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Research Article

Cytotoxic effect of *Laurus nobilis* leave extracts on bacterial growth and MCF-7 cancer cell line *in vitro*

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Abstract

Background: Medicinal plants play an important role in preventing as well as treating various types of diseases throughout the ages. There are many species of medicinal plants in the world used as herbal drugs. *Laurus nobilis* has been extensively used as an herbal plant in traditional medicine to treat numerous disorders.

Objectives: The study tested the antibacterial properties of *L. nobilis* leaves extracts against various species of bacteria besides the cytotoxic effects of ethanol extract on the MCF7 cancer cell line.

Methods: Different concentrations of ethanolic and aqueous extracts were tested for antibacterial efficacy against *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus faecalis*. Agar well diffusion method was used to determination of inhibition zone for all bacteria. Trypan blue and the MTT assay were used to investigate the cytotoxic effect of the ethanolic extract on the MCF7 cell line.

Results: The aqueous extract revealed high inhibitory effect against gram-positive bacteria only. While the ethanolic extract showed inhibition zone on all the bacterial species were highest in gram-positive bacteria than gram-negative bacteria. The rate of proliferation in cells treated with ethanol extract shows a significant reduction depending on concentration; the more cells were decreased as the dose was increased.

Conclusions: Our study concluded that *Laurus nobilis* could serve as a source of beneficial medications because of the existence of diverse phytochemical components. These phytoconstituents appeared to possess both antibacterial and anticancer activities and may serve as promising anticancer agent in the future.

Keywords: *Laurus nobilis;* Antibacterial activity; cytotoxic; MTT assay

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Introduction

Medicinal plants play an important role in preventing as well as treating various types of diseases throughout the ages. There are many species of medicinal plants in the world used as herbal drugs [1]. The WHO [2] evaluates that 80 percent of some Asian and African populations currently use herbal

therapies in specific areas of primary health care. Plant-derived extracts have been historically considered important alternative medicines for enhancing immune status by prevention and treatment of chronic diseases [3]. One of these plants is *Laurus nobilis* which is also called laurel leaves or Bay that belongs to *Lauraceae* family, Laurales order, Magnoliopsida class, and Magnoliophyte division [4]. It includes 32 genera and about 2,000 - 2,500 species [5]. This plant is locally found in the South of the Mediterranean area and is cultivated in Algeria, Morocco, Portugal, Spain, Italy, France, and Turkey [6]. *Laurus nobilis* tree is length ranged 2-10 m high, and its leaves contain different amounts of essential oils, polar flavonoids, alkaloids, glycosylated, Tannins, phenolic components, megastigmane, sesquiterpenic alcohol, minerals, and vitamins [7]. *L. nobilis* is used as culinary spices in the preparation of various foods such as meat, fish and soup [8], as well as a treatment of several neurological, dermatological, and urological disorders [9]. Recently it has been used in treating diabetes [10]. Furthermore, the dry leaves of *L. nobilis* are used in treatment of some disorders of digestive system as epigastric pain [8] and anti-viral infections [11]. Many studies revealed that *L. nobilis* has various pharmacological effects, including antimicrobial properties against some microorganisms [12]. The inhibitory effect of bay extracts against different bacterial species makes their use as antibiotics to treat fungal and bacterial infections by reducing the growth of pathogenic bacteria [13]. In recent years, investigators have detected many species of medicinal plants can inhibit the growth of various kinds of cancer cells [14]. The herbal medicine has few side effects, is less expensive and has long-lasting effects, which can be alternatives to traditional medications [15]. Cancer has quickly become one of the leading causes of death in developed countries. However, in the last few decades, the incidence of breast cancer has increased considerably in developing countries [16]. There are many studies on antitumor ability of aqueous and ethanol extracts of *L. nobilis, and* many of the important compounds have been shown to have cytotoxic activity [17].

The aims of this study were designed to:

1. Determine the inhibitory effect of *L. nobilis* leaves aqueous and ethanolic extracts on gram-positive bacteria and gram-negative bacteria.
2. Investigate the possible beneficial effects of the ethanol of *Laurus nobilis* extract (bay leaf) on MCF7 human breast cancer cell line in an attempt to identify its cytotoxic activity.

