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# Nutritional Value, Cytotoxic and Antimicrobial Activities of *Stevia rebaudiana* Leaf Extracts

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## Abstract

*Stevia rebaudiana*Bertoni (*Stevia*), is a plant species in the genus *Stevia*. This study aimed to explore the main active constituents of dried *Stevia* leaves. Besides, the antimicrobial and cytotoxic properties of 6 *Stevia* extracts (aqueous, methanol, ethanol, acetone, chloroform, and petroleum ether) were assessed. *Stevia* leaves were dried by solar energy then the proximal chemical analysis and the total antioxidant capacitywere conducted. The antimicrobial effects of the 6 different solvent extracts of *Stevia* leaves were assessed against 7 different pathogens using agar well diffusionmethod. Furthermore, the cytotoxic effect of the 6 *Stevia* extracts was assessed against human breast carcinoma cell line MCF7. *Stevia* leaves have good nutritional value as well as have a good amount of total antioxidant capacity, total phenols and total flavonoids (20.788mg AAE/g, 29.5 mg GAE/g and 7.105 mg QE/g, respectively). All *Stevia* extracts decreased the growth of MCF7 cells. The $IC_{50}$ of acetone, chloroform, water, ethanol, petroleum ether, and methanol were 150, 100, 374, 180, 79, and 228 µg/ml, respectively. The petroleum ether *Stevia* leaves extract exerted the most potent cytotoxic activity against MCF7. The aqueous extract of *Stevia* leaves was the most effective against *Bacillus cereus, Salmonella typhi, Pseudomonasaeruginosa, Listeria monocytogenes, Escherichiacoli*, and *Bacillus cereus*. The methanolic extract was the most effective extract against *Staphylococcus aurous* and*Aspergillusflavus*. In conclusion, *Stevia* leaves contain considerable amounts of several macro and micronutrients. The aqueous extract of *Stevia* leaves has a significant potential antimicrobial action. Besides, the petroleum ether extract of *Stevia* leaves was the most potent cytotoxic extract against MCF7 cancer cells.

**Keywords:***Steviarebaudiana*, antimicrobial, cytotoxic, cells, MCF7, active constituents

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## Introduction

Natural pharmacological plant compounds are used to prevent or treat several diseases. They constitute significant importance where the pathogens have built their resistance with costly treatment systems with the conventionally used medicines. They also devoid the severe adverse effects of chemical drugs (Nitta et al., 2002).*Stevia rebaudiana*Bertoni (*Stevia*), family Asteraceae is a plant species in the genus *Stevia*. It is usually recognized as sugar leaf, sweet leaf, or candy leaf. *Stevia* is a tiny seasonal tree of 1–2 feet in height. It possesses outspread leaves that extend over the stems and are arranged versus each other (Hossain et al., 2017). *Stevia* plant is grown in the universe for the often-featured sugary glycosides, diterpene.One*Stevia*feddan harvested about 400 kg of *Stevia* sugar per year, which could cope with the lack of sugar production worldwide (Alaam, 2007).

*Stevia,* being as natural, zero caloric, and high-intensity sweeteners proved to be the best sugar alternative in a wide range of beverages, foods, and medicines industry, which substitute for artificial and calorie-dense sweeteners (Chughtai et al., 2020). *Stevia*, leaves, stems, and flowers have a complex combination of glycosides such as diterpene glycosides, including stevioside, isosteviol, steviolbioside, rebaudioside, and dulcoside A ( Rajasekaran et al., 2008; Goyal et al., 2010). The sweetness of both rebaudioside A and stevioside exceeds that of sucrose by 250–300 times (Debnath, 2008; Pradhan, 2016). Besides, *Stevia* leaves comprise another bioactive constituent, like, phenolics, flavonoids, fatty acids, and vitamins( Milani et al., 2017; Chughtai et al., 2020). Steviol glycosides usage is currently supported to limit the calorie-rich sugar from beet sugar, sugarcane, and nectar thus may decrease the metabolic disorders and the prevalence of overweight and obesity, which are the main causes of several health difficulties ( Romo-Romo et al., 2017; Chughtai et al., 2020).

