www.pharmaerudition.org

ISSN: 2249-3875

International Journal of Pharmaceutical Erudition

Research for Present and Next Generation

MAY 2018

Vol: 08 Issue:01

(66-76)

ISSN 2249-3875

International Journal of Pharmaceutical Erudition

Research Paper

IN VITRO ANTIOXIDANT ACTIVITY OF DIFFERENT EXTRACTS OF PLANTS BY USING REDUCTION OF 1, 1- DIPHENYL- 2- PICRYLHYDRAZYL (DPPH)

Vasudev Seeram[1] and Dr. Anil Midha[2]

*[1] Research Scholar, Department of Biochemistry, OPJS, University, Rajasthan
[2] Professor, Department of Biochemistry, OPJS, University, Churu, Rajasthan

Diabetes mellitus is a metabolic disorder characterized by resistance in the action of insulin, insufficient insulin secretion or both. It is becoming one of the most common diseases of the world. Type II diabetes in young has increased 30 fold over the last 20 years concomitant with increase in obesity. Studies have revealed that all incidences of diabetes in this young age group are 2.5% and alarmingly 25% of their young adults have abnormalities of blood glucose. Being a major heterogeneous endocrine and metabolic disorder, Diabetes mellitus leads to hyperglycaemia and several other complications, such as hyperlipidemia, hypertension and atherosclerosis. And the oxidative stress, caused by hyperglycemia induced free radicals, contributes to the development and progression of diabetes along with various secondary complications.The antidiabetic and/or hypoglycemic activity study of the extracts at the tested dose levels were assessed on normoglycaemic, glucose loaded and alloxan induced hyperglycemic rats in both single and multi-dose treatment (for 11 days) models. Others associated study parameters aretest of; body weight variation, serum lipid profile, Serum biochemical parameters like urea and creatinine, AST, ALT, ALP levels in the 11 days treated diabetic animals using standard experimental procedures.

Key words: Diabetes mellitus, creatinine, AST,Hyperglycaemia.

INTRODUCTION

Hyperglycemia promotes auto-oxidation of glucose to form free radicals. The generation of free radicals beyond the scavenging abilities of endogenous antioxidant defenses results in macro- and microvascular dysfunction. Antioxidants such as N-acetylcysteine, vitamin C and α-lipoic acid are effective in reducing diabetic complications, indicating that it may be beneficial either by ingestion of natural antioxidants or through dietary supplementation. However, while antioxidants are proving essential tools in the investigation of oxidant stress-related diabetic pathologies and despite the obvious potential merit of a replacement style therapy, the safety and efficacy of antioxidant supplementation in any future treatment, remains to be established

Diabetes and Oxidative Stress

A number of complications arise as a consequence of macro and microvascular complications that result from diabetes; these deficits have a central role in the tissue-damaging effects of chronic hyperglycemia.

ISSN 2249-3875

Since endothelial cells (as well as renal mesangial and Schwann cells) are unable to limit glucose transport as well as other cells do, they are more vulnerable to the toxic effects of hyperglycemia.Oxidative stress results from an imbalance between radical- generating and radical-scavenging systems, i.e. increased free radical production or reduced activity of antioxidant defenses or both. Hyperglycemia-induced oxidative stress has also been associated with increased endothelial cellapoptosis *in vitro* and *in vivo*. Several studies have shown that diabetes mellitus (types 1 and 2) is accompanied by increased formation of free radicals and decreased antioxidant capacity, leading to oxidative damage of cell components.