Materials and methods

Extraction of plant material

The *Laurus nobilis* plant's leaves (figure 1) were acquired from a local supermarket. For aqueous extract, the leaves were ground using an electric blender, and about 5 g of the powder plant was refluxed with 250 ml distilled water under 100°C for 4 hrs., moreover, mixed at 30 ºc for 6 hrs. After that, paper filters were used to filter the mixture to remove all the residual material. Then, it dried at room temperature and kept at 4˚C until use [18]. The extraction yield was obtained by the following equation:

$$\text{Extraction yield (\%, w/w)} = \frac{Initial\ weight\ (g) - Final\ weight\ (g)}{Sample\ powder\ weight\ (g)} * 100\%$$

Crude ethanol extract of *Laurus nobilis* leaves were prepared according to Pieroni *et al.* [19]. For 4 hours, 5 g of plant powder was refluxed with 250 ml of absolute ethanol at boiling point. The supernatant was then separated from the precipitate using Whatman Number 32 filter paper and stored at 4 C until use as before.

Figure1: *Laurus nobilis A:* tree, B: fresh leaves and Flowers. C: Dried leaves

Phytochemical Screening

Extracts have been evaluated for the presence of active compounds by different chemical tests. Wagner's, Dragendorff's, Mayer's, and Marquis' tests were used to identify alkaloids and tannins in plant extracts. Saponin was identified according to [20]. Benedict test was used for carbohydrates. Flavonoids and the presence of glycosides before and after hydrolysis were detected by Auwal *et al.* [21]. Protein was detected using the biuret test. Peptides and free amino group tests were utilized to identify peptides, and the ninhydrin test has been employed to identify primary and secondary amino groups. For triterpenoids, the Liebermann-Burchard test was used. Finally, Ferric chloride and ammonia vapor were used for polyphenol [22]. The stock solution of ethanolic and aqueous extracts was prepared by redissolving 10mg/mL in 5% DMSO and 8mg/mL in water respectively for antimicrobial and antitumor activity evaluation [23]. Usually, the stock was made fresh and used immediately. The final solutions were diluted to various concentrations for bioactivity testing.

Antibacterial activity assay

The inhibitory effect of *Laurus nobilis* leaves ethanolic and aqueous extracts was tested against gram-negative and gram-positive bacteria (*Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Staphylococcus aureus, and Streptococcus faecalis)* respectively. The bacterial species were obtained from the microbiology laboratory at Al-Sader Teaching Hospital. The species of bacteria were grown in Mueller Hinton Agar. Several test tubes were prepared each of them containing 4ml of 0.9% normal saline. To each, 3-10 colonies of the tested bacterial species were then added to turbidity-matched McFarland tube number 0.5 (1.5 108 CFU/ ml) [24]. Agar well diffusion was method used for determination of antibacterial activity of aqueous and ethanolic against various bacterial species [25]. Cultures from the standardized broth for each species of bacteria were swabbed on the surface of Mueller Hinton Agar plates. Five wells of 6 mm were punched in each inoculated plate using a sterile borer. The crude ethanolic extract was diluted by using Dimethyl sulphoxide (DMSO) to prepare two- fold serial dilution concentrations of (200, 100, 50, 25)mg/ml. The crude aqueous extract was diluted in the same manner. Then, about 0.1 ml of these concentrations was transferred into four wells [26]. The fifth well was used as a control with similar concentrations of DMSO solvent for comparison. All plates were then inoculated overnight in the standard method. The diameter of the inhibition zone around the well was used to assess antibacterial activity for *Laurus nobilis* leaves extracts.

Cell line and culture

The MCF-7 cell line was obtained from Tehran University, Iran. MCF7 cells were extracted from the pleural effusion of a female patient, age 69, who had breast cancer [27]. The cells were seeded in a T-75 flask with ten percent fetal bovine serum and one percent penicillin-streptomycin antibiotic in the RPMI-1640 medium. After filling the flask surface with cells, the cells were scraped with trypsin EDTA solution. After that, the cell suspension was centrifuged for 5 minutes at 1100 rpm. The supernatants of the cells were removed carefully after centrifugation, and the precipitate was suspended in the new culture medium. Trypan blue staining on the Neubauer slide was used to count the cells under the microscope.

