

Acknowledgements

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ABSTRACT

The aim of this study was to determine the phytochemical screening and an evaluation of the antioxidant and antibacterial activity of leaves of a new variety of *Stevia rebaudiana* in Algeria. Stevia has many pharmacological and therapeutic properties including antimicrobial, antifungal, antioxidant and anticarcinogenic activity. Due to this importance the leaf was analyzed for preliminary phytochemical studies. In this study, the crude plant extract from three different methods of extractions (ethanol, methanol and water) were collected and tested for the presence of phytochemicals. The tests revealed the presence of flavonoïdes, tannins, sterols, triterpenes and glycosides in all extracts. The qualitative analysis using TLC showed the presence of flavonoids in different extracts. This result was confirmed by a quantitative analysis based on the dosage of phenolic compounds. The results proved that the extracts represent a significant source of phenolic compounds. The value varied between 110.2 and 371.50 mg equivalent Gallic acid/g. DPPH free radical scavenging method was used for antioxidant activity analysis. All the extracts exhibited a good antioxidant activity; the ethanolic extract of maceration exhibited the highest radical scavenging activity with an IC_{50} of 13.47 μ g/ml. The extracts were also tested for their possible antibacterial activity *in vitro* against two bacterial strains (*Escherichia coli* and *Staphylococcus aureus*) using the disc diffusion method. The results on antibacterial activity showed that the highest activity was attributed to decoction extract with a maximum zone of inhibition was 21 mm against *Staphylococcus aureus*. There is a need to elucidate the actual compounds in the plant leaf extracts responsible for the antimicrobial and antioxidant activity so that can be used in drug development.

Keywords: *Stevia rebaudiana*, phenolic compounds, TLC, antioxidant activity, antibacterial power.

ملخص

كان الهدف من هذه الدراسة هو تحديد الفحص الكيميائي النباتي وتقييم النشاطية المضادة للأكسدة والمضادة للجراثيم لأوراق ستيفيا ريبودانا من منطقة جديدة في الجزائر. ستيفيا لها العديد من الخصائص الدوائية والعلاجية بما في ذلك مضادات الميكروبات ، مضادات للفطريات ، ومضادات الأكسدة والنشاط المضاد للسرطان. بسبب هذه الأهمية تم تحليل الأوراق للدراسات الكيميائية النباتية الأولية. في هذه الدراسة ، تم جمع واختبار مستخلص النبات الخام بواسطة ثلاث طرق مختلفة للاستخراج (الإيثانول، الميثانول و الماء) حيث تم جمعه و اختباره للكشف عن المواد الكيميائية النباتية. كشفت الاختبارات عن وجود فلافونويدات ، التانينات ، الستيرويدات ، التربينات، الجلوسيدات في جميع المستخلصات، أظهر التحليل الكيفي باستخدام TLC وجود فلافونويد في مختلف المستخلصات. تم تأكيد هذه النتيجة من خلال تحليل كمي يعتمد على جرعة المركبات الفينولية. أثبتت النتائج أن جميع المستخلصات تمثل مصدرا هاما للمركبات الفينولية. وتراوحت القيمة بين 110.2 و 371.50 مغم مكافئ لحمض الغاليك / غم. تم استخدام طريقة كسح الجذور الحرة DPPH لتحليل نشاط مضادات الأكسدة. جميع المستخلصات أظهرت نشاط جيد مضاد للأكسدة. أظهر المستخلص الإيثانولي للنقع أعلى نشاط للمسح الجذري بتركيز مثبط IC50 بمقدار 13.47 ميكروغرام / مل. كما تم اختبار المستخلصات لنشاطها المضاد للبكتيريا في المختبر ضد سلالتين بكتيرية (*Escherichia coli* و *Staphylococcus aureus*) باستخدام طريقة انتشار القرص. وأظهرت النتائج على النشاطية المضادة للجراثيم أن أعلى نشاط كان يعزى إلى مستخلص المائي مع الحد الأقصى لمنطقة تثبيط كان 21 ملم ضد *Staphylococcus aureus*. هناك حاجة لتوضيح المركبات الفعلية في مستخلصات أوراق النبتة المسؤولة عن النشاط المضاد للميكروبات ومضادات الأكسدة بحيث يمكن استخدامها في تطوير الأدوية.

كلمات مفتاحية: مركبات الفينولية، النشاط المضاد للأكسدة، القدرة المضادة للجراثيم، ستيفيا ريبودانا

ABBREVIATIONS

HCL	Hydrochloric
H₂SO₄	sulfuric acid
NH₄OH	Ammonia solution
TLC	Thin layer chromatography
UV	Ultraviolet-Visible
GAE	Gallic acid equivalent
DPPH	2,2'-diphenyl-1-picrylhydrazyl
IC₅₀	Inhibitory concentration for 50% of activity
DMSO	Dimethyl sulfoxide
AI	Activity Index
InfE	infusion extract
DecE	decoction extract
SMeE	soxhlet methanol extract
SEtE	soxhlet ethanol extract
MMeE	maceration methanol extract
MEtE	maceration ethanol extract
Tol	Toluene
AcOEt	Ethyl acetate
MeOH	Methanol
CHCl₃	Chloroform
H₂O	Water
R²	correlation coefficient
IZ	Inhibition zone

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Introduction

Introduction

The therapeutic properties of various medicinal plants have been used for centuries to treat many human diseases. As estimated, between 60-90% of the population of developing countries use traditional herbal medicines considering them to be a normal part of primary healthcare (1). The demand of herbal medicine has been increasing day by day by consumers as they perceive these forms of healing as safe and effective than the synthetic drugs. This trend of using alternative and complimentary healthcare has prompted scientists to investigate the various biological activities of medicinal plants (2). These plant-derived products contain a great diversity of phytochemicals such as phenols, flavonoids, tannins and other phytoconstituents that possess numerous health related effects such as antibacterial, antidiabetic and anticarcinogenic properties.(3). *Stevia rebaudiana* is one such plant that contains such properties.

Stevia rebaudiana is a plant of the Asteraceae family and native to Paraguay. Its sweetness and calorie free property has increased its demand commercially all over the world as a sugar substitute in food beverages and medicines. Stevia has many pharmacological and therapeutic properties including antimicrobial, antifungal, antioxidant and anticarcinogenic activity. Due to this importance the leaf was analyzed for preliminary phytochemical studies to find the presence of phytoconstituents such as alkaloids, flavonoids, steroidal compounds, saponins, tannins, phenols.

The aim of this study was to present the phytochemical screening, and antioxidant and antibacterial potential of leaves from a new variety of *Stevia rebaudiana*. The concentration of these bioactives in different extracts prepare by different extraction methods was also carried out.

BIBLIOGRAPHIC SYNTHESIS

1. Plant profile

1.1. History of *Stevia rebaudiana*

Stevia rebaudiana, commonly known as the sweet leaf of Paraguay[®] is an herbaceous perennial plant of Paraguay. It is a small, semi-bushy, perennial shrub of the Asteraceae family originated in Paraguay (4). The plant grows for about 30 cm in height (Fig 1). Natives of Paraguay centuries ago used the leaves of the herb to sweeten their bitter drinks. Stevia is native to the valley of Rio Monday in highlands of North-eastern Paraguay in South America where it grows in sandy soils near streams on the edges of marshland, acid infertile sand or muck soils. It is one of the 154 members of the genus *Stevia*, which produces sweet stevioside, diterpenoid glycoside in the leaves of this plant (5). It was first brought to attention of Europeans in 1887 when M.S Bertoni learned of its unique properties from the Paraguayan Indians and Mestizos (6). Dating back to the Guarani Indians of South America this plant has been used to sweeten tea for centuries. Its extracts are used today as a food additive by the Japanese and Brazilian and as a non-caloric sweetener but in the US it has been used as a supplement only (7).

1.2. Distribution

Stevia rebaudiana has been grown on an experimental basis in Ontario, Canada since 1987 to determine the feasibility of commercial cultivation. Duke University researchers developed a strategic plan to assist farmers and exporters in Paraguay to compete in the global market for stevia. Today, *Stevia rebaudiana* is cultivated and used to sweeten food elsewhere in East Asia including China (since 1984), Korea, Taiwan, Thailand, and Malaysia. It can also be found in Saint Kitts and Nevis, Brazil, Colombia, Peru, Paraguay, Uruguay, and Palestine.



Figure 1: Zone of distribution of *Stevia rebaudiana*.

1.3. Botanical description

This perennial semi-shrub grows up to 30 cm in height. Its leaves are sessile, 3-4 cm long, elongate-lanceolate or spatulate shape with blunt tipped lamina, serrate margin from the middle to the top and entire below. The upper surface of the leaf is slightly glandular pubescent. The stem is weak pubescent at bottom and woody. The rhizome has slightly branching roots. Flowers of this plant are composite surrounded by an involucre of epicalyx. The capitulate are in loose, irregular sympodial cymes. Flowers are purple colored and the fruits of this plant are generally five-ribbed spindle-shaped achene (7).



Figure 2: *Stevia rebaudiana*.

1.4. Scientific classification

Kingdom: Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order:Asterales

Family:Asteraceae

Genus: Stevia

Species: rebaudiana

Binomial name: *Stevia rebaudiana* (Bertoni) Bertoni

1.5. Chemical constituents of *Stevia*

The presence of biologically important secondary plant products in *Stevia* leaf contributes to its medicinal value, since they exhibit physiological activity (8-13). These secondary plant constituents include flavonoids, sterols, triterpenoids, chlorophylls, organic acids, mono-disaccharides, and inorganic salts (14-15). *S. rebaudiana* is rich in terpenes and flavonoids, volatile oil components, pigments, gums and inorganic constituents. The phytochemicals present in *S. rebaudiana* are austroinullin, b-carotene, nilacin, riboflavin and thiamine (9-12).