In vitro antioxidant activity of different extracts of plants by using Reduction of 1, 1- diphenyl- 2- picrylhydrazyl (DPPH)

In vitro antioxidant activity of potentfraction by using :

- Reduction of 1, 1- diphenyl- 2- picrylhydrazyl (DPPH)
- Nitric oxide scavenging activity
- ABTS scavenging activity Super oxide radical Scavenging

In vivo antioxidant activity of fractions by Determination of Enzymatic antioxidantsin pancreas, liver and kidney homogenates

- Superoxide dismutase (SOD)
- Super oxide radical Scavenging

In vivo antioxidant activity of fractions by Determination of Enzymatic antioxidantsin pancreas, liver and kidney homogenates

- Superoxide dismutase (SOD)
- Catalase (CAT)
- Reduced Glutathione
- Glutathione-S-transferase (GST)
- Total Protein level
- ***In vitro* antioxidant activity of *A. mexicana* by using DPPH (α, α- diphenyl α-picrylhydrazyl) Free Radical Scavenging Activity**
- **Principle:** Free radical scavenging potentials of the extracts were tested (*in vitro*) against a methanolic solution of α,α-diphenyl-β-picrylhydrazyl (DPPH). Antioxidants react with DPPH and convert it to α,α-diphenyl-β-picryl hydrazine (Fig. 4.1). The DPPH is stable free radical which is purple in colour and upon reaction with an antioxidant; it becomes colourless and the degree of discoloration indicates the scavenging potentials of the antioxidant extract. The change in the absorbance produced at 517nm has been used as a measure of antioxidant activity.

  The IC_{50} value, defined as the amount of the sample sufficient to elicit 50% reduction of the initial DPPH concentration, was calculated from the linear regression of plots of

ISSN 2249-3875

concentration of test compounds against the mean percentage of antioxidant activity

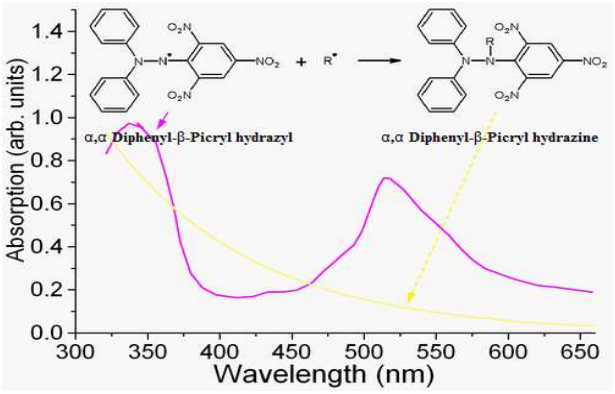


Fig. 1: Reduction of DPPH free radical

obtained from the three replicate tests. The free radical scavenging activity of ascorbic acid (Vitamin C) was also measured under the same condition to serve as +ve control.

Procedure:

DPPH stock solution (0.3 mM): 11.8 mg of DPPH was dissolved in 100 ml of analytical grade ethanol.

Preparation of stock solutions of test fraction:

The stock solutions of extracts/fraction and standard were prepared with analytical grade ethanol and further test solutions of different concentration of test fractions of *A. mexicana* (5-160 µg/ml) were prepared. All solutions were prepared with ethanol.

Method: The DPPH scavenging activity was done using the method of Changet al, 2002 and Umamaheswari et al, 2008.A total 1ml of 0.3mM DPPH solution was added to 2.5 ml of sample/standard solution of different test concentrations and allowed to react at room temperature. After 30 min, the absorbance values were measured at 517nm and converted into the percentage antioxidant activity using the following equation.

$$\% \text{ Anti radical activity (I)} = \frac{\text{Control Abs} - \text{Sample Abs}}{\text{Control Abs}} \text{ x } 100$$

Ethanol (1ml) plus plant test fraction solution (2.5 ml) was used as a blank, while DPPH solution plus methanol was used as a negative control. The positive controls were DPPH solution plus each ml of standard (Ascorbic acid). The IC_{50}values were calculated by linear regression of plot, where the abscissa represents the concentration of tested plant extracts/standard and the ordinate the average % of scavenging capacity from three triplicates. Each experiment was carried out in triplicate and IC_{50} (µg/mL) of the chloroform fraction of *A. mexicana*were reported.