Over the years, no harm has been accounted for to be related to *Stevia;* subsequently, it can be viewed as safe for human utilization (Chranioti et al., 2016). It proved to have a major effect in blood pressure regulation, glucose modulation, renal functions, obesity management, and dental diseases (Benford et al., 2006; Chughtai et al., 2020). It also possesses a high free radical scavenging effect (Abdalbasit et al., 2014). *Stevia* has anticancer, anti-mutagenesis, antioxidant, antihypertensive, and anti-inflammatory activities (Jayaraman et al., 2008; Takasaki

et al., 2009; Yildiz-Ozturk et al., 2015; Gupta et al., 2013; Chughtai et al., 2020). In addition, it inhibits the growth of specific bacteria (Tadhani and Group, 2006).

This investigation aimed to explore the chemical composition of *Stevia* leaves. Besides, the antimicrobial and cytotoxic effects of different extracts prepared from dried *Stevia* leaves (water, ethanol, methanol, acetone, chloroform, and petroleum ether) were studied.

## Materials and Methods

*Plants and chemicals*

*Stevia* leaves were obtained from the Agricultural Research Centre (ARC), Giza, Egypt.Ethanol, methanol, acetone, chloroform, and petroleum ether (P. ether) were obtained from El-Gomhoria Company for Chemical, Cairo, Egypt.

*The microbes strain*

Certified strains including *Salmonella typhi*, *Bacillus cereus*, *Pseudomonas aenginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, and *Aspergillusflavus* were obtained from Regional Center for Food and Feed, Agriculture Research Center.

*Human breast carcinoma cell lines*

Human breast cancer cell lines MCF7 was purchased from the American Type Culture Collection (Minnesota, USA). Successive culture has maintained the cell lines at the National Institute of Cancer, Egypt). Dimethyl sulfoxide (DMSO), trypan blue, penicillin/streptomycin antibiotic, RPMI-1640 medium, fetal bovine serum, and trypsin-EDTA were purchased from Sigma Aldrich Chemical Co., St. Louis, Mo, USA. Tris buffer was obtained from Appli. Chem, Germany.

*Preparation of Stevia leaves extracts*

*Stevia* leaves were washed in distilled water and spread on trays after filtering from water. Solar energy at the (National Research Center, Dokki, Egypt) was used to drying *Stevia*leaves.Dried*Stevia* 750 g was extracted by shaking with (1 L) from 6 different solvents (water, ethanol, P. ether, methanol, acetone, and chloroform) at room temperature (28-30 °C) for 72 hours. The extract has been filtered *via* Buchner funnel, and the residue extracted has been replicated twice in the same way. Collected filtrate was concentrated to dryness in a hot air oven at 40 °C and then freeze-dried until further analysis (Jahan et al., 2010).

*Chemical analysis of Stevia leaves*

Dried stevialeaves were chemically analyzed to determine the macro-nutrients including moisture, protein, crude fiber, fat, ash, and carbohydrate/ 100 g dry weight of the sample according to the described methods by AOAC (2019). The micro-nutrients /1000 g including mineral; iron (Fe), potassium (K), phosphorus (P), magnesium (Mg), sodium (Na), calcium (Ca), manganese (Mn), and selenium (Se) were determined using the method of AOAC (2019); vitamins; α-tocopherol (Vit. E), thiamin (Vit. B1), riboflavin (Vit. B2), and ascorbic acid (Vit C) were determined according to Hossain et al. (2010).

*Analysis of Stevia leaves antioxidant contents*

The total antioxidant of the dried *Stevia* leaves expressed as ascorbic acid equivalent (mg AAE/g) was determined by the method of Prieto et al. (1999), total phenolic content expressed as gallic acid equivalent (mg GAE/g) was determined by the Folin–Ciocalteu method (Singleton et al., 1999), total flavonoid content expressed as Quercetin equivalent (mg QE/g) was determined using the colorimetric method of Sarikurkcu et al. (2009) utilizing aluminum.