In addition to these compounds, *Stevia* extracts were also reported to contain the natural sweeteners, steviol glycosides, are diterpenes, isolated and identified as stevioside, steviolbioside, rebaudioside A, B, C, D, E, F and dulcoside (10-12). Stevioside was reported to be the most abundant stevia glycoside (4–13% w/w) found in the plant leaves. It is followed by rebaudioside A (2–4% w/w), rebaudioside C (1–2% w/w) and dulcoside A (0.4–0.7% w/w) (11). Steviolbioside, rebaudioside B, D, E and F were also identified in the leaf extracts, but as minor constituents (12).

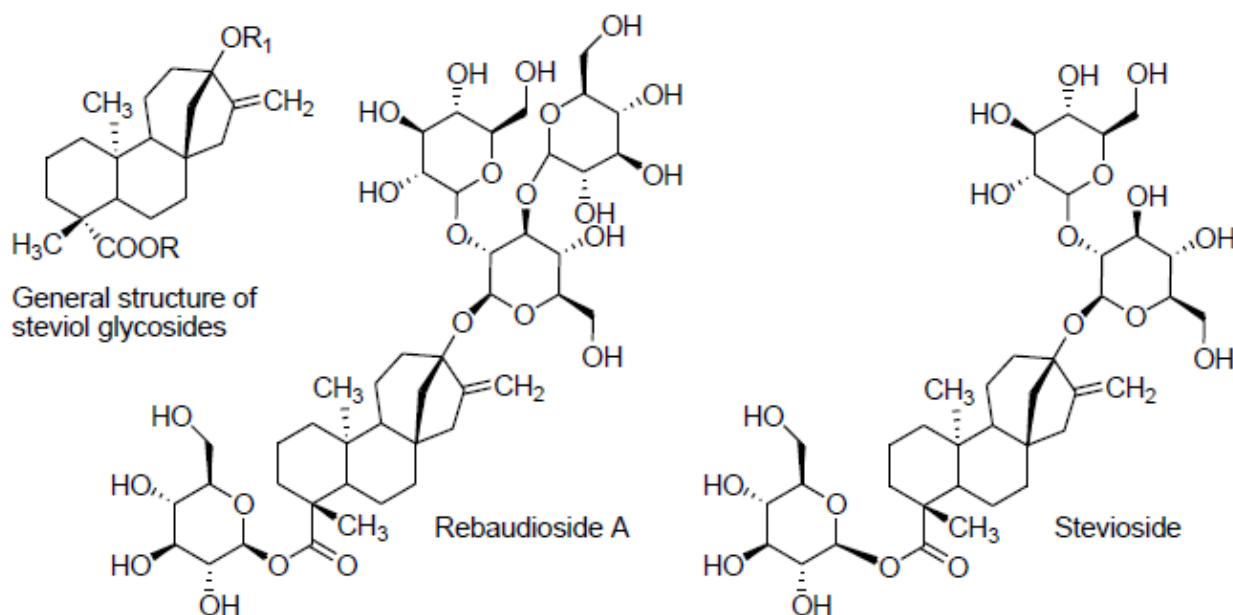


Fig3: General structure of steviol glycoside and representative structure of rebaudioside A.

1.6. Therapeutic values of *Stevia rebaudiana*

The ancient medicinal system of medicine has a long history regarding the use of *S. rebaudiana* (16). Leaves of *S. rebaudiana* has been recommended as a treatment against various chronic and non-chronic diseases like diabetes, cardiovascular disease, cancer, renal disease, obesity, inflammatory bowel disease and dental caries. Stevia leaf extract has been used traditionally in the treatment of diabetes (16-5). Their ingestion causes a slight suppression of plasma glucose levels and significantly increased glucose tolerance in normal adult humans (17).

The effects of *Stevia* leaves and its extracted polyphenols and fiber on streptozotocin induced diabetic rats were studied besides its hypoglycemic effect and it also reduce the risk of oxidative stress (18). Overall, *Stevia* possess the ability to increase the insulin effect on cell membranes, increase insulin production, stabilize glucagon secretion and blood sugar levels, and improve glucose tolerance to ingested carbohydrates and lower post-prandial blood sugar levels in both animals and humans (19). Studies have demonstrated the inhibitory effects of *Stevia* leaf extracts and their polyphenolic constituents on tumor promotion and initiation (20).

1.7. Industrial applications

Stevia sweeteners, extracts from the leaves of this herb, are commercially available in Japan, Korea, China, South-East Asia and South America, where they have been used for some decades to sweeten a variety of foods (10). In these countries stevioside is being used to sweeten foodstuffs and beverages. In the USA powdered *Stevia* leaves and their extracts are used only as a dietary supplement and a skin care product, but not as a sweetener.

The steviol glycosides are currently in use as a sweetener in a number of industrial foods, such as soft drinks or fruit drinks (22-9-23), desserts, cold confectionery, sauces, delicacies, sweet corn, breads, biscuits, table-top sweetener. They replace saccharose, for example in ready-to-eat cereals (23), pickles (10), yoghurt (15-24), candies (22-10), soju, soy sauce (15-24) and sea foods (22-10).

2. Phytochemicals in medicinal plants

2.1. Introduction

Plants produce a vast and diverse assortment of organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances, traditionally referred to as **secondary metabolites**, often are differentially distributed among limited taxonomic groups within the plant kingdom. Their functions, many of which remain unknown, are being elucidated with increasing frequency. The **primary metabolites**, in contrast, such as phytosterols, lipids, nucleotides, amino acids, and organic acids, are found in all plants and perform metabolic roles that are essential and usually evident (25). Secondary plant metabolites (Phytochemicals) have been extensively investigated as a source of medicinal agents (24).

2.2. Classification

Secondary metabolites can be classified on the basis of chemical structure (for example, having rings, containing a sugar), composition (containing nitrogen or not), their solubility in various solvents, or the pathway by which they are synthesized. Three large molecule families are generally considered: Phenolics, Terpenes and Steroids, and Alkaloids. Some of them can have severe consequences (26).

2.2.1. Polyphenols

Phenolics are characterized by having at least one aromatic ring with one or more hydroxyl groups attached. In excess of 8000 phenolic structures have been reported and they are widely dispersed throughout the plant kingdom, are widespread groups of substances in flowering plants, occurring in all vegetative organs, as well as in flowers and fruits, vegetables, cereals, grains, seeds and drinks. Phenolics range from simple, low molecular-weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols. They can be classified based on the number and arrangement of their carbon atoms and are commonly found conjugated

to sugars and organic acids. Phenolics can be classified into two groups: Tannins and flavonoids (27).

2.2.1.1. Tannins

Tannin is astringent vegetable product found in a wide range of plants parts ranging from the barks, roots, seeds, fruits, leaves, galls and roots (28). They occur naturally in plants and are water soluble phenolic compounds of the higher molecular weight of about 500 - 3000 containing phenolic hydroxyl groups that make them to effectively cross-link with proteins and other macromolecules (28).

They are divided into two groups, hydrolyzable and condensed tannins. Hydrolyzable tannins are based on gallic acid, usually as multiple esters with D-glucose, while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers (Cowan, 1999).

Tannins are generally found in plants and they are thought to function as chemical defenses against pathogens and herbivores (29). They have been commercially used primarily in the preservation of leather, making glue stains and mordant (30). It has also been used in the vegetable industry in different concentration in pickling process to provide protection against bacteria, mold, and yeasts (31).

Tannins have been found not only effective against pathogenic microbes but also have a significant value as a cytotoxic and an antitumor agent (32). Studies carried out have shown that the carcinogenic activity associated with tannin consumption might be related to other components associated with tannins. This anticarcinogenic potential of tannins was related to their antioxidative properties and protection against cellular peroxidation damage and inhibition of generation of superoxide radicals (33).

2.2.1.2. Flavonoids

Flavonoids are secondary metabolites of plants that chemically have a general structure of 15 carbon skeleton consisting of two phenyl rings and a heterocyclic ring (34). There are over 500 groups of flavonoids that have been characterized from various plants according to their chemical structure (35). They are usually subdivided into flavanones, flavanols, flavans, and anthocyanidin (36). In plants they are responsible for floral pigmentation, ultraviolet ray's filtration in higher plants and symbiotic nitrogen fixation (37). They are also known to have inhibitory activities against organisms that cause plant diseases for example *Fusariumoxysporum* (37).

Flavonoids and their derivatives are considered as therapeutic agents in studies focused on degenerative diseases and brain aging processes, and serve as possible neuroprotective agents in progressive neurodegenerative disorders such as Parkinson's and Alzheimer's diseases. High flavonoid intakes lead a decrease in LDL oxidation (38). Flavonoids have been known to possess antimicrobial activity against bacterial, fungal and viral microorganisms (39). They are usually known for their antimicrobial activity of inhibiting the synthesis of the nucleic acids, tampering with the integrity of the cytoplasmic membrane function and the energy metabolism process (40).

2.2.2. Alkaloids

They are a group of naturally occurring compounds that contain nitrogen and can be neutral or have weakly acidic properties (34). They may also sometimes contain oxygen more rarely other elements such as chlorine, bromine, and phosphorus (41). They are mainly secondary metabolites of plants but can also be produced by a variety of organisms including bacteria, fungi, and animals (42). They dissolve in water poorly but readily dissolve in organic solvents. They are divided into five major groups namely: true alkaloids (contain nitrogen in heterocyclic and originate from amino acids), proto alkaloids, polyamine alkaloids, peptide and cyclopeptidesalkaloids and pseudoalkaloids (43). They have a wide range of pharmacological activities such as antiasthma, antimalarial, anticancer, cholinomimetic, vasodilatory, antiamyhyrithic, analgesic, antibacterial and antihyperglycemic activities (44). Some alkaloids

have been known to possess psychotropic and stimulant activities and have been used as recreational drugs and entheogenic rituals (45). Alkaloids have great antimicrobial activity against bacterial pathogens such as *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus* and *Pseudomonas aureginosa*(46).

