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Original Article

Evaluation of Antibacterial, Antibiofilm and Cytotoxicity Effect of Crude Ethanolic Extract of *Cuminum cyminum* Against *Streptococcus mutans*

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ABSTRACT

Although, oral health is important for its role in the overall health but oral health care does not have an integrated model. So, today meeting the needs of oral health requires strong interactions between health research policy and oral health researchThe aim of this study investigates the effects of ethanolic crud extract of *Cuminum cyminum* L. on *S. mutans* in terms of antibacterial, antibiofilm and, its cytotoxicity properties. The present experimental study which has conducted in 2019-2020. The crude alcoholic extract of *C. cyminum* seeds was prepared by the maceration method. Then, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of crude ethanolic extract *of C. cyminum* was determined by micro-dilution method according to CLSI protocol on *Streptococcus mutans* (PTCC1683). In addition, its antibiofilm effect was investigated by the previous method using crystal violet. As well, an MTT test was performed to evaluate its cytotoxicity on SW480 cells. Statistical analysis was done by SPSS V.22 software. MIC and MBC concentrations of *C. cyminum* extract on *S. mutans* were found to be 0.62 mg/mL and 1.25 mg/mL. The effect of inhibiting biofilm production was also observed at a concentration of 40 mg/mL. In MTT assay showed a greater cytotoxic effect on SW480 cancer cells at higher concentrations for longer periods of time. The present study showed that the *cumin* ethanolic crude extract has antimicrobial properties, inhibits the growth of *S. mutans* biofilms and has no toxic properties.

MIC: minimum inhibitory concentration. MBC: minimum bactericidal concentration. MTT: 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl-2H- tetrazolium bromide. DMSO: dimethyl sulfoxide

INTRODUCTION

Although, oral health is important for its role in the overall health but oral health care does not have an integrated model. So, today meeting the needs of oral health requires strong interactions between health research policy and oral health research [1]. Break down of tooth enamel caused dental decay or tooth decay [2]. Treatment of high-prevalence dental caries, which accounts for 100% of adults and 60 to 90% of children, along with significant treatment costs that make it one of the most expensive diseases after diabetes and cardiovascular diseases [3,4]. It is also of particular importance because of its association with cervical infections and cardiovascular diseases [5]. The occurrence and development of dental caries is closely related to oral microorganisms [4]. In fact, oral microbiota is involved in the formation of dental caries by forming biofilm [3]. Biofilm formations are usually chronic in nature and greater resistance to antibiotics and the immune system activity is a major feature of the bacteria which are present in the biofilm [6]. Biofilm

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formation is effective in bacterial pathogenesis in different ways, including causing inflammation and even helping to cause cancer [6] . Biofilm is an extremely complex ecosystem full of active and secreted materials known as EPS (extracellular polysaccharides). During biofilm formation on teeth, a salivary glycoprotein film (called dental pellicle) is coated on the tooth surface [7]. Antimicrobial agents cannot penetrate deep into layers of biofilm due to the biofilm structure. So, biofilm formation not only increases bacteria's tolerance to antibiotics and resistance to the host's immune system, but also results an infection [8]. Biofilm is a polymicrobial community and *S. mutans* play an important role in the early stages of dental biofilm formation [3]. Tolerance to acidic environment, acid production and ability to communicate with other bacterial species highlight the role of *S. mutans* in the formation of dental biofilms. So, as discussed above it is important to find a suitable treatment strategy with low adverse side effects and financial burden [9,10]. Herbal medicine usage in dentistry has always been considered as one of the useful treatment options, among the applications of these medicines can be used to relieve toothache, canker sores, and gum inflammation [11]. *C. cyminum* is a member of *Apiaceae* or Umbelliferae family and is the most cultivated and the second most popular seed with many medicinal and nutritional properties. Traditionally, it has several uses such as anticoagulant and epilepsy, toothache, whooping cough, Anti-inflammatory, antibacterial and antifungal properties have been observed in this plant [12]. The anti-biofilm effects of *C. cyminum* have been proven in gram-negative bacteria [1]. The antibacterial effect of *cumin's* ethanolic extract on *Staphylococcus aureus* has also been reported before [13]. The aim of this study was evaluation of antibacterial, antibiofilm and cytotoxicity effect of crude ethanolic extract of *C. cyminum* against *S. mutans*.

