

Citrullus colocynthis (bitter apple) extracts shows strong antibacterial effects against dental infection pathogens

Sayyad Ahmad Reza Amini¹, Behdokht Jamali¹, Katayoun Aliyari², Hadi Shakerin³, Roohollah Zarei koosha^{4*}

1. Department of microbiology, kherad institute of higher education, Bushehr, Iran

2 Dr. aliyaripathobiolab, borujerd, iran

3. Research Center for Prevention of Oral and Dental Diseases, Baqiyatallah University of Medical Sciences, Tehran, Iran

4. Applied microbiology research center, systems biology and poisonings institute. baqiyatallah university of medical sciences. Tehran, Iran

* Corresponding author: Roohollah Zarei Koosha, Email: rohollah.kosha@yahoo.com



Sayyad Ahmad Reza Amini

M.Sc. of Microbiology -
Pathogenic microorganisms

Article Type:
Research article

Article Info:

Received: 9 May . 2025

Revised: 17 May .2025

Accepted: 22 April .2025

ePublished: 25 April .2025

Key words: Extract, bitter apple, Flavonoid, dental infection.

Abstract

Background: One of the factors involved in tooth decay is pathogenic bacteria, which due to the development of antibiotic resistance in them, it is necessary to invent new treatment options. One of these therapeutic approaches is to investigate the potential of medicinal plant extracts with antibacterial properties against pathogenic bacteria that cause tooth decay.

Objectives: Therefore, in the present study, the antimicrobial effects of aqueous, ethanolic and methanolic extracts of *Citrullus colocynthis* (bitter apple) were studied on pathogens isolated from dental infections.

Methods: Aqueous, ethanolic and methanolic extracts of bitter apple fruit were prepared and their total phenol content, total flavonoid content and antioxidant capacity were studied. In the next step, two clinical isolates of *Streptococcus mutans* and *Bacillus subtilis* were exposed to different extracts of bitter apple fruit to check the antibacterial effects based on disc diffusion method. Broth microdilution method was used to determine MIC.

Results: Ethanol extract of bitter apple fruit showed the highest content of total phenol, total flavonoid and antioxidant capacity equivalent to ascorbic acid. All the extracts showed antibacterial effects on the isolates in the disc diffusion test. The MICs of aqueous, ethanolic and methanolic extracts of bitter apple fruit against the clinical isolate of *S. mutans* were estimated to be 4000, 1000 and 2000 $\mu\text{g/ml}$, respectively, and all these extracts showed an MIC equal to 2000 $\mu\text{g/ml}$ against *B. subtilis*. The MBCs of aqueous, ethanolic and methanolic extracts of bitter apple fruit were estimated to be 8000, 2000 and 4000 $\mu\text{g/ml}$ against the clinical isolate of *S. mutans* isolated from decayed teeth, and against *B. subtilis* MBC was measured to be 8000, 4000 and 8000 $\mu\text{g/ml}$ respectively.

Conclusions: Ethanolic extract of bitter apple has the potential to be used in combating bacteria caused dental infection.

1. Introduction

One of the most common dental diseases is dental caries, and cariogenic bacteria such as *Streptococcus mutans* are an important factor in the onset and progression of this disease (1). This pathogen, with its ability to synthesize sucrose

from water-insoluble glucan by Gtf enzyme and consequently, the accumulation of acid-producing bacteria such as *Lactobacillus acidophilus*, reduces the pH of the mouth, which in turn causes oral caries (2, 3). Treatment of caries at the beginning of the disease is of great importance, and the prescription of antibiotics is

of great importance, and the prescription of antibiotics is recommended by dentists.

Treatment of caries at the beginning of the disease is of great importance, and the prescription of antibiotics is recommended by dentists. However, these bacteria have become resistant to many antibiotics in recent years (4), and therefore the development of new therapeutic approaches is of great importance.

