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IN *VITRO* AND IN *VIVO* EVALUATION OF ANTIMICROBIAL AND ANTIOXIDANT POTENTIAL OF STEVIA EXTRACT.

Said S. Moselhy [1,2,3,4] , Magdy A. Ghoneim [1,5] and Jehan A. Khan[6]

[1]Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia.[2]Bioactive Natural Products Research Group, KAU.[3] Experimental Biochemistry Unit, King Fahd Medical Research Center, KAU.[4]Department of Biochemistry, Faculty of Science, Ain Shams University, Cairo, Egypt.[5]Department of Biochemistry Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.[6]Department of Biology (Genomic Division), Faculty of Science, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia.

Corresponding author Email: seldesouky@kau.edu.sa

Abstract

Background: The current trend globally is the utilization of natural products as therapeutic agents given its minimum side effects. The leaves of *Stevia* contain several active ingredient compounds such as rebaudioside. *Stevia* extract have been used for many purposes. Active oxygen radicals can induce base modifications, DNA breakage, and intracellular protein crosslink's**.** This study was done to evaluate the potential of *stevia* extract as antibacterial and antioxidants actions.
Materials and methods*:* Antibacterial activity of different extracts of *stevia* was tested in vitro against different species of bacteria and hepato-protective efficacy was testes in rats injected with CCl_4 as hepatotoxic.
Results: Acetone extract exhibited antibacterial activity against selected five bacteria species. The acetone extract suppressed the elevation of serum ALT ($p < 0.05$) and AST ($p < 0.001$) activities induced by CCl_4. Animals given stevia extract showed prevention against deleterious effects of CCl_4 by lowering lipid peroxidation and enhancement of antioxidant activities as SOD and CAT. The protection trial is better than treatment trial. Total phenolic content of aqueous and acetone extracts were found 30 mg and 85 mg gallic /gm extract respectively. While the total flavonoids were 40 mg and 80 mg quercetin/g respectively. The GC-MS analysis showed that monoterpene and indole are the main components. Aqueous extract don't show any antibacterial activity against the tested strains. The antioxidant properties were attributable to its phenolic content to scavenge free radicals.
Conclusion: Acetone extract possess a potent antimicrobial and activity against deleterious effect of CCl_4-caused liver damage.

Key words: antibacterial, antioxidant enzymes, hepatic damage, *stevia*???

Introduction

Oxygen radicals including hydroxyl, nitrate and peroxide can induce mutation of DNA and adducts (Alia et al., 2006).These radicals can directly or indirectly damage cellular components (Anzai et al., 2005), as nucleic acid, proteins and lipid that are implicated in genesis of different diseases as cancer (Amzad et al., 2010). Nowadays, complementary and replacement therapeutic agents that contain bioactive compounds as polyphenols have the ability to remove these radicals and protect the cells against this damage upon their absorption. (Atassi and Casali, 2008). Practically carbon tetrachloride (CCl_4) forms highly reactive peroxyl radicals (Bauer et al., 2003), which causes lipid peroxidation and cell aging. Also, this radical can bind protein, enzymes, hormones leading to covalent damage of these molecules (Babu etal.,2002).
Stevia rebaudiana is a sweet herb used as low calorie sweetener. Stevioside is the major sweet compound of this plant (5-10 %). It is 350 times sweeter than sucrose (Bamias and Cominelli, 2007). The leaves of *Stevia* contain several active ingredient compounds such as rebaudioside. *Stevia* extract have been used for in lowering of blood glucose worldwide (Brandle etal., 1998). It was also found that stevioside have blood pressure–lowering effects in hypertensive patients.

It has been proved that, stevia leaves were traditionally used as folk medicine in the treatment of many diseases such as spasmolytic, anti constipation, analgesic and anti-inflammatory (Buege and Aust, 1978).

