Correspondence: Mohsen Yazdanian, Research Center for Prevention of Oral and Dental Diseases, Baqiyatallah University of Medical Sciences, Tehran, Iran. Tel.: +989122163963. E-mail: drmyazdanian@yahoo.com

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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 causative 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.5,6

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. Abujahli 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 parts 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-sitosterol and flavonoids 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 \textit{C. colocynthis} extracts on the growth of \textit{S. mutans} and \textit{C. albicans} and their cytotoxic effects on normal gingival fibroblast cells and breast cancer cells \textit{in vitro}.

**Materials and Methods**

**Sampling**

\textit{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 \textit{C. colocynthis} were dried in a hot air oven (Behdad 3490, Sari, Iran) at 50°C to a constant weight. All samples were then ground into a fine powder with a kitchen blender (Pars khazar, Iran). Dried powder of \textit{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.20,21

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

The aqueous and ethanolic extracts of \textit{C. colocynthis} were inoculated by positive culture of \textit{S. mutans} ATCC 35668 (Pasteur Institute, Tehran, Iran) and \textit{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 \textit{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 \textit{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 1-(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 \textit{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 \textit{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 \textit{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 \textit{Citrus colocynthis} fruit pulp.**

|                      | \textit{Streptococcus mutans} | \textit{Candida albicans} |
|----------------------|--------------------------------|--------------------------|
|                      | MIC  | MBC  | MIC | MBC  |
| 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 effects 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 extract 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.

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![Figure 1](image1.png)

*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](image2.png)

*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.

References

1. Islam B, Khan SN, Khan AU. Dental caries: from infection to prevention. Med Sci Monit 2007;13:RA196-RA203.
2. Zero DT, Fontana M, Martinez-Mier EA, et al. The biology, prevention, diagnosis and treatment of dental caries: scientific advances in the United States. J Am Dental Assoc 2009;140:25S-34S.
3. Loesche WJ. The specific plaque hypothesis and the antimicrobial treatment of periodontal disease. Dent Update 1992;19:68-74.
4. Barbieri DiSAV, Vicente VA, Fraiz FC, et al. Analysis of the in vitro adherence of Streptococcus mutans and Candida albicans. Braz J Microbiol 2007;38:624-31.
5. Jarosz LM, Deng DM, van der Mei HC, et al. Streptococcus mutans competence-stimulating peptide inhibits Candida albicans hypha formation. Eukaryotic Cell 2009;8:1658-64.
6. Kuboniwa M, Tribble GD, Hendrickson EL, et al. Insights into the virulence of oral biofilms: discoveries from proteomics. Expert Rev Proteomics 2012;9:311-23.
7. Yazdianian M, Moradi K, Sobhani V, et al. Assessing the prevalence of musculoskeletal disorders and posture conditions during work time of dentists in a dentistry clinic affiliated to a military college in Tehran in 2017. J Mil Med 2018;20:222-30.
8. Sofowora A. Medicinal plants and traditional medicine in Africa. Paris, France: Karthala; 1996.
9. Lozoya M, Lozoya X. Pharmacological properties in vitro of various extracts of Mimosa pudica Linn. Tepescohuite Arch Invest Mex 1989;87:93.
10. Karthikeyan A, Shanthi V, Nagasathaya A. Preliminary phytochemical and antibacterial screening of crude extract of the leaf of Adhatoda vasica. L. Int J Green Pharm (IJGP) 2009:3.
11. Delazur A, Gibbons S, Kosari AR, et al. Flavone C-glycosides and cucurbitacin glycosides from Citrullus colocynthis. DARU J Pharm Sci 2006;14:109-14.
12. Lloyd JU, Cincinnati O. Citrullus colocynthis. Chicago: Engelhard; 1898.
13. Wasylikowa K, Van der Veen M. An archaeobotanical contribution to the history of watermelon, Citrullus lanatus (Thumb.) Matsum. & Nakai (syn. C. vulgaris Schrad.). Veget History Archaeobotany 2004;13:213-7.
14. Tamnin-Spitz T, Grossman S, Dovrat S, et al. Growth inhibitory activity of cucurbitacin glucosides isolated from Citrullus colocynthis on human breast cancer cells. Biochem Pharmacol 2007;73:56-67.
15. Kumar S, Kumar D, Saroha K, et al. Antioxidant and free radical scavenging potential of Citrullus colocynthis (L.) Schrad. methanolic fruit extract. Acta Pharm 2008;58:215-20.
16. Bendjeddou D, Lalaoi K, Satta D. Immunostimulating activity of the hot water-soluble polysaccharide extracts of Anacyclus pyrethrum, Alpinia galanga and Citrullus colocynthis. J Ethnopharmacol 2003;88:155-60.
17. Marzouk B, Marzouk Z, Haloui E, et al. Screening of algalsic and anti-inflammatory activities of Citrullus colocynthis from southern Tunisia. J Ethnopharmacol 2010;128:15-9.
18. Lakshmi B, Sendrayapenumal V, Subramanian S. Beneficial effects of Citrullus Colocynthis seeds extract studied in alloxan-induced diabetic rats. Int J Pharm Sci 2013;19:47-55.
19. Taherian A, Fazilati M, Moghadam AT, et al. Optimization of purification procedure for horse F(ab’2) antivenom against Androctonus crassicauda (Scorpion) venom. Trop J Pharm Res 2018;17:409-14.
20. Ebrahimzadeh MA, Nabavi SM, Nabavi SF, Eslami B. Antioxidant activity of the bulb and aerial parts of Ornithogalum sintensis L. (Liliaceae) at flowering stage. Trop J Pharm Res 2010;9:141-8.
21. Khojaste M, Yazdianian M, Tahmasebi E, et al. Cell toxicity and inhibitory effects of Cyperus rotundus extract on Streptococcus mutans, Aggregatibacter actinomycetemcomitans and Candida albicans. Eur J Transl Myol 2018;28:362-9.
22. Beree P, Gaillard J, Simonet M. Bactériologie-Bactéries des infections humaines [Bacteriology-Bacteria of human infections], Paris, France: Médecine Sciences Publications, Collection PCEM Flammarion; 1991.
23. Marzouk B, Marzouk Z, Décor R, et al. Antibacterial and anti-candidal screening of Tunisian Citrullus colocynthis Schrad. from Medine. J Ethnopharmacol 2009;125:344-9.
24. Stimpson H. Colocynthis. J Am Inst Homeopath 1926;19:501-10.
25. Elawad A, Abdel EB, Mahmoud O, et al. The effect of Citrullus colocynthis on sheep. Vet Human Toxicol 1984;26:481-5.
26. AL-Qarawi A, Adam S. Effect of combination of Capsicum frutescens and Citrullus colocynthis on growth, haematological and pathophysiological parameters of rats. Phytother Res 2003;17:92-5.
27. Shaiikh J, Shaiikh D, Rahman AB, et al. Antimicrobial and toxicological studies on fruit pulp of Citrullus colocynthis L. Pak J Pharm Sci 2016;29:9-15.
28. Chawech R, Jarraya R, Girardi C, et al. Cucurbitacins from the leaves of Citrullus colocynthis (L.) Schrad. Molecules 2015;20:18001-15.
29. Haddad MHF, Mahbodfar H, Zamani Z, et al. Antimicrobial evaluation of selected medicinal plant extracts used in Iranian traditional medicine. Iran J Basic Med Sci 2017;20:415-22.