Prevention of dental decay by medicinal plants has attracted the attention of researchers in recent years, as they have fewer side effects and reduce the risk of developing drug resistance (5). Bitter apple, scientifically known as *Citrullus colocynthis*, is a member of the Cucurbitaceae family that grows mostly in arid regions of the world, especially in Iran, and has a wide range of pharmacological effects due to its bioactive compounds (6). For example, this plant is used in the treatment of diseases such as diabetes, asthma, jaundice, cancer, bronchitis, and joint pain (6). The most abundant bioactive component found in various parts of the plant, including the fruit, is quercetin (7). Moreover, this plant is rich in phenolic compounds, coumarins, and alkaloids (8), and its antimicrobial effects against various pathogens have been reported. For example, ethanolic extract of bitter apple showed antibacterial effects against *Bacillus subtilis* and *Staphylococcus aureus*, as well as *Klebsiella pneumoniae* (9). Interestingly, the ethyl acetate extract of this plant exerted antibacterial effects against a range of Gram-positive and Gram-negative bacteria and had an MIC of 0.0625 mg/mL against *Bacillus cereus* (10). Therefore, it seems that the fruit extract of the bitter apple plant has the potential for therapeutic use in combating a variety of bacterial infections leading to dental caries. Therefore, the present study was conducted to investigate the antimicrobial effects of aqueous, ethanolic, and methanolic extracts of the bitter apple on pathogens isolated from dental infections.

2. Materials and Methods

2-1 Preparation of Plant Materials

The fruits of Bitter apple (*C. colocynthis*) plant were obtained from the Research Institute of Forests and Rangelands in Iran. They were then dried in a room at 25°C under shaded conditions for one week and stored.

2-2 Preparation of aqueous, methanolic and ethanolic extracts

2.2.1. Preparation of aqueous extract

To prepare the aqueous extract, 20 g of bitter apple fruit powder was added to 150 ml of distilled water and gently heated for 15 minutes. Then, the mixture was filtered through Whatman filter paper and the solution was centrifuged at 5000g for 10 minutes and the supernatant was collected.

2.2.2. Preparation of ethanolic and methanolic extracts

To prepare the ethanolic extract, 20 g of dried fruit powder was extracted separately with 150 ml of ethanol or 80% methanol as the solvent for 8 hours using Soxhlet equipment. The extract was then filtered and allowed to evaporate in an oven at 45°C. The dried extract was dissolved in dimethyl sulfoxide (DMSO) and stored in the refrigerator for further use.

2-3 Total phenol content

The total phenol content was determined using the Folin–Ciocalteu method (11). A 125 µl aliquot of the extract was mixed with 375 µl of water and 2.5 ml of 10% Folin reagent. After six minutes, 2 ml of 7.5% sodium carbonate was added to the resulting mixture. The absorbance of the reaction mixture was measured after 90 minutes of storage in the absence of light at a wavelength of 765 nm using a spectrophotometer (Unico, USA). Finally, the total phenol content was calculated from the standard curve and expressed in terms of milligrams of gallic acid equivalent per gram of dry matter.

2-4 Total flavonoid content

A colorimetric method using $AlCl_3$ reagent was employed to estimate the total flavonoid content (TFC) (12). Two mL of aluminum trichloride (2% w/v) was mixed with 2 mL of the extract solution. After a 10-minute incubation at room temperature, the absorbance was read at 415 nm against a blank (a mixture of 2 mL of extract solution and 2 mL of ethanol). A quercetin calibration curve (10–100 µg/mL) was used to determine the TFC of each extract.

2-5 Antioxidant capacity of the extracts

The ability of the extracts to scavenge stable DPPH radicals was determined as described by the Blois method with minor modifications (Blois, 1958). For this purpose, 2 mL of DPPH ethanol solution (0.2 mM) was added to 0.1 mL of each extract solution at different concentrations. The reaction mixture was incubated for 30 minutes at 28°C in the dark. Absorbance was measured spectrophotometrically at 517 nm. The percentage of DPPH inhibition was estimated using the following formula:

$$\text{Effectiveness} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A₀ and A₁ are the absorbance of blank and extracts, respectively. Ascorbic acid was used as a positive control.