Stevia leaf extract contains a variety of constituents besides the steviosides and volatile oil rich in sterols, flavonoids, and tannins (Buege and Aust, 1978). These unidentified constituents may probably have biological impact on human and might assist in explaining some of the therapeutic uses of *stevia*. However, little work has been performed either to prove its potential as having antioxidants and antimicrobial actions. Therefore, the aim of the present study is to investigate the chemical composition of the essential oils of *Stevia* leaves by GC–MS and to evaluate the antimicrobial activity of acetone extract and the antioxidants activity of water extract of these ingredients.

Material and methods

Plant material and Preparation of *stevia* extract:

The green leaves of *Stevia rebaudiana* were purchased from Jeddah markets and identified by a taxonomy specialist at the biology department, KAU. Fifty grams of dry powdered leaves were ground and mixed separately with 500 ml acetone and 500 ml distilled water, boiled for 20 minutes, and then cooled. Afterwards, it was filtered through whatmann #1 filter paper and dried using a rotary vacuum pump. Both extracts (water and acetone) were stored at - 4°C till use and analysis.

10.21010/ajtcam. v13i6.4

Assay of Total Phenolic

The total phenolic content was determined by Folin's ciocalteau reaction according to (Cadet et al., 2002). 2 ml of each extract was added to 8 ml of distilled water. 2ml of Folin's ciocalteau reagent (1:1) dilution was added to it, incubation for 5 minutes, 5 ml of 10 % sodium carbonate solution was added to the mixture, and absorption was measured at 750nm spectrophotometer. Gallic acid was used as standard.

Assay of **Total flavonoids**

The total flavonoid content was determined by method of (Chatsudthipong and Chatchai, 2009). 2 ml of each extract was added to 0.3 ml of 5% sodium nitrite incubated for five mintutes. 0.3 ml of 10% Aluminum chloride and 0.2ml of 1M sodium hydroxide were added. Absorbance was measured at 510nm in spectrophotometer. Quercetin was used as standard

Gas chromatography and mass spectroscopy analysis of the extract

Variant GC-MS analysis was used after extraction immediately on a Hewlet Packard mass spectrometer interfaced with Gas Chromatograph. The capillary column (30 m×0.25 i.d. mm film thickness 0.25 µm, Varian, USA).The flow rate of carrier gas was 1ml/min. The initial temperature was 40°C and elevated to 240°C at a rate of 5°C /min. The chemical structure was detected by MS/MS system (Collins and Lewis, 1971).

Evaluation of Antibacterial activity of acetone extract (*In vitro* study)

Antibacterial activity of either acetone or water extracts (10, 20, 30, 40 and 50 mg/ml) were tested against different species of bacterial strain [*Staphylococcus aureus, Salmonella typhimurium, and Escherichia coli, Klebsiella pneumoni and Bacillus cereus*]. The strains were obtained from microbiology department, faculty of science, KAU using the agar diffusion assay method (Diallo et al., 1999). Chloramphenicol (200 mg/ml) was used as a positive control. After incubation at 37 ^{o} C for 24 h, the diameters of growth inhibition zones was measured in mm.

Evaluation of antioxidant activity of water extract (In *vivo* study)
Animal

In this study four groups of male albino rats (each 10 rats) weighting (180- 200gm) were used. Group I; served as control. Group II rats were received *i.p* 0.5ml/kg b.w CCl_4 for 7 days. Group III (Protected) rats were given orally 200mg/kg b.w of *stevia* extract for 14 days and then will give *i.p* 0.5ml/kg b.w $CCl_4$for 7 days. Group IV (Treated) rats were given *i.p* 0.5ml/kg b.w carbon tetrachloride for 7 days then daily orally 200mg/kg b.w of *stevia* extract for 14 days. The animals handling was according to the guidelines of the Animal Care Committee of King Abdulaziz University.

Sample preparation (Serum and tissue)

The rats were fasted for 12 hours .The blood samples was collected on plain tube and centrifuged at 3000RPMfor 15min for serum separation. Liver was excised from the rats and extracted in cold 0.25M sucrose (1:5 w/v) (Dimayuga and Garcia, 19991) centrifuged at 8000 RPM for 30 minutes. The supernatant will be used for the enzyme assay.