Nitric oxide scavenging activity

ISSN 2249-3875

Principle: Nitric oxide (NO) is an extremely unstable free radical generated from sodium nitroprusside at physiological pH, which can be determined by Griess reagent. It reacts with O_2 to produce the stable product nitrates and nitrite through intermediates like NO_2, N_2O_4 and N_3O_4. In the presence of test compound, which is a scavenger, the amount of nitrous acid will decrease. The degree of decrease will reflect the amount of scavenging, which is measured at 546 nm (Shirwaikar et al, 2006).

ABTS scavenging activity

Principle: In this method scavenging of ABTS [2, 2‟‟ azinobis (3- ethylbenzthiazoline-6-sulfonic acid) diammonium salt] radical cation take place. The principle involves the reaction between ABTS and sodium persufate, ABTS is converted to its radical cation by addition of sodium persulfate. In the presence of antioxidant reductant, the coloured radical is converted back to colourless ABTS; the absorbance is read at 745 nm (Shirwaikar et al, 2006).

Procedure for preparation of ABTS radical cation: The ABTS radical cation was prepared by reacting ABTS solution (7mM) with 2.45mM of ammonium persulphate and the mixture was allowed to put in dark at room temperature for 12-16 hrs prior to use.

Method: 1.5ml of different concentration of chloroform fraction of *A. mexicana*(5-160 µg/ml) was added with 0.9ml of ABTS radical cation. The absorbance was read at 745nm and all experiments were performed in triplicate (Shirwaikar et al, 2006).

Super oxide radical scavengingactivity

Principle: Superoxide radical is known to be very harmful to the cellular components.The assay was based on the capacity of sample to inhibit farmazan formation by scavenging the superoxide radicals generated in riboflavin-light- nitro blue tetrazolium (NBT) system(Kumaran and Karunakaran, 2007).

Method: Each 3ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 µg riboflavin, and 12 mM EDTA and 0.1 mg NBT and 1ml of sample solution. Reaction was started by illuminating the reaction mixture with different concentrations of test fractions and standard ascorbic acid solution (5-160 µg/ml) for 5min. Immediately after illumination, the

Method: The reaction solution comprising of 2.5 ml of different concentrations of chloroform fraction of *A. mexicana* (5-160 µg/ml) and 0.75ml of 5mM of sodium nitroprusside. The test tubes were incubated at 25^0C for 5hours. After 5 hrs, 0.5ml of Griess reagent was added. For control, Griess reagent and sodium nitroprusside solution was quantified spectrophotometrically at 546nm. The experiment was performed in triplicate (Shirwaikar et al, 2006).

ISSN 2249-3875

absorbance was measured at 590 nm. Identical tubes with reaction mixture and 1ml of methanol were kept in the dark along and served as control. The % scavenging activity at different concentrations was determined and the IC_{50} value of the chloroform fraction of *A. mexicana* was compared with that of ascorbic acid, which was used as the standard.

The percentage inhibition of superoxide anion generation was calculated from

$$\% \text{ Scavenging} = \frac{\text{Absorbance of Control - Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

Estimation of *in vivo* antioxidant activity:

The chloroform and aqueous soluble fractions from *A. mexicana*has also been studied for their antioxidant potential *in vivo* in the alloxan induced diabetic rats at the end of 21 days of treatment (daily dosing) with the plant fractions keeping the standard drug as glibenclamide.

Preparation of tissue homogenate

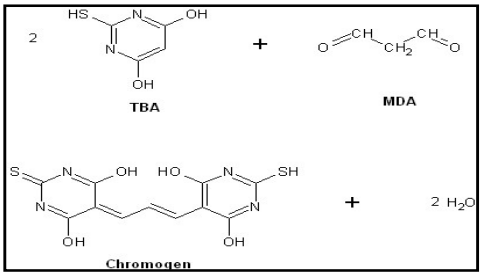
After sacrificing the animals on 21st day the liver, kidney and pancreas tissues from the control and experimental groups of rats were excised and rinsed with ice-cold saline. The preparation of tissue homogenates was done by a known amount of the liver and kidney tissues, homogenized in 0.1 M Tris-HCl buffer, pH 7.4 at 4°C, in a Remi homogenizer with a Teflon pestle at 600 rpm for 30 min. The homogenates were centrifuged at 3,000 × g for 10 min at 4°C using refrigerated centrifuge. The supernatant was collected as tissue homogenate, and the same was used for the antioxidant enzymes estimations.