Some of the bioactive components of alkaloids such as morphine and cordine have been found to be active not only against bacterial and fungal pathogens but also trypanosomes and plasmodia (47-48).

2.2.3. Saponins

They are a class of chemical compounds found in various plant species and they are amphipathic glycoside grouped structurally by having one or more hydrophilic glycosides moieties combined with lipophilic triterpene. In plants, saponins are known to provide protection against microbes and fungi (49). Saponins have been used by a wide range of commercial therapeutic claims for natural products whereby in organism or human benefit are often based on preliminary biochemical and cell biology studies (50).

There has been evidence of the presence of saponins in traditional medicine preparations where the administration is through oral means that is expected to lead to the hydrolysis of glycosides from terpenoids (51). Studies carried out have shown medicinal plant extracts fractions rich in saponins are effective against microorganisms such as *Escherichia coli*, *Salmonella typhi*, *Aeromonashydrophilia* and other fungal pathogens such as *Candida albicans*(52). Saponins antimicrobial activity is attributed mainly to its capability of lysing microorganism's membranes rather than the surface tension of the extracellular medium. Apart from antimicrobial activity, saponins have shown other biological properties with its cytotoxic activity on cancer or tumor cells being considered the most important one (53).

2.2.4. Terpenoids

The terpenoids are a class of natural products which have been derived from five-carbon isoprene units. Most of the terpenoids have multi cyclic structures that differ from one another by

their functional groups and basic carbon skeletons. These types of natural lipids can be found in every class of living things, and therefore considered as the largest group of natural products (54). Many of the terpenoids are commercially interesting because of their use as flavours and fragrances in foods and cosmetics examples menthol and sclareol or because they are important for the quality of agricultural products, such as the flavour of fruits and the fragrance of flowers like linalool(55). Terpenes are widespread in nature, mainly in plants as constituents of essential oils. Their building block is the hydrocarbon isoprene, $\text{CH}_2=\text{C}(\text{CH}_3)-\text{CH}=\text{CH}_2$. Terpene hydrocarbons therefore have molecular formula $(\text{C}_5\text{H}_8)_n$ and they are classified according to the number of isoprene units (56).

2.2.5. Steroids

Steroids are a group of cholesterol derived lipophilic, low-molecular weight compounds found in derived from a variety of different marine, terrestrial, and synthetic sources. Steroid family includes the sterols,, bile acids (57-58), a number of hormones both gonadal and adrenal cortex hormones) and some hydrocarbons (59). All steroid classes and their metabolites play important roles in the physiology and biochemistry of living organisms in which these are found. A number of synthetic steroids are being extensively used as anti-hormones (60), contraceptive drugs (61), anti-cancer agents (62) cardiovascular agents (63), osteoporosis drugs (64) anti-biotics, anesthetics, anti-inflammatories and anti-asthmatics (65). All steroids are derived from cholesterol and therefore different classes of steroids resemble closely since all of them have the same basic perhydro-1,2-cyclopentenophenanthrene skeleton 1 and a slight variation in this skeleton or the introduction of functional groups result in various classes of steroids (66).

3. Biological activity

3.1. Antimicrobial activity

There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action due to an alarming increase in the incidence of new and re-emerging infectious diseases and development of resistance to the antibiotics in current clinical use (39). The screening of plant extracts has been of great interest to scientists in the search for new drugs for effective treatment of several diseases. Therefore, plant extracts and phytochemicals with known antimicrobial properties can be of great significance in therapeutic treatments (9). The results of an investigation performed in the late 19th and 20th century and the advent of streptomycin and other antibiotics provide the ground for experimentation of a vast number of plants for antibiotic or antimicrobial activities that are useful to man (67).

Many plant leaves have antimicrobial principles such as tannins, essential oils and other aromatic compounds. In addition, many biological activities and antibacterial effects have been reported for plant tannins and flavonoids. Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. These compounds protect the plant from microbial infection and deterioration. Some of these phytochemicals can significantly reduce the risk of cancer due to polyphenol antioxidant and anti-inflammatory effects. Some preclinical studies suggest that phytochemicals can prevent colorectal cancer and other cancers (9). Stevia is thought to inhibit the growth of certain bacteria and other infectious organisms. Some people even claim that using Stevia helps to prevent the onset of colds and flu. The ability of Stevia to inhibit growth of certain bacteria helps to explain its traditional use in treating wounds, sores and gum disease. It may also explain why the herb is advocated for anyone who is susceptible to yeast infections or reoccurring streptococcal infections, two conditions that seem to be aggravated by white sugar consumption (68).

3.2. Antioxidant activity

3.2.1. Free radicals

Free radicals, namely reactive oxygen species (ROS) and reactive nitrogen species (RNS), The ROS and RNS include diverse reactive entities namely superoxide O_2^- , hydroxyl (OH \cdot), peroxy (ROO \cdot), peroxynitrite (ONOO) and nitric oxide (NO \cdot) radicals as well as non free radical species as hydrogen peroxide (H₂O₂), nitrous acid (HNO₂) and hydrochlorous acid (HOCl) (69, 70).

3.2.1.1. Production of free radicals in the human body

Free radicals and other ROS are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants and industrial chemicals (71). Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non enzymatic reactions. Enzymatic reactions, which serve as source of free radicals, include those involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis and in the cytochrome P-450 system. Free radicals can also be formed in non enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing reactions.

3.2.1.2. Beneficial effects of free radical

ROS and RNS are produced in a well regulated manner to help maintain homeostasis at the cellular level in the normal healthy tissue and play an important role as signalling molecules. Most of cells can produce superoxide, hydrogen peroxide and nitric oxide (72).

3.2.1.3. Harmful effect of free radical

Free radicals, ROS and RNS cause irreversible damage to biomolecules such as fatty acid, amino acid, and DNA. Oxidative modification / damage of these molecules lead to the onset of several degenerative diseases including inflammation, cancer, atherosclerosis, diabetes, liver

injury, Alzheimer, Parkinson, and coronary heart pathologies are due to these free radicals and oxidative stress. Several evidences indicate that oxidative stress can lead to cell and tissue injury

3.2.2. Antioxidants

Interestingly, aerobic organism developed antioxidant defense mechanisms that arrest the damage caused by ROS and RNS entities. The defense mechanism can be both enzymatic and non enzymatic. In the enzymatic mechanisms, several enzymes such as superoxide dismutase, catalase, glutathione reductase, peroxidase and nitric oxide synthase are involved. Whereas non enzymatic mechanisms are restricted to antioxidants and trapping agents such as ascorbic acid, α – tocopherol, β – carotene, glutathione, flavonoids, uric acid, cysteine, vitamin – K, serum albumin, bilirubin, and trace elements such as zinc and selenium (69).

Besides natural antioxidants, some synthetic antioxidants are also reported such as, butylatedhydroxytoluene and butylatedhydroxyanisole. But these synthetic antioxidants possess greater risks of side effects; therefore, investigations on identifying the natural antioxidants have become very important issue (70). In the past few years, natural antioxidants have generated considerable interest in preventive medicine (73). Plants produce a huge amount of antioxidants and they can represent a potential source of new compounds having antioxidant properties (69).

MATERIALS AND METHODS

1. Plant sample collection

A total of 200 g of powdered *Stevia rebaudiana* leaves was collected from GHARDAIA, Algeria. The samples leaves were finely powdered without any impurities mixed in them. After collection they were stored at the laboratory.

2. Preparation of extracts

A total of five types of extracts using three different solvents ethanol, methanol and water were collected from the samples of *Stevia rebaudiana*

2.1. Extraction of phenolic compounds by soxhlet

For collection of ethanolic and methanolic extracts 30g of stevia powder was packed in thimble and extracted in Soxhlet apparatus using 300ml of ethanol 96% (Fig 4). The temperature was kept between 40-60°C. The samples in the thimble of the soxhlet apparatus was kept boiling for approximately 4 hours till the solution becomes clear and the dark colored extract was collected at the bottom of the apparatus. The extract was filtered and dried in a rotary evaporator (Heidolph) at 42°C under vacuum. This was then collected in petri dishes and left to dry for 24 hours. The dried extract having a sticky appearance was stored in 25 ml McCartney bottles at temperatures at 4 °C in the refrigerator for further use.

