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

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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 tested in rats injected with CCl4 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 CCl4. Animals given stevia extract showed prevention against deleterious effects of CCl4 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 monoterpane and indole are the main compounds. 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-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 (CCl4) 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 et al., 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 (Barnias 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 et al., 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 spasmodic, 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.
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 (Chatsudhipong and Chatchai, 2009). 2 ml of each extract was added to 0.3 ml of 5% sodium nitrite incubated for five minutes. 0.3 ml of 10% aluminum chloride and 0.2 ml 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 Anti-bacterial 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 pneumonia and Bacillus cereus]. The strains were obtained from microbiology department, faculty of science, KAU. The GC-MS analysis was used as standard. Chloramphenicol (200 mg/ml) was used as a positive control. After incubation at 37°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 CCl4 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 CCl4 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 3000RPM for 15 min 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 malondialdehyde was evaluated (Hoerudin, 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 carophyllene 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).
ion by stevia water extract

4h oxygen to give trichloromethyl peroxy radical. This radical binds covalently to-

reactive CCL species induced by pro-

reductase may be an antioxidant enzyme

reactive CCL were signif-

decrease in MD

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acute antimicrobial compounds are terpenes (10%), it would seem reasonable that their antimicrobial mode of action might be related to that compounds. Carbon tetrachloride (CCl4) 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. CCl4 induce hepatic damage in experimental animals (Rajalaksham and Geervani, 1990; Sankhala et al., 2005). The toxic metabolite CCl3 radical was mediated by the action of cytochrome p450 which further reacts with oxygen to give trichloromethyl peroxo 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 CCl4. The protection trail is better than treatment one. CCl4 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 CCl4 administration and subsequently stimulates the release of cytokines from macrophages and induces phagocyte oxidative metabolism and NO production (Sahishkumar et al., 2008). The NO is a highly reactive oxidant and it can augment oxidative stress by reacting with ROS and forming peroxynitrite (Hoerudin, 2004). Another mediator of CCl4 - 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 CCl4. This may be due to mobilization of these enzymes to scavenge the reactive CCL3. 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 CCl4 treatment attenuate membrane lipids liability to deleterious actions of reactive oxygen species and free radical. The measurement of lipid peroxidation was a

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

| Pathogen       | S. aureus | Salmonella typhimurium | Escherichia coli | Klebsiella pneumonia | Bacillus cereus |
|----------------|-----------|------------------------|-----------------|---------------------|----------------|
| Extract (mg/ml)|           |                        |                 |                     |                |
| 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 malondialdehyde (MDA) and hepatic antioxidant activities superoxide dismutase (SOD) and catalase, of different groups (Mean ± SD).

| Animal groups Parameters | Group I Control | Group II CCl4 Treated | Group III | Group IV Protected |
|--------------------------|-----------------|-----------------------|-----------|-------------------|
| Serum ALT (IU/l) Mean ±SD | 28.4±3.56       | 54.0±5.86*            | 33.9±4.14b | 32.6±4.08ab       |
| Serum AST (IU/l) Mean±SD  | 36.9±3.56       | 64.0±5.86*            | 39.8±4.14b | 35.0±3.08b        |
| MDA (mmol/mg protein) Mean±SD | 5.11±0.14   | 10.14±0.37a           | 6.94±0.47 b | 5.30±0.62b        |
| SOD (MU/mg protein) Mean±SD | 246.8±23.8 | 180.5±30.0a            | 212.7±29.8ab | 259.3±29.2ab      |
| Catalase(nmol/mg protein/min) Mean±SD | 889.6±67.7 | 319.0±46.6*           | 758.6±48.5 ab | 528.3±35.3 ab     |

a:p<0.05 compared with control. b: significant compared with CCl4

The protective effects of acetone stevia extracts against CCl4-intoxicated rats are shown in Table 2. In the CCl4 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 CCl4 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 CCl4-intoxicated group, while it was significantly elevated in treated group. The protection was better than treatment effect. Stevioside was used as low calorie sugar replaceable and as commercial sweetener used in a variety of foods and products (Marinova et al., 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 flavonoids 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 inhibition growth against selected five bacteria species. The antibacterial activity could be attributed to presence of high flavonoids 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 (CCl4) 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.

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convenient method to monitor oxidative stress damage. There was an increase in MDA formation following CCl₄ injection. Increase in lipid peroxidation may possibly 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 aceton 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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