International Journal of Aquatic Science
ISSN: 2008-8019
Vol 12, Issue 02, 2021

Evaluation of Anti Microbial and Antioxidants Potential of Blueberry Extracts.

Surabhi Tripathi[1], Rakhi Mishra[2], Avishikta Ray[3], Anju Singh[4], Shailesh Kumar Ghatuary[5], Reenu Yadav[6]

[1,2,3,4,5]*Bhabha Pharmacy Research Institute, Bhabha University, Bhopal, (M.P.)- India*
[6]*IITM (Department of Pharmacy), IES University, Bhopal, (M.P.)- India*

Abstract:
Objective
To investigate anti-microrial and anti-oxidant activity of the leaf extract of tropical medicinal herb and food plant Vaccinium corymbosum L. (V. corymbosum).
Methods
Free radical scavenging activity on DPPH, ABTS, and nitrites were used to analyse phenoic and flavonoid contents of leaf extract. Other focuses included the determination of antioxidant enzymatic activity (SOD, CAT and GPx), metal chelating activity, reduction power, lipid peroxidation inhibition and the prevention of oxidative DNA damage. Antibacterial activity was determined by using disc diffusion for seven strains of bacteria.
Results
Results found that V. corymbosum leaf extract had significant antibacterial activity. The tested extract displayed the highest activity (about 23.18 mm inhibition zone) against Salmonella typhymurium and the lowest antibacterial activity was observed against Enterococcus faecalis (about 14.08 mm inhibition zone) at 10 mg/ disc. The IC_{50}values for DPPH, ABTS and radical scavenging activity were 0.120, 0.049 and 1.160 mg/mL, respectively. V. corymbosum leaf extract also showed dose dependent reduction power, lipid peroxidation, DNA damage prevention and significant antioxidant enzymatic activity.
Conclusions
These findings demonstrate that leaf extract of V. corymbosum could be used as an alternative therapy for antibiotic-resistant bacteria and help prevent various free radical related diseases.

Keywords: Vaccinium corymbosum, Antioxidant, Free radicals, Phenolic compound, Flavonoid, DNA damage, Antibacterial

1. INTRODUCTION

Reactive oxygen species (ROS) are constantly produced in cells by cellular metabolism and by exogenous agents.They are essential for life because they are involved in cell signalling and are used by phagocytes for bactericidal action[1]. Recently, increasing evidence highlights that overproduction of ROS and oxygen-derived free radicals may contribute to a variety of pathological effects (*e.g.* DNA damage, carcinogenesis and cellular degeneration) and induce many diseases including aging, cancer, atherosclerosis, diabetes and rheumatoid arthritis[2]–[3].Currently, plant sourced antioxidant agents have been attracting special interest because they can protect the human body from diseases induced by free radicals with little or no side

effects[4]. In the last few years, as well, the number of bacteria resistant to current antibiotics has increased dramatically, creating a great need for discovering new anti-microbial agents[5]. *Vaccinium corymbosum* (*V. corymbosum*), blueberry, are flowering plants belonging to the large genus of*Vaccinium*. Various members of the *Vaccinium* genus have been used extensively as traditional medicines for the treatment of diabetic symptoms by Quebec traditional practitioners, along with fenugreek[6],[7]. Blueberry leaves are primary plant parts that have been used medicinally for generations[8].

Blueberries are a good source of chlorogenic acid, quercetin, kaempferol, myricetin, procyanidins, catechin, epicatechin, resveratrol, and vitamin C to contribute to antioxidant activity[9]. Recent studies showed that leaf extract of *Vaccinium angustifolium* (*V. angustifolium*) was abundant in chlorogenic acid, several flavanols and glycosides, as well as catechin and epicatechin[10],[11]. Blueberry leaves may be considered as one of the richest and most readily available sources of 5-CQA, even compared to green coffee beans[8].

Blueberries (*Vaccinium* sp.) are rich in anthocyanins, polyphenolics--recognized for their ability to provide and activate cellular antioxidant protection, inhibit inflammatory gene expression, and consequently protect against oxidant-induced and inflammatory cell damage and cytotoxicity[12]–[14]. *Vaccinium myrtillus* L. leaves may prove potentially useful for treatment of dyslipidemia associated with impaired plasma triglyceride-richlipo protein clearance[15]. Anti-diabetic properties of the Canadian low bush blueberry *V. angustifolium* fruit, leaf and stem was studied by Martineau *et al*[10]. Piljac-Zegarac *et al.* reported antioxidant capacity and phenolic content of blueberry (*V. corymbosum* L.) leaf infusions[16].

The aim of the present study was to examine the antioxidant and antibacterialactivity of extracts of the *V. corimbosum* leaf. Little to no information exists currently in scientific literature on the antibacterial and antioxidant activities of hydroalcoholic leaf extract of *V. corimbosum*.

2. MATERIALS AND METHODS

2.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), ABTS (2, 20-azinobis-3-ethylbenzothiazoline- 6-sulphonic acid), gallic acid, sodium nitrite, Folin-Ciocalteu reagent, trichloroacetic acid, butylated hydroxyluene, ascorbic acid, α-tocopherol, potassium persulphate, ferrous chloride, ammonium thiocyanate, ethylenediaminetetraacetic acid (EDTA), linoleic acid, anhydrous sodium phosphate (dibasic), anhydrous sodium phosphate (monobasic), 5,5′-dimethyl pyrroline-1-oxide, ferrous sulphate ($FeSO_4$) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Sodium hydroxide and ferric chloride were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The catalase and superoxide dismutase assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The pBR322 DNA and 6X DNA loading dye were purchased from Fermentas Inc. (Cromwell Park, Glen Burnie, USA). Agarose A was purchased from Bio Basic Inc. (Ontario, Canada). Roswell Park Memorial Institute medium (RPMI 1640) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from WElGENE Inc. (Republic of Korea). All the other solvents and chemicals used were of analytical grade.