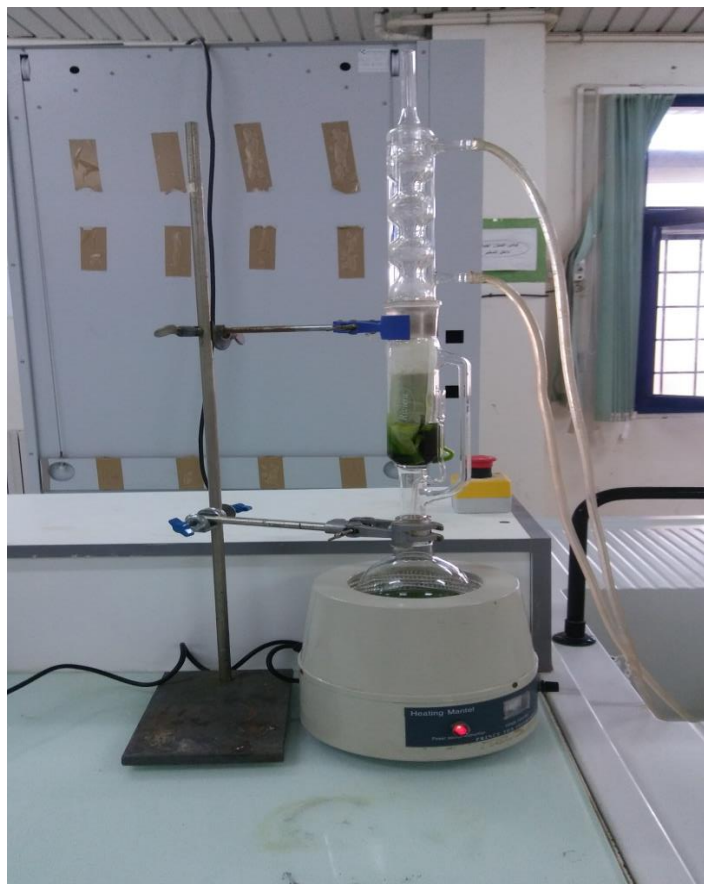


Fig4: Extract collection through Soxhlet Apparatus

2.2. Extraction of phenolic compounds by maceration

The extraction procedure for phenolic compounds was conducted as described (74). The dried plant material (40 g) was ground to a coarse powder and soaked in 1200 ml of methanol-water and ethanol-water solution (80%). The solution was agitated and kept for 24h in the dark at room temperature, to obtain the first fraction. The same procedure was repeated by using 600 ml of ethanol or methanol 80%. The extracts was filtered and dried in a rotary evaporator (Heidolph) at 42°C under vacuum. This was then collected in petri dishes and left to dry for 24 hours the. The dried extract was stored at temperatures at 4 °C in the refrigerator for further use.

2.3. Decoction

Ten g of *Stevia rebaudiana* powder was measured and mixed with 100 ml of distilled water and boiled for 1 hour. The liquid extract was then cooled, filtered and dried in the oven at 40 ° C (ref).

2.4. Infusion

In this process the collection of aqueous extract using 10g of stevia powder placed in 100 ml of boiling water for 3 hours. The liquid extract was then filtered and dried in the oven at 40 ° C (ref).

3. Phytochemical screening

Preliminary screening of all for extracts was done for testing various phytochemicals found in plants. The extracts were tested for the presence or absence of secondary metabolites such as alkaloids, steroidal, flavonoids, tannins and saponins compounds.

These tests were performed according to the method described by Sofowora (1985) and Trease (2002). The tests were based on the extraction with suitable solvents of increasing polarity and the visual observation of color change or formation of a precipitate after the addition of specific reagents.

3.1. Tests for alkaloids (Dragendraff's Test)

0.2 ml of extract was taken in a test tube with 1.5ml dilute HCL 2% and 1 ml of Dragendraff's reagent and left for a few mins. A positive result is indicated by the presence of an orange brown precipitate.

3.2. Test for steroidal compounds (Salkowaski's test)

0.5g of extracts was dissolved in acetic anhydride and then 2ml 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 color at the interface indicated the presence of a steroid ring.

3.3. Test for Tannins

1 ml of extract was added in 2 ml of water in a test tube. 2 to 3 drops of diluted ferric chloride (1%) solution was added and observed for green to blue-green (Cathechic tannins) or a blue-black (Gallic tannins) coloration.

3.4. Test for flavonoids

Test for anthocyanins

To the 5% infused with a more or less dark coloration, 5 ml of 10% H₂SO₄ are added, followed by 1 ml of 10% NH₄OH. If the color increases with acidification, then turns to purplish blue in basic medium.

Free flavonoids

Introduced in a test tube 5 ml of extract, and added 5 ml of hydrochloric alcohol (95% alcohol, distilled water: concentrated HCl) in equal parts, then a few chips of magnesium and 1 ml of isoamyl alcohol. The appearance of a pink-orange (flavones) or purplish pink (flavonones) or red (flavonols, flavononols) indicates the presence of a free flavonoid (genin).

3.5. Test for saponins

0.5g of each type of extracts was dissolved in 10ml distilled water. The test tube was stoppered and then shaken vigorously for 30 secs. It was then allowed to stand for 15 mins. Froth above the surface that stays after 15 minutes is taken as a positive result.

4 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is a chromatographic technique used to separate the components of a mixture using a thin stationary phase supported by an inert backing. TLC functions on the same principle as all chromatography: a compound will have different affinities for the mobile and stationary phases, and this affects the speed at which it migrates. The goal of TLC is to obtain well defined, well separated spots. Then remove the plates and allow them to dry. The sample spots can now be seen in a suitable UV light chamber.

The extracts were screened on silica gel plates (0.25 mm thickness). Development was carried out with various solvent systems: Tol/AcOEt/MeOH (80/18/2), CHCl₃/MeOH (90/10) To/acetone/ Formic acid (3/5/1), AcOEt/MeOH/H₂O(100/13.5/1).

After development in the different solvents, the detection was carried out visually in visible light and under UV light (365 nm).

5. Determination of total polyphenols content in plant extracts

Total polyphenols content was determined spectrophotometrically, using the Folin–Ciocalteu reagent according to the method described by Boumerfeg and Baghiani (75-76). This method is based on the reduction of a phosphowolframate– phosphomolybdate complex by phenolics to blue reaction products. This reaction occurring under alkaline conditions is carried out with sodium carbonate. Blue coloration is measured at 765 nm and reflects the quantity of polyphenols usually expressed as gallic acid equivalent (GAE).

Briefly, a volume of 200 µl of the extract was mixed with 1000 µl of Folin–Ciocalteu reagent (diluted 10% in distilled water). After 4 min, 800 µl of sodium carbonate solution Na₂CO₃ (75 g/l) was added to the mixture, the reaction mixture was incubated at room temperature for 1h 30 min and the absorbance of the mixture was read at 765 nm, Gallic acid (0-160 µg/ml) was used as standard for the calibration curve. The total polyphenols content was expressed as microgram of gallic acid equivalents (GAE) per milligram of extract (µg GAE/mg of extract). All samples were analyzed in three replications.

6. DPPH free radical-scavenging activity of plant extracts

The molecule, 2, 2 – Diphenyl – picrylhydrazyl (DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecule do not dimerise, as would be the case with most other free radicals. The delocalization gives rise to the deep violet colour, characterized by an absorption band in methanol solution centered at about 517 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives reduced form with the loss of the violet colour. The pale colour may appear at the end due to still presence of pecryl residue (74).

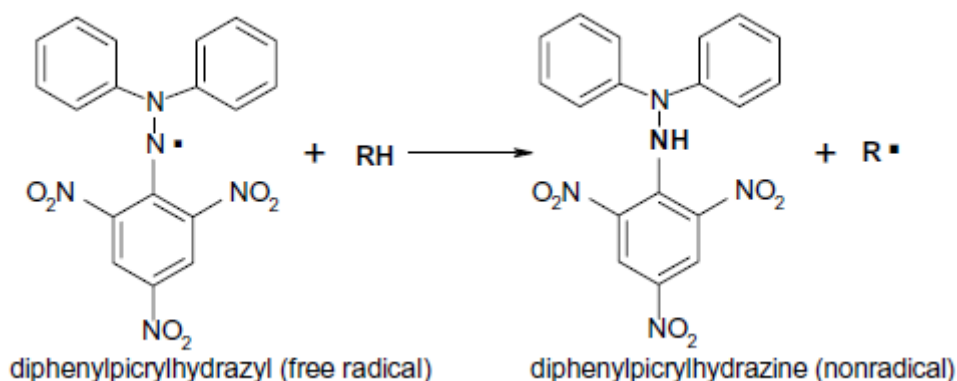


Figure5: Structure of DPPH and its reduction by the antioxidant RH.

After dissolving the plant extracts, the solution of DPPH in methanol (0.04 mg/mL) was prepared and 2500 μ L of this solution was added to 100 μ L of extracts solution at different concentration. The mixture was shaken vigorously and then kept in the dark for 30 minutes at room temperature. Then, the absorbance was measured at 517 nm in a spectrophotometer. All tests were performed in triplicate (77). Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula I %.

$$I (\%) = \{(A \text{ blank} - A \text{ sample}) / A \text{ blank}\} \times 100.$$

A blank: Absorbance of the control.

A sample: Absorbance of the reagent with extract.

The scavenging ability of the extracts was expressed as IC₅₀ value, which is the concentration of substrate that causes 50% loss of DPPH activity (color). Low IC₅₀ value indicates strong ability of the extract to act as DPPH scavenger. The three standards antioxidants were using rutin, quercetin and gallic acid.

7. Antibacterial activity test

To test the antibacterial property of the sample well diffusion method was used. A positive and negative control was used to compare the results.

7.1. Test organisms

Staphylococcus aureus

Escherichia coli

7.2. Preparation of extract solution for antibacterial activity test

All for types of the dry extracts (100 mg) of *Stevia rebaudiana* that were collected were dissolved in DMSO to make extract solutions for the antibacterial activity test. For inoculation during the test the stock solution of extract was freshly prepared.

7.3. Method of antibacterial activity test

In sterile Petri dishes previously poured by media (gelose), a bacterial suspension to be tested was deposited on the surface of the agar plates using a sterile swab. Under aseptic conditions and using a sterile forceps, disks of sterile 6 mm diameter filter paper impregnated with 15 µl of the various extracts (100 mg) were sterilely deposited on the surface of the media,

previously inoculated. The negative control discs are impregnated with distilled water and DMSO. Standard disks containing the reference antibiotic (gentamycin, 10 µg per disc) serve as positive controls. Petri dishes are incubated at 37 ° C for 24 hours (78).