*Analysis of Stevia leaves active constituents by gas chromatography-mass spectroscopy (GC-MS)*

This assay was performed utilizing a GC-MS (Agilent Technologies 7890A) connected to a mass-specific detector (MSD, Agilent 7000). Helium was the carrier gas. The recognition of constituents was carried out by comparing their mass spectra and retention time with the library of authentic compounds (NIST and WILEY) (Santana et al., 2013).

*Evaluation of the cytotoxic effect of Stevia extracts*

All samples were prepared by dissolving the six different *Stevia* extracts with a stock solution at the ratio (1:1), then stored in DMSO100 mM at -20 ◦C. Briefly, diverse concentrations of the six *Stevia* extracts (0, 62.5, 125, 250, 500 µg/ml) were used, 3 wells for each treatment, then the plates were left for incubation for 48 h. Then, trichloroacetic acid 10 % (50 µl) was used to fix the cells for 1 h at 4 °C. The cells were stained with sulforhodamine-B (50 µl 0.4 %). The optical density (O.D.) of the plate was determined at 570 nm using a microplate reader (Sunrise Tecan reader, Germany). The survival % of MCF7 cells was calculated using the following equation:

$$\text{Survivingfraction} = \frac{\text{O. D. (TreatedCells)}}{\text{O. D. (ControlCells)}}$$

The values of the IC50 were then computed for the 6 extracts (Skehan et al., 1990; Vanicha and Kanyawim, 2006).

*Evaluation of the antimicrobial effect of Stevia extracts*

The agar well diffusionmethod was adopted (Ghosh et al., 2008). The concentration of 50 µl/well form the six extracts of *Stevia* leaves was used in each well to evaluate their antimicrobial activity against pathogenic bacterial for nutrient agar media including *Salmonella typhi*, *Pseudomonas aenginosa*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes* also fungal for rose Bengal

agar media *Aspergillusflavus* on Petri dishes, then incubated at 37 ℃ and 28 ℃, respectively. The growth readings were counted after 24–48 h and 3–5 days, respectively using inhibition zone (mm).

*Statistical analysis*

The obtained data were analyzed using SPSS program, version 24. Data were represented as mean ± SE for3 replicates. ANOVA test followed by Duncan's test was used to compare results among samples. $P < 0.05$ was significant.

## Results

*Chemical composition of Stevia leaves*

The results indicated that *Stevia* leaves powder (100 g) contains a high carbohydrate amount (53.33 g) which provides 305.73 Kcal energy. It also contains 16.5 g protein, 9.8 g crude fiber, and 2.69 g fat. *Stevia* leaves contain many minerals the predominant are potassium (K), calcium (Ca), and magnesium (Mg) (2964.3, 2117.4, and 1324.2 mg/1000 g, respectively). In addition, *Stevia* leaves have a considerable quantity of vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin C (ascorbic acid), and vitamin E (α-tocopherol) of which vitamin B1 is the most predominant (5, 25.1, 1.3, 0.12 mg/1000 g, respectively (Table 1).

**Table 1:**Phytoconstituents of *Stevia* leaves.

| Components | Mean ± SE |
|---|---|
| **Macronutrient (g/100 g)** | |
| Moisture | 9.1 ± 1.15 |
| Protein | 16.5 ± 0.87 |
| Fat | 2.69 ± 0.58 |
| Crude fiber | 9.8 ± 0.57 |
| Ash | 8.13 ± 1.15 |
| Total carbohydrate | 53.33 ± 5.78 |
| Energy (Kcal /100 g) | 305.73 ± 5.77 |
| **Minerals (mg/1000 g)** | |
| Ca | 2117.4 ± 9.81 |
| P | 313.5 ± 7.51 |
| Fe | 200.0 ± 11.54 |
| K | 2964.3 ± 17.30 |
| Mg | 1324.2 ± 11.55 |
| Mn | 31.0 ± 2.39 |
| Na | 291.0 ± 2.89 |
| Se | 34.0 ± 1.73 |
| **Vitamins (mg/1000 g)** | |
| Vitamin B1 (Thiamin) | 5.0 ± 0.58 |
| Vitamin B2 (Riboflavin) | 25.1 ± 2.88 |
| Vitamin C (Ascorbic acid) | 1.3 ± 0.23 |
| Vitamin E (α-tocopherol) | 0.12 ± 0.01 |