2-6 Preparation of dental caries pathogens

In the present study, two clinical isolates of *Streptococcus mutans* and *Bacillus subtilis* bacteria isolated from dental caries samples were used. The *S. mutans* isolate was resistant to penicillin, amoxicillin, gentamicin, imipenem, and doxycycline antibiotics, and the *B. subtilis* isolate was resistant to all of the aforementioned antibiotics except doxycycline.

2-7 The antibacterial activity of Extracts

The disk diffusion method was used to study the antibacterial effects of bitter apple fruit extracts. For this purpose, after culturing bacteria in broth agar medium and allowing colony formation, a colony was selected from the culture and placed on Mueller-Hinton agar. Then, 50 µL of concentrations (10, 500, 1000, 2000, and 4000 µg/mL) of extracts were applied to blank disks. After one day, these disks were placed on Mueller-Hinton agar at equal intervals. The plate was then incubated at 37°C. Ciprofloxacin was used as a positive control.

2-8 Minimum inhibitory and bactericidal concentrations

The determination of the minimum inhibitory concentration (MIC) of the fruit extracts was determined according to the standard microdilution method following the guidelines of the Clinical and Laboratory Standards Institute (13). A colony of the test strain was inoculated into 2 mL of sterilized autoclaved Luria-Bertani (LB) medium and

incubated at 37°C with shaking at 200 rpm overnight. The culture was then grown to the exponential growth phase, and the bacteria were diluted to 10⁸ CFU/mL in LB medium.

Next, the fruit extracts of bitter apple were added in serial concentrations (500 to 8000 µg/mL) in 96-well plates (127.71 mm x 85.43 mm). The 96-well plates were prepared by distributing 50 µL of Mueller-Hinton broth for bacteria into each well. 150 µL of the stock solution of the tested extracts (concentration 8000 mg/mL) was added to the first row of the plate. Twofold serial dilutions were then performed using a micropipette. The concentration range obtained was from 500 to 8000 mg/mL, and 10 µL of inoculum was added to each well, except the positive control. The plant extract alone was used as the positive control, and the inoculum alone was used as the negative control. The test plates were incubated at 37°C for 18 hours.

After 18 hours, 50 µL of 0.01% 2, 3, 5-triphenyltetrazolium chloride (TTC) solution was added to the wells, and the plate was incubated for another hour. Since the colorless tetrazolium salt is converted to a red product by biologically active bacteria, growth inhibition was detectable when the solution in the well remained clear after incubation with TTC. The MIC was defined as the lowest concentration of the sample without color change (clear), indicating complete growth inhibition.

Finally, 10 µL of a well that showed no bacterial growth and higher concentrations were taken and cultured on an agar-containing medium for one night. No bacterial growth at the lowest concentration was considered the minimum bactericidal concentration (MBC).

2-9 Statistical Analysis

GraphPad Prism version 8 software was used for data analysis and graphing. All data are presented as mean ± standard deviation. The ANOVA test was used for data analysis.

3. Result

3-1 Total phenols and flavonoids of extracts

The total phenol content of the ethanolic extract of bitter apple (34.34 ± 6.06 mg gallic acid/g dry weight of extract) was significantly higher than that of the aqueous extract (22.89 ± 7.94 mg gallic acid/g dry weight of extract; P=0.049).

However, there was no significant difference in the total phenol content of the ethanolic extract of bitter apple fruit compared with the methanolic extract ($P= 0.105$; 24.78 ± 6.14 mg gallic acid/g dry weight of extract, Figure 1a).

The results also indicated that the total flavonoid content of the ethanolic extract of bitter apple was significantly higher than both its aqueous ($P<0.001$) and methanolic ($P<0.0001$) extracts. However, no significant difference was observed between the aqueous and methanolic extracts in terms of total flavonoid content ($P=0.804$; Figure 1b).

3-2 Antioxidant activity

In this study, the DPPH method was used to determine the antioxidant potential of aqueous, ethanolic, and methanolic extracts, and the results are shown in Figure 2. The results indicated that there was a concentration-dependent antioxidant activity of extracts, as the concentration of the extracts increased, the free radical scavenging activity in-

creased. Also, comparing the antioxidant activity of the extracts showed that ethanolic extract of bitter apple fruit at low concentrations led to greater inhibition of DPPH free radicals and had a DPPH free radical scavenging potential equal to ascorbic acid (Figure 2).