7.4. Measuring zones

Following 24 hours of incubation of the test plates the clear zones were measured using a ruler. This was done by measuring the entire diameter of the clear zone and the results were recorded.

7.5. Measuring the activity index

The inhibitory effects of the methanolic, ehtanolic, infusion and decoction extracts were calculated and compared by measuring the activity index. This was done by using the following formula:

Activity Index (AI) = Zone of inhibition of extract/ Zone of inhibition of antibiotic

RESULTS AND DISCUSSION

1. Amount of extracts collected

The extraction was done using 3 different solvents; methanol, ethanol and water. Different amount of extracts were collected during the experiment. Table 1 shows the amount of extracts collected in grams after drying them in petri dishes.

Table 1: Amount of extracts collected from each experiment..

Extracts	Plant mass (g)	Extracts mass(g)	Yield (%)
<i>InfE</i>	10 g	1.2 g	12
<i>DecE</i>	10 g	1.7 g	17
<i>SMeE</i>	30 g	11.8 g	39.33
<i>SEtE</i>	30 g	9.3 g	31
<i>MMeE</i>	40 g	22.3 g	55.75
<i>MEtE</i>	40 g	19.5 g	48.75

The ethanolic and methanolic extracts gave the highest yields (55.75 and 48.75%), followed by the sohxlet methanolic and ethanolic extracts (39.33 and 31%). The water-extract show the lowest yields for both methods with 12% and 17% respectively for infusion and decoction. These substances represent used by traditional medicine because they uses only water as extraction solvent.

From these results we find that the alcoholic mixture gives the best extraction yields compared to aqueous mixtures. In most plants studied alchoolic extracts represent higher yields than aqueous extracts.

2. Phytochemical screening

The phytochemical assays were done to check the secondary metabolites present in the sample of *stevia rebaudiana* that was collected. A total of seven phytochemical tests were performed to see the presence of alkaloids, steroids, flavonoids, phenols, saponins, tannins and cardiac glycosides.

The phytochemical characteristics of this medicinal plant investigated are summarized in Table 1 and 2 and fig 3.

Table2: Results of characterization reactions of the main secondary metabolites contained in *stevia rebaudiana* leaves.

compounds		Results	
alkaloids		-	
Tannins	Globales	++	++
	Cathechic	++	++
	Gallic	+++	+++
flavonoids	free	++	++
	anthocyanins	++	++
Steroids and triterpenes		++	
Saponins		+	
Oses		++	
Mucilage		-	
Coumarin			

Table3 : Results of the characterization reactions of the main secondary metabolites contained in the extracts of stevia rebaudiana.

compounds		Results					
		EInf	EDéc	EMMe	EMEt	ESMe	ESEt
alkaloids		-	-	-	-	-	-
Tannins	Globales	++	++	++	++	++	++
	Cathechic	++	++	++	++	++	++
	Gallic	++	++	+++	+++	+++	+++
flavonoids	free	++	++	+++	+++	+++	+++
	anthocyanins	++	++	++	++	++	++
Steroids		++	++	++	++	++	++
Saponnins		+	+	+	+	+	+
Oses		++	++	++	++	++	++
Mucilage		-	-	-	-	-	-
Coumarin							

Negative result (-) and Positive result(+)

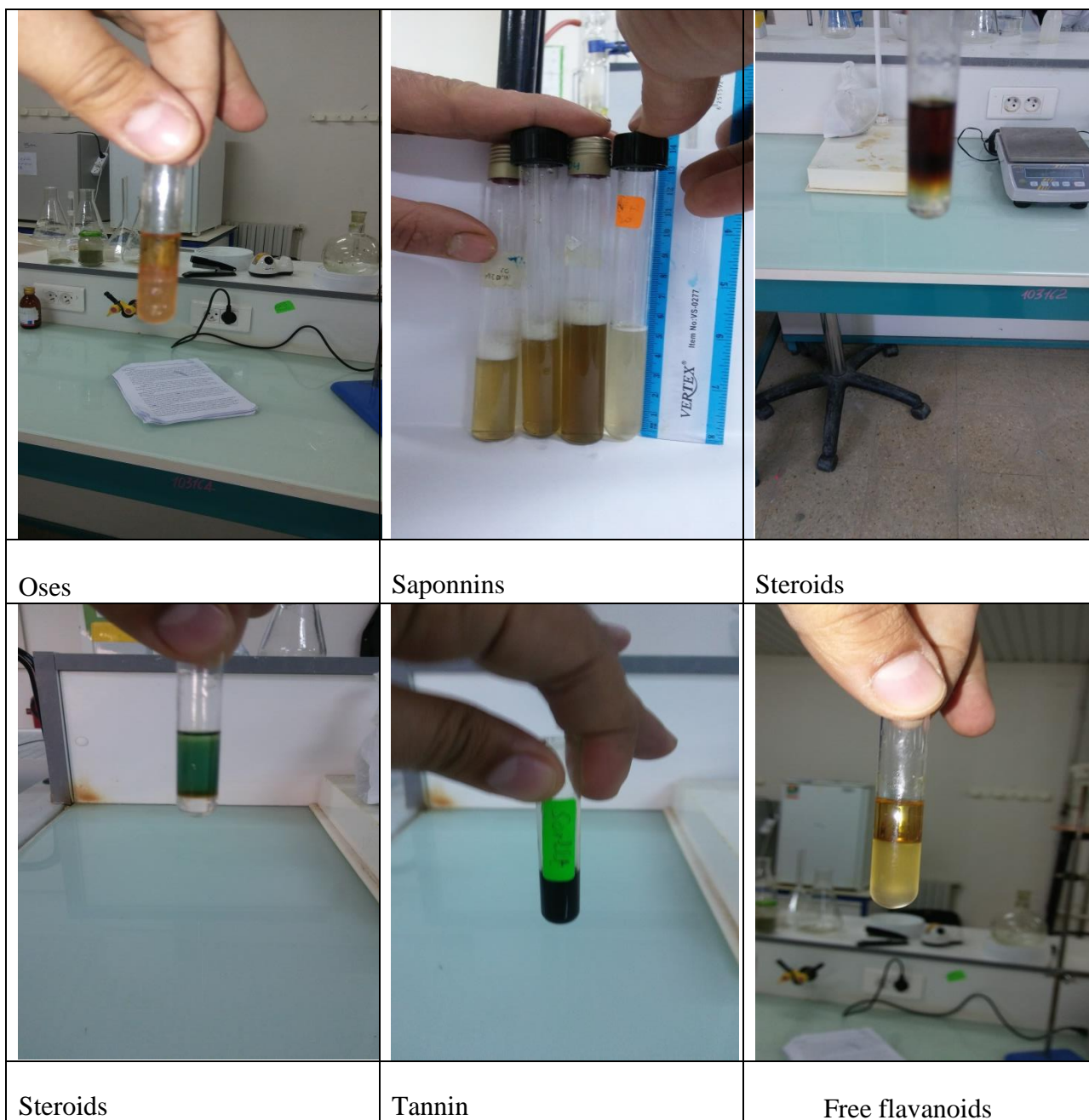


Fig6: The results images of the characterization reactions of the main secondary metabolites contained in stevia rebuadiana extracts

The results reveal the presence of medicinally active constituents like tannins, flavonoids, saponins, glycosides and steroids in the various extract of plant. These metabolites have been shown to be responsible for therapeutic activity of plants (79). Also plants containing these metabolites usually demonstrate stronger antioxidant and antimicrobial properties than others. Flavonoids, the major group of phenolic compounds are reported for their antimicrobial, antiviral and spasmolytic activity. Flavonoids are able to scavenge hydroxyl radicals, superoxide anion radicals and lipid peroxy radicals, which highlights many of the flavonoid health-promoting functions in organism (80-81). Tannins have been reported to inhibit growth of microorganisms by precipitating microbial pattern and making nutritional proteins unavailable for them (82-83). Tannins have been found to have antiviral, antibacterial, antiparasitic effects, anti-inflammatory, antiulcer and antioxidant property for possible therapeutic applications (84-85).

While mucilage, coumarin and alkaloids were absent in this plant.

3. Thin Layer Chromatography (TLC)

The analysis results of the various extracts obtained from the leaves of *stevia rebaudiana* by TLC are presented in fig 7. Solvent systems used to separate several compounds in the extracts (Table 4). This is the information on their behavior with UV light (365 nm). Examination under ultraviolet light provides very important information on the structure of molecules. It provides particular indications concerning substitutions. The following table summarizes the relationship between the structure of a flavonic compound and its fluorescence under UV (table 4).