Values were presented as the mean of three replicates ± SE.
Ca: Calcium, P: Phosphorus, Fe: Iron, K: Potassium, Mg: Magnesium, Mn: Manganese, Na: Sodium, Se: Selenium.

*Total antioxidants, flavonoids, and phenols contents of Stevia leaves powder*

Total antioxidants content of *Stevia* leaves powder is 20.78 mg AAE/g, the total phenols is 29.56 mg GAE/g, and the total flavonoids is 7.11 mg QE/g (Table 2).

**Table 2:** Total antioxidants, phenols, and flavonoids contents of *Stevia* leaves powder.

| Antioxidant constituents | Mean ± SE |
|---|---|
| Total antioxidant (mg AAE/g) | 20.78 ± 0.87 |
| Total phenols (mg GAE/g) | 29.56 ± 1.44 |
| Total Flavonoids (mg QE/g) | 7.11 ± 1.04 |

Values were presented as the mean of three replicates ± SE.
AAE: ascorbic acid equivalent, GAE: gallic acid equivalent, QE: Quercetin equivalent.

*Chemical active constituents of Stevia leaves powder analyzed by GC-MS*

TheGC-MS analysis of *Stevia* leaves was presented in Figure 1 and Table 3. The results revealed that there were several active compounds present in *Stevia* leaves the most predominant are, furfural (55.45%), hexadecanoic acid, ethyl ester (7.82%), 17-octadecenal (5.43%), dodecane 1-methoxy (3.82%), and 2-pentanol (3.15 %).

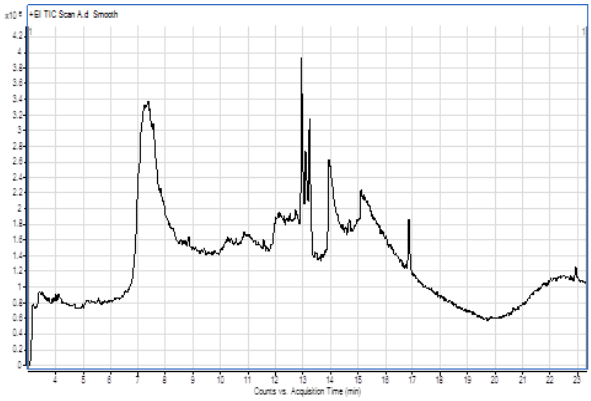

**Figure 1:** Gas chromatography-mass spectroscopy (GC-MS) spectra of the *Stevia*leavespowder.

*Cytotoxic effect of different Stevia extracts against MCF7 cell line*

Results in Figure 2 revealed the effect of different *Stevia* extracts concentrations on the growth of MCF7 cells. It was revealed that all *Stevia* extracts lowered the cell viability of MCF7 cells in a dose-dependent manner. Besides, P. Ether *Stevia* extract was more effective for the reduction of the viability of MCF7 cells followed by chloroform, acetone, ethanol, methanol, and water extract, respectively.
The $IC_{50}$ value of different *Stevia* extracts in MCF7 cells was presented in table 4. The $IC_{50}$ of acetone was 150 µg/ml, for chloroform was 100 µg/ml, for water was 374 µg/ml, for ethanol was 180 µg/ml, for P. Ether was 79 µg/ml, and for methanol was 228 µg/ml. The P. ether extract possesses the most potent cytotoxic activity against MCF7 cell line.