The *in vivo* antioxidant activity of the plant fractions were started by the determination of the activities of lipid peroxidation products (oxidants) like Thiobarbituric Acid Reacting Substances (TBARS) and estimating the activities of different liver antioxidant enzymes like Reduced Glutathione (GSH), Glutathione-S-Transferase, Superoxide Dismutase (SOD), Total Protein level,and Catalase (CAT) were also quantified using regular experimental procedures.

Determination of the lipid peroxidation

Principle: This assay is based on the reaction of a chromogenic reagent, 2-thiobarbituric acid, with Malondialdehyde (MDA) at 25°C. One molecule of Malondialdehyde (MDA) reacts with 2 molecules of 2-thiobarbituric acid via a Knoevenagel-type condensation to yield a chromophore with an absorbance maximum at 532 nm.

ISSN 2249-3875



Procedure: Acetic acid 1.5ml (20%, pH 3.5), 1.5 ml of thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulphate (8.1%) was added to 0.1ml of supernatant and heated at 100°C for 60min. Mixture was cooled and five ml of n-butanol pyridine (15:1) mixture was added with one ml of distilled water and vortex vigorously. After centrifugation at 1200 x g for 10 minutes, the organic layer was separated and absorbance was measured at 532 nm. It was calculated using a molar extinction coefficient of 1.56 x 105 M-1 cm-1 and expressed as nanomoles of TBARS, mg of protein (Mukherjee et al, 2007).

Calculations were made as per the formula

Inhibitory rate = [1 - (A1 – A2) / A0] X 100

Where A0 was the absorbance of control (without extract) and A1 was the absorbance in the presence of the extract, A2 was the absorbance without tissue homogenates.

Estimation ofreduced glutathione (GSH)

Principle: Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to other unstable molecules. Reduced glutathione is a main cellular antioxidant. It upholds the normal structure of red blood cells and remains hemoglobin in the ferrous state. It is involved in the detoxication process. The toxic material (organophosphate, nitro compound) is converted into mercapturic acid. Glutathione is implicated in maintaining normal brain function (Lushchak, 2012).

Reagents:

1. TCA (Tri-chloro Acetic acid) 20%
2. DTNB (5, 5 di thio bis 2 nitro benzoic acid) 0.6mM
3. Phosphate Buffer (pH 7.4)

Procedure: The same quantity of tissue homogenate (supernatant) and 20%TCA were mixed. The precipitated fraction was centrifuged and to 0.01ml of supernatant, 2ml of phosphate buffer 0.5M DTNB (5, 5 di thiobis 2 nitro benzoic acid) and 0.4mL double distilled water was added. Mixture was vortexed and the absorbance read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as µg/mg of protein (Sedlak and Lindsay, 1968; Ellaman, 1959).

ISSN 2249-3875

Calculation:

$$\text{GSH (µmol/ml)} = \frac{\text{Absorbance} \times V \times \text{dilution factor}}{E_{mM} \times V_{enzyme}}$$

Where,

E_{mM}(Molar Absorption coefficient) = 13.60

Dilution Factor = 30

V = Reaction Volume

V_{Enzyme}= The volume of sample (0.01)

Estimation of superoxide dismutase (SOD)

Principle: SOD activity can be measured by activity assays. In the biochemical method, xanthine-xanthine oxidase is used to generate superoxide radical (O_2-) and nitrobluetetrazolium (NBT) reduction is used as an indicator of superoxide radical (O_2-) production. Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide radical (O_2-) into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2) and as such affords an important defense against the toxicity of the superoxide radical. In this assay, superoxide ions (O_2-) generated by xanthine oxidase (XOD) which convert xanthine to uric acid and hydrogen peroxide and nitrobluetetrazolium (NBT) to NBT-diformazan, quantified at 560 nm. SOD will compete with NBT for $O_2^{\bullet-}$; the percent inhibition of NBT reduction is a measure of the amount of SOD present.