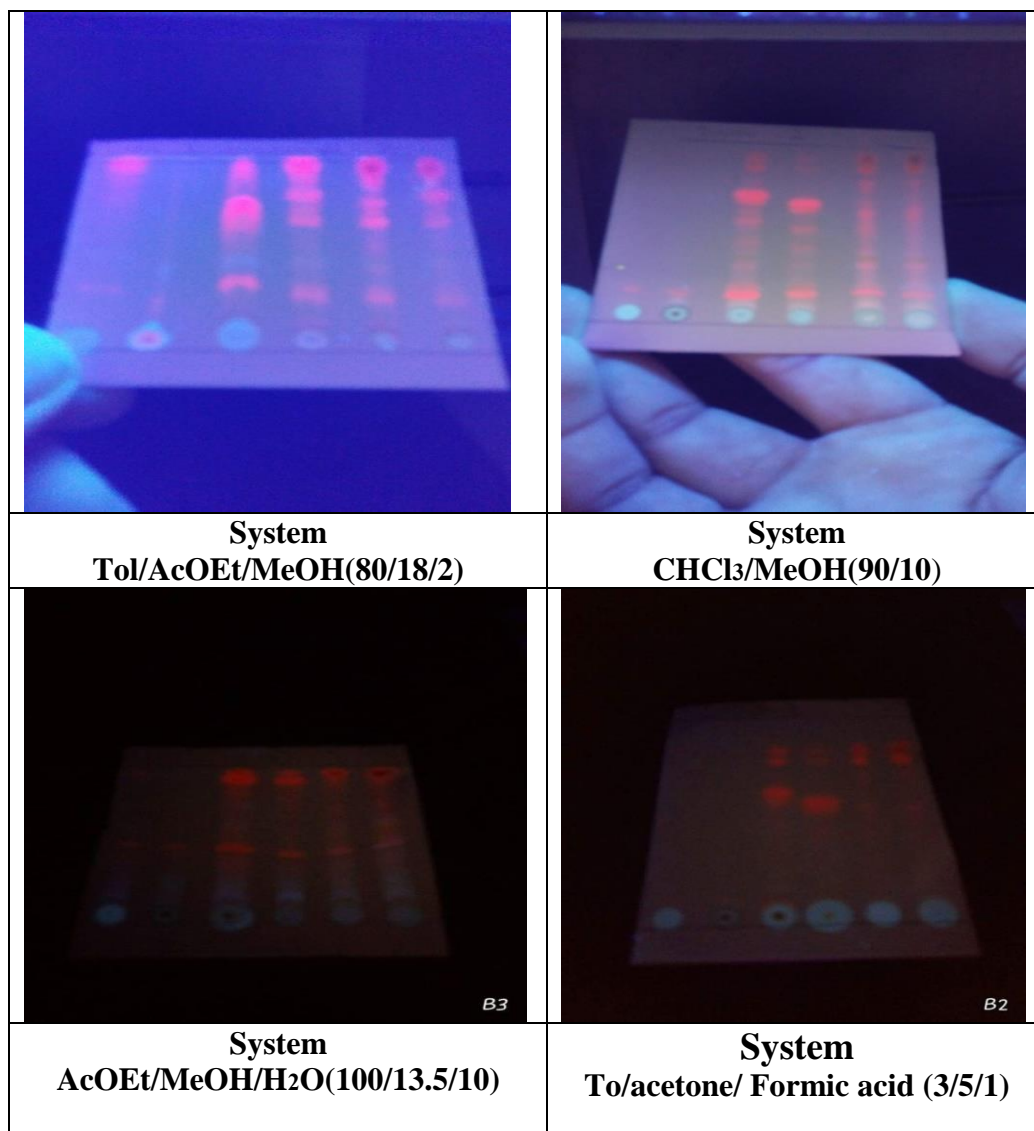


Fig7: Chromatograms observed under UV at 365 nm extracts.

Table4: Results of thin layer chromatography of the extracts.

Extracts	System 1 Tol/AcOEt/MeOH (80/18/2)		System 2 CHCl ₃ /MeOH (90/10)	
	Couleur	Interpretation	Couleur	Interpretation
<i>Infusion</i>	Bleu Red	Flavon Flavonol Anthocyanidin	Bleu Red	Flavon Flavonol Anthocyanidine
<i>Decoction</i>	Bleu Red	Flavon Flavonol Anthocyanidin	Bleu Yellow Red	Flavon Flavonol Free flavonol Anthocyanidin
<i>EMMe</i>	Blue Red Purple	Flavon Flavonol Anthocyanidin Dihydroflavanol Isoflavon Chalcons flavanon	Bleu Yellow Red	Flavon Flavonol Free flavonol Anthocyanidin
<i>EMEt</i>	Blue Red	Flavon Flavonol Anthocyanidin	Bleu Yellow Red	Flavon Flavonol Free flavonol Anthocyanidine
<i>ESMe</i>	Blue Red purple	Flavon Flavonol Anthocyanidin Dihydroflavanol Isoflavon Chalcons flavanon	Bleu Yellow Red	Flavon Flavonol Free flavonol Anthocyanidin
<i>ESEt</i>	Blue Red purple	Flavon Flavonol Anthocyanidin Dihydroflavanol Isoflavon Chalcons flavanon	Bleu Yellow Red	Flavone Flavonol Free flavonol Anthocyanidin

Table 5: Results of thin layer chromatography of the extracts.

Extracts	System 3 AcOEt/MeOH/H ₂ O(100/13.5/1)		System 4 To/acetone/ Formic acid (3/5/1)	
	Couleur	Interpretation	Couleur	Interprétation
<i>Infusion</i>	Bleu Red	Flavon Flavonol Anthocyanidin	Bleu	Flavon Flavonol
<i>Decoction</i>	Bleu Red	Flavone Flavonol Anthocyanidin	Bleu	Flavon Flavonol
<i>EMMe</i>	Blue Red purple	Flavon Flavonol Anthocyanidin Dihydroflavanol Isoflavon Chalcons Flavanon	Bleu Red	Flavon Flavonol Anthocyanidin
<i>EMEt</i>	Blue Red purple	Flavon Flavonol Anthocyanidin Dihydroflavanol Isoflavon Chalcons Flavanon	Bleu Red	Flavon Flavonol Anthocyanidin
<i>ESMe</i>	Blue Red purple	Flavon Flavonol Anthocyanidin Dihydroflavanol Isoflavon Chalcons flavanon	Bleu Red	Flavon Flavonol Anthocyanidin
<i>ESEt</i>	Blue Red purple	Flavon Flavonol Anthocyanidin Dihydroflavanol Isoflavon Chalcons Flavanon	Bleu Red	Flavon Flavonol Anthocyanidin

The chemical screening on TLC plates made it possible to demonstrate the presence of several flavonoids in all the extracts studied.

4. Total phenolic content assay

The evaluation of total phenolic content of different extracts of plant is value in equivalent terms of Gallic, different concentrations of the standards (20 to 160 $\mu\text{g}/\text{mL}$) were plotted versus absorbance. The equation obtained from the linear calibration graph in the studied concentration range for Gallic acid is, $y=0.006x+0.003$, with a correlation coefficient of $R^2=0.986$ (Fig8).

Table 5: Standard curve preparation by using Gallicacid.

Gallic acid[$\mu\text{g}/\text{ml}$]	Absorbance
20	0.096
30	0.185
40	0.242
60	0.444
70	0.516
80	0.591
100	0.78
120	0.895
140	0.99
160	1.075

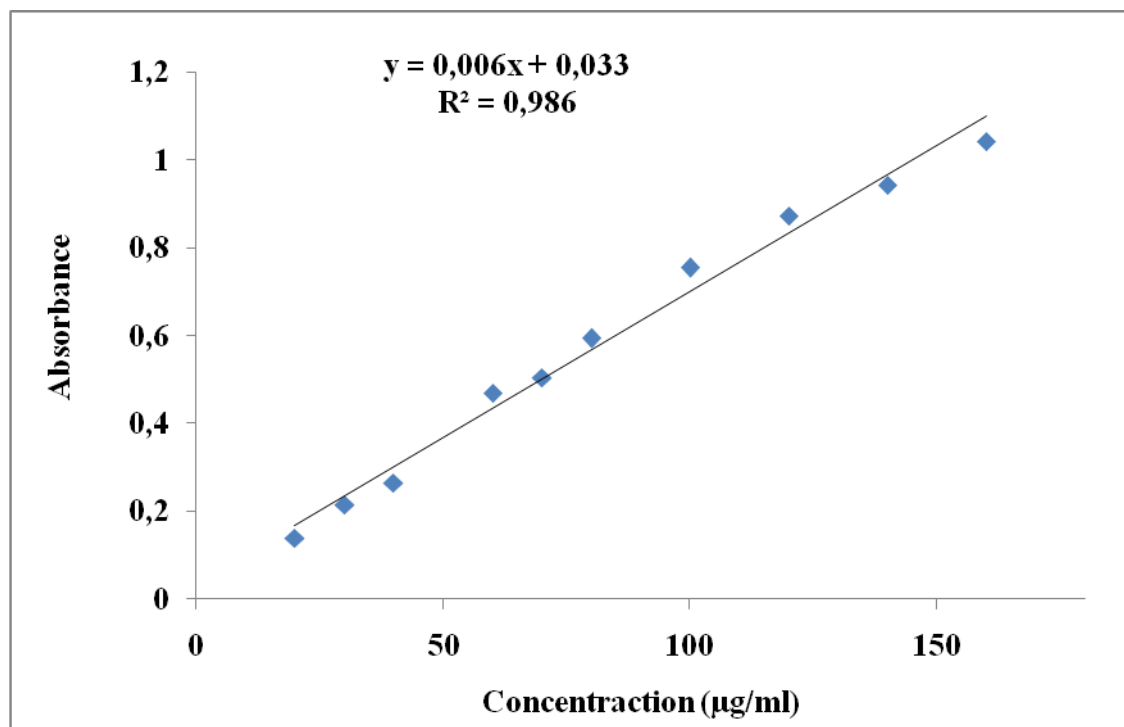


Figure 8: The calibration curve obtained.