3-3 Antibacterial activity

The antibacterial effects of aqueous, ethanolic and methanolic extracts of the bitter apple against clinical isolates of *S. mutans* and *B. subtilis* isolated from dental caries were studied by the disk diffusion method and the results are given in Table 2. As can be seen, all three extracts showed antibacterial effects against clinical isolates. However, the ethanolic extract of this plant at a concentration of $4000 \mu\text{g/ml}$ had the greatest effect on *S. mutans* (diameter of the zone of inhibition: 17 mm) and the methanolic extract at a concentration of $4000 \mu\text{g/ml}$ showed the greatest antibacterial effects on *B. subtilis*.

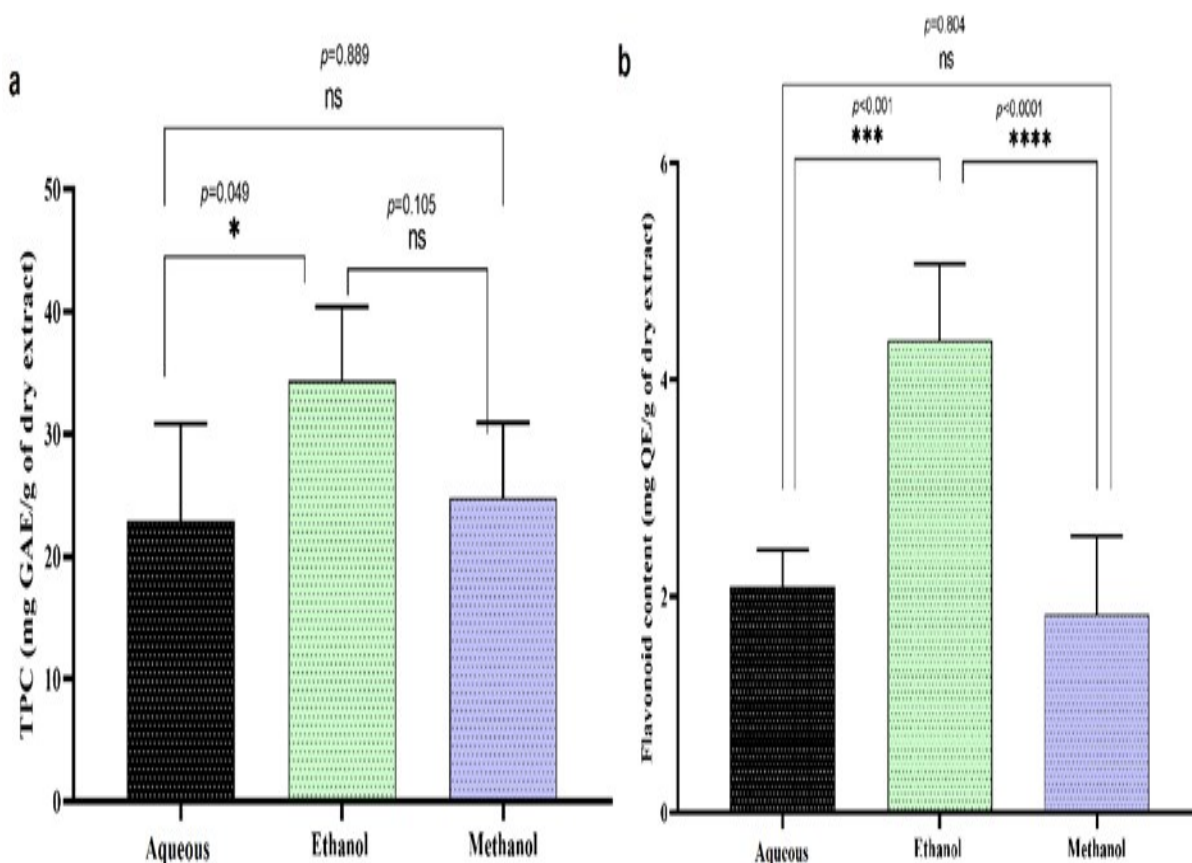
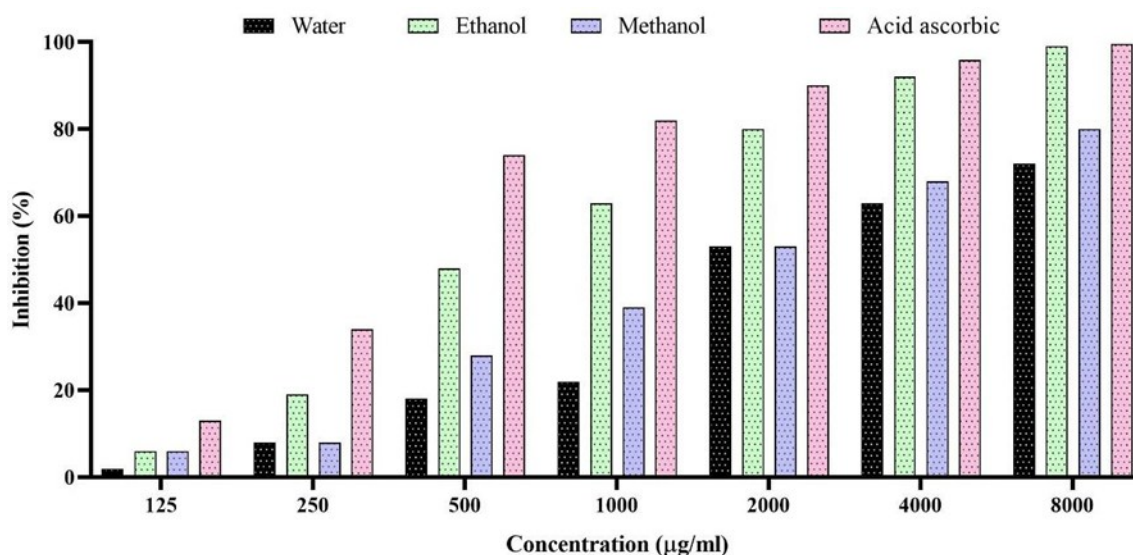