2.2. Plant material collection and extraction

V. corymbosum leaves were collected from Blueberry Suite, Chae-Hyang-Won, Gangwon-do, Korea. Hydroalcoholic extract of *V. corymbosum* leaf was prepared according to method of Abirami *et al.* with some modification[17]. About 100 g grinded powder was extracted with

500 mL of 75% (v/v) ethanol at room temperature for 24 h. Then the extracts were filtered through Whatman filter paper (GE Healthcare UK Limited, UK). These extracts were evaporated under vacuum rotary evaporator. The residual leaf extract was freeze dried the yield was calculated. Finally, the extracts were stored in a refrigerator at -20 °C until used.

2.3. Determination of total phenolic content

Total phenolic content of leaf extract was measured by the Folin-Ciocalteu method described by Oueslati *et al*with some modification[18]. Briefly, 10 mg of each extract was dissolved in 1 mL of distilled water and different concentrations of gallic acid (0.007 8–1.000 0 mg/mL) were prepared in water. A total of 40 µL of each sample, 20 µL of 1 mol/L Folin-Ciocalteu reagent and 60 µL of Na_2CO_3 (20%, w/v) were mixed. Mixtures were kept in dark at room temperature for 30 min to complete the reaction. The absorbance of the reaction mixture was read at 700 nm. Gallic acid was used to calculate the standard curve and the results were expressed as mg of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/100g dw).

2.4. Determination of total flavonoid content

Total flavonoid content was measured using a colorimetric assay developed by Pereira *et al.* with some modification[19]. Briefly, 25 µL of each sample (10 mg/mL) or standard reagent was mixed with 125 µL of distilled water and then 8 µL of 5% sodium nitrate solution was added. After 5 min incubation 15 µL of 10% (w/v) aluminum chloride solution was mixed with the above mixture and mixture was incubated at room temperature for 6 min. After 50 µL of NaOH (0.1 mol/L) and 27 µL of distilled water were added with the mixture, The intensity of pink colour was measured at 517 nm. (+)-Catechin was used to calculate the standard curve (0.015–1.000 mmol/L) and the results were expressed as mg of (+)-chatequin equivalents per 100 g of extract.

2.5. DPPH radical scavenging activities

The DPPH scavenging activity of the leaf extract was measured by colorimetric method Farvin *et al*[20]. In brief, an aliquot of 80 µL of sample solution at different concentrations (0.12–2.00 mg/mL) was mixed with 80 µL of DPPH solution (0.3 mmol/L in methanol). The reaction mixture was incubated for 30 min in the dark at room temperature. The absorbance of the resulting solution was measured at 517 nm with a spectrophotometer (Sunrise-Basic Tecan, Austria). Controls were prepared in a similar way as for the test group except for the replacement of the test sample with the corresponding extraction solvent. The radical scavenging capacity of the tested samples was measured using the following equation. All determinations were performed intriplicate.

DPPH radical scavenging activity(%)=(1-Absorbance of sample/Absorbance of control)×100

2.6. ABTS radical-scavenging activity

Determination of the $ABTS^{\bullet+}$ radical scavenging activity was performed according to the method of Yadav *et al*[21] with some modifications. The $ABTS^{\bullet+}$ radical was generated by reaction of 5 mL of 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS, 7 mmol/L) in water, with 88 µL of potassium persulphate (2.45 mmol/L). The mixture was left to stand for 12–16 h in the dark at room temperature. Absorbance of the reactant was later adjusted to 0.70±0.02, at a wave length of 734 nm. Different concentrations of leaf extracts were mixed with 0.7 mL of $ABTS^{\bullet+}$ solution and the mixture was shaken for 5 min. The reduction of the $ABTS^{\bullet+}$ radical was determinate by reading the absorbance at 734 nm. The controls contained the extraction solvent instead of the test sample. The scavenging activity of ABTS free radical was calculated as:

ABTS radical scavenging activity (%)=(1-Absorbance of sample/Absorbance of control)×100

2.7. Nitrite-scavenging activity

Nitrite scavenging activity was assessed as reported by Patel *et al*[22]. Briefly, 1 mL of each sample or standard reagent at various concentrations, from 0.125–2.000 mg/mL, was mixed with 1 mL of 1 mmol/L $NaNO_2$. Then 8 mL of 0.2 mol/L citrate buffer (pH 3) was added to the mixture and incubated in 37 °C water bath for 1 h. After incubation, 1 mL of the reaction mixture was withdrawn and added to a mixture of 2 mL (2% (v/v)) acetic acid and 0.4 mL of Griess reagent. Solution was vigorously mixed with a vortex mixer and placed at room temperature for 15 min.The absorbance was measured at 520 nm. The control contained water instead of the antioxidant solution. Ascorbic acid was used as a positive control. The scavenging activity of each sample/standard solutionwas calculated by using the following equation:

Scavenging activity(%)={1-(Absorbance of sample-Absorbance of sample or standard)/Absorbance of control}×100

2.8. Chelating activity

The chelation of iron (II) ions was estimated as described by Locatelli *et al*[23]. Extracts (0.12–2.0 mg/mL) were incubated with 50 µL of 2 mmol/L $FeCl_2$ for 5 min. The reaction was initiated by adding 200 µL of 5 mmol/L ferrozine and left standing for 10 min at room temperature. The absorbance of the mixture was recorded at 562 nm against the blank performed in the same way using $FeCl_2$ and water. EDTA (100 µg/mL) served as the positive control, and a sample without extract or EDTA served as the negative control. The Fe^{2+}-chelating activity was calculated using the equation below:

Chelating activity (%)=(1-Absorbance of sample/Absorbance of control)×100

2.9. Assay of reducing power

The reducing power was determined according to the method of Dutta*et al*[24] with some modifications. Reaction was carried out in a mixture containing 2.5 mL of sample (0.12–2.0 mg/mL), 2.5 mL of 0.1 mol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ (10 mg/mL) by incubating at 50°C for 20 min. After addition of 2.5 mL trichloroacetic acid (100 mg/mL), the mixture was centrifuged at 5000 r/min for 10 min. Then 0.25 mL of supernatant was mixed with 0.25 mL of distilled water and finally 0.5 mL of $FeCl_3$ (0.1% (w/v)) was added with the mixture. The absorbancewas measured at 700 nm.