Cytotoxicity Assay (MTT Assay)

Ten thousand MCF-7 cells were seeded into a 48-well micro-titration plate. For 48 hours, the cells were cultivated in a 37 ° C incubation medium, with 10% humidity and 5% CO2. After 48 h, the cells were replaced and incubated with the new medium containing different concentrations (62.5, 125, 250, 500, and 1000) µg/ml of crude ethanolic extract of *Laurus nobilis* leaves for 48 h. The cell sample without ethanol crude extracts of *Laurus nobilis* leaves was considered a control sample. After 48 hours of incubation with the extract, the supernatants of the cells were decanted and MTT solution (0.5 mg/ml) was added to the 48-well plate samples and incubated for 3.5 hours in the dark at 37°C. After completion of the mentioned time, the MTT solution was eliminated from the plate and the resulting purple dye was solubilized in DMSO. The purple absorbance (optical density) at 531 nm was read by an ELISA reader. The experiment was conducted three times for each concentration and cell viability was expressed as mean optical density (OD) using the formula [28]:

$$\text{Viability of cells} = \frac{\text{sample OD}}{\text{control OD}} \text{x}100$$

Statistical analysis

All data has been presented as mean ± standard deviation of three replicates. For each parameter, the mean of three wells was calculated. The medium-treated cells served as the control, and the remaining samples were expressed as a viability percentage decrease or increase when compared to the control. A P value of less than 0.05 was regarded as statistically significant. SPSS was used for statistical analysis and was performed by One-way ANOVA.

Results

Chemical composition

The nature and color of dried ethanol and aqueous *Laurus nobilis* leaf extract solutions, and the yield of extraction (g) are listed in table (1). The dried products of ethanolic and aqueous extracts were solid dark brown and viscous dark brown, respectively. In the present study, the ethanolic content (yield) was 0.9 g, and 1.5 g for the aqueous extract. Extraction yields significantly varied with different solvents; aqueous extract gave higher yields than ethanol extract

Table 1: The nature and color of dried and solutions of *Laurus nobilis* extracts as well as the extraction yield (g)

Part of plant	Type of extract	Nature &color of extract	Color of Solution	Yield of Extraction (g)
Leaves	Ethanol	Solid → dark brown	Dark brown	0.9 g
Leaves	Aqueous	Viscous → dark brown	Dark brown	1.5 g

Table (2) shows the chemical composition of Laurus nobilis leaf extracts. The chemical constituents, like glycosides, carbohydrates, alkaloids, saponins, flavonoids, proteins, tannins, free amino acids, and polyphenols were found in the crude aqueous and ethanolic extracts from leaves of *Laurus nobilis* whereas the triterpenoid gave negative results in both extracts.

Table (2): The screening of active compounds for aqueous and ethanolic of *Laurus nobilis* leaves extracts

Compound group	Aqueous extract	Ethanolic extract	Remarks
Alkaloids			
a- Wagner′s test	+	+	Reddish brown
b-Dragendorff’s test	+	+	Orange-reddish brown

c-Mayer's test d-Marquis' test	+ +	+ +	Immediate creamy white ppt. Yellow-purple participate
Saponins	+	+	Thick foam
Carbohydrate Benedict test	+	+	Purple ring
Glycosides (Benedict) a-before hydrolysis b- after hydrolysis	+ +	+ +	orange precipitate orange precipitate more than in the first state
Flavonoid test alcoholic KOH 5N	+	+	yellow precipitate
Protein Biuret test	+	+	violet color
Tannins a-Lead acetate b-Ferric chloride	+ +	+ +	white jelly precipitate turn blue
Peptides-free amino group Ninhydrin	+	+	blue or violet color
Triterpenoid (Liebermann-Burchard test)	-	-	
Polyphenol Ferric chloride and ammonia vapor	+	+	Green or blue

Antibacterial Activity of ethanolic and aqueous extracts on bacterial species

The extracts of *Laurus nobilis* leaves showed different grades of inhibitory effects on the growth of gram-negative and gram-positive bacteria. Ethanolic crude extract showed inhibition zone on gram-negative and gram-positive tested bacteria (*Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Streptococcus faecalis)*. As well as *Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* revealed resistance to the aqueous extract, while *Staphylococcus aureus* and *Streptococcus faecalis* revealed sensitive to the aqueous extract, whereas control solution DMSO showed no inhibitory effect on all tested bacteria as shown in (table 3).