Procedure:SOD activity was estimated by Beauchamp and Fridovich . The reaction mixture consisted of 0.5 ml of homogenate, 1 ml of 50 mM sodium carbonate, 0.4 ml of 25 µM NBT and 0.2 ml of 0.1mM EDTA. The reaction was initiated by the addition of 0.4 ml of 1mM hydroxylamine- hydrochloride. The change in the absorbance was recorded at 560 nm. The control was simultaneously run without tissue homogenate. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% in mg/ml (Beauchamp and Fridovich, 1971).

Calculation:

Formula for SOD activity:

SOD U/ml = (A-B) × 100/A × 50

A = absorbance of control

B = absorbance of sample

ISSN 2249-3875

International Journal of Pharmaceutical Erudition

Characterization of isolated phytoconstituent from chloroform fraction of *A. mexicana* using spectroscopic techniques

The various absorption spectrums of the isolated compound from chloroform fraction of *A. mexicana*showed peaks as follows:

Compound AM **:** Isolated from chloroform fraction

Synonym : Stigmasta-5, 22-dien-3β-ol

Molecular formula : $C_{29}H_{48}O$

Molecular weight : 412.69 g/mol

Description : white amorphous solid

Solubility : Soluble in Petroleum ether and Chloroform

R_f value : 0.54 (Chloroform)

M. P. : 169-170^{0}C

Phytochemical Test : The compound gave a red colour for Salkowski‟s test while a green colour in case of Liebermann- Burchard‟s tests.

Spectroscopic data:

UV (λ_{max}) : 257 nm [Petroleum ether (60-80)]

IR (ranges in cm^{-1}) :3428 (O-H stretching), 2937(C-H stretching), 2852(C-H stretching), 1642 (C=C stretching), 1465 (C-H bend.), 1460 (C-H bend.), 1192 (O-H bend.), 1053 (C-C str.), 739 (CH_2 rocking), 699 (O-H bend.).

^{1}HNMR (DMSO): δ 5.24 (m, 1H, H-6), δ 4.57 (s, 1H), δ 4.14 (s, 1H), 3.20 (tdd, OH, H-3), δ 1.23 (s, 3H), δ 1.19 (s, 3H), δ 1.06 (s, 3H), δ 0.98 (s, 3H), δ 0.91 (s, 3H).

^{13}CNMR (DMSO) : δ 140.8 (C-22), δ 130.1 (C-5), δ 129.1 (C-23), δ 121(C-6),δ 71.6 (C-3), δ 56.1 (C-4), δ 55.1 (C-5), δ 52.2(C-24), δ 50.10,(C-17), δ 43.8 (C-9), δ 41.2 (C-13),δ 39.4 (C-10), δ 37.7, (C-10), δ 33.4 (C-20), δ 31.7 (C-25), δ 29.1 (C-21),δ 28.1 (C-23), δ 25.1 (C-12), δ 21.8 (C-11, C-25, C-26), δ 15.1 (C-29), δ 12.8(C-27).

EI MS :412 [M+, $C_{29}H_{48}O$] 355(101), 311 (49), 301 (49), 279 (71), 219 (60), 200 (65), 175 (95)