The results for total polyphenol content of *stevia rebaudiana* are presented in fig 9

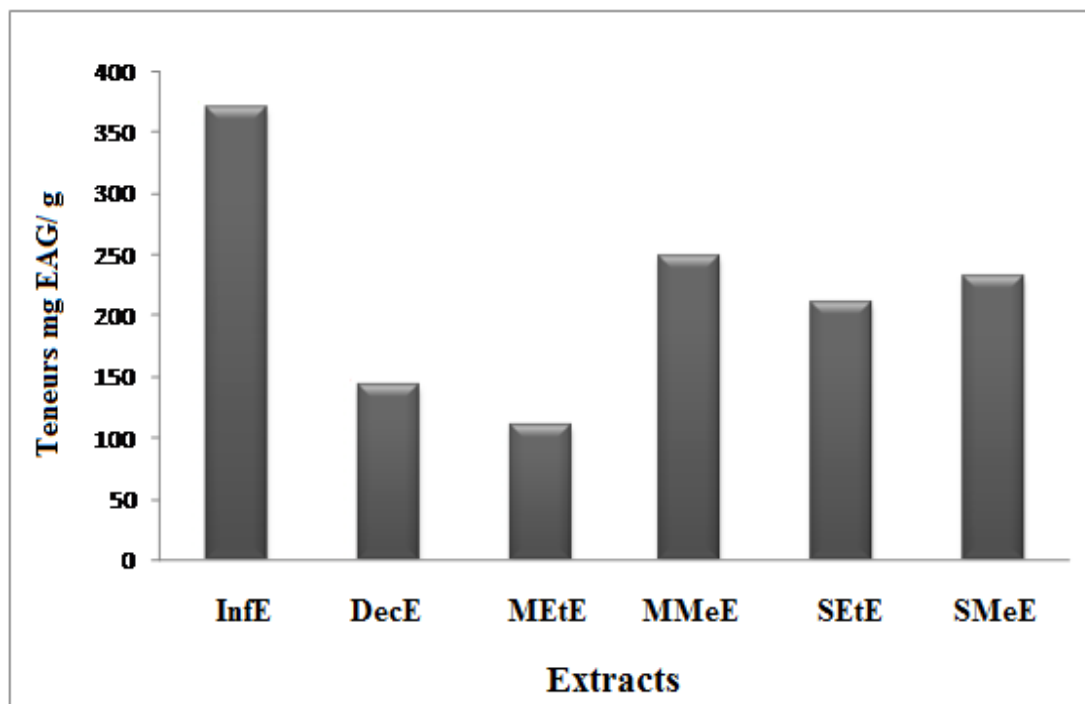


Fig 9: Comparison total phenolic content of different extracts of *s.rebaudiana*.

The quantitative analysis of total polyphenol in mg GAE/g of various extracts of *stevia rebaudiana* revealed that the infusion extract contained highest amount (371.5 mg GAE/g), followed by MMeE and SMeE (249.4 and 233.1 mg GAE/g respectively) and SEtE (211.9 mg GAE/g). While DecE and MEtE gave lower total polyphenol values (143.4mg and 110.2 mg GAE/g respectively)

The results showed that the water is the best solvent for extracting most of the chemical constituents responsible for different biological activities, demonstrating the relevance using of the traditional form.

5.DPPH radical scavenging assay

Renewed interest in plant antioxidant has emerged during the recent years, probably due to the appearance of undesirable side effects of certain commercial antioxidant. In medicinal plants world, there are a huge number of different types of bioactive compounds with antioxidant activity that play an significant role in terminating the generation of free radical chain reactions. Hence in the present study, DPPH radicals were used in the test to investigate the scavenging effects of *stevia rebaudiana* extracts (Fig 10).

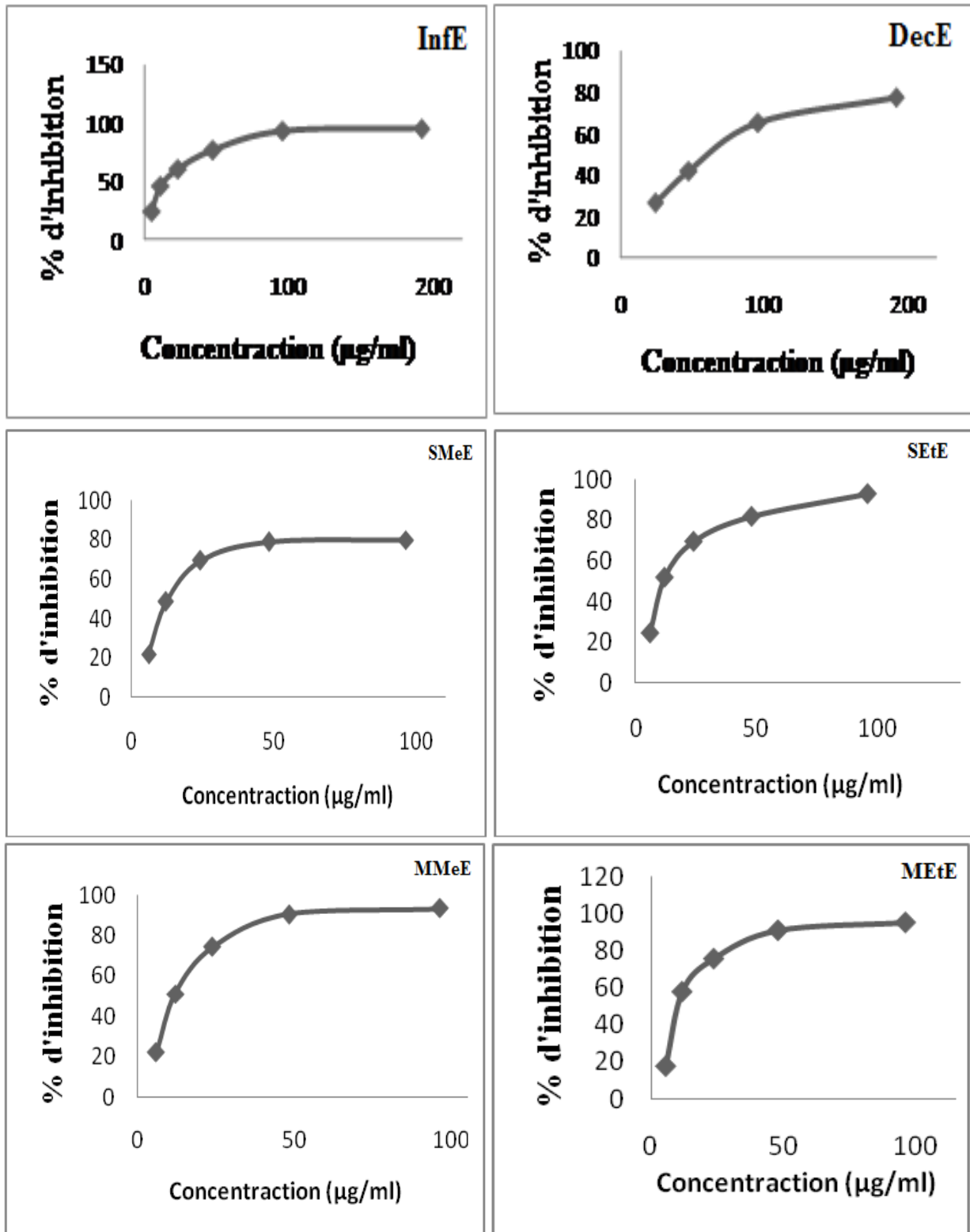


Fig10: Graphical representation of The DPPH scavenging of extracts of *Stevia rebaudiana*.

IC₅₀ was calculated as amount of antioxidant present in the sample necessary to decrease the initial DPPH concentration by 50 %. IC₅₀ value was used to reflect the capability. Basically, IC₅₀ is defined as the capability of an antioxidant and necessary concentration to reach 50% of scavenging DPPH free radical activity. In other word, the small IC₅₀ value means great free radical scavenging capability while large EC₅₀ value means relatively low scavenging capability.

The scavenging activity of extracts from *Stevia rebaudiana* against the stable radical (DPPH) has been evaluated and the results are illustrated in Fig 11.

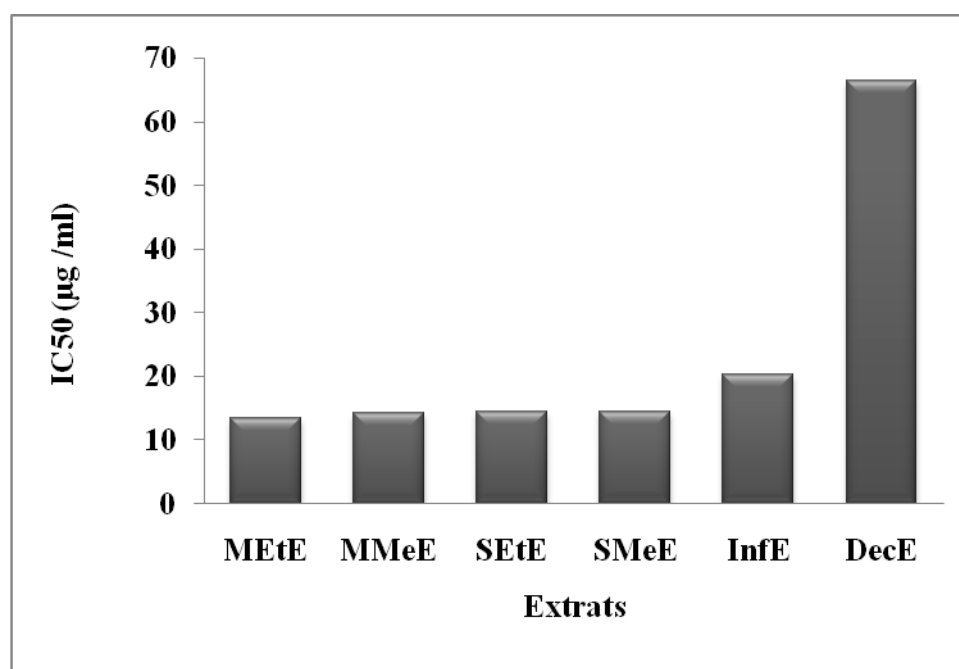


Fig11: IC₅₀ values of plant extracts for free radical scavenging activity by DPPH method.

