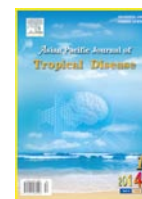




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## Phytochemical screening and comparative antimicrobial potential of different extracts of *Stevia rebaudiana* Bertoni leaves

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### PEER REVIEW

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#### Comments

This is an interesting study in which authors evaluated the phytochemical screening and antimicrobial activity of *S. rebaudiana* Bertoni leaves extracts using various solvents. The paper revealed that the plant has some bioactive compounds which are useful to prepare herbal medicine.

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### ABSTRACT

**Objective:** To evaluate *in vitro* antimicrobial potential and phytochemical screening of the crude extracts of leaves of *Stevia rebaudiana* Bertoni.

**Methods:** The essential oil and crude extracts were prepared by using different usual method. Antimicrobial and antifungal activities were measured by the well established methods.

**Results:** Highest antifungal index [(12.13±0.08) mm] and lowest antifungal index [(9.13±0.04) mm] as well as highest antibacterial index [(11.89±0.07) mm] and lowest antibacterial index [(7.24±0.03) mm] were obtained for extracts B, H, A and F, respectively. Invariably extract C, E, I, J and H did not show antimicrobial activity. The extract F showed all antifungal and antibacterial activity except *Bacillus cereus* and *Bacillus megaterium*.

**Conclusions:** The above findings support the idea that plant extracts of *Stevia rebaudiana* Bertoni leaves may have a role to be used as pharmaceuticals or preservatives.

### KEYWORDS

*Stevia rebaudiana* Bertoni, Antimicrobial property, Plant extracts, Antibacterial index, Antifungal index

## 1. Introduction

The screening of plant extract has been of great interest to scientists in the search for new drugs for greater effective treatment of several diseases[1]. *Stevia rebaudiana* (*S. rebaudiana*), a natural alternative to artificial sweetener is found to contain over 100 phytochemicals including well characterized stevioside and rebaudioside A[2]. Besides, it is well known for its application in treatment of many diseases like diabetes, high blood pressure and weight loss in various traditional systems of medicine. Plant extracts and

phytochemicals with known antimicrobial properties can be of great significance in therapeutic treatments[3-5]. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolic compounds[6]. Many plant leaves have antimicrobial principles such as tannins, essential oils and other aromatic compounds[7,8]. In addition, many biological activities and antibacterial effects have been reported for plant tannins and flavanoids[9-11].

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Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives<sup>[12]</sup>. These compounds protect the plant from microbial infection and deterioration<sup>[13]</sup>. Some of these phytochemicals can significantly reduce the risk of cancer due to polyphenol antioxidant and anti-inflammatory effects. Some preclinical studies suggested that phytochemicals could prevent colorectal cancer and other cancers<sup>[14–16]</sup>. One of the potent members of the Asteraceae family is *S. rebaudiana* (commonly referred to as honey leaf, candy leaf or sweet leaf). It is rich in terpenes and flavanoids. The phytochemicals present in *S. rebaudiana* are austroinullin, vasodilator cardiogenic, anesthetic and anti-inflammatory. The plant used in the present study was *S. rebaudiana* Bertoni (Asteraceae), which is used traditionally for the source of natural sweetener. The dry extract from the leaves of *S. rebaudiana* Bertoni contains sweet diterpene glycosides, flavonoids, alkaloids, water-soluble chlorophylls and xanthophylls hydroxycinnamic acids (caffeic, chlorogenic, etc.), neutral water-soluble oligosaccharides, free sugars, amino acids, lipids, essential oils and trace elements<sup>[17]</sup>. To the best of our knowledge, none has investigated the potential antimicrobial effect of *S. rebaudiana* Bertoni leaves in Bangladesh till now.

The present study was carried out to evaluate the phytochemical screening and the antimicrobial activity of *S. rebaudiana* Bertoni leaves extracted using various solvents.

