SINAI Journal of Applied Sciences 12 (3) 2023 423-432

Available online at www.sinjas.journals.ekb.eg

SCREENED BY SINAI Journal of Applied Sciences

iThenticate Professional Plagiarism Prevention

Print ISSN 2314-6079
Online ISSN 2682-3527

ARU-EGYPT SJAS Trust Science | It Creates

ANTIOXIDANT, ANTIDIABETIC, ANTIBACTERIAL, AND ANTICANCER ACTIVITIES OF EGYPTIAN FENUGREEK

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ARTICLE INFO

Article history:
Received: 13/03/2023
Revised: 14/04/2023
Accepted: 26/05/2023

Keywords:
Fenugreek,
antioxidant,
antidiabetic,
antibacterial, anticancer.

ABSTRACT

Fenugreek (FG) flour is a common plant used in Egypt to add flavour and colour to food, keep food fresh, and as a medicine. The current study tested FG for its antioxidant, antidiabetic, antimicrobial and anticancer activities. FG showed significant contents of phenolic and flavonoid compounds, reflecting their nutraceutical behaviors. The EC_{50} of FG when tested for its DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging was 2476 ± 62.9 µg/mL, while the FG extract's concentration that inhabited ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) was 37.13 ± 1.24 µg/mL. FG could lower blood sugar, as it showed an alpha-glucosidase inhibiton at EC_{50} > 1000 µg/mL. Almost 99% of breast cancer cells were damaged by FG extract concentrations > 100 µg/mL. FG extract was successful in antimicrobial activity against *Escherichia coli* ATCC 8739 at a 1000 µg/mL concentration. The current study showed the promising usage of FG as a functional additive to different foods.

INTRODUCTION

In Egypt, adding a small amount of fenugreek (FG) (*Trigonella foenum-graecum*) flour to wheat flour makes bread healthier and tastes better **(Wani and Kumar, 2018**). It is common to mix FG flour with other types of flour when making bread **(Ahmed, 2015**). According to World Health Organization, **Zhang (2002**) demonstrated that around 70% of the world's population uses nutraceutical plants in some way for primary health care. Different parts of the plant showed pharmacological effects, such as antimicrobial, anticancer, analgesic, antioxidant and many other effects **(Al-Snafi, 2015**).

Different herbs and spices have been used by many cultures and acknowledged for thousands of years. Some of those plants have been shown to have the antimicrobial properties of spices. In many parts of the world, they could be used to make medicine, cosmetics, perfume, and liquorice **(Al-Snafi, 2016**). It has been said that FG is a common plant used in very small amounts to add flavour and colour to food, to keep food fresh, and as a medicine **(Sachan *et al.*, 2018**). In Egypt, FG was a festival drink at official Muslim events when it was still a green seedling plant. It was also used as an ingredient in a local bread called "Batawy," particularly in the middle of the country **(Mehdawy and Hussein, 2010**). Some antioxidants in FG are 3,4,5-tri hydroxybenzoic acid, dihydroxybenzoic acids, catechin, polyphenol and ester **(Muslim, 2023**). EC_{50} for FG seed extracted by methanol was found to be 350 µg/mL and 117 µg/mL in DPPH and ABTS radical tests, respectively **(Kaviarasan *et al.*, 2007**). In 2019, 9.3 percent of adults around the world had diabetes, which is a metabolic disease. It is

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https://doi.org/10.21608/sinjas.2023.199738.1195

thought that its existence with COVID-19 will cause more deaths. Diabetes is mostly treated with drugs that must be treated for ages. These medications are often costly and have undesirable side effects **(Dietrich *et al.*, 2023**). So, FG is a plant worldwide that fights diabetes **(Przeor *et al.*, 2020; Elsaadany *et al.*, 2022; Madhu *et al.*, 2023**). Previous research has examined how FG leaves lower blood sugar or fights diabetes. This study aims to determine if the local Egyptian FG plant has antibacterial, anticancer, and antioxidant properties that could be used to make functional foods.

MATERIALS AND METHODS

Materials Description and Preparation

The Agricultural Research Center (ARC) at Giza, Egypt, provided local Egyptian fenugreek (*Trigonella foenum-graecum* L.; cultivar Giza-30; Arabic name: Hilba). Fenugreek seeds were ground in-house using a grain mill (Kenwood Chef XL Stand Mixer, 1200 - UK) to pass a 250 μm sieve. FG flour sample extract was prepared by mixing 375 g with 1.5 L ethanol and was allowed to macerate for one day before filtration. This process was repeated twice. Then the residues were dried under vacuum conditions at 45°C producing (green) solid extract weighing 44.68 g. This extract was used to characterize FG flour by measuring total phenolic and flavonoid compounds, antioxidant activity, antimicrobial activity and anticancer activity of FG. The extract was dissolved in water for future analysis.

Antioxidant Assay

Total phenolic components in FG extract samples were measured using Attard's technique **(Attard, 2013).** Antioxidant activity plays a crucial role in protecting the body from oxidative stress and related diseases. In this study, we will use two common antioxidant assays, namely the DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assays, to evaluate the antioxidant activity of plant extract**.** An aliquot of 80 μL of 1.0 M Na_2CO_3 was used and kept in the dark (25°C) for 20 min. A complex blue colour was developed, and its intensity was recorded at a wavelength of 630 nm. The total flavonoid content of FG extract samples was determined using $AlCl_3$ method modified by **Kiranmai *et al.* (2011).** A 15 μL of each sample was arranged in a 96-well microplate (FluoStar Omega reader). Methanol and 1.25 percent $AlCl_3$ were added, then, a proportion of 30 μL of $C_2H_3NaO_2$ with a concentration of 0.125 M was added and left for 5 min. the developed golden colour was measured at a wavelength of 420 nm.

The extracts were tested for their antioxidant activity by scavenging the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical **(Faso, 2016**) and ABTS according to the method of **Arnao *et al.* (2001**). FG different concentrations were used at 500, 1000, 1500, 3000, and 4000 μg/ml. A methanolic solution of DPPH (100 μgL in 0.1% methanol) was swiftly combined with the FG seed extract (100 μL) in a 96-well plate. Samples were swirled and incubated for 30 min in dark. FluoStar Omega microplate reader was used to measure the absorbance of the mixture at a wavelength of 540 nm. ABTS was dispersed in double distilled water (192 mg), relocated to a volumetric flask with 50 mL capacity, and then filled with distilled water. An aliquot of one mL of the SBTS working solution was added to 17 μL of 140 mM $K_2S_2O_8$ and incubated in the dark for 24 hours. An aliquot of 190 μL of freshly produced ABTS reagent and 10 μL of the sample were combined in a 96-well plate (n=6) and incubated at room temperature for 30 minutes. Using microplate reader FluoStar Omega, ABTS obtical density was measured at 734 nm. ABTS/DPPH inhibition percent = $[(A_c - As)]/ (Ac) \times 100$, where Ac is ABTS/DPPH + methanol absorbance, and As is ABTS/DPPH radical + sample absorbance (sample or standard).

The EC_{50} value (µg/mL), the effective concentration at which 50% of DPPH or ABTS radicals are scavenged, was calculated as described by **Chen *et al.* (2013**).

Antibacterial Activity

A disc of each bacteria, *Escherichia coli* ATCC 8739 and *Salmonella typhimurium* ATCC 14028 was inoculated into 100 ml of tryptic soy broth medium and incubated at 37.0°C±1.0 for 24.0 hr. A loopful of broth was spread onto an appropriate non-selective medium (Tryptic soy agar) and incubated at a similar temperature for a new (18–24 hr) culture agar plate. The suspension was adjusted to 0.5 McFarland strain standard using DensiCHEK optical instrument to inoculate 3-4 colonies into a sterile saline solution. That modification yields 1-2 X 10^8 CFU/mL suspension. Agar well diffusion modified from the method described by **Al-Timimi (2019**) was used to evaluate the antibacterial activities of FG extract against the two selected pathogenic bacteria. Briefly, A sterile brush was streaked three times over the agar surface and turned 60 degrees each time to evenly distribute the prepared inoculum. The agar was punched to a diameter of about 10 mm to make a well for each sample concentration and control, and then 100 µl of the FG extract (125, 250, 500, and 1000 µg/ml) was poured into the well. All dishes were incubated for 24.0± 2.0 hr at 35.0°C±1.0°C. After the incubation period, the inhibition zone was measured and calculated: X=a-b, where "a" is the inhibition zone diameter, and "b" is the well's diameter (10 mm).

Anticancer Activity

MCF-7 Breast Adenocarcinoma cell line was purchased from a private, scientific laboratory (NS Inc, El-Mokatam, Cairo, Egypt). Cells were preserved in Dulbecco's Modified Eagle media with 100 mg of streptomycin per mL, penicillin (100 units/ mL) and humidified (5% *V/V* CO_2 at 37°C) of heat-inactivated fetal bovine serum (10%). Cytotoxicity assay and EC_{50} were conducted as described by **Skehan *et al.* (1990**) and **Allam *et al.* (2018**). Cell viability was evaluated by Sulforhodamine B (SRB) test. Cells were exposed to 100 µL media containing FG extract at different concentrations (0.0, 0.01, 0.1, 1, 10, 100 and 1000 µg/ml). Cells were fixed with 150 µL of 10% trichloroacetic acid at 4 °C for one hr, followed by 5 distilled water rinses and 70 µL SRB solution. Protein-bound SRB stain was dissolved with TRIS ((tris (hydroxymethyl) aminomethane)) and recorded at a wavelength of 540 nm using BMG LABTECH®-FLUOstar.

In vitro Antidiabetic Activity

The α-glucosidase inhibition activity was determined using the method described by **Gutiérrez-Grijalva *et al.* (2019) and Abdallah *et al.* (2022)**. The enzyme α-glucosidase was purchased from Sigma-Aldrich from *Saccharomyces cerevisiae*. CAT number: G5003. The substrate para nitrophenyl β-D-glucopyranoside was purchased from Sigma-Aldrich CAT number: N7006. 25 µL of samples/blank incubated with α-glucosidase and 3 mM pNPG for 5 min at 37 °C. Sample solutions were prepared by the final concentration of 2 mg/mL in DMSO, then, by dilution, five concentrations: 0.1, 1.0, 10, 100, and 1000 µg/ml were prepared and used. Enzyme activity was determined by measuring the release of p-nitrophenol from the pNPG substrate at 405 nm using a microplate reader (microplate reader FluoStar Omega, USA). The percentage of inhibition of α-glucosidase was computed as follows:

% inhibition= $[(A_b - A_S) / A_b] \times 100$, where A_b is the absorbance of the control (blank, without inhibitor), and A_S is the absorbance with the inhibitor.

RESULTS AND DISCUSSION

Total Phenolic and Flavonoids Compounds

The Folin-Ciocalteau technique using gallic acid as a standard, evaluated the FG extract's total phenolic content. FG contained total phenols of 13.4 ± 1.26 µg GA E/mg (Table 1). In a study undertaken by **Al-Dabbagh *et al.* (2018**), they reported that the total phenolic compound in FG was 9.7 ± 0.008 µg GA E/mg extract. The flavonoid contents of FG are listed in Table 1. The total amount of flavonoid in the FG extract was 15.2 g R E/mg. The current result was in harmony with **Al-Dabbagh *et al.* (2018**), who reported that the total flavonoid compound in FG was 14.6 ± 0.21 mg Q E/g extract. Flavonoids in herbs contribute significantly to their antioxidant properties **(Muflihah *et al.*, 2021**).

Ability to Scavenge DPPH and ABTS Radicals of FG Extract

The tested sample was evaluated for its antioxidant activity by measuring its capacity to neutralize the stable DPPH radical, as described by **Faso (2016**). The scavenging activity of the extracts against DPPH radicals was measured as EC_{50}. The most effective scavenging of free radicals is associated with the lowest EC_{50} values. FG, which had a high total phenol concentration (13.4 µg/mL, Table 1), was the active scavenger at EC_{50}level of 2476 ± 62.9 µg/ mL. These results are almost in agreement with their total polyphenol contents. Thus, phenolic component content may be linked to these plants' antioxidant activity, as the published research suggested. **Al-Dabbagh *et al.* (2018**) investigated the relationship between total phenol concentration and anti-free radicals using EC_{50}. They found that total phenol concentration and anti-free radical activity (EC50) are associated with the herbs' high antioxidant capacity levels, as previously thought to be linked to the lowering antioxidant ability of various herb extracts. This aligns with the findings reported by **La Mantia *et al.* (2023**). DPPH scavenging was absent in several ABTS-scavenging compounds. This research showed otherwise. The extracts' components cannot scavenge free radicals by the measuring technique utilized in the present investigation, according to the ABTS scavenging results in terms of EC_{50}. However, the concentration of inhabits ABTS was 37.13 ± 1.24 µg/ml (Table 1). It is widely believed that free radicals, which are a part of the process of lipid peroxidation, play a significant part in the development of a wide variety of chronic pathologies, cancer and cardiovascular illnesses **(Dorman *et al.*, 2003; Roby *et al.*, 2013**).

In vitro Antidiabetic Activity of Fenugreek

An alpha-glucosidase inhibitory test was done to determine if fenugreeks can lower blood sugar. As shown in Table 1, the alcohol extract of fenugreek exhibited an EC_{50} value of more than 1000 µg/mL. Studies have shown that fenugreek seeds contain antidiabetic proteins, amino acids, alkaloids, coumarins, saponins, flavonoids, phenolic compounds, and polysaccharides **(Fuller and Stephens, 2015**). Because of this, fenugreek's bioactive compounds need to be improved to be used more effectively in pharmaceutical or nutraceutical applications.

Anticancer Activity of Fenugreek Extract

When treating cancer, natural drugs, particularly those derived from plants, are frequently more well tolerated than synthetic analogues **(Newman and Cragg, 2016**). These plants, which contain beneficial secondary metabolites, are used in integrative cancer prevention and therapy **(Block *et al.*, 2015**).

Table 1. Total phenolic and flavonoid compounds, EC_{50} for DPPH and ABTS assays, and inhibition effect on α-glucosidase enzyme as affected by FG extracts

Analyses	Value	SD
Total phenolic compound µg GA E/mg	13.4	1.26
Total flavonoids µg R E/mg	15.23	1.17
Free radical scavenging activity (DPPH Assay) EC_{50} µg/ml	2476	62.9
Free radical-scavenging activity (ABTS) EC_{50} µg/ml*	37.13	1.24
Inhibition of α-glucosidase µg /ml	> 1000	nd

*EC_{50} did not detectable, and the value represents the concentration that inhabits ABTS

FG extract was evaluated on MCF-7 breast cancer cells. FG extract reduced cell viability (almost 99% reduction; Fig. 1a). Extract EC_{50} was 27.27 µg/mL (Fig. 1b). FG extract at 100 µg/mL caused a total elimintation of the cancer cell. Fenugreek extract and its active components are being studied as supplements in dietary preventive/therapeutic measures to improve health **(Li *et al.*, 2010**). A linear regression study undertaken by **Al-Dabbagh *et al.* (2018**) has investigated FG extracts' anticancer (cell viability) capabilities. The authors stated that FG extract had a favourable connection (R^2 = 0.797, P-value < 0.05). The cytotoxicity experiment showed that ethanol fenugreek extract inhibited the MCF-7 cell line by more than 99 percent at concentrations > 100 µg/mL (Fig. 1). An image of a cancer cell line (Fig. 1 c, d, e and f) nearly completely incubated with FG extract for 72 hr., indicated almost 0% cell viability at FG extract concentration > 100 µg/ml. Fenugreek's unique action on transformed and untransformed cells explains these antithetic outcomes. Our findings suggest that FG may reduce breast cancer, in accordance with results obtained by **Al-Timimi (2019**). Including FG in food, additives may offer a strategy for treating multiple forms of cancer. Based on these findings, it is recommended that FG be added to foods like bread to utilize its health benefits.

Antibacterial Activity of Fenugreek Extract

New infectious illnesses and overuse of current antibiotics necessitate the development of newer antibacterial drugs. Thus, plant chemical extraction is making interesting development. Plants produce many biologically active chemical substances. Medicinal plant antibiotics have eradicated numerous bacterial illnesses. These plants can be developed to target drug-resistant bacterial infections, unlike pharmaceutical antibiotics **(Amenu, 2014**). Fenugreek seed extract's inhibition zone (IZ) against two pathogenic bacteria was investigated. Figure 2 shows IZ findings. Ethanol extract significantly impacted the inhibiton zone area of *Escherichia coli* ATCC 8739 but not *Salmonella typhimurium* ATCC 14028 at varied concentrations of FG extracts. Only the IZ with the greatest FG extract content (Figure 2 at 1000 mg/L; IZ: 1mm) was shown. These findings are noteworthy since a modest quantity of fenugreek seed extract inhibited bacteria growth. Developing concentration and extraction procedures might make it worthwhile.

FG extract showed no IZ values at other concentrations. Literature yielded conflicting findings. FG seed ethanol extract was more active than aqueous extract against most bacteria except *E. coli*. **Alwan *et al.* (2017**) are in harmony with this statement. However, they disagree, whereas **Al-Abdeen *et al.* (2010**) and **Sharma *et al.* (2016**) found no impact on bacterial species from ethanol or aqueous extraction.

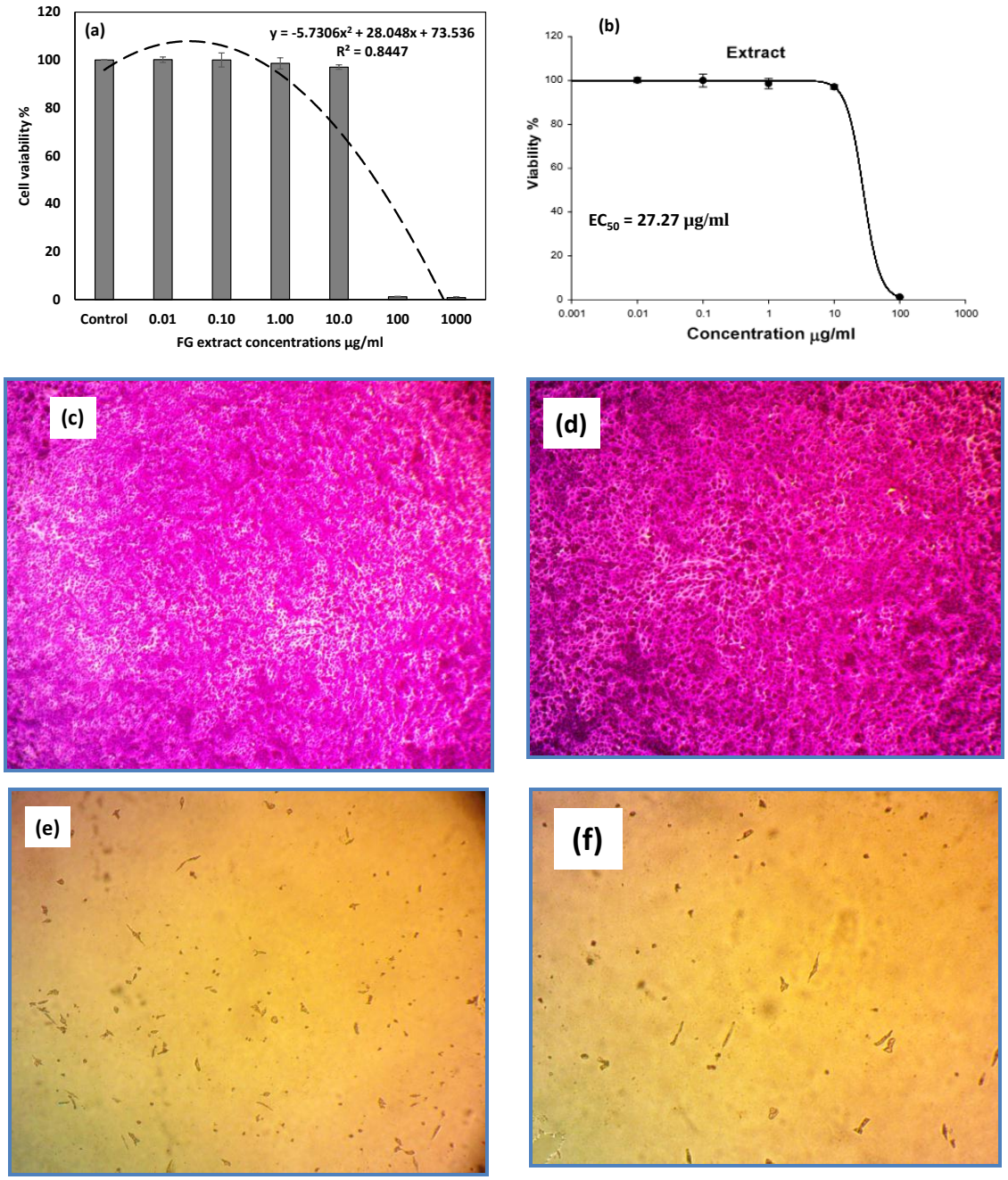

Fig. 1. Assessment of the cytotoxic effects of different concentrations of FG extracts on MCF-7 viability (%) after 72 hr (a). Determination of EC_{50} of FG on breast cancer cell line (b). Assessment of morphological changes of the cell at a concentration of 0.0 µg/ml (control) at 40x magnification (c) and 1000x magnification (d) and at 1000 µg/ml (highest concentration) at 40x magnification (e) and 1000x magnification (f).

Fig. 2. Inhibition zone area on pathogenic bacteria of *Escherichia coli* ATCC 8739 and *Salmonella typhimurium* ATCC 14028 as affected by different concentrations of FG extracts

Conclusion

This study demonstrated that fenugreek (FG) is a rich source of phenolic and flavonoid compounds, indicating its potential as a nutraceutical agent. The FG extract exhibited strong antioxidant activity as evidenced by its ability to scavenge free radicals in DPPH and ABTS assays. Furthermore, FG showed antidiabetic effects by inhibiting alpha-glucosidase, and exhibited significant anticancer activity against breast cancer cells. Additionally, FG extract exhibited antimicrobial activity against *Escherichia coli*. These findings suggest that FG could be utilized as a functional additive to enhance the nutritional and health benefits of various food products.

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الملخص العربي

الأنشطة المضادة للأكسدة، لمرض السكرى، للبكتيريا وللسرطان في الحلبة المصرية

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دقيق الحلبة (FG) هو نبات شائع يستخدم في مصر لإضافة نكهة ولون إلى الطعام، والحفاظ على الطعام طازجا، وكدواء. في الدراسة الحالية، تم اختبار الحلبة للأنشطة المضادة للأكسدة والمضادة لمرض السكرى ومضادات الميكروبات والمضادة للسرطان. أظهرت الحلبة محتويات كبيرة من مركبات الفينول والفلافونويد، مما يعكس سلوكياتها العلاجية. أظهر قيم السمية لتثبيط الشوادر الحرة من نوع DPPH قيم 2476 ± 62.9 ميكروجرام / مل بينما كان التركيز الذي يمثله النوع ABTS قيم 37.13 ± 1.24 ميكروجرام / مل. يمكن لمستخلص الحلبة أن يخفض نسبة السكر في الدم، حيث أظهر مثبط انزيم الالفا كلوكسيديز قيمة أكثر من 1000 ميكروغرام / مل. تم قتل ما يقرب من 99٪ من الخلايا السرطانية للثدى (معمليا) بسبب مستخلص الحلبة بتركيزات أعلى من 100 ميكروغرام / مل. كان مستخلص الحلبة فعالا كنشاط مضاد للميكروبات ضد بكتريا القولونية بتركيز 1000 ميكروغرام / مل. أظهرت الدراسة الحالية الاستخدام الواعد لنبات الحلبة كإضافات وظيفية للأطعمة المختلفة.

الكلمات الإسترشادية: الحلبة، مضادات الأكسدة، مضادات السكرى، مضاد للبكتيريا، مضاد للسرطان.

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