Biochemical assay

Liver enzymes including transaminases were assayed by kits from Bio system (Girish et al., 2004). Antioxidant enzymes including superoxide dismutase Kakkar and Visvanathan, 1972), catalase (Smna ,1972),were measured in live tissue. In addition lipid peroxidation marker as malondialdhydewas evaluated (Hoeruudin,2004)

Statistical analysis

The significant difference between different groups was calculated by ANOVA test and using SPSS version 16, $p < 0.05$ was considered as significant.

Results and Discussion

The results obtained showed that, the total phenolic content of aqoues and acetone extracts were 30mg and 85mg gallic/gm extract respectively. While the total flavonoids were 60 mg and 80 mg/gm respectively. The GC-MS analysis showed mainly monoterpene and caryophyllene oxide. ledene oxide-(II) and β-guaiene, indole.

The antibacterial activity of acetone extract in table (1) showed the effect of different acetone *stevia* extracts against selected five bacteria species. The extracts showed variable inhibitory effects at different concentrations on bacteria as calculated by the inhibition zone diameter (mm).

10.21010/ajtcam. v13i6.4

Table 1: Antibacterial activity of acetone *stevia* extracts at different concentration (Inhibition expressed as millimeter)

Pathogen / Extract (mg/ml)	*S. aureus*	*Salmonella typhimurium*	*Escherichia coli*	*Klebsiella pneumonia*	*Bacillus cereus*
10	3	--	--	5	7
20	4	12	--	8	9
30	8	10	12	5	7
40	10	6	7	3	-
50	12	-	-	8	12

Results indicated that acetone extract had variable activity against different species. The maximum activity against *S. aureus* at 50 mg/ml, *Salmonella typhimurium* at 20 mg/ml, *Escherichia coli*at 30 mg/ml and *Bacillus cereus*at 50 mg/ml.

Table 2: Serum aminotransferase enzymes (ALT and AST) and lipid peroxide product malondialdhyde (MDA) and hepatic antioxidant activities superoxide dismutase (SOD) and catalase, of different groups (Mean ± SD).

Animal groups / Parameters	**Group I Control**	Group II CCI_4	Group III Treated	Group IV Protected
Serum ALT (IU/l Mean ±SD	28.4±3.56	54.0±5.86[a]	33.9±4.14[b]	32.6±4.08[a,b]
Serum AST (IU/l) Mean±SD	36.9±3.56	64.0±5.86 [a]	39.8±4.14[b]	35.0±3.08[b]
MDA (mmol/mg protein) Mean ±SD	5.11± 0.14	10.14± 0.37 [a]	6.94± 0.47 [b]	5.30± 0.62 [b]
SOD (MU/mg protein) Mean ±SD	246.8±23.8	180.5± 30.0 [a]	212.7± 29.8 [a,b]	259.3± 29.2 [a,b]
Catalase(nmol/mg protein/min) Mean ±SD	889± 67.7	319.0± 46.6 [a,]	758.6±48.5 [a,b]	528.3±35.3 [a,b]

a:$p<0.05$ compare with control. b: significant compared with CCl_4

The protective effects of acetone stevia extracts against CCl_4-intoxicated rats are shown in Table 2. In the CCl_4 group rats serum AST and ALT were significantly elevated as compared with control ($p<0.001$). However it was significantly decreased in the rats treated with stevia acetone extract. Protection trail was observed to be better than treatment trail. Results obtained revealed a significant increase in liver MDA level, a marker of lipid peroxidation and a significant decrease in the antioxidant activities in CCl_4- group rats compared with control. Treatment or protection by stevia water extract significantly reversed this action. The activities of SOD and CAT have significantly reduced in CCl_4-intoxicated group, while it was significantly elevated in treated group. The protection was better than treated effect. Stevioside was used as low calorie sugar replaceable and as commercial sweetener used in a variety of foods and products (Marinova etal., 2005). The sweetness of stevioside was observed to be 350 times more than that of sucrose (Mohan and Janardhanan, 2005). Preliminary analysis of water and acetone extract showed that, acetone extract contain phenolic and flavanoides higher than aqueous extract. For this reason, the acetone extract was tested as antibacterial and antioxidant. It was found that, the antibacterial activity of acetone extract showed different growth inhibition against selected five bacteria species. The antibacterial activity could be attributed to presence of high flavonoides content. The active antimicrobial compounds are terpenes (10%), it would seem reasonable that their antimicrobial mode of action might be related to that compounds. Carbon tetrachloride **(**CCl_4) was being used as hepatotoxic in vitro to investigate protective activity of novel medicinal plants (Misra and Fridovich, 1972). The major defense in living cell include Zn-SOD, catalase and reduced glutathione that to remove these radicals.