## 2. Materials and methods

### 2.1. Plant material

Fresh leaves were collected from the matured plantlets grown in Bangladesh Sugarcane Research Institute (basic soil, pH 7.7), Ishurdi, Pabna, Bangladesh, and packed in polythene bags and stored at  $-4^{\circ}\text{C}$  until its use. *S. rebaudiana* Bertoni (popularly called as stevia and synonymously known as 'sugar substitute') leaves were washed, dried in an oven at  $40^{\circ}\text{C}$  for 3 d, ground into powder.

### 2.2. Preparation of plant crude extract

A total of 100 g weighed powder was taken into a Clevenger type apparatus and heated with water for 48 h, then filtered and the filtrate was partitioned with different solvents like *n*-hexane, dichloromethane, acetone and ethyl alcohol successively. Eight milligrams of each partition was collected into the vials labeled A, B, C, D and E.

A total of 250 g weighed powder was taken for extraction in the Soxhlet apparatus using MeOH as solvent. After completion the extraction MeOH was evaporated by a rotary

evaporator under reduced pressure. The dried extracts were further portioned by using the solvents *n*-hexane, dichloromethane, and ethyl acetate successively. A weight of 8.0 mg of each portion were collected into the vials and were labeled as F, G, H and I.

After completion of extraction with methanol the residue left in the thimble was dried in air and the dried residue was further extracted with water under reflux in a boiling water bath. After filtration the extracts were concentrated in a rotary evaporator under reduced pressure. The extract was collected into a vial labeled as J.

### 2.3. Preliminary phytochemical screening (I)

The crude extracts were subjected to classical tests for the detection of the presence or absence of unsaturation, carbonyl group, phenolic group, acetyl group, carboxylic acids and sugar molecules.

#### 2.3.1. Test for unsaturation using Baeyer's test

Alcoholic extract *S. rebaudiana* was taken in a test tube to which very dilute  $\text{KMnO}_4$  solution was added. The disappearance of the purple color of very dilute  $\text{KMnO}_4$  solution was observed. This test indicates the presence of unsaturation.

#### 2.3.2. Test for carbonyl group

The extract (10 mL) of *S. rebaudiana* was treated with a few millilitres of 2, 4-dinitrophenylhydrazine and then 2 drops of concentrated HCl was added in that solution, a yellow precipitate was formed. This test indicates the possibility of the presence of an aldehydic compound.

#### 2.3.3. Test for phenolic OH

The extract (10 mL) was taken in a test tube to which 2 mL of freshly prepared ethanolic  $\text{FeCl}_3$  solution was added and greenish blue color appeared. This test confirmed the presence of phenolic compound.

#### 2.3.4. Test for carboxylic acid

The extract was taken in a test tube and sodium bicarbonate ( $\text{NaHCO}_3$ ) was added. The bubbles were evolved in the extract. So carboxylic acid group is present.

#### 2.3.5. Test for acetyl group using iodoform test

Alcoholic extract was taken in a test tube to which 2 mL of  $\text{I}_2$  in KI solution was added and warmed in a hot water bath for 10 min. The aqueous NaOH solution was added dropwise until the disappearance of  $\text{I}_2$  color and a creamy yellow precipitate deposited at the bottom of the test tube. This test indicates the presence of either  $\text{CH}_3\text{CH}_2\text{O}-$  or  $\text{CH}_3\text{CO}-$ .

### 2.3.6. Test for sugar

The methanol and aqueous extract was taken in a test tube to which 2 mL of 1:1 Fehling's solution–I and Fehling's solution–II was added and found that a brick red precipitate appeared. This indicates that a sugar molecule (monosaccharide) is present in the plant.

## 2.4. Preliminary phytochemical screening (II)

Standard screening test of the extract was carried out for various plant constituents. The crude extract was screened for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, tannins and anthraquinones using standard procedures (Hymete, 1986).

### 2.4.1. Test for alkaloids

About 0.5 g of the extract was stirred with 5 mL of 1% hydrochloric acid on a steam bath and filter. Treat 1 mL of the filtrate a few drops of each of the Dragendorff's and Mayer's reagents separately. We got a creamy white and orange–red precipitate that confirmed the presence of alkaloids.