Table (3): Effect of crude ethanolic and aqueous extracts on the growth of gram-positive and gram-negative bacteria

Microorganisms	Ethanolic extract	Aqueous extract	DMSO
Escherichia coli	Sensitive	Resistant	Resistant
Klebsiella pneumonia	Sensitive	Resistant	Resistant
Pseudomonas aeruginosa	Sensitive	Resistant	Resistant
Staphylococcus aureus	Sensitive	Sensitive	Resistant
Streptococcus faecalis	Sensitive	Sensitive	Resistant

The antibacterial effect of the aqueous extract according to the concentrations on the different tested bacteria is shown in (table 4). The aqueous extract exhibited varying degrees of inhibitory effect on the bacteria. At the 200mg/ml concentration, it was observed that inhibition zone diameter of gram-positive bacteria *Staphylococcus faecalis* and *Streptococcus aureus* was ranged 22-20mm, in addition, the inhibitory effect found on these bacteria at 50 mg/ml and 25 mg/ml. While gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia*) not revealed inhibition zone of the aqueous extract.

Table (4): Inhibitory effect of different concentrations of aqueous extracts of *Laurus nobilis* leaves on gram-positive and gram-negative bacteria

Microorganisms	Concentrations of aqueous extract)			
	200mg/ml	100mg/ml	50mg/ml	25mg/ml
	inhibition zone (mm)			
Escherichia coli	0 mm	0 mm	0 mm	0 mm
Klebsiella pneumonia	0 mm	0 mm	0 mm	0 mm
Pseudomonas aeruginosa	0 mm	0 mm	0 mm	0 mm
Staphylococcus aureus	20 mm	9 mm	8 mm	0 mm
Streptococcus faecalis	22 mm	12 mm	7 mm	0 mm

The antibacterial effect of ethanolic extract of *Laurus nobilis* leaves according to the concentrations on the different types of bacteria is shown in (table 5). The ethanolic extract exhibited a high effect of antibacterial activity at 200mg/ml concentration and the lowest effect at 25mg/ml concentration. It was also observed that the inhibition zone diameter of the ethanol extract at 200 mg/ml concentration was highest in gram-positive bacteria as compared with gram-negative bacteria. The inhibition zone diameter among *Staphylococcus aureus* and *Streptococcus faecalis* ranged 25-23 mm, while among *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia* was ranged 22-18 mm. On the other hand, the ethanolic extract at the lowest concentration 25 mg/ml revealed a higher effect on *Staphylococcus aureus* (10 mm) while no effect on *Klebsiella pneumonia*. These results revealed the antibacterial effect ethanolic extract of Laurus nobilis was highest in gram-positive bacteria as compared with gram-negative bacteria. In addition, the ethanolic extract has higher effective antimicrobial activity as compared to the aqueous extraction.

Table (5): Inhibitory effect of different concentrations of ethanolic extracts of *Laurus nobilis* leaves on gram-positive and gram-negative bacteria

Microorganisms	Concentrations of ethanolic extract			
	200mg/ml	100mg/ml	50mg/ml	25mg/ml
	inhibition zone (mm)			
Escherichia coli	20 mm	14 mm	10 mm	8 mm
Klebsiella pneumonia	18 mm	12 mm	8 mm	0 mm
Pseudomonas aeruginosa	22 mm	10 mm	9 mm	7 mm
Staphylococcus aureus	25 mm	18 mm	12 mm	10 mm
Streptococcus faecalis	23 mm	16 mm	12 mm	8 mm

Cytotoxic effect of *Laurus nobilis* ethanolic extract on MCF-7 breast cancer cells (MTT Assay)