MATERIALS AND METHODS

This experimental study was done in Shahid Beheshti University of Medical Sciences (SBMU) Tehran/Iran during 2019-2020. The proposal was approved in ethical committee and received the code (IR.SBMU.RIDS.REC.1396626).

Bacterial preparation

S. mutans PTCC1683 was obtained from the Iranian research organization for science and technology. Based on the manufacturer's protocol, the lyophilized bacterial strain was treated by BHI broth and then was cultured to a sheep blood agar plate and incubated at 37 °C for 24 hours in a 5% CO2.

Crude Alcoholic Extraction

The seeds were purchased from reputable sources and received herbarium code PF12 from pharmaceutical department, Pharmacy school, Shahid Beheshti University of Medical Sciences. The plant extract was made by soaking of 350g of plant seed powder in 2 liter of ethanol (96%) for 72 hours at room temperature based on the maceration method [10]. The created liquid extract was dried using rotary apparatus and final 10 g of dried ethanolic extract was kept at dark bottle and placed in dry environment. A syringe filter (0.45µL) was used to sterilize the prepared extract.

Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay

The micro-dilution broth method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanolic crude extract of *C. cyminum* against *S. mutans*. In a 96-microtiter plate, a serial dilution of alcoholic extract of *C. cyminum* ranging from 40 mg/mL to 0.31 mg/mL using DMSO 2% was prepared. The 96-well plates were incubated at 37 °C for 24 hours after being inoculated with 10 µL of bacterial suspension with turbidity equivalent to a 0.5 McFarland standard ($1.5x10^5$ CFU/mL). According to CLSI guidelines, MICs were determined visually in the last tube with no turbidity [11]. Sub-culturing 0.01 mL of the medium drawn from the culture wells after 48 hours on Mueller Hinton agar and incubating further for bacterial growth yielded the minimum bactericidal concentration (MBC). The MBC was determined to be the lowest concentration of the antimicrobial agents that caused negative growth. These tests were done twice. Amoxicillin (Mast, UK) and DMSO 2% were used as positive and negative controls respectively.

Biofilm Assay

The biofilm of *S. mutans* was created using the crystal violet method. Each well received 100 µL of BHI broth containing 3% sucrose. Then 100 µL of different dilutions of the cumin ethanolic extract

based on the MIC result were added to each well, followed by 100 µL of bacterial suspension (equivalent to 0.5 McFarland). After incubating for 24 hours, the 96-well plate was rinsed twice with PBS (pH 7.0) and stand for 5 minutes with 0.1 percent of crystal violet (Sigma). Then distilled water was used to remove the remaining dye. After the biofilm had dried slightly, 200 µL of absolute ethyl alcohol was added and the plate was shaken for 30 minutes at room temperature (23 °C). At 575 nm wave length, the absorbance of extracting crystal violet in absolute ethyl alcohol was measured. The tests were conducted twice, and the results were averaged. The anti-biofilm assessment according to OD spectrophotometry interpreted based on Kolter and Otoole as follows: OD ≤ ODc (OD in negative control) (non-adherent), ODc <OD ≤ 2ODc (weakly adherent), 2ODc <OD ≤ 4ODc (moderately adherent) and 4ODc <OD (strongly adherent) [12].

Cytotoxicity Assay

Cell Culture

The SW480 colon cancer cells and NIH3T3 (mouse embryo fibroblast cells) were purchased from the Pasteur Institute of Iran. The cells were cultivated in RPMI medium that contained 1% fetal bovine serum (Gibson USA) and 1% penicillin-streptomycin antibiotic (Gibson USA), incubated at 37 °C and 5% CO2 incubator. Trypsin (Gibson USA) was used for passage the cells after 3 days.