### 2.4.2. Test for steroidal compounds using Salkowski's test

Initially 0.5 g of the alcoholic extract was dissolved in 2 mL chloroform in a test tube. Concentrated sulfuric acid was carefully added on the wall of the test tube to form a lower layer. A reddish brown colour at the interface indicated the presence of a steroid ring (*i.e.* the aglycone portion of the glycoside).

### 2.4.3. Test for phenolic compounds

Three drops of a freshly prepared mixture of 1 mL of 1% ferric chloride and 1 mL of potassium ferrocyanide was added to 2 mL of filtered solution of the aqueous macerate of the plant material. Formation of bluish–green color was taken as positive indication.

### 2.4.4. Test for flavonoids

A volume of 5 mL of ethyl acetate was added to a solution of 0.5 g of the crude extract in water. The mixture was shaken by hand, allowed to settle and inspected for the production of yellow colour in the organic layer, which was taken as positive test for free flavonoids.

### 2.4.5. Test for saponins using Froth test

Initially 0.5 g of the alcoholic extract was dissolved in 10 mL of distilled water in a test tube. The test tube was stoppered and shaken vigorously for about 30 seconds. The test tube was allowed to stand in a vertical position and observed over a 30 min period of time. If a “honey comb”

froth above the surface of liquid and persists after 30 min, the sample is suspected to contain saponins.

### 2.4.6. Test for tannins using Ferric chloride test

The alcoholic extract was dissolved in water. The solution was clarified by filtration. Ferric chloride solution (10%) was added to the clear filtrate. This was observed for a change in colour to bluish black, which indicated the presence of tannins.

### 2.4.7. Test for phlobatannins

Deposition of a red precipitate when an aqueous extract of the plant part was boiled with 1% aqueous hydrochloric acid was taken as an evidence for the presence of phlobatannins.

### 2.4.8. Test for free anthraquinones (Borntrager's test)

The hydro–alcoholic extract of the plant material (equivalent to 100 mg) was shaken vigorously with 10 mL of benzene, filtered and 5 mL of 10% ammonia solution was added to the filtrate. Shaken the mixture and the absence of a pink, red or violet color in the ammonia (lower) phase indicated the absence of free anthraquinones.

### 2.4.9. Test for O–anthraquinone glycosides (modified Borntrager's test) for combined anthraquinone

Initially 5 g of the plant extract was boiled with 10 mL 5% sulphuric acid for 1 h and filtered while hot. The filtrate was shaken with 5 mL benzene; the benzene layer separated and half its own volume of 10% ammonia solution was added. The absence of a pink, red or violet color in the ammonia phase (lower layer) indicated the absence of anthraquinone derivatives in the extract.

## 2.5. Microorganisms

Thirteen bacteria [*Escherichia coli* (ATCC 25922), *Bacillus subtilis* (QL 40, *B. subtilis*), *Bacillus cereus* (QL 29, *B. cereus*), *Bacillus megaterium* (QL 38, *B. megaterium*), *Sarcina lutea* (QL 166), *Pseudomonas aureus*, *Salmonella paratyphi* A (AM 16590), *Salmonella typhimurium* (AM 16406, *S. typhimurium*), *Shigella boydii* (ATCC 13147), *Shigella dysenteriae* (ATCC 26131, *S. dysenteriae*), *Vibrio mimicus* (N 196, *V. mimicus*), *Vibrio parahemolyticus* (AM 16362), *Staphylococcus aureus* (ATCC 25923, *S. aureus*)] and three fungi [*Aspergillus niger* (ATCC 10864, *A. niger*), *Candida albicans* (ATCC 10231), *Saccharomyces cerevisiae* (AB 972)] isolates obtained from Biochemical Research Centre, Faculty of Pharmacy, University of Dhaka, Bangladesh [Microbial type culture collection & gene bank (MTCC)] were stored at –20 °C. These microorganisms were selected for microbial assay study as these are common pathogens either to plants or animals or cause food spoilage.