2.10. Lipid peroxidation inhibition assay

Lipid peroxidation was estimated by measuring thiobarbituricacid-reactive substances, as described by Dias *et al*[25]. Briefly, brain tissues werehomogenized with a Polytronin ice-cold Tris-HCl buffer (20 mmol/L, pH 7.4) to produce a 1:2 (w/v) brain tissuehomogenate which was centrifuged at 3000 r/min for 10 min. Malondialdehyde (MDA) contents in the supernatant were measured using the corresponding assay kit. Briefly, an aliquot (0.1 mL) of the supernatant was incubated with the different concentrations of the sample (0.12–2.00 mg/mL; 0.2 mL) inthe presence of $FeSO_4$ (10 µmol/L; 0.1 mL) and ascorbic acid (0.1 mmol/L; 0.1 mL) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and the mixturewas then heated at 80 °C for 20 min. After centrifugation at 3000 r/min for 10 min the precipitated protein was removed.The colour intensity of the MDA-TBA complex in the supernatant was measured at 532 nm. The inhibition ratio (%) was calculated using thefollowing formula:

Inhibition ratio (%)=(1-Absorbance of sample /Absorbance of control)×100

2.11. Cellular antioxidant enzymes assays

Cellular antioxidant enzymes assays were determined as described by Jeong *et al.* with minor modifications[26]. Briefly, RAW264.7 cells were plated at adensity of 2×10^6 cells in 6 well plates with DMEM. After 24 h incubation at 37 °C in 5% CO_2, the medium was removed fromthe dish leaving cells on the bottom. New media containing different concentrations of the freeze-dried extracts were added to the dish. After 24 h incubation, 1 mL of ice cold lysis buffer (containing 50 mmol/LTris-HCl (pH 7.4) and 10 mmol/L EDTA) was added and cells were collected. The cell pellet was homogenised for centrifuged at 12 000 r/min for 10 min at 4 °C. The supernatant was collected and total protein contents were quantified by Lowry's method using BSA standard (Lowry, Rosebrough, Farr, & Randall, 1951). Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities were measured according to each manufacturer'sinstructions of the kits. Enzyme activity was expressed as unit/mg proteins.

2.12. Prevention of oxidative DNA damage

The determination of the oxidative DNA damage preventive activity was performed as described by Chandrasekara and Shahidi with some minor modifications[27]. Briefly, 1 µL of plasmid pBR 322 DNA (0.5 µg/µL) was treated with 3 µL of $FeSO_4$ (0.08 mmol/L), 4 µL of 30% H_2O_2 (v/v), 3 µL distilled water and 2 µL test extracts at different concentrations (0.5–2.0 mg/mL). The mixture was incubated at 37 °C for 1 h. Then 2 µL of DNA loading dye (6X) were added with the mixture. The DNA band was analysed on 0.8% (w/v) agarose gel usingethidium bromide staining. Gels were scanned on a Gel documentation system (Nextep, Korea) and bands were quantified using NEXTEP analysis software.

2.13. Antimicrobial activity

2.13.1. Microbial strains and culture conditions

The reference strains *Escherichia coli* ATCC 25922 (*E. coli*), *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*), *Salmonella typhimurium* KCCM 11862 (*S. typhimurium*), *Acetobacter baumannii* ATCC 19609 (*A. baumannii*), *Klebsiella pneumoniae* ATCC 10031(*K. pneumoniae*), *Staphylococcus aureus* ATCC 29213 (*S. aureus*) and *Enterococcus faecalis* ATCC 29212 (*E. faecalis*) were obtained from Korean Culture Center of Microorganisms (Seoul, South Korea). Microorganisms were cultured aerobically at 37 °C in Nutrient Agar medium and Tryptic Soy Agar medium.

2.13.2. Determination of inhibitory effect

The antimicrobial activity of *V. corymbosum* leaf extract was determined by Disc diffusion method. Twenty millilitres of agar medium were poured into each Petri dish. Approximately 200 µL of suspension (2×10^8 cfu/mL) of the test microorganisms was smeared onto the agar after ithad solidified. Paper disc of 8 mm diameter were placed on it, and the extract was added (50 µL) and the same volume (50 µL) of ethanol was used as a negativecontrol. Standard antibiotics were used as positive controls. The inoculated plates were incubated for 24 h at 37 °C. After incubation the diameters (mm) of the inhibition zone were measured.

2.14. Statistical analysis

Experiments were performed in triplicate and the results wereexpressed as mean±SD. The statistical analysis was carried out by using SPSS 15.0, Sigma plot 10.0, GraphPad Prism 5 and Microsoft Excel 2007. A value of$P<0.05$ was considered to indicate statistical significance.

3. RESULTS

3.1. Total polyphenol and flavonoid contents

Total phenolic and flavonoid contents of hydroalcoholic extract of *V. corymbosum* leaf are expressed as mg GAE/100 g dry weight and mg CE/100 g of dry weight, respectively. The results are shown in Table 1. The present study showed that the leaf extract exhibited significant amount of phenols (9.25 mg GAE/100g of dry weight) and flavonoids (12.66 mg CE/100 g of dry weight).The extraction yield was 30% for the hydroalcoholic extract of *V. corymbosum* leaf.