MTT Assay Test

The viability of the cells was evaluated by the MTT test. The process was based on the reduction of dimethyl thiazole diphenyl tetrazolium bromide by mitochondrial dehydrogenases into insoluble and purple crystals. There is an important correlation between the intensity of the purple color produced and the number of metabolically active cells. We cultured 10^3 SW480 cells per well in 96-well plates. First, 96 well plates were treated with various concentrations of alcoholic crude extract of *cumin* (0, 50, 100, 300, 400, 600, 800, 1000, 2000, 4000, 8000, and 10000 µg/ml). Then, the plates were incubated at 37 °C for 24 hours. As a control, medium and bovine serum with no extract was used. Afterward, the cells were washed with PBS and then 20 µL of MTT solution was added to each well. The microplate was incubated for another 3 hours at 37 °C. Subsequently, 100 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany) was added to each well, and all these wells were gently shaken. An ELISA reader (Bio-Rad, USA) was used to measure the optical density (OD) at 570 nm wavelength. In parallel, the three substances were tested in a noncancerous NIH 3T3 (mouse embryo fibroblast), from Pasteur institute, Tehran, Iran, cell line as a normal cell line.

RESULTS AND DISCUSSION

MIC and MBC

Based on the CLSI 2020 test micro- dilution broth was performed in two times and the same results were observed in both times. According to the instructions amoxicillin (Mast, UK) was used as a control with MIC equal to 0.3 mg/mL. Due to the results, the MIC and MBC of ethanolic cumin extract were detected at 0.62 mg/mL and 1.25mg/mL respectively.

Antibiofilm effect of alcoholic crude extract of *C. cyminum* on *S. mutans*

Examination of the effect of different concentrations (40, 20, 10, 5, 2.5, 1.25, 0.62, and 0.31 mg/mL) of *cumin* extract on biofilm formation showed that the alcoholic extract of this plant at a concentration of 20 mg/mL led to the formation of a weak biofilm and at a concentration of 40 mg/mL prevented the formation of a biofilm.

Anti-proliferative effect of alcoholic extract of *C. cyminum*

Cumin alcoholic extract was tested for its inhibitory effects on SW480 colorectal cancer cells using MTT assay. A statistical analysis of MTT assays revealed a significant reduction in cancer cell growth when treated with an alcoholic extract of *cumin*. *Cumin* alcohol extract showed only a minor toxic effect on NIH 3T3 (mouse fetal fibroblasts) (P> 0.05).

For 24 hours at concentrations of 50, 100, 300, 600, 800, 1000, 2000, 4000, 8000 and 10000 µg/mL, the cell viability was 92, 80, 74, 60, 54, 49, 40, 38, 30 and 20% respectively. The IC50 was 800 µg/mL for 24 hours. Similarly, the IC50 value at 24, 48, and 72 hours was 800, 700 and 500 µg/mL respectively. It can be said that alcoholic extract of *cumin* has therapeutic potential and it has a greater cytotoxic effect on cancer cells at higher concentrations and for longer periods. This includes colorectal cancer cells SW480, which is the most resistant of all cancer cells. Dental caries is an infectious microbial disease. *S. mutans* is important in the development of this disease, and dental caries occurs in a specific area when the amount of *S. mutans* in that area occupies half of the microbial population. As a result,

changing the microbial ecology of the mouth by reducing the amount of *S. mutans* is a critical issue [3,16]. On the other hand, the emergence of drug resistance in a wide range of pathogenic microorganisms has emerged as a significant challenge in both health and treatment. As a result, there is an ongoing need to discover new antimicrobial compounds to reduce drug resistance in microorganisms and use them as alternatives to chemical preservatives [17]. Essential oils and extracts of medicinal plants containing antimicrobial, anticancer, antioxidant and, free radical scavenging compounds have a wide range of applications [3].