*Antimicrobial and antifungal activities of different Stevia extracts*

Results presented in Figure 3 illustrated that *Stevia* extracted with distilled water was the most effective against *Bacillus cereus, Salmonella typhi, Pseudomonasaeruginosa, Listeria monocytogenes, Escherichiacoli,* and*Bacillus cereus*. The most effective extract against *Staphylococcus aurous* and*Aspergillusflavus*wasthe methanolic extract. All the extracts exerted a nearly equivalent antimicrobial activity against *Pseudomonasaeruginosa.Escherichiacoli* was resistance to the action of the ethanolic, methanolic, and chloroform extracts of *Stevia*. The results also showed that the chloroform extract of *Stevia* is the least effective against all types of bacteria and fungi under this study.

**Table 3:**Chemical composition of *Stevia* leaves powder analyzed by GC-MS.

| Compounds | RT* (min) | Area (%) |
|---|---|---|
| 2-Pentanol | 4.009 | 3.15 |
| Diallyl sulfide | 5.531 | 1.0 |
| Furfural | 7.31 | 55.45 |
| 2-Eicosanol | 8.526 | 1.96 |
| Nobiletin | 8.814 | 0.59 |
| Oxamyl | 9.381 | 1.44 |
| 1-Nitro-β-d-arabinofuranose, tetraacetate | 10.372 | 0.69 |
| 2-Propanone, 1,1-diethoxy- | 10.552 | 1.25 |
| 2-Nonadecanone 2,4-dinitrophenylhydrazine | 10.84 | 1.78 |
| Melezitose | 11.552 | 1.64 |
| Trans-2-Hexadecenoic acid | 12.096 | 1.29 |
| Stevioside | 12.29 | 0.89 |
| Desulphosinigrin | 12.502 | 0.79 |
| Dodecanoic acid, 2-(acetyloxy)-1-[(acetyloxy) methyl] ethyl ester | 12.727 | 0.88 |
| 17-Octadecenal | 12.939 | 5.43 |
| 1-Heptatriacotanol | 13.083 | 1.42 |
| Phytol | 13.218 | 2.37 |
| Oleic Acid | 13.438 | 0.97 |
| 2-Tridecenoic acid, (E) | 13.659 | 0.77 |
| Hexadecanoic acid, ethyl ester | 13.934 | 7.82 |
| Pseduosarsasapogenin-5-en methyl ether | 14.416 | 1.06 |
| 3-Octadecanone | 14.672 | 0.69 |
| Eicosanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester | 15.095 | 0.92 |
| 9-Octadecen-12-ynoic acid, methyl ester | 16.825 | 1.91 |
| Dodecane, 1-methoxy | 22.917 | 3.82 |
| Non-identified compounds | > 23.02 | 0.02 |

*RT: Retention time.