Table 1
Total phenolic and flavonoid content, DPPH, ABTS and Nitrite radical scavenging activities of the *V. corymbosum* leaf extract.

Extract	Total phenolic (mg GAE/100 g of dry mass)	Total flavonoid (mg CE/100 g of dry mass)	DPPH radical		ABTS radical		Nitrite radical	
			Scavenging activity(%) (2 mg/mL)	IC_{50} value mg/mL	Scavenging activity(%) (2 mg/mL)	IC_{50} value mg/mL	Scavenging activity(%) (2 mg/mL)	IC_{50} value mg/mL
GLBR	9.25 ±0.13	12.66 ±0.56	93.07±0.43[a]	0.12± 0.003[a]	97.67±1.009[a]	0.049±0.009[a]	79.82±1.056[a]	1.16± 0.027[a]
Positive control			96.05±0.74[b] (Ascorbic Acid)	0.11± 0.002[b]	99.78±0.34[b] (Ascorbic Acid)	0.013±0.0004[b]	98.12±0.57[b] (Ascorbic Acid)	0.16± 0.003[b]

All data are expressed as mean ±Standard deviation (n=3). Different letters in each column denote statistically significant difference compare to the positive control group at$P$<0.05.

3.2. DPPH, ABTS and Nitrite radical scavenging activity

The leaf extract of *V. corymbosum* showed scavenging activity on DPPH, ABTS and nitrite radicals. The scavenging effects of the extract and standard (ascorbic acid) on the DPPH radical were expressed as half maximal inhibitory concentration (IC_{50}) values. As shown in Table 1, the IC_{50} value of ascorbic acid was 0.11mg/mL and the IC_{50} value of the extracts was 0.12 mg/mL. This revealed the high level of ABTS radical scavenging ability of the leaf extract (Table 1). 2 mg/mL leaf extract scavenged the ABTS radical 97.67%, which is significantly different from that of the ascorbic acid standard (99.78%). IC_{50} values were 0.049 mg/mL and 0.013 mg/ml, for leaf extract and ascorbic acid, respectively (P<0.05 compared to a positive control).

The nitrite-scavenging activity of the leaf extract was increased in a dose-dependent manner, using a concentration ranging from 0.12 to 2.00 mg/mL. The IC_{50} values were 1.16 and 0.16 mg/mL, respectively, for tested extract and ascorbic acid standards, indicating thatthe

scavenging activity of ascorbic acid was significantly stronger than that of the extract (P<0.05, Table 1).

3.3. Chelating activity

The metal chelating ability in terms of ferrous ion chelating capacity is claimed as an important mechanism in antioxidant activity. Fe^{2+} chelating ability is summarised in Figure 1. In this study, *V. corymbosum* leaf extract showed a relatively modest chelating ability with ferrous ions in comparison to the positive control. At 2 mg/mL, the tested extract showed 41.46% Fe^{2+} chelating ability. EDTA was used as a reference in this assay and its Fe^{2+}chelating activity was 91.55% at 100 µg/mL (P<0.05).

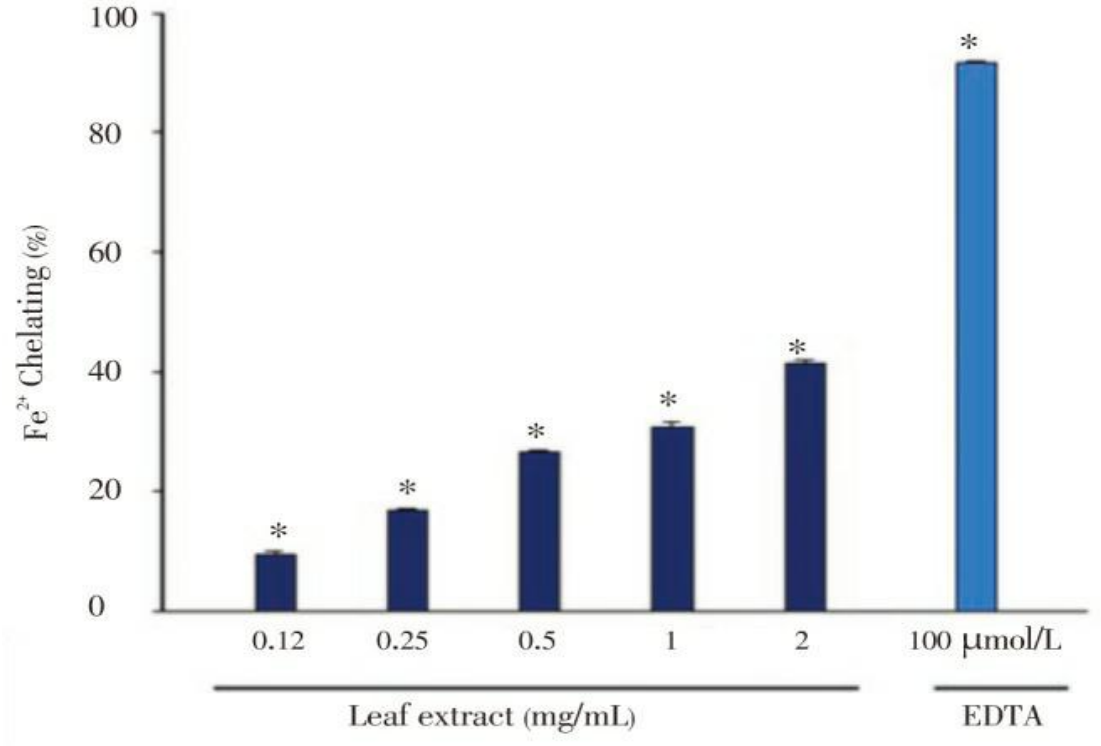

Figure 1.