Cumin, scientifically known as *C. cymimum*, is an annual, delicate, and fragrant herbaceous plant of the Umbelliferae family. This plant can be found in the Mediterranean, the Southwest and Central Asia. *Cumin* is one of our country's most important and cost-effective medicinal plants too. It is grown in a variety of locations including Tabriz, Yazd and Kerman. In Germany, it is combined with bread, while in the Netherlands, it is combined with cheese. In medical science, there have been reports of its antibacterial and antifungal activity of medicinal herbs [10, 18-19]. Because most studies have looked at the antimicrobial effect of *cumin* essential oil, this study looked at the antimicrobial effect of alcoholic extract of *cumin* seeds on *S. mutans* in vitro. The MIC and MBC concentrations of *cumin* extract on *S. mutans* in the current study were 0.62 and 1.25 mg/mL, respectively that is in accordance with study of Ghazi *et al* [20]. Soleimani *et al*. who investigated the effect of *cumin* on gram-positive and gram-negative bacteria (*E. coli*, *P. aeruginosa*, *S. aureus*, *S. flexneri*, *B. cereus*, *E. faecalis* and *S. typhimurium*) discovered that *cumin* has a stronger effect on *E. coli*. Additionally, *cumin* seems to boost gentamicin's antimicrobial properties. *Cumin* was mentioned as a possible treatment on drug-resistant bacteria in the Soleymani *et al* study [21]. Contrary to Soleymani's study we used micro-both dilutions; by removing the inhibitory effect of agar by the micro-broth dilution method, we were able to improve the antibacterial effect against *S. mutans* by decreasing the diameter of the growth inhibition zone. Golestannejad *et al*, who studied the effect of eucalyptus, *cumin* and savory essential oils on *S. mutans*, showed that all three essential oils had an antibacterial effect, but the best effect of savory essential oil was based on both MIC and diameter of growth inhibition zone [22]. Erturk *et al*. in their study demonstrated the antimicrobial effect of *cumin* against *E. coli*, *S. aureus*, *S. epidermidis*, *Bacillus subtilis*, *P. aeruginosa*, *Aspergillus niger* and *Candida albicans*. Similar to our study *cumin* showed the good effect on inhibition of *S. mutans* [23]. In the study of Vignesh *et al*. *cumin* with silver nanoparticles showed a good inhibitory effect against oral bacteria [24]. In the study of Motamedifar *et al*, the aqueous and alcoholic extract of *C. cyminum was* effective on methicillin resistant *S. aureus (MRSA) and methicillin susceptible S. aureus (MSSA)* with MIC of 75±35.4 mg/mL [25]. Moradi B *et al* showed antimicrobial effect of *C. cyminum* oil on *B. cereus* in a food model of barley soup with MIC of 300 ppm at 10 °C and with MIC of 450 ppm at 25° C respectively [26]. Although, in the recent study *B. cereus* and *S. aureus* were not evaluated but the results of Motamedifar *et al* and Moradi *et al* study showed, the alchoholic extract and essence oil of *cumin* was effective on *S. aureus* and *B. cereus* both members of gram positive bacteria in similar to *S. mutans* in the recent study respectively. This may have related to the structure of gram positive cell wall which may be affected by temperature such as Motamedifar et al study.

Based on our best knowledge, the anti-biofilm ability of ethanolic extract of *C. cyminum on* a gram positive was not evaluated except of Derakhshan study showed the anti- ofilm effect of cumin essential oil on *K. pneumonia* which is a gram negative bacteria [27,28]. So, in this study the effect of crude ethanolic extract of *C. cyminum* was evaluated on *S. mutans* with evaluation of cytotoxicity of this extract with MTT assay for the first time. One of the limitations of this study is that it is laboratory data and its results cannot be generalized to the clinic.

Finally, using of cumin extract in oral health products such as mouth wash, tooth paste or gum needs more studies on mutagenicity and other in vivo evaluation in the future before entering to commercial application.

CONCLUSION

The results showed that *cumin* ethanolic extract has an antibacterial effect against *S. mutans* and can also affect the biofilm formation of this bacterium. Considering that *cumin* ethanolic extract does not exhibit toxicity, it may be useful in treating bacterial infections in the mouth. Although, further studies are needed.

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Conflict of Interests

There is nothing to declare.

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