Figure 1: Total phenols (a) and flavonoids (b) contents of aqueous, ethanolic and methanolic extracts of bitter apple fruit (n=3).



Figure

2: The antioxidant activities of different concentrations of aqueous, ethanolic and methanolic extracts of bitter apple against DPPH free radical. Ascorbic acid was used as control.

Table 1: The diameters of the growth inhibition zone of clinical isolates of *S. mutans* and *B. subtilis* exposed with the aqueous, ethanolic and methanolic extracts of bitter apple fruit

Extract/concentration (µg/ml)	<i>Streptococcus mutans</i>	<i>Bacillus subtilis</i>
Aqueous		
10	0	0
500	0	0
1000	3	2
2000	7	8
4000	13	11
Ethanolic		
10	0	0
500	2	4
1000	6	5
2000	12	10
4000	17	14
Methanolic		
10	0	0
500	0	2
1000	4	6
2000	8	11
4000	13	15

3-4 MIC and MBC

The MICs of aqueous, ethanolic and methanolic extracts of the bitter apple against clinical isolates of *S. mutans* were estimated to be 4000, 1000 and 2000 µg/mL, respectively, and all these extracts showed an MIC of 2000 µg/mL against *B. subtilis* (Table 2).

The MBCs of aqueous, ethanolic and methanolic extracts of bitter apple against clinical isolates of *S. mutans* were estimated to be 8000, 2000 and 4000 µg/mL, respectively, and the MBC against *B. subtilis* was measured to be 8000, 4000 and 8000 µg/mL, respectively (Table 2).