Fe2+ chelating ability of *V. corymbosum* leaf extract.

Values represent the mean±SE (n=3). Values marked with * indicate significant differences (P<0.05) between tested sample and EDTA Standard

3.4. Estimation of reducing power

The reducing power of the extract was determined using a modified Fe^{3+} to Fe^{2+} reduction assay, whereby the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of the samples. The reducing power of leaf extract, ascorbic acid and BHT increased with the increase of concentrations. The reducing power of the leaf extract increased from 0.12 to 0.92 at 0.125 to 2.000 mg/mL. However, the reducing power of leaf extract was lower than that of ascorbic acid and BHT (P<0.05). The reducing power of the positive control, BHT and ascorbic acid were more pronounced than that of the tested extract (Figure 2).

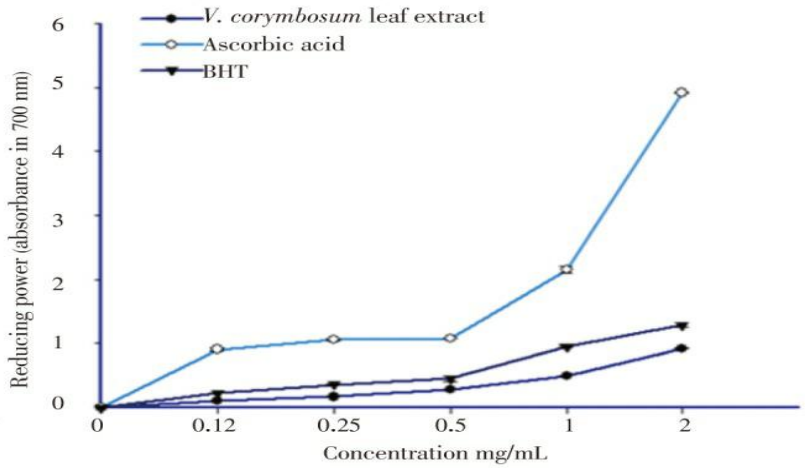

Figure 2.

Reducing power of *V. corymbosum* leaf extract, ascorbic acid and BHT standard.
All data are expressed as mean±Standard deviation (n=3). $P<0.05$, compare to the positive control group

3.5. Lipid peroxidation inhibition

Lipid peroxidation is known to result in the formation of malonaldehyde and other structurally similar compounds, which react with thiobarbituric acid (thiobarbituricacid-reactive substances, TBARS) to produce achromophore that absorbs at 532 nm. The inhibition of TBARS formation provides sound evidence concerningthe potency of a compound for protecting against lipid peroxidation. The inhibition of Fe^{2+} induced lipid peroxidation in the brains of mice homogenates by *V. corymbosum* leaf extract is shown in Figure 3. *V. corymbosum* leaf extract significantly reduced the MDA content in the brains of mice homogenates compared to controls. The tested extract showed a moderate capacity to reduce the Fe^{2+}-induced formation of MDA. At 2 mg/mL, leaf extract and ascorbic acid inhibit 59.55 % and 90.08 % MDA production, respectively ($P<0.05$)

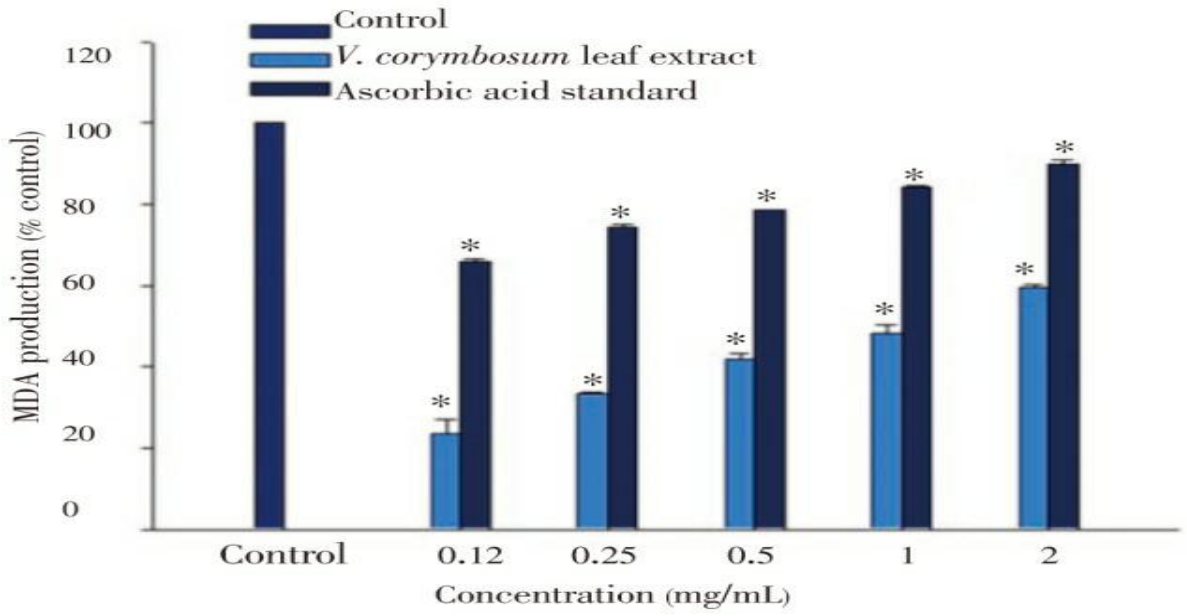

Figure 3.

Effects of *V. corymbosum* leaf extract on Fe^{2+}-induced lipid peroxidation in mouse brain homogenates (*in vitro*).