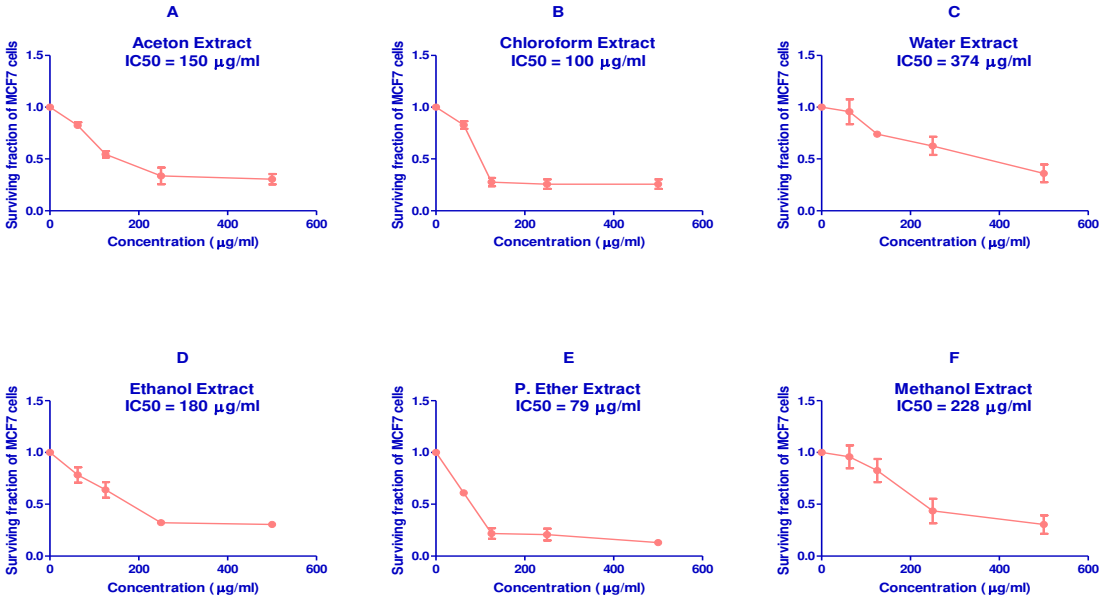


**Figure 2:***In vitro* cytotoxicity of various *Stevia* extracts against MCF7 cells.
A: Acetone, B: Chloroform, C: Water, D: Ethanol, E: P. Ether, and F: Methanol.

**Table 4:** The IC50 values of different *Stevia* extracts in MCF7 cells.

| *Stevia* Extract | IC50 (µg/ml) |
|---|---|
| **Acetone** | 150 |
| **Chloroform** | 100 |
| **Water** | 374 |
| **Ethanol** | 180 |
| **P. Ether** | 79 |
| **Methanol** | 228 |

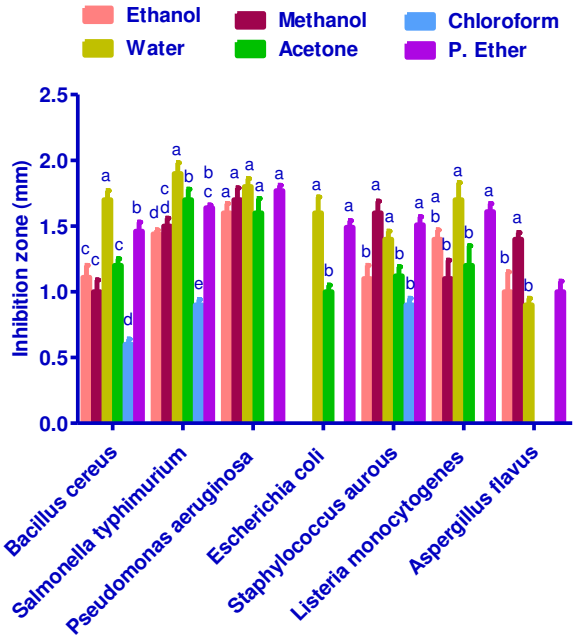


**Figure 3:**Antimicrobial and antifungal activities of different*Stevia*extracts. Inhibition zones values are represented as mean ± SE. Values with different superscript letters against the same microbial species are significant different (P≤ 0.05).

## Discussion

The results of this research showed that *Stevia* is a good source of crude protein, carbohydrates, fiber, and energy. Like our results (Segura-Campos et al., 2014). Moreover, energy value provide in this work was 305.73 kcal/100 g powder. The obtained results are harmony with Savita et al. (2004), who proved that *Stevia* leaves contain 269 Kcal/100 g, which could be attributed to the intense sweetness of *Stevia* in comparison to other available low-calorie sweeteners (Johnson et al., 2018).