4. Discussion

In this study, the antibacterial effects of aqueous, ethanolic, and methanolic extracts of bitter apple fruit were studied on two clinical isolates of *S. mutans* and *B. subtilis*, and the findings indicated that all three extracts had antibacterial effects against pathogens causing dental infections. However, *S. mutans* was more sensitive to the ethanolic extract and the methanolic extract affected strongly *B. subtilis* isolate. The antibacterial and bactericidal effects of bitter apple have been reported in other studies (14, 15), which are similar to the findings of the present study. It seems that the presence of flavonoids in bitter apple extract can explain the antibacterial effects observed in this study. In addition, flavonoids in bitter apple fruit extracts were also shown in other studies (16), and the findings of the present study also confirmed the presence of flavonoids in aqueous, ethanolic, and methanolic extracts of bitter apple. However, Chawech et al. (2015) reported that flavonoids were not detected in the ethanolic extract of bitter apple fruit (14), which is contrary to the findings of the present study. These differences can be attributed to the

different variety of bitter apple in the present study compared to the variety used in their study. Also, some chemical compounds belonging to the iridoids and alkaloids categories can be involved in the antibacterial activity of bitter apple fruit extracts. It is worth noting that in the present study, it was found that the type of bitter apple extract affects the antibacterial activity and it was found that the ethanolic extract can be more strongly associated with the inhibition of *S. mutans*. This finding is also similar to the findings of the study by Najafi et al. (2010) in which they showed that the ethanolic extract of bitter apple fruit has stronger inhibitory effects compared to the aqueous extract against *Staphylococcus aureus* (17). In another study, the acetone extract of bitter apple leaves inhibited the growth of gram-positive and gram-negative bacteria such as *S. marcescens*, *K. pneumoniae*, and *S. aureus*, as well as *Escherichia coli* (18). However, the MIC and MBC of the bitter apple fruit extracts measured in the present study against the two pathogens were lower than those in the above study, which can be attributed to the type of bacterial species.

In the present study, the ethanolic extract showed stronger antibacterial activity against clinical isolate of *S. mutans*, which could reflect the preferential solubility/extraction properties of the phytochemicals presented in ethanol versus aqueous/methanol solvents. However, the methanolic extract was more active against *B. subtilis* compared to the aqueous extract.

Table 2: The minimum inhibition and bactericidal concentrations of the aqueous, ethanolic and methanolic extracts of bitter apple fruit against clinical isolates of *S. mutans* and *B. subtilis*

Pathogens	Ethanolic	Aqueous	Methanolic
MIC			
<i>S. mutans</i>	1000	4000	2000
<i>B. subtilis</i>	2000	2000	2000
MBC			
<i>S. mutans</i>	2000	8000	4000
<i>B. subtilis</i>	4000	8000	8000

5. Conclusion

In general, it is concluded that the ethanolic extract of the bitter apple fruit has strong antibacterial effects against *S. mutans* and its methanolic extract has strong antibacterial effects against *B. subtilis*. Therefore, these extracts, especially the ethanolic extract, can be prescribed as a strategy for preventing dental infections. However, clinical studies are needed in this regard.

Reference

- Gao Z, Chen X, Wang C, Song J, Xu J, Liu X, et al. New strategies and mechanisms for targeting *Streptococcus mutans* biofilm formation to prevent dental caries: A review. *Microbiological Research*. 2024;278:127526. <https://doi.org/https://doi.org/10.1016/j.micres.2023.127526>.
- Dhruw C. Isolation of Dental Caries Bacteria from Dental Plaque and Effect of Tooth Pastes on Acidogenic Bacteria. *Open Journal of Medical Microbiology*. 2012;02:65-9. <https://doi.org/10.4236/ojmm.2012.23009>.
- Atta L, Mushtaq M, Siddiqui AR, Khalid A, Ul-Haq Z. Targeting glucosyltransferases to combat dental caries: Current perspectives and future prospects. *International Journal of Biological Macromolecules*. 2024;278:134645. <https://doi.org/https://doi.org/10.1016/j.ijbiomac.2024.134645>.
- Datta S, Soma N, and Roy DN. Biofilm-producing antibiotic-resistant bacteria in Indian patients: a comprehensive review. *Current Medical Research and Opinion*. 2024;40(3):403-22. <https://doi.org/10.1080/03007995.2024.2305241>.
- Tzimas K, Antoniadou M, Varzakas T, Voidarou C. Plant-derived compounds: A promising tool for dental caries prevention. *Current Issues in Molecular Biology*. 2024;46(6):5257-90.
- Rao V, Poonia A. *Citrullus colocynthis* (bitter apple): bioactive compounds, nutritional profile, nutraceutical properties and potential food applications: a review. *Food Production, Processing and Nutrition*. 2023;5(1):4. <https://doi.org/10.1186/s43014-022-00118-9>.
- Li Q-Y, Munawar M, Saeed M, Shen J-Q, Khan MS, Noreen S, et al. *Citrullus colocynthis* (L.) Schrad (Bitter Apple Fruit): Promising Traditional Uses, Pharmacological Effects, Aspects, and Potential Applications. *Frontiers in Pharmacology*. 2022;Volume 12 - 2021. English. <https://doi.org/10.3389/fphar.2021.791049>.
- Gupta SC, Tripathi T, Paswan SK, Agarwal AG, Rao CV, Sidhu OP. Phytochemical investigation, antioxidant and wound healing activities of *Citrullus colocynthis* (bitter apple). *Asian Pacific Journal of Tropical Biomedicine*. 2018;8(8):418-24.
- Hameed B, Ali Q, Hafeez M, Malik A. Antibacterial and antifungal activity of fruit, seed and root extracts of *Citrullus colocynthis* plant. *Biological and Clinical Sciences Research Journal*. 2020;33:1-5.
- Chawech R, Jarraya R, Girardi C, Vansteelandt M, Marti G, Nasri I, et al. Cucurbitacins from the Leaves of *Citrullus colocynthis* (L.) Schrad. *Molecules*. 2015;20(10):18001-15. [eng. \[PubMed ID:26437392\]. \[PubMed Central ID:PMC6332406\]. https://doi.org/10.3390/molecules201018001](https://doi.org/10.3390/molecules201018001).
- Singleton VL, Orthofer R, Lamuela-Raventós RM. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology*: Academic Press; 1999. p. 152-78.
- Chander PA, Sri HY, Sravanthi NBM, Susmitha UV. In vitro anthelmintic activity of *Barleria buxifolia* on Indian adult earthworms and estimation of total flavonoid content. *Asian Pacific Journal of Tropical Disease*. 2014;4:S233-S5. [https://doi.org/https://doi.org/10.1016/S2222-1808\(14\)60445-X](https://doi.org/https://doi.org/10.1016/S2222-1808(14)60445-X).
- Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*. 2008;3(2):163-75. <https://doi.org/10.1038/nprot.2007.521>.
- Chawech R, Mhalla D, Trigui M, Mihoubi M, Fabre N, Jarraya R. Chemical composition and antibacterial activity of extracts and compounds isolated from *Citrullus colocynthis* (L.) Schrad. *Journal of Pharmacognosy and Phytochemistry*. 2015;4(4):197.
- Bourhia M, Bouothmany K, Bakrim H, Hadrach S, Salamatullah AM, Alzahrani A, et al. Chemical profiling, antioxidant, antiproliferative, and antibacterial potentials of chemically characterized extract of *Citrullus colocynthis* L. seeds. *Separations*. 2021;8(8):114.
- Marzouk B, Marzouk Z, Décor R, Edziri H, Haloui E, Fenina N, et al. Antibacterial and anticandidal screening of Tunisian *Citrullus colocynthis* Schrad. from Medenine. *Journal of Ethnopharmacology*. 2009;125(2):344-9. <https://doi.org/https://doi.org/10.1016/j.jep.2009.04.025>.
- Najafi S, Sanadgol N, Nejad BS, Beiragi MA, Sanadgol E. Phytochemical screening and antibacterial activity of *Citrullus colocynthis* (Linn.) Schrad against *Staphylococcus aureus*. *Journal of Medicinal Plants Research*. 2010;4(22):2321-5.
- gowri SS, Priyavardhini S, Vasantha K, Umadevi M. Antibacterial activity on *Citrullus colocynthis* Leaf extract. *Ancient Science of Life*. 2009;29(1).