The percent inhibition of MDA production was expressed with respect to basal brain tissue (without leaf extract). Values represent the mean±SE (n=3). All data are expressed as mean±Standard deviation (n=3).*P*<0.05, compare to the positive control group

3.6. Antioxidant enzymatic activity

In order to investigate whether the antioxidant activities of leaf extract were mediated by an increase in antioxidant enzymes, CAT, SOD and GPx activities in RAW264.7 cells weremeasured. SOD and catalase are two key enzymes in detoxifying intracellular O^{-2} and H_2O_2. As shown in Table 2, cellular SOD, CAT, and GPx activity were increased at 250 µg/mL tested extract. The tested extract showed significantly higher CAT, and GPx activity (9.6 U/mg protein, 54.16 U/mg protein, respectively) than the control (*P*<0.05). Cellular SOD activity was not statistically significant compared to the control at the indicated concentration (250 µg/mL). One unit for SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. One unit for GPx activity was defined as the amount of the enzyme oxidising 1.0 nmol of NADPH to NADP+ per minute.

Table 2
Effect of *V. corymbosum* leaf extract on activities of SOD, CAT and GPx in cells.

Extract	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
Control	36.01±0.15	7.4±0.09	44.35±0.68
Leaf extract	44.71±0.25	9.6±0.09*	54.14±0.93*
Positive control	96.43±1.49 (Bovine erythrocyte SOD)	13.90±0.15 (Bovine liver catalase)	78.24±0.93 (Glutathione Peroxide)

Cells are treated with 250 µg/mL leaf extract. Values are expressed as enzyme units per mg of protein ±SE (n = 3).
* Significantly different from control, *P*<0.05.

3.7. Oxidative DNA damage preventive activity

In this study, free radical induced DNA strand scission inhibition was tested for three concentrations of crude phenolic extract: 0.5, 1.0 and 2.0 mg/mL. In general, hydroalcoholic extracts of *V. corymbosum* leaf demonstrated effective inhibitory activity against peroxyl radical induced DNA scissionat all concentrations tested. Figure 4 shows the inhibitory effect of the extractson pBR322 plasmid DNA cleavage caused by H_2O_2. The plasmid DNA was mainly of the supercoiled form in the absence of Fe^{2+} and H_2O_2 (Lane 1, control). During the addition of Fe^{2+} and H_2O_2, the supercoiled form of DNA decreased and converted to the relaxed circular and linear forms. However, the further fragmentation of the linear form decreased in the presence of the extracts. At the concentrations of 0.5, 1.0 and 2.0 mg/mL, a dense supercoiled form was observed rather than an open circular and linear form (Lane 3–5).

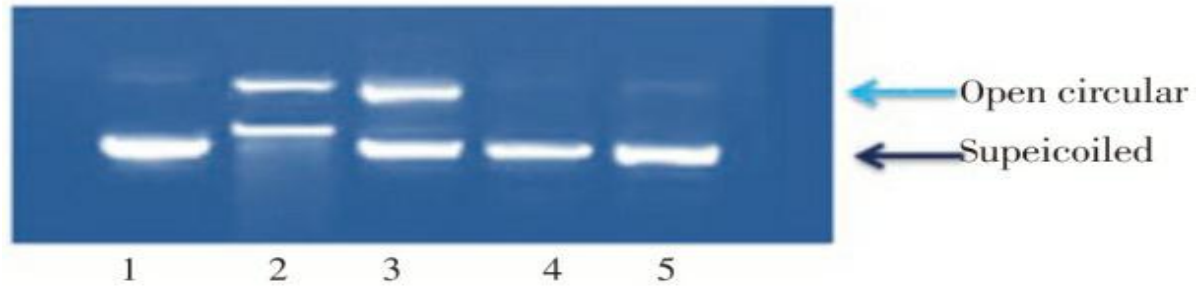

Figure 4.

Electrophoretic pattern of pBR 322 DNA breaks by •OH generated from the Fenton reaction and prevented by Vacciniumcorymbosum leaf extract. Lane 1: untreated control DNA, Lane 2: $FeSO_4$+H_2O_2(DNA damage control), Lane 3: $FeSO_4$+H_2O_2+DNA in the presence of *V. corymbosum* leaf extract (0.5, 1.0 and 2.0 mg/mL, respectively).

3.8. Antibacterial activity

The antimicrobial effect of *V. corymbosum* leaf extract was tested with seven species of bacteria. Potency was assessed qualitatively and quantitatively by the presence or absence of inhibition zones and zone diameters. Results showed that all the tested bacteria are sensitive to leaf extract. Among the bacteria strains tested, at 5 mg/disc the diameters (mm) of the inhibition zone were 8.37, 10.96, 16.67, 13.62, 11.49, 12.94 and 11.43 for *E. coli, P. aeruginosa, S. typhimurium, A. baumannii, K. pneumonia, S. aureus, E. faecalis*, respectively. The diameters of the inhibition zone increased as the extract concentration was increased (Table 3).

Table 3
Antibacterial activity of *V. corymbosum* leaf extract.

Pathogenic bacteria	Inhibition zone (mm) 5 mg/disc	Inhibition zone (mm) 10 mg/disc	Antibiotic (Control)	Blank
Gram negative bacteria				
E. coli	8.37±0.68	20.59±3.22	18.07±0.11A	-
P. aeruginosa	10.96±0.09	16.92±0.74	13.36±0.45B	-
S. typhimurium	16.67±1.54	23.18±1.46	25.12±0.17B	-
A. baumannii	13.62±0.51	16.02±1.31	28.57±0.63B	-
K. pneumoniae	11.49±0.79	15.32±0.31	10.38±0.04A	-
Gram-positive bacteria				
S. aureus	12.94±10	15.88±1.35	20.52±0.17C	-
E. faecalis	11.43±1.28	14.08±1.39	33.02±1.37A	-

Values are the mean of three determinations±standard deviation.
(-) no inhibition. A: Ampiclilin 10 µg/disc, B: Tetracycline 50 µg/disc, C: Oxacillin 1 µg/disc.