## 2.6. Bioassay for antibacterial activity

### 2.6.1. Preparation of medium

Initially 21.2 g nutrient agar was taken in a conical flask containing 400 mL of distilled water. The contents were heated in a water bath to make a clear solution. The pH was adjusted at 7.2–7.6. Volumes of 18.0 mL and 5.0 mL of the medium were then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized by autoclaving at 15 lbs pressure at 121 °C for 20 min. The slants were used for making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

### 2.6.2. Sterilization procedures

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in laminar hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the laminar hood. Petridishes and other glassware were sterilized by autoclaving at a temperature of 121 °C and a pressure of 15 lbs/sq. inch for 20 min. Micropipette tips, cotton, forceps, blank discs *etc.* were also sterilized.

### 2.6.3. Preparation of subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 h at 37 °C for their optimum growth. These fresh cultures were used for the sensitivity test.

### 2.6.4. Preparation of the test plates

The test organisms were transferred from the subculture to the test tubes containing about 10.0 mL of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial and fungal suspension was immediately transferred to the sterilized petridishes. The petridishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

### 2.6.5. Standard discs

In this investigation, kanamycin (30 µg/disc) standard disc was used as the reference.

### 2.6.6. Preparation of sample discs with test samples

A weight of 8.0 mg of each test sample was dissolved in 200 µL methanol. Sterilized metrical filter paper discs (6 mm) were taken in a blank petridishes under the laminar hood. Then discs were soaked with 10 µL solutions of test samples

and dried. Disc concentration is now 400 µg/disc.

### 2.6.7. Diffusion and incubation

The sample discs and the standard antibiotic discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4 °C for about 24 h upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then kept in an incubator at 37 °C for 24 h.

### 2.6.8. Determination of antimicrobial activity by measuring the zone of inhibition

The antimicrobial potency of the test agents was measured by their activity to prevent the growth of the microorganisms surrounding the discs which gave clear zone of inhibition. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

### 2.6.9. Determination of minimum inhibitory concentration

Minimum inhibitory concentration was determined for extracts A, B and G of different concentrations.

## 3. Results

The signs '+' or '-' against different tests indicate the presence and absences of respective functional groups or compounds shown in Table 1. The number of positive signs indicates the quantity present. Preliminary phytochemical screenings showed the most abundant compound in the stevia leaves were alkaloids and steroids followed by tannins, saponins and flavonoids.

**Table 1**

Phytochemical screening of *S. rebaudiana* Bertoni leaves (from basic soil, pH 7.7).

Phytochemical screening	Test name/Reagents	Response
(I) Unsaturation	Baeyer's test	+
Carbonyl group	2, 4-dinitrophenylhydrazine and HCl	+
Phenolic group	Ethanollic FeCl <sub>3</sub> solution	+
Carboxylic acids	Iodoform test	+
Acetyl group	Sodium bicarbonate	+
Sugar molecules	Fehling's solution-I and II	+
(II) Alkaloids	Dragendorff's	+++
Steroids	Salkowski's test	+++
Saponins	Froth test	++
	Ferric chloride test	++
Tannins	Phlobatannins	++
Flavonoids	Free flavonoids	++
	Borntrager's test	-
Anthraquinones	Modified Borntrager's test	-

-: Negative result +: Positive result ++: Low concentration +++: High concentration.