The lipid peroxidation can lead to cell damage as DNA adduct, cell lysis, inactivation of many enzymes. In the present study serum ALT, AST were used as a biomarker of hepatic damage. $CC1_4$ induce hepatic damage in experimental animals Rajalakshm and Geeravani, 1990; Sankhala et al., 2005). The toxic metabolite $CC1_3$ radical was mediated by the action of cytochrome p_{450} which further reacts with oxygen to give trichloromethyl peroxy radical. This radical binds covalently to macromolecule and causes peroxidative degradation of lipid membrane of the adipose tissue. For this reason, administration of aqueous extract of *stevia* revealed hepatoprotective activity against the toxic effect of CCl_4.The protection trail is better than treatment one. CCl_4 produces free radicals that not only directly cause damage to tissues, but also initiate inflammation. Kupffer cells produce subsequently pro inflammatory cytokines, and activate other non-parenchymal cells involved in liver inflammation. TNF-α is produced by resident macrophages after CCl_4 administration and subsequently stimulates the release of cytokines from macrophages and induces phagocyte oxidative metabolism and NO production (Sathishkumar et al., 2008). The NO is a highly reactive oxidant and it can augment oxidative stress by reacting with ROS and forming peroxy nitrite (Hoerudin, 2004). Another mediator of CCl_4 - induced hepatic inflammation which was induced by pro inflammatory cytokines, leading to formation of pro inflammatory substrates from arachidonic acid (Taniguchi et al., 1978). Scavenging of free radicals was one of the major anti-oxidation mechanisms to inhibit the chain reaction of lipid peroxidation. Reduced lipid peroxidation was revealed by significant decrease in MDA level in treated or protected groups with simultaneously a significant elevation in SOD and CAT enzymes activity. Results obtained showed that, protection treatment was more sound potent impact than treated. The activities of SOD and catalase were significantly decreased following treatment with CCl_4. This may be due to mobilization of these enzymes to scavenge the reactive CCL_3. This decrease could result in the deficiency of necessary antioxidant enzyme to prevent the cell from damage by reactive oxygen species. Treatment or protection with *stevia extract* enhanced these enzymes significantly. The restoration of the antioxidant enzymes indicate an evidence for *in vivo* antioxidant potential of *stevia* extract. The stimulation of antioxidant enzyme may be attributed to the phenolic and quercetin as this component found to induce catalase, superoxide dismutase, and glutathione reductase and glutathione peroxidase (Yagi and Rastgi, 1979). Pre-treatment with *stevia* before CCl_4 treatment attenuate membrane lipids liability to deleterious actions of reactive oxygen species and free radical. The measurement of lipid peroxidation was a

convenient method to monitor oxidative stress damage. There was an increase in MDA formation following CCl_4 injection. Increase in lipid peroxidation may possible account for the increase of serum aminotransferase activities.

Attenuation of the accumulation of MDA by stevia extract indicates that it acted as antioxidant *in vivo* and protects bio membrane against oxidative stress damage.

Conclusion

This study suggested that acetone stevia extract possess a potent antimicrobial and hepatoprotective activity against CCl4-induced liver injury in rats. These observations were documented by biochemical results that supporting the potential clinical use of stevia in the hepatic protection from various diseases.

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