4. DISCUSSION

Blueberries are of interest in health and nutritionalscience because of their high phenolic content compared to other fruit crops[28]. In contrast, the leaves of the plant blueberry have not been given attention. The present study investigated antioxidant and antimicrobial activity of *V. corymbosum* leaves.

It iswidely believed that the antioxidant activity of plant phenolics (e.g., flavonoids, tannins, phenolic acids, etc.) resides mainly in their ability to donate hydrogen atoms or electrons and there by scavenge free radicals[29]. Hydroalcoholic leaf extract of *V. corymbosum* leaves contained significant amount of phenolic and flavonoid compounds. An important study in Psidiumguajava significantly puts forward the high content of phenols, flavonols, etc., which gave a clear-cut property against free radicals[30].

The DPPH assay is simple, quick and commonly used to assess the antioxidant activity of plants and natural compounds,which act as free radical-scavengers or hydrogen donors *in vitro*[31]. The extracts demonstrated appreciable scavenging properties against DPPH radicals. DPPH scavenging activity was significantly (P<0.05) lower than ascorbic acid. Oliveira *et al*[32] reported strawberry leaves exhibit strong free radical scavenging activity on DPPH assay at very low concentrations.

$ABTS^{\bullet+}$ is a well-known nitrogen-centred synthetic radical and is widely used to determine antioxidant activity. In this study, the antioxidant ability to scavenge the radical $ABTS^{\bullet+}$ has been compared to ascorbic acid. A gradual increase in ABTS radicals was found as concentration increased. Nitrite and amine compounds are present in protein-containing foods and both leafy and root vegetables; they can produce nitrosamine and are responsible for cell damage and cancer.[22]. The high phenolic and flavonoid content ofthe leaf extract might be responsible for the increased nitrite scavenging activity. In this study, the tested extract exhibited significant nitrite radical scavenging activity. The scavenging activity of ascorbic acid was significantly stronger than those of extracts (P<0.05). This assay was also performed to investigate antioxidants in *Brassica juncea* by Jung *et al*[33], which showed noticeable activity against the NO_3- radical.

Free or poorly liganded iron may be the basis of many diseases involving cell death and apoptosis[34]. Thus, the chelating ability of the extracts in regards to ferrous ions was investigated. Even though the extracts were not as strong Fe^{2+} chelators as EDTA, the investigated extract demonstrated notable chelating properties. Ng *et al*[35]found that *Rhamnus nakaharai* is a source of phenolic compounds with prominent chelating activity. Literature reports suggest that the antioxidant activity of plant herbs is associated with their reducing power, which terminates free radical chain reactions[36].

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity[37]. In the present study, the reducing power of the *V. corymbosum* leaf extract was evaluated measuring the conversion of a Fe^{3+} ferricyanide complex to the ferrous form. The reducing power of the investigated extract linearlyincreased with concentration. Ganie*et al*[38] reported similar results for *Podophyllum hexandrum* 70% ethanol extract with a strong relationship between the phenolic compound and the reducing power of the extract.

Inhibition of TBARS was also determined, as an index of inhibition of lipid peroxidation, using mice brain homogenate as real animal tissue. As discussed above, the leaf extract was an efficient free radical scavenger, suggesting the prevention of Fe^{2+}-induced lipid peroxidation in mice brain homogenates *in vitro*. Dandlen *et al*[39] demonstrated the capacity of *Thymus* spp. to protect lipid membranes against oxidative processes.

Some compounds demonstrate antioxidant activity when levels of antioxidant related enzymes increase[40]- [42]. SOD and catalase are two key enzymes in detoxifying intracellular O_{-2} and H_2O_2[43]. GPx are critically involved in the detoxification of ROS and have been suggested to be protective against various forms of oxidative injuries[44]. The catalase assayis based on the reaction of the enzyme with methanol in the presence of an optimum concentration of hydrogen peroxide (H_2O_2). Incubation of raw 264.7 cells with 250 µg/mL of tested extract for 24 h resulted in a significant elevation of cellular SOD, CAT and GPx content. Yoo *et al*[45] reported that at a concentration of 100 µg/mL lemon verbena and green tea increased SOD and CAT activities 60.9 and 60.0 unit/mg proteins, respectively.

This study demonstrates that the tested leaf extract (at 0.5, 1.0 and 2.0 mg/mL) was able to protect plasmid DNA against oxidative damage. Burdulis *et al*[46] observed that natural antioxidants such as carotenoids and flavonoids were effective in preventing DNA damage caused by singlet oxygen. Ramos *et al*[47] have shown that *Salvia fruticosa* and *Salvia officinalis* aqueous extracts protected from DNA damage and increased the DNA repair rate in cultured human cells treated with H_2O_2. Kalim *et al*[48] reported DNA damage induced by the Fenton reaction was prevented because of the presence of hydroxyl radical scavenging flavonoids. Thus, the present results suggest that the tested extract may be used for preventing diseases mediated by ROS-induced DNA damage.