**Table 2**Antimicrobial activity of different extracts of *S. rebaudiana* Bertoni leaves (from basic soil, pH 7.7).

Organisms	A		B		C	D	E	F	G		H	I	J	Kan
	DZI (mm)	MIC ( $\mu\text{g}/\text{disc}$ )	DZI (mm)	DZI (mm)	DZI (mm)	DZI (mm)	DZI (mm)	DZI (mm)	DZI (mm)	MIC ( $\mu\text{g}/\text{disc}$ )	DZI (mm)	DZI (mm)	DZI (mm)	DZI ( $\mu\text{g}/\text{disc}$ )
B. C	12.14 $\pm$ 0.15	100	12.04 $\pm$ 0.04	100	–	–	–	–	9.18 $\pm$ 0.03	150	–	8.13 $\pm$ 0.05	–	34.00
B. M	12.09 $\pm$ 0.08	100	12.08 $\pm$ 0.04	100	–	–	–	–	9.05 $\pm$ 0.04	150	–	8.15 $\pm$ 0.04	–	34.00
B. S	12.14 $\pm$ 0.04	100	13.02 $\pm$ 0.03	100	–	–	–	10.05 $\pm$ 0.05	10.08 $\pm$ 0.03	125	–	8.15 $\pm$ 0.04	–	34.00
S. A	13.24 $\pm$ 0.24	100	13.06 $\pm$ 0.03	100	–	–	–	9.05 $\pm$ 0.04	12.04 $\pm$ 0.04	100	–	9.16 $\pm$ 0.05	–	34.00
S. L	10.15 $\pm$ 0.06	125	10.04 $\pm$ 0.04	125	–	–	–	8.13 $\pm$ 0.05	10.08 $\pm$ 0.03	125	–	9.05 $\pm$ 0.04	–	34.00
E. C	12.08 $\pm$ 0.04	100	11.12 $\pm$ 0.05	100	–	–	–	8.02 $\pm$ 0.03	12.07 $\pm$ 0.04	100	–	9.07 $\pm$ 0.04	–	34.00
P. A	12.17 $\pm$ 0.04	100	10.05 $\pm$ 0.05	125	–	–	–	8.15 $\pm$ 0.04	12.08 $\pm$ 0.04	100	–	9.12 $\pm$ 0.05	–	34.00
S. P	12.14 $\pm$ 0.05	100	12.18 $\pm$ 0.04	100	–	–	–	8.12 $\pm$ 0.04	10.04 $\pm$ 0.04	12	–	8.15 $\pm$ 0.04	–	34.00
S. T	12.04 $\pm$ 0.04	100	14.05 $\pm$ 0.04	100	–	–	–	8.04 $\pm$ 0.05	10.15 $\pm$ 0.06	125	–	9.10 $\pm$ 0.01	–	34.00
S. B	10.08 $\pm$ 0.03	125	12.07 $\pm$ 0.04	100	–	–	–	9.15 $\pm$ 0.04	12.18 $\pm$ 0.04	100	–	9.18 $\pm$ 0.05	–	34.00
S. D	12.07 $\pm$ 0.04	100	10.04 $\pm$ 0.04	125	–	–	–	8.15 $\pm$ 0.04	13.06 $\pm$ 0.03	100	–	9.07 $\pm$ 0.04	–	34.00
V. M	14.05 $\pm$ 0.05	100	12.04 $\pm$ 0.04	100	–	–	–	9.08 $\pm$ 0.05	12.14 $\pm$ 0.05	100	–	9.10 $\pm$ 0.01	–	34.00
V. P	12.18 $\pm$ 0.04	100	10.05 $\pm$ 0.05	125	–	–	–	8.15 $\pm$ 0.04	12.09 $\pm$ 0.15	100	–	8.04 $\pm$ 0.05	–	34.00
C. A	10.04 $\pm$ 0.04	125	12.08 $\pm$ 0.04	100	–	–	–	9.16 $\pm$ 0.05	12.04 $\pm$ 0.04	100	–	9.04 $\pm$ 0.04	–	34.00
A. N	10.08 $\pm$ 0.03	125	12.18 $\pm$ 0.04	100	–	–	–	9.04 $\pm$ 0.04	12.08 $\pm$ 0.04	100	–	9.17 $\pm$ 0.03	–	34.00
S. C	10.21 $\pm$ 0.03	125	12.14 $\pm$ 0.15	100	–	–	–	9.18 $\pm$ 0.03	12.07 $\pm$ 0.04	100	–	9.18 $\pm$ 0.05	–	34.00