This study results indicated that *Stevia* leaves powder (100 g) contains carbohydrate (53.33 g), protein (16.5 g), crude fiber (9.8 g), and fat (2.69 g). In agreement with our results, both the carbohydrate and lipids contents per 100 g dry leaves of sweet *Stevia* leave were previously reported (35.2-61.9 g and 1.9-6.13 g, respectively) (Boonkaewwan et al., 2006; Esmat Abou-Arab et al., 2010; Gibson et al., 2017). In another study demonstrated the total protein, fat, ash, crude fiber and carbohydrates in *Stevia* leaves powder were recorded 9.63, 3.47, 3.08, 17.12, and 66.50 g/100 g, respectively (El-Nassag et al., 2019). Our results indicated that *Stevia* contains a considerable quantity of vitamin B (3 mg/100 g) and ascorbic acid (0.13 mg/100 g). Conversely, sweet *Stevia* leave is a source of ascorbic acid (14.98 mg/100 g) and slight amounts of vitamins B (Boonkaewwan et al., 2006; Jahangir Chughtai et al., 2020).

Gaweł-Bęben et al. (2015) mentioned that *Stevia* extracts contain substantial quantities of antioxidant bioactive constituents. Moreover, Jahan et al. (2010) suggested that Stevia leaves have a considerable potency to be utilized as a natural antioxidant. Savita et al. (2004) proved that *Stevia* leaves contain a high amount of antioxidant activity (2295) mg. Furthermore, Ruiz et al. (2015) reported that the leaves of *Stevia* could be used as antioxidants as the total phenolic and flavonoids contents of the *Stevia* extracts ranged between 28.7-28.4 mg/g and 39.3-36.7 mg/g, respectively. Polyphenols content and antioxidant activity are presented in both *Stevia* leaf powder and commercial stevioside, but the higher polyphenols were in the leave powder (Taleie et al., 2012; Rao, 2014).

Dietary polyphenols and flavonoids can preserve cell constituents from oxidative damage and thus reduce the risk of various oxidative stress-relative degenerative diseases and cancers (Scalbert et al., 2005; Crozier et al., 2009; Gupta et al., 2013; Jahangir Chughtai et al., 2020). This could explain the cytotoxic effect of *Stevia* leaves extracts presented in this study against MCF7 (human breast cancer cell line).The ethanolic extract of *Stevia* was cytotoxic against three cell lines, the highest activity was observed versus HeLa cell line (cervix cancer cells) (López et al., 2016). Besides, stevioside encourage reactive oxygen species-induced apoptotic cell death in MCF7 culture (Paul et al., 2012). Moreover, it was also reported that steviol exerted a powerful cytotoxic effect against the human gastrointestinal tumor cells (Chen et al., 2018).

The obtained results agreed with Debnath (2008) who reported that the growth of many pathogenic bacteria was inhibited by *Stevia* extracted with various solvents. Like our results Tomita et al. (1997) also showed a bactericidal effect of the fermented hot water *Stavia* extract versus enter hemorrhagic *Escherichia coli* and other food borne pathogenic bacteria. In contrast to this study results of Vlietinck et al. (1995) stated that the extracts of water have little bacterial activity. This may be due to the different culture media that proposed to exert an essential function in the settlement of bactericidal action (Lin et al., 1999). Our results were also disagreed with Jayaraman et al. (2008) who reported the largest zones of inhibition were detected for acetone extract against *Staphylococcus aureus* while the chloroform and water extracts have respectively been marginally effective against the test species. Similar to our results, *Stevia* extracted with methanol was more effective against *Aspergillusflavus* fungal than both ethanol and water respectively. This result was agreement with Tomita et al. (1997) and Debnath (2008) who reported that, the methanolic extract was more effective against all the fungi. This may be attributed to the stability of the active ingredient of *Stevia* extract in this solvent for a longer period.

## Conclusion

It could be concluded that *Stevia* leaves contain considerable amounts of several macro and micronutrients, and it could provide high caloric energy. The aqueous extract of *Stevia* leaves has a significant potential antimicrobial action against several types of pathogenic bacteria, while the methanolic extract showed the highest antifungal activity. Besides, the petroleum ether extract of *Stevia* leaves was the most potent cytotoxic extract against MCF7 cancer cells.

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