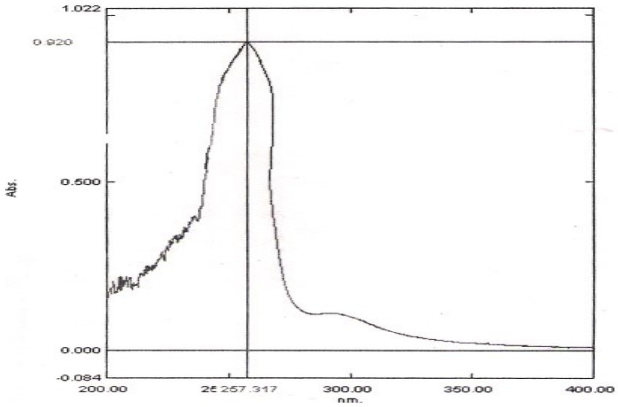
Isolated compound is positively to the Salkowski's test and Lieberman-Burchard test for steroids and triterpenes. The melting point of compound AM was 169°C; the UV λ_{max} value of compound AM was 257 nm. Mass spectrum of isolated compound AM showed parent molecular ion [M$^+$] peak at mlz 412 which corresponds to the molecular formula $C_{29}H_{48}O$ (Fig. 6.06).

In the IR spectrum of isolated compound a very intensely broad peak at 3428 cm^{-1} and

ISSN 2249-3875

moderately intense peak at 1192 and 699 cm^{-1} were observed for the O-H bond vibrations of hydroxyl group. In the ^{1}H-NMR spectrum of isolated compound, H-3 proton appeared as a triplet of a double doublet (tdd) at δ3.20 and, H-6 olefinic proton showed a multiplet at δ5.24. Two olefenic protons appeared downfield at δ 4.57 m and δ 4.14 m. Six methyl protons also appeared at **δ**1.23, **δ**1.19, **δ**1.06, **δ** 1.00, **δ**0.98 and **δ**0.91 (3H each, s, CH_3).

The ^{13}C-NMR has shown recognizable signals at 140.8 and 121 ppm, which corresponds to double bond at C-22 and C-6 double bonds respectively as well as it also represent signals at 130.1 and 129.1 ppm, which shows one more double bond in between C-5 and C-23. The δ value at 71.6 ppm is due to C-2 β-hydroxyl group. The signal at δ 31.7 and δ 12.8 ppm corresponds to angular carbon atom at C-25 and C-27 respectively.



- **Fig.2: UV spectra of the isolated compound from chloroform fraction of *A. mexicana***

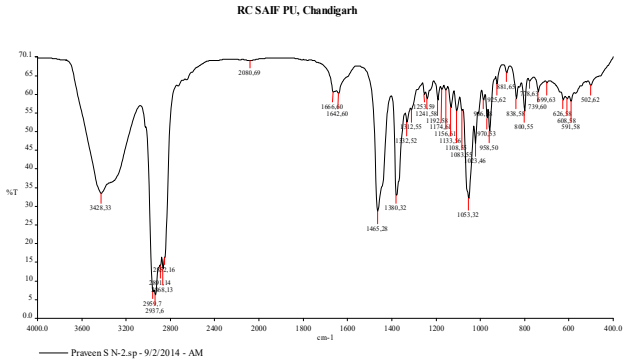


Fig.3: IR spectra of the isolated compound from chloroform fraction of *A. mexicana*

ISSN 2249-3875

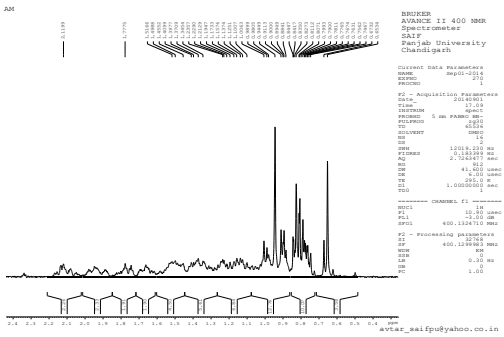


Fig.4:^{1}H-NMR spectra of the isolated compound from chloroform fraction of *A. mexicana*

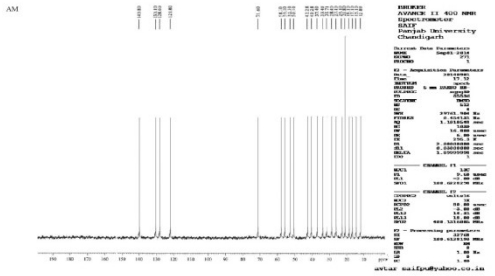


Fig.5:^{13}C- NMR spectra of the isolated compound from chloroform fraction of *A. mexicana*

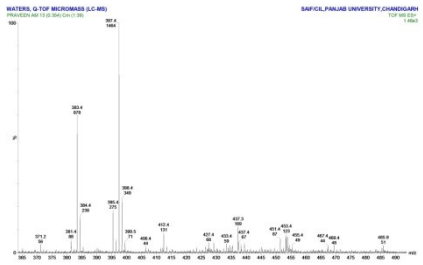


Fig.6: Mass spectra of the isolated compound from chloroform fraction of *A. mexicana*

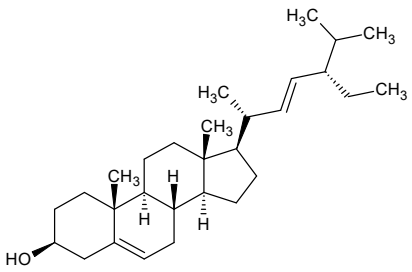


Fig. 7: Structure of Stigmasterol isolated from chloroform fraction of *A. mexicana*

ISSN 2249-3875

Discussion: The anti-oxidant activity studies were carried out in *in-vitro* models in case of test extracts and in both *in-vitro* and *in-vivo* models in case of fractions of the plants. The *in-vitro* parameters include determination of the total Phenolic & Flavonoid content; DPPH, hydrogen peroxide free radicals scavenging activities in case of test extracts, where as DPPH, superoxide, ABTS radical cation, and nitric oxide free radicals scavenging activities in case of test fractions. Similarly, *in-vivo* study was assayed by using the parameters of lipid peroxidation products in the liver, where as antioxidant enzymes, such as superoxide dismutase, catalase, Glutathione-S-transferase, reduced glutathione, total protein levelwere estimated inthe liver, kidney and pancreas and used as marker of the evaluation of antioxidant potential.

The critical analyses of experimental results of the entire study protocol, as illustrated in the thesis describe the following conclusions in support of the anti-diabetic and/or hypoglycemic activitypotential of the selected plant; *A. mexicana.*

- The preliminary phytochemical screening resultsinferredethanolic and aqueous extract of *A. mexicana*showed the presence of most of the phytochemicals analyzed such as: alkaloids, carbohydrates, triterpenoids, steroids, tannins, and saponins. Whereas, chloroform and aqueous fractions of *A. mexicana*shows the presence of alkaloids, carbohydrates, triterpenoids, phytosterols, saponins, and tannins except carbohydrates in the aqueous fraction.

REFERENCES

1) Aebi H, Wyss SR, Scherz B, Skvaril F (1974). Heterogeneity of erythrocyte catalase II. Isolation and characterization of normal and variant erythrocyte catalase and their subunits. *European Journal ofBiochemistry***48**:137-145.

2) Al-Abrash AS, Al-Quobaili FA, Al-Akhras GN (2000). Catalase evaluation in different human diseases associated with oxidative stress. *Saudi Medical Journal***20**: 826-830.

3) Alexander SA, Kolodgie FD, Munn DH, Gerrity RG (2004). Regulation of macrophage foam cell formation by αVβ3 integrin. *American Journal of Pathology***165**: 247-258.

4) Amer MA, Ghattas MH, Abo-ElMatty DM, Abou-El-Ela SH (2012). Evaluation of glutathione S-transferase P1 genetic variants affecting type-2 diabetes susceptibility and glycemic control. *ArchivesMedical Science* **8**: 631-636.

5) Amer MA, Ghattas MH, Abo-Elmatty DM, Abou-El-Ela SH (2011). Influence of glutathione S-transferase polymorphisms on type-2 diabetes mellitus risk. *Genetics and Molecular Research***10**: 3722-3730.