A result of different concentrations of ethanolic leaves of *Laurus nobilis* extracts and control on the proliferation of MCF-7 cells after 48 hours has been shown in table (6). The present investigation showed a significantly different effect between cells treated with ethanolic crude extract and non-treated control at P-value ($P \leq 0.05$). The toxicity effect of ethanolic leaves of *Laurus nobilis* extracts is diverse with different concentrations. According to the L.S.D test, there is no significant effect between the two concentrations (1000^{A} and 500^{A} µg/ml), and among 250^{B}, 125^{B}, and 62.5^{B} µg/ml concentrations after 48 hours. However, the two groups had a significant effect on the breast cancer cell line. Cell proliferation is inhibited by the ethanolic extract in a dose-dependent manner. As a result, increasing extract concentrations reduced cell viability 79.730, 77.680, 77.330, 55.256, and 51.857 respectively.

Table (6): Mean and SD for the effect of control and different concentrations of ethanolic leaves of *Laurus nobilis* extract, on the proliferation of MCF-7breast cancer cells after 48 hours

Ethanolic extract			P value ($p \leq 0.05$) between extracts and control
Conc.(µg/ml)	Absorbance (mean and std.)	Cell viability%	0.019732*
1000 ^{A}	0.493±0.121944	51.857	
500 ^{A}	0.526 ±0.027683	55.256	
250 ^{B}	0.736 ±0.025794	77.330	
125 ^{B}	0.739 ± 0.04564	77.680	
62.5 ^{B}	0.759 ± 0.030406	79.730	
(0)control ^{C}	0.951 ±0.085921	100.00	

Figure 2 (a, b, c, d, e, and f) reveals the increased toxicity of the ethanolic extract on MCF-7 cell proliferation after 48 hours as compared with a control group. The microscopic observations of ethanolic extract-treated cells revealed inactive proliferation of malignant cells at 1000 µg/ml concentration compared with untreated control cells (Fig.2 a).

Discussion

One of the main objectives of medical science is to improve human life .For many years, medicinal plants have been used to inhibit and treat cancer, and many therapeutic plants with anti-cancer activity have been reported [29]. Additionally, many herbs exhibit different antibacterial activities against different bacteria. In the present study, the aqueous extract showed inhibitory effects against gram-positive bacteria, but not gram- negative one, these results agree with other studies which revealed that the aqueous extract of *Laurus nobilis* has antimicrobial activity against gram-positive bacteria [30] particularly, Staphylococcus *aureus*. Another study by Vijayakumar *et al*. [31] showed that aqueous extract of *Laurus nobilis* has antibacterial activity against gram-positive (*Staphylococcus aureus*) bacteria greater than gram-negative (*Pseudomonas aeruginosa*) bacteria. On the other hand, our study revealed that the antibacterial effect of ethanolic extract was highest in gram-positive bacteria as compared with gram-negative bacteria. In addition, the ethanolic extract has higher effective antimicrobial activity as compared to the aqueous extraction. This also mirrored in other study of Mohamed *et al*.

[32] that revealed that methanolic extract was a better inhibitor of bacterial growth than the aqueous extract. As well as Rizwana *et al*. [33] who showed the ethyl and methanol extracts exhibit high inhibitory effect against all the bacterial isolates than aqueous extract. Sakran *et al*. [34] revealed that ethanol extract of Laurus nobilis leaves exhibited a significant antimicrobial effect against *Escherichia coli* and *Staphylococcus aureus*. Aldhaher *et al*. [35] demonstrated the flavonoid compounds have antibacterial properties against *Escherichia coli* and *Staphylococcus aureus*, which influence on cytoplasm and DNA of bacteria. Concerning the anticancer activity of *Laurus nobilis*, our findings are consistent with earlier research carried out by Maria *et al.* [36] and Rizwana *et al.* [33] which reported high antiproliferative activity of laurel extracts against MCF7 tumor cell line. Many proposed mechanisms for antiproliferative effects of herbs, the study of Abotaleb *et al.* [37] demonstrated that bay leaves have anti-proliferative effects on breast cancer cell lines, as the crude extract is rich in flavonoids and terpenoids. Phenolic and tannins compounds have redox properties that enable them to serve as antioxidants [38]. Essentially carcinogenic materials reduce cell proliferation via several ways; apoptosis is one of these mechanisms that allow cells to die actively [39], that is due to the presence of flavonoids by elevation of reactive oxygen species, and DNA damage induction [40]. Rajesh *et al.* [41] found that the major goal of cancer chemotherapy medications work by causing apoptosis in cancerous cells. According to Azevedo *et al*. [42], the medicinal plant contains a protease inhibitor that can decrease MCF-7 breast cancer cells' vitality and growth by inducing apoptosis and lysosome membrane permeabilization. Elvia *et al.* [43] reported that alkaloids, terpenes, flavonoids, and terpenoids prompt toxicity of breast cancer cells via the intrinsic route, resulting in cell death. An additional study examined the process of radical scavenging and antioxidant capabilities of bay leaves [44]. Somayeh *et al.* [45] suggested another mechanism by inducing S phase inactivation in adenocarcinoma was associated with a decrease in Cdk1, in a p53-independent route. A previous study reported that the mitotic index decreased, indicating that the extract had an antiproliferative effect, due to the presence of alkaloids , flavonoids, saponins, and phenol in the phytochemical components [46].