Values are means $\pm$ SD ( $n=3$ ). DZI: Diameter of zone of inhibition, MIC: Minimum inhibition concentration. B. C: *B. cereus*; B. M: *B. megaterium*; B. S: *B. subtilis*; S. A: *S. aureus*; S. L: *Sarcina lutea*; E. C: *Escherichia coli*; P. A: *Pseudomonas aureus*; S. P: *Salmonella paratyphi*; S. T: *S. typhi*; S. B: *Shigella boydii*; S. D: *S. dysenteriae*; V. M: *V. mimicus*; V. P: *Vibrio parahemolyticus*; C. A: *Candida albicans*; A. N: *A. niger*; S. C: *Saccharomyces cerevisiae*. Kan: Kanamycin, –: No sensitivity.

The antibacterial activities of the solvent extracts of *S. rebaudiana* showed significant variations as shown in Table 2. Among the ten extracts tested, extract A had greater antibacterial potential followed by extract B and then G. Extract A showed greater antibacterial activity against *V. mimicus* with the largest zones of inhibition (14.05 $\pm$ 0.05) mm and for *S. aureus* the zone of inhibition (13.24 $\pm$ 0.24) mm. Extract B showed the largest zone of inhibition (14.05 $\pm$ 0.04) mm, (13.02 $\pm$ 0.03) mm and (13.06 $\pm$ 0.03) mm against *S. typhimurium*, *B. subtilis* and *S. aureus* respectively. Extract G was very effective against *S. dysenteriae* with the zone of inhibition of (13.06 $\pm$ 0.03) mm. Extract F was inactive against *B. cereus* and *B. megaterium*. Extract C, E, I, J, and H showed no activity.

#### 4. Discussion

Antimicrobial activities of various herbs and spices in plant leaves, flowers, stems, roots, or fruits have been reported by many workers<sup>[18–20]</sup>. We just want to highlight some points that the extracts C, E, I, J and H showed no antimicrobial activity but A and F showed a great antimicrobial activity. There are another point is that those two (A and F) are of *n*-hexane partition but F did not show any activity against *B. cereus* and *B. megaterium* where A showed a greater activity. The present investigations endow with the basic information that *n*-hexane extract of stevia leaves which is found to be potent enough in exhibiting substantial antimicrobial activity against dreaded

animal pathogens like *S. aureus*. Possession of sizeable antimicrobial activity against food spoiling fungi like *A. niger* and bacteria *S. aureus* may be explored for a value addition as natural food preservative to sugar substituting property of stevia used nowadays for diet restricted package food products. And stevia leaves are good source of protein and carbohydrate. We can also conclude that stevia leaf extracts may be an ideal candidate for further research into their uses for pharmaceutical and natural plant-based products.

#### Conflict of interest statement

We declare that we have no conflict of interest.

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## Comments

### Background

*S. rebaudiana*, a natural alternative to artificial sweetener is found to contain over 100 phytochemicals. The most important bioactive compounds of this plant are alkaloids, flavanoids, tannins and phenolic compounds. The other antimicrobial principles such as tannins, essential oils and aromatic compounds are also present. To the best of our knowledge, none has investigated the potential antimicrobial effect of *S. rebaudiana* Bertoni leaves in Bangladesh till now.

### Research frontiers

The present study is to evaluate the phytochemical screening and antimicrobial activity of *S. rebaudiana* Bertoni leaves extracts using various solvents.

### Innovations & breakthroughs

The scientific work was done by the authors and it gives new information and data to the scientific community.

### Applications

This plant is used worldwide as a sweet. According to the paper, there are so many bioactive compounds that can be used to prepare herbal medicine.

### Peer review

This is an interesting study in which authors evaluated the phytochemical screening and antimicrobial activity of *S. rebaudiana* Bertoni leaves extracted using various solvents. The paper revealed that the plant has some bioactive compounds which are useful to prepare herbal medicine.

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