It can be seen that, in general, all extracts showed important antioxidant potential. The free radical scavenging activities of all extracts of *stevia rebaudiana*. Were in the following order: MEtE (IC₅₀ 13.47 µg/ml) > MMeE (IC₅₀ 14.13 µg/ml) > SEtE (IC₅₀ 14.54 µg/ml) > SMeE (IC₅₀ 15.31 µg/ml) > infE (IC₅₀ 20.33 µg/ml) > decE (IC₅₀ 66.47 µg/ml). So it is found that the effect of DPPH radical scavenging extracts of the plant is inferior compared to the levels of standards: Rutin (4.25 µg/ml), quercetin (3.22 µg/ml) and gallic acid (0.58 µg/ml).

Poor correlation ($R^2=0.104$) found between total phenolic and values of IC_{50} . Indicates that the antioxidant activity of extracts could not be explained just on the basis of their phenolic content but also required their proper characterization. This lack of relationship is in agreement with other literature. It is known that only flavonoids with a certain structure and particularly hydroxyl position in the molecule can act as proton donating and show radical scavenging activity. Furthermore, the extracts are very complex mixtures of many different compounds with distinct activities. Our study is in agreement with Sengul et al. which reported no correlation between total phenolic content and antioxidant capacities of a number of medicinal plant extracts no correlation between total phenolic content and antioxidant capacity in our plant samples is possible owing to the presence of the following antioxidant capacity observed was not solely from the phenolic contents, but could possibly be due to the presence of some other phytochemicals such as ascorbic acid, tocopherol and pigments etc which also contribute to the total antioxidant capacity (86).

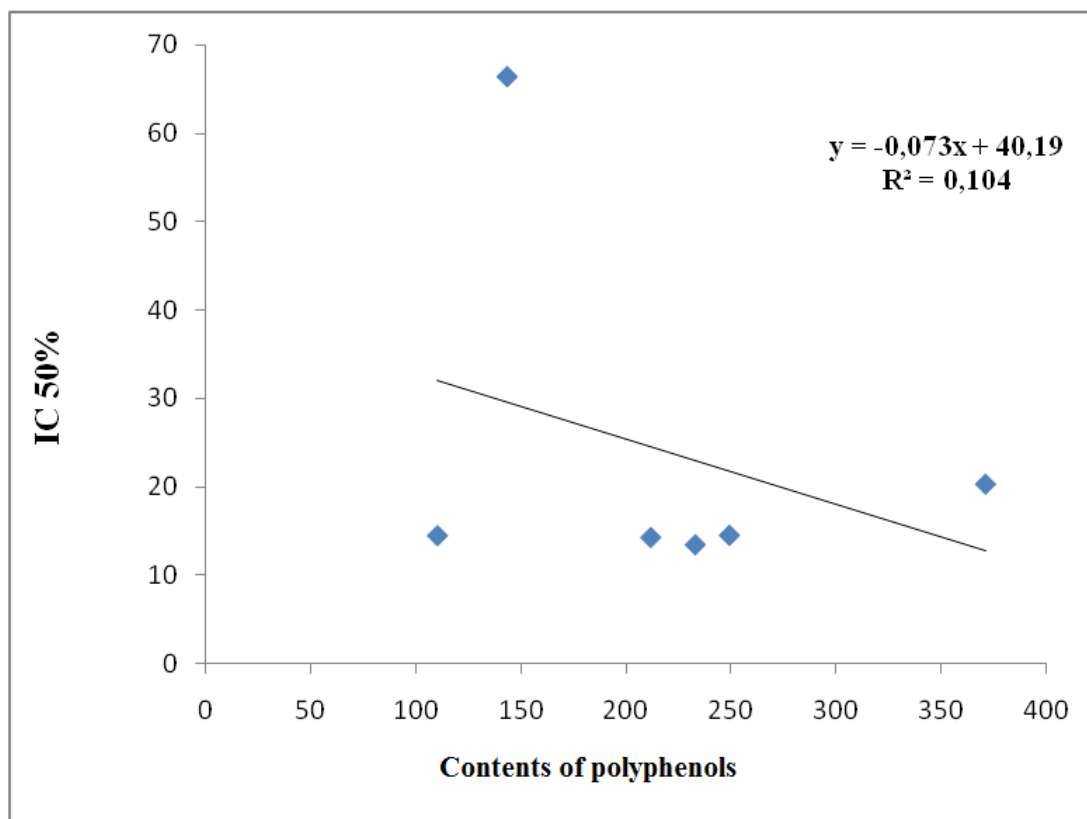


Fig12: Relationship between total phenolic content and values of IC50.

6. Antibacterial activity test results

Antibacterial activity test for all four types of extract ethanolic, methanolic, infusion and decoction were performed using agar diffusion method. The zone of inhibitions were measured in millimeters (mms) and compared with the zone of inhibitions of antibiotics which were used as positive control. Antibacterial activity of the four different extracts with positive control has been shown in table 6, and the comparison of zone of inhibition of extracts.

Table 6: Antibacterial activity of aqueous extract.

Microorganisms		MeE	EtE	InfE	DecE
<i>Staphylococcus aureus</i>	IZ	10 mm	11mm	10 mm	21 mm
	AI	0.4	0.44	0.4	0.84
<i>Escherichia coli</i>	IZ	-	-	-	-
	AI	-	-	-	-

IZ= Inhibition zone (in mm); AI- activity index; Standards: Gentamycin (10 µg /disc)

- showed no significant effect of extract.

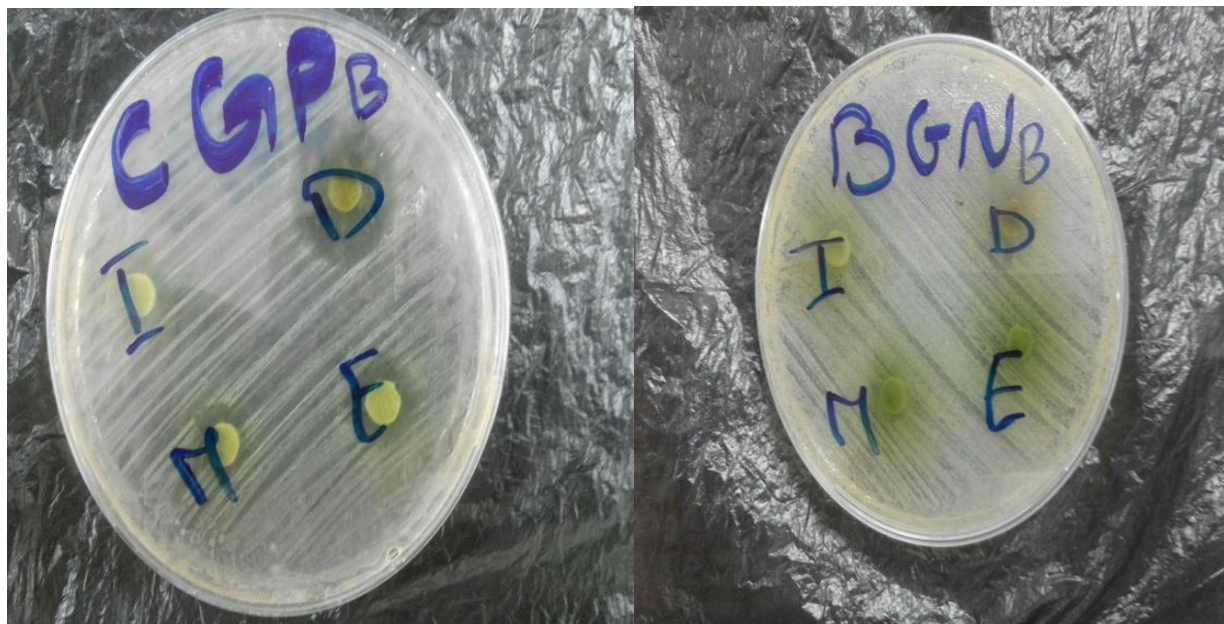


Fig13: Antibacterial activity of all extract.

The results show that **DecE** extract shows maximum inhibition with 21 mm of inhibition zone. While moderate activity was shown by other extract (**InfE**, **EtE** and **MeE**) against *Staphylococcus aureus* with inhibition zone between 10 to 11mm.

However all extracts show no sensitivity against *Escherichia coli*.

CONCLUSION

Conclusion

The extract of *Stevia* leaves has been subjected to various pharmacological, clinical and toxicological investigations and results revealed interesting therapeutic applications. In addition there has been an immense interest in utilization of natural plant extracts as antimicrobial and antioxidant activity due to the increase in outbreak of food borne diseases and to minimize the health causing diseases over synthetic drugs. The purpose of this study was to collect crude extracts and investigate the presence of phytoconstituents in *Stevia rebaudiana* and antibacterial and antioxidant activity that serves as an affect agent to treat many diseases.

The primary phytochemical analysis of ethanolic, methanolic and water extracts revealed the presence of flavonoids, saponins, tannins, steroids, phenols compounds.

Various chemical tests with by thin-layer chromatography (TLC) showed that the major chemical constituents found in this plant were flavonoids. This result was confirmed by a quantitative analysis based on the dosage of phenolic compounds. The qualitative analysis showed that all extracts are the richest in these molecules.

In result of this study *Stevia rebaudiana* plant bear a potent antioxidant activity at a concentration level which is related to constituents. Their constituents scavenge free radicals and exert a protective effect against oxidative damage induced to cellular macromolecules because it has shown the presence of number of polyphenols, which are related to their chemical structure and may be responsible of the antioxidant activities

Furthermore this plant also showed promising antibacterial activity, and tests show that the plant extracts may be used effectively as an antibiotic agent against microorganisms such as *Staphylococcus aureus*.

Eventually, the present study reveals the presence of potential antioxidants and antimicrobial properties in the plant extract which could be exploited for pharmaceutical application.

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