were calculated as equivalent to trolox from the equation obtained from the calibration curve of the trolox standard graph given in Figure 3. The obtained graph equation was found as y = 0.0031x-0.0012 (R2=0.9999).

Figure 3. *Trolox standard graph*



The results of FRAP antioxidant power of Viburnum opulus L. extracts obtained from ethanol, water, ethyl acetate and n-hexane solvents are given in Table 5.

Table 5. *FRAP values*

	Water	Ethanol	Ethyl acetate	n-hexane
µg/mL	189.86	178.08	215.98	165.87

Cell Culture

In the study, Viburnum opulus L. extracts prepared in water, ethanol, ethyl acetate and n-hexane solvents were applied to colon cancer in cell culture with 24 hours incubation. IC50 values were calculated and given below (Table 6 and Table 7).

Table 6. *IC_{50} values of Viburnum opulus L. calculated in different solvents in colon cancer*

	Water	Ethanol	Ethyl acetate	n-hexane
µg/mL	189.73±2.65	108.98±1.93	135.87±2.07	152.98±1.04

Table 7. *IC50 values of Viburnum opulus L. calculated in different solvents in healthy cell linecancer*

	Water	Ethanol	Ethyl acetate	n-hexane
µg/mL	276.56±1.96	250.72±1.90	300.72±2.73	350.82±3.98

DISCUSSION

Various parts of different species of the genus Viburnum opulus L. are widely used in Chinese medicine due to numerous pharmacological properties such as antimicrobial, anti-inflammatory, antiallergic and antioxidant activities. In the literature, the antimicrobial effect of leaf and flower essential oil of Viburnum opulus L. is observed[6]. Antioxidants can protect human cells from reactive oxygen species (ROS). Antioxidants can convert free radicals into non-radical compounds by methods such as chain reaction of lipid oxidation, inhibition of pro-oxidative enzymes and chelation of metal ions. Thus, antioxidants present in the diet may have a significant impact on disease prevention and progression. Water-soluble vitamin C is found in cellular fluids such as cytosol or cytoplasmic matrix, whereas lipid-soluble vitamin E and carotenoids are predominantly found in cell membranes[7]. Vitamin C may act directly by reacting with aqueous peroxyl radicals and indirectly by restoring the antioxidant properties of fat-soluble vitamin E. Vitamin C content in VO fruit ranged from 12.4 to 164 mg/100 g fresh weight, depending on growing location and genotypes. The vitamin C content of VO fruits grown in Turkey ranged from 25.0 to 59.5 mg per 100 g of fresh fruit [8, 9, 10, 11, 12, 13].

In a study, the essential oils of the leaves and flowers of Viburnum opulus L. were extracted and analysed. Of the 16, 53 and 35 compounds identified in the stem, leaf and flower of Viburnum opulus L., only 5 were found to be present in all three segments of the plant. The essential oil in the plant stem was found to consist mainly of spathulenol, carvacrol, santolina alcohol and trans-caryophyllene oxide. The main constituents of the leaf oil were 1,8-cineole, camphor, ascaridol, trans-isoacaridole and piperitone oxide, while the main constituents of the flower oil were ascaridol, trans-isoacaridol, 1,8-cineole, p-cymene and camphor. These factors provide strong anticancer and antiproliferative properties of Viburnum opulus L.

In our study, when the DPPH free radical scavenging activity of Viburnum opulus L. extract was studied, ascorbic acid, which is a strong antioxidant, was 4.67±0.09, while 44±1.98 n-hexane solvent was the highest free radical scavenging. Viburnum opulus L. ethanol solvent 53±2.76, water solvent 62.5±1.23, ethyl acetate solvent 65±2.34. In our study, the antioxidant power of the extract of n-hexane solvent was found to be high. The highest total flavonoid content was 30.75±0.12 µg/g in n-hexane solvent. In water solution 28.67±1.56 µg/g, in ethanol solution 29.87±2.97 µg/g and in ethyl acetate 25.98±2.34 µg/g. It was observed that total polyphenol content was low. Iron reducing power was found as 215.98±2.78 mg/g in n-hexane solvent. Iron reducing power of Viburnum opulus L. Extract was found to be at medium level in our study.

Colorectal cancer is one of the most common malignancies. Colorectal cancer is the 4th most common cancer and the 2nd most common cause of death in the United States of America (USA). The lifetime probability of a person developing colorectal cancer is 6%. From this point of view,

it is a serious public health problem. More than 90% of the patients are over the age of 50 years and 75% have no other known risk factors other than age[14,15].Although screening programmes in colorectal cancer are widely used in western societies, this point has not been given the necessary importance in our country. In a study, it was confirmed that commercially prepared gilaburu juice (80 µl/mL) showed a cytotoxic effect against HeLa and Caco-2 cells, while no inhibition of metabolic activity was observed in normal HUVEC cells[16]. In another study, the preparation obtained from defatted VO fruit pulp after extraction with pressurised ethanol reduced the proliferation of human colon adenocarcinoma HT-29 cells (IC50=0.39 mg/mL) without toxic effect on Caco-2 cells[17].

Viburnum opulus L. After the extract was obtained in different solvents, it was applied to colon cancer cell line and the proliferation effect of colon cancer was examined. In our cell culture study, the IC50 value of ethanol extract was found to be the most effective. The IC50 values of Viburnum opulus L. water extract were 189.73±2.65, ethanol 108.98±1.93, ethyl acetate 135.87±2.07, n-hexane 152.98±1.04 ug/mL. While an extract kills cancer cells, it should not damage healthy cells. For this reason, in our study, healthy cells were applied as a positive control and it was observed that the doses affecting cancer had a low effect on healthy cells. Our study seems to be supportive of the literature.

CONCLUSION

In this study, Viburnum opulus L. can be considered as one of the promising aromatic plants on colon cancer in the future. Considering the results of this study; the effect of Viburnum opulus L. on colon cancer should be investigated at different incubation times and new studies should be included to elucidate apoptotic and cancer mechanisms.

Conflict of interest statement

The authors declare that they have no conflicts of interests.

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None

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