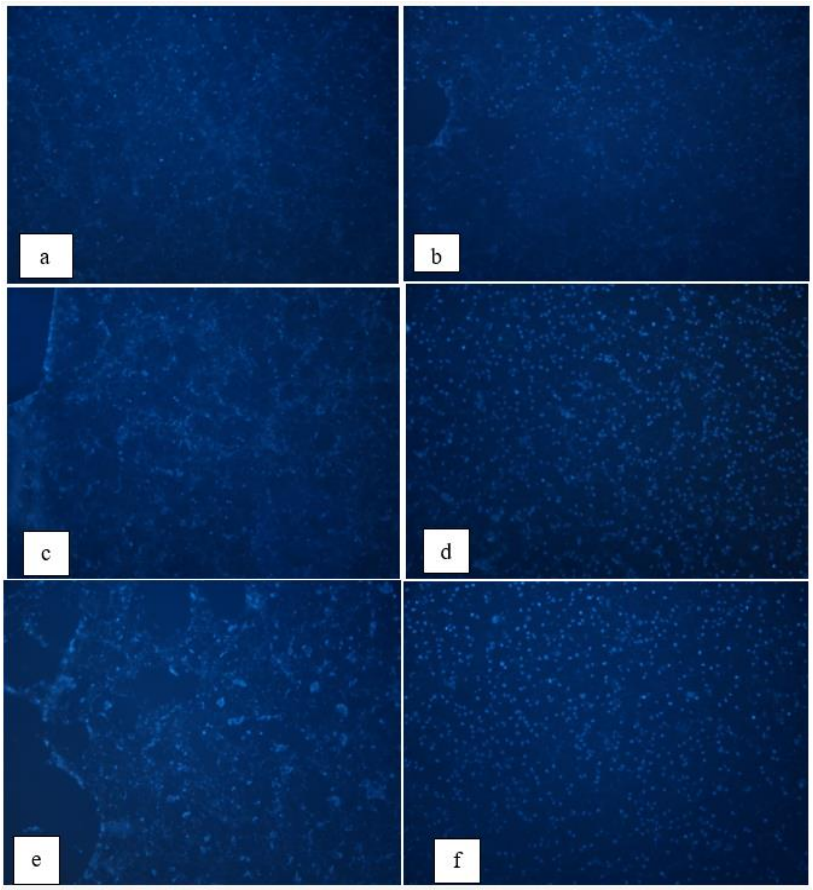

Figure 2: Proliferation of MCF-7 cell lines after incubated 48 hours: (a) control (b) 62.5 µg/ml (c) 125 µg/ml (d) 250 µg/ml (e) 500 µg/ml (f) 1000 µg/ml leaves of *Laurus nobilis* extract

Conclusion

Our study concluded that *Laurus nobilis* could serve as a source of beneficial medications because of the existence of diverse phytochemical components such as flavonoids, alkaloids, phenol, terpenoids, Saponin, and carbohydrates. These phytoconstituents appeared to possess both antibacterial and anticancer activities and may serve as promising anticancer agent in the future.

Conflicts of interest

The authors declare no conflicts of interest

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