Plant polyphenols are considered to possess anti-bacterial activity[49]. The antimicrobial activities of the investigated extract were evaluated by determining the zone of inhibition against two gram-positive and five gram-negative bacteria using a disc diffusion method. The disc diffusion method is quite usefulin obtaining preliminary information. The effects of the leaf extract on the tested microorganisms are shown. The tested extract showed a broad spectrumof activity against all the susceptible microorganisms and exerted astronger antimicrobial effect against gram-negative bacteria than gram-positive bacteria. The antimicrobial activity of berry phenolics has been studied previously[50]–[52] with the ethanol extracts of berry and berry skins showing inhibitory effects on the growing of gram-positive and gram-negative test cultures, *S. aureus, E. faecalis, Listeria monocytogenes, Bacillus subtilis, E. coli, P. aeruginosa, S. typhimurium, Citrobacter freundii*[46]. In a recent study, Sener *et al*[53] reported *Verbascum sinuatum* leaf extracts had a strong antimicrobial activity against *E. faecalis, E. coli, K. pnemoniae, P. aeruginosa, Proteus mirabilis* and *Candida albicans*.

According to the results of this study, it was clearly indicated that *V. corymbosum* leaf extract had significant antibacterial as well as antioxidantactivity against various antioxidant systems *in vitro*. Administrationof leaf extract could significantly enhance the activitiesof antioxidant enzymes (SOD, CAT and GPx). The pronounced antioxidant activity of *V. corumbosum* leaf extract was possibly due to its high phenolic content. Further work is needed to identify bioactive molecules. These results suggested that *V. corymbosum* leaf can be regardedas a natural source of antimicrobials and antioxidants and maybe considered for future use in replacing synthetic antioxidants and antimicrobial agents in pharmaceutical products.

5. REFERENCES

[1] Halliwell B, Gutteridge JMC. *Free radicals in biology and medicine.* 2nd ed. Oxford: Oxford University Press; 1999.

[2] Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med.*2010;48(6):749–762.

[3] Jeong EM, Liu M, Sturdy M, Gao G, Varghese ST, Sovari AA, et al. et al. Metabolic stress, reactive oxygen species, and arrhythmia. *J Mol Cell Cardiol.* 2012;52:454–463.
[4] Oyedemi SO, Afolayan AJ. Antibacterial and antioxidant activities ofhydroalcoholic stem bark extract of*Schotia latifolia* Jacq. *Asian Pac J Trop Med.* 2011;4(12):952–958.
[5] Theuretzbacher U. Resistance drives antibacterial drug development. *Curr Opin Pharmacol.* 2011;11(5):433–438.
[6] Jellin JM, Gregory PJ, Batz F, Hitchens K. *Pharmacist's Letter/Prescriber's Letter.* Stockton: Therapeutic Research Faculty; 2005. Natural medicines comprehensive database; p. 2239.
[7] Haddad PS, Depot M, Settaf A, Chabli A, Cherrah Y. Comparative study on the medicinal plants most recommended by traditional practitioners in Morroco and Canada. *J Herbs Spices Med Plants.* 2003;10:25–45.
[8] Kim SM, Shang YF, Um BH. Preparative separation of chlorogenic acid by centrifugal partition chromatography from highbush blueberry leaves (*Vaccinium corymbosum* L.) *Phytochem Anal.* 2010;21:457–462.
[9] Giovanelli G, Buratti S. Comparison of polyphenolic composition and antioxidant activity of wild Italian blueberries and some cultivated varieties. *Food Chem.* 2009;112(4):903–908.
[10] Martineau LC, Couture A, Spoor D, Andaloussi AB, Harris C, Meddah B, et al. et al. Anti-diabetic properties of the Canadian lowbush blueberry *Vaccinium angustifolium* Ait. *Phytomedicine.* 2006;13:612–623.
[11] Harris CS, Burt AJ, Saleem A, Le PM, Martineau LC, Haddad PS, et al. et al. A single HPLC-PAD-APCI/MS method for the quantitative comparison of phenolic compounds found in leaf, stem, root and fruit extracts of *Vaccinium angustifolium*. *Phytochem Anal.* 2007;18:161–169.
[12] Williams RJ, Spencer JP, Rice-Evans C. Flavonoids: antioxidants or signalling molecules? *Free Radic Biol Med.* 2004;36:838–849.
[13] Zafra SS, Yasmin T, Bagchi M, Chatterjee A, Vinson JA, Bagchi D. Berry anthocyanins as novel antioxidants in human health and disease prevention. *Mol Nutr Food Res.* 2007;51:675–683.
[14] Lau FC, Bielinski DF, Joseph JA. Inhibitory effects of blueberry extract on the production of inflammatory mediators in lipopolysaccharide-activated BV2 microglia. *J Neurosci Res.* 2007;85:1010–1017.
[15] Cignarella A, Nastasi M, Cavalli E, Puglisi L. Novel lipid-lowering properties of *Vaccinium myrtillus* L. leaves, a traditional antidiabetic treatment, in several models of rat dyslipidemia: a comparison with ciprofibrate.*Thromb Res.* 1996;84:311–322.
[16] Piljac-Zegarac J, Belscak A, Piljac A. Antioxidant capacity and phenolic content of blueberry (*Vaccinium corymbosum* L.) Leaf infusions. *J Med Food.* 2009;12(3):608–614.
[17] Abirami P, Gomathinayagam M, Panneerselvam R. Preliminary study on the antimicrobial activity of*Enicostemma littorale* using different solvents. *Asian Pac J Trop Med.* 2012;5(7):552–555.
[18] Oueslati S, Ksouri R, Falleh H, Pichette A, Abdelly C, Legault J. Phenolic content, antioxidant, anti-inflammatory and anticancer activities of the edible halophyte *Suaeda fruticosa* Forssk. *Food Chem.*2012;132(2):943–947.
[19] Pereira E, Barros L, Martins A, Ferreira ICFR. Towards chemical and nutritional inventory of Portuguese wild edible mushrooms in different habitats. *Food Chem.* 2012;130(2):394–403.

[20] Farvin FHS, Jacobsen C. Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast. *Food Chem.* 2013;138(2–3):1670–1681.
[21] Yadav SA, Raj AJ