The effect of *Citrullus colocynthis* extracts on *Streptococcus mutans*, *Candida albicans*, normal gingival fibroblast and breast cancer cells

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Abstract

Tooth decay is one of the most common chronic diseases in humans. The aim of this study was to investigate the inhibitory effects of *Citrullus colocynthis* extracts on the growth of *Streptococcus mutans* and *Candida albicans* and their cytotoxic effects on normal gingival fibroblast cells and breast cancer cells. The aqueous and alcoholic extracts of *C. colocynthis* fruit were evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay, minimum inhibitory concentration (MICs) and minimal bactericidal concentration (MBCs)/minimal fungicidal concentration (MFCs) tests. The MICs and MBCs/MFCs were obtained from the aqueous extract (MIC 0.37 mg/mL and MBCs 1.5 mg/mL against *S. mutans* and MIC 0.37 mg/mL and MFCs 3.0 mg/mL against *C. albicans*) and ethanolic extract (MIC 0.75 mg/mL and MBCs 1.5 mg/mL against *S. mutans* and MIC 3.0 mg/mL and MFCs 12.0 mg/mL against *C. albicans*). The growth of *S. mutans* and *C. albicans* were effectively inhibited by extracts of *C. colocynthis*. The LC50 values of *C. colocynthis* on HGF1-PI cells were 4589.19 µg/mL and 3933.84 µg/mL by aqueous and ethanolic extracts, respectively. The LC50 values of *C. colocynthis* on MCF-7 cells were 4589.19 µg/mL and 3933.84 µg/mL by aqueous and ethanolic extracts, respectively. The extracts of *C. colocynthis* significantly decreased the growth of breast cancer and normal gingival fibroblast cells. The results of the study showed that the extracts may be used to treat oral mucosal diseases and prevent dental caries but future research is needed.

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Key words: Citrullus colocynthis; Tooth decay; Cytotoxic effects; Bacterial infections.

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Introduction

Tooth decay (dental caries) is one of the most common chronic diseases in humans.[1,2] Dental caries have a multifactorial etiology, the main causal factor being cariogenic microorganisms. In particular, *Streptococcus mutans* (*S. mutans*) is considered the principal responsible microorganism and is actively involved in the disease.[3] Some studies have shown that *Candida albicans* (*C. albicans*) cause dental caries as an opportunistic agent and increasingly contributing to the *S. mutans* exacerbate and accelerate tooth decay.[4,5] Bacterial causes of dental caries can also be translocation of other parts of the body and cause systemic diseases such as coronary artery disease. Therefore, these factors will contribute general health in addition to the effect on oral health.[6,7]

Traditional medicine, often based on the use of plants, has been an ancient root stone in the prevention and treatment of many infections and diseases from centuries ago to now. The effects of these plants that are caused by the metabolites present in them are shown in various *in vitro* and *in vivo* studies.[8-10] *Citrullus colocynthis* (*C. colocynthis*; other common name, *i.e.* Abujahl water melon, Bitter Apple) is a desert Viny plant that grows in sandy, arid soils. It is native to the Mediterranean Basin and Asia, and distributed among other part of the world.[11,12] Its fruit contains various bioactive compounds including alkaloids, saponins, flavonoids, carbohydrates, glycosides, fatty acids and essential oils.[13]

The plant contains cucurbitacins A, B, C and D, α-aelaterin and flavononids that have antioxidant activity.[14] The fruit of this plant was used in traditional medicine for various therapeutic (*i.e.* treatment of bacterial infections, jaundice, cancer) and pharmacological (*i.e.* antimicrobial, anti-inflammatory, anti-oxidant and immunostimulatory activity) purposes.[15-19] The aim of this study was to investigate the inhibitory effects of *C. colocynthis* extracts on the growth of *S. mutans* and *C. albicans* and their cytotoxic effects on normal gingival fibroblast cells and breast cancer cells *in vitro*.

Materials and Methods

Sampling

C. colocynthis plants were collected (autumn 2018) from Yazd province, Iran (3"22'54˚ E and 50"53'31˚ N). The plant species were approved by the experts at Sari Agricultural Sciences and Natural Resources University, Iran. The *C. colocynthis* were dried in a hot air oven (Behdad 3490, Sari, Iran) at 50ºC to a constant weight. All samples were then ground into a fine powder with a kitchen blender (Pars khazar, Iran). Dried powder of *C. colocynthis* was placed in polyethylene bags and transported to the laboratory for further investigation.

Aqueous extract approach

Two hundred grams of dried fruit pulp powder were added to 250 mL of distilled water for preparation of aqueous extracts. The mixture were filtered using Whatman no.1 filter paper under the vacuum of a water pump and lyophilized (Christ Alpha 1-2) for 24 hours.[20,21]

Alcoholic extract approach

The alcoholic (ethanolic) extract was prepared by soaking. Three hundred grams of dried fruit pulp powder were added to 200 mL of 70% ethanol and remained at room temperature for 5 days. The mixture was stirred twice a day to make its compounds enter the solvent, during 5 days. The mixture was filtered using Whatman no.1 filter paper. Finally, the solvent was extracted using a rotary machine (Laborota 4000, Heidolph, Germany) at a temperature of 35-40˚C and lyophilized (Christ Alpha 1-2) for 24 hours to obtain the desired extract in powder form. The extracts were exposed with UV light (Philips) for 45 minutes to ensure the absence of any contamination.[20,21]

Minimum inhibitory concentration, minimal bactericidal concentration and minimal fungicidal concentration tests

The aqueous and ethanolic extracts of *C. colocynthis* were inoculated by positive culture of *S. mutans* ATCC 35668 (Pasteur Institute, Tehran, Iran) and *C. albicans* ATCC 10231 (Pasteur Institute, Tehran, Iran) for antibacterial and antifungal investigation, respectively. The minimum inhibitory concentration (MIC) preventing visible bacterial or fungal growth were measured by the broth dilution method (microdilution using 96-well microplates), following the procedure of Berche *et al.*[22] All extracts stock solution were prepared by dissolution in 20% dimethyl sulfoxide (Sigma, St Louis, USA). The fruit extracts concentrations tested ranged from 0.02 to 24.00 mg/mL. The MIC of each extract were defined as the lowest concentration, which inhibited either bacterial or candidal growth, at 37˚C after incubation 24 h. The minimal bactericidal concentration (MBC) and the minimal fungicidal concentration (MFC) were determined by subculture on blood agar and sabouraud dextrose agar at 37°C for 24 h, respectively (Table 1).

MTT assay

The cytotoxic effects of aqueous and ethanolic extracts of *C. colocynthis* on HGF1-PI cells (normal gingival cells) ATCC-CRL-2014 (Pasteur Institute, Tehran, Iran) and MCF-7 (breast cancer cells) ATCC-HTB-22 (Pasteur Institute, Tehran, Iran) were measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay method (ISO 10993-5). Cells were incubated 24 h prior to adding into 96 plates. Cells cultured without extracts were used as a control. After 24 and 48 hours, the solution was replaced with 5 mg/mL MTT. After that, the MTT solution was removed and after 3 hours, 0.1 mL isopropanol was added to dissolve Formazan product. Their absorption was monitored at 570 nm on a micro-plate reader. The findings are presented as percentages (control value=100%). These tests were performed three times.

Results

The LC50 values of *C. colocynthis* on HGF1-PI cells were 4589.19 µg/mL and 3933.84 µg/mL by aqueous and ethanolic extracts, respectively. The LC50 values of *C. colocynthis* on MCF-7 cells were 4589.19 µg/mL and 3933.84 µg/mL by aqueous and ethanolic extracts, respectively.

In vitro cytotoxicity analysis

The cytotoxic potential of aqueous and ethanolic extracts of *C. colocynthis* were showed in Figures 1 and 2 after 24 hours incubation period on HGF1-PI cells (normal gingival cell line) and MCF-7 cells (breast adenocarcinoma cell line), respectively. The percentage of surviving cells decreased in comparison to the control group with increasing the concentration of aqueous and ethanolic extracts. Figure 1 shows that more than 50% of HGF1-PI cells were decreased in concentrations of 5000 µg/mL. Figure 2 shows that more than 50% of MCF-7 cells were decreased in concentrations of 500 µg/mL and 1000 µg/mL for ethanolic and aqueous extracts, respectively (Figures 1 and 2).

Table 1. Antibacterial and antifungal [MIC (mg/mL) and MBC or MFC (mg/mL)] of aqueous and ethanolic extracts of *Citrullus colocynthis* fruit pulp.

	Streptococcus mutans		*Candida albicans*	
	MIC	MBC	MIC	MFC
Aqueous extracts	0.37	1.5	0.37	3.0
Ethanolic extracts	0.75	1.5	3.0	12.0

MIC, minimum inhibitory concentration; MBC, minimal bactericidal concentration; MFC, minimal fungicidal concentration.

Discussion

Tooth decay due to *S. mutans* and *C. albicans* is one of the most common chronic diseases in humans.[1,2] Our hypothesis is that the use of *C. colocynthis* fruit as a traditional medicine, is involved in the prevention and treatment of tooth decay. In the present study, the antimicrobial effects of *C. colocynthis* fruit pulp on one of the most important bacteria and fungi in the oral environment showed that both types of aqueous and alcoholic extracts had positive effectives on *S. mutans* and *C. albicans*. Interestingly, the alcoholic extract inhibited bacterial growth in high concentrations, contrary to some of the hypotheses and according to Marzouk *et al.*[23] The MBC of *S. mutans* were observed in the same concentrations of 1.5 mg/mL for both aqueous and alcoholic extracts, indicating the proximity of the concentration of the effect of these extracts.

Marzouk *et al.*[23] evaluated antimicrobial and antifungal inhibitory effects of aqueous and acetone extracts of various parts of the Tunisian *C. colocynthis* plant including roots, stems, leaves and three maturation stages of its fruit and seeds. The maximum effect of the aqueous extracts from the plant was on *C. albicans* (MIC 0.10 mg/mL) and *Escherichia coli* (MIC 0.2 mg/mL). According to their study, the premature fruit of *C. colocynthis* showed the lowest MIC against different fungi and bacteria.[23]

Today, many studies have focused on the anti-diabetic effects and other therapeutic effects of *C. colocynthis* plant, but it is also necessary to use many tests to determine its side effects for use in clinical form. Therefore, the cytotoxicity of this extract should be investigated in clinical conditions. In the present study, the cancer cells in comparison with normal cells were exposed to the aqueous and alcoholic extracts of this fruit in the lowest concentrations. In both cell lines, the alcoholic extract was more toxic than the aqueous one. The toxic effects of this fruit have been shown in various studies on different animals.[24-26] Recently, another study showed the toxicity of *C. colocynthis* fruit pulp based on the evaluation of LC50 values and histopathology of various organs of mouse.[27]

Tannin-Spitz *et al.*[14] showed that cucurbitacin glycoside extracted from *C. colocynthis* plant leaves inhibits the growth of breast cancer cells in humans. The cucurbitacin glycoside is effective in treating breast cancer cells by apoptosis and stopping the cell cycle.[14] In another study, French researchers investigated the cytotoxic effects of cucurbitacin extracted from the *C. colocynthis* plant leaves on two types of colon cancer cell lines (HT-29 and Caco-2) and a normal epithelial cell line of rat intestine (IEC6). The results showed that, one of the existing cucurbitacin compounds had a marked and significant cytotoxic effects on cancer cell lines.[28]

The study of Haddad *et al.*[29] in 2017 was conducted to investigate the antimalarial effects of herbal extracts used in Iranian traditional medicine. In Haddad *et al.*[29] study, the cytotoxic effects of *C. colocynthis* showed that the fruit of this plant had a significant cytotoxicity on 3D7 and K1 cell lines, but there was no a significant toxicity on Raji Cells.[29] In this study we evaluate a normal and a cancer cell lines. The difference between the present study and the study of Haddad *et al.*[29] was about choosing cell type, type of extract and toxicity study. The present study showed that cancer cells are subjected to lowest concentrations than normal cells under the influence of aqueous alcoholic extract of this fruit, and alcoholic extracts have a slightly higher toxicity than alcoholic extract. Finally, the extracts may be used to treat oral mucosal diseases and prevent dental caries but future research is needed. Future research of present study can be completed by fragment analysis and testing wide verity of oral cells and microbial for clinical application.

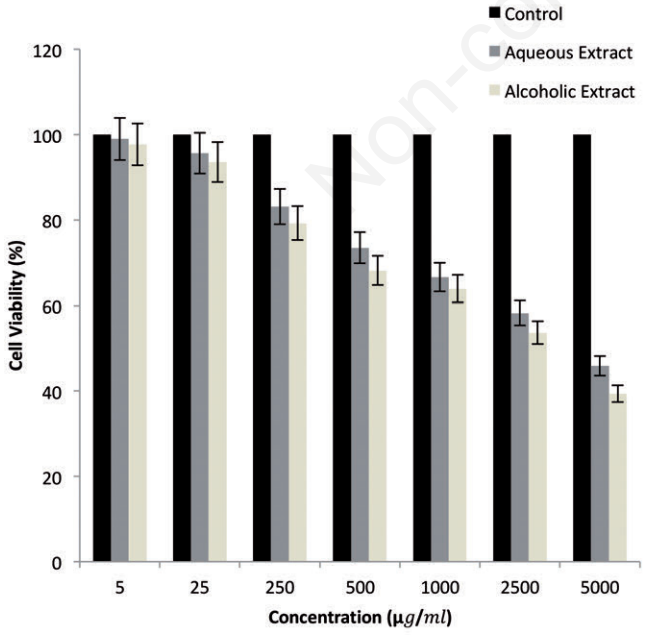

Figure 1. The percentage of survival of HGF1-PI cells exposed to aqueous and alcoholic extracts of *Citrullus colocynthis* fruit pulp during 24-hour incubation period.

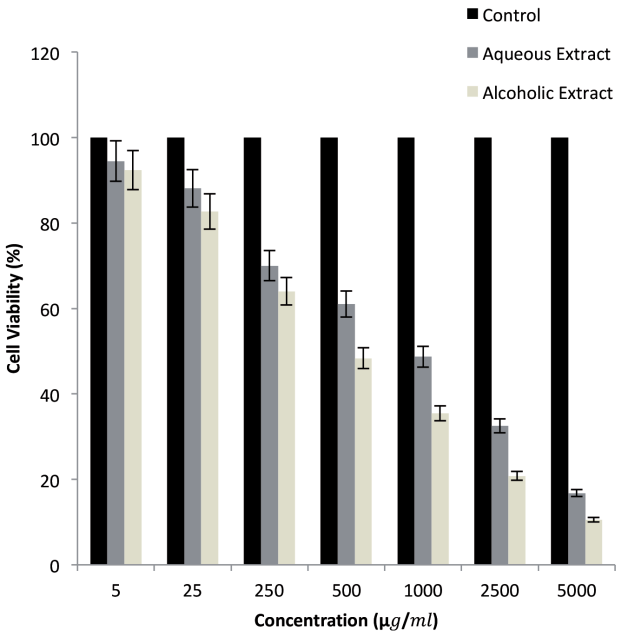

Figure 2. The percentage of survival of minimal fungicidal concentration-7 cells exposed to aqueous and alcoholic extracts of *Citrullus colocynthis* fruit pulp during 24-hour incubation period.

Conclusions

The growth of *S. mutans* and *C. albicans* were effectively inhibited by aqueous and alcoholic extracts of *C. colocynthis* fruit pulp. This property may be used to treat oral mucosal diseases and prevent dental caries. Also, the aqueous and alcoholic extracts of *C. colocynthis* in low and high concentrations caused the loss of breast cancer cells and normal gingival fibroblast cells, respectively. For this reason, it is possible to use these extracts to treat cancer, but because of their effects on normal cells, more and more precise studies are needed for future clinical use. Totally, the aqueous extracts had the lowest toxic and the most antibacterial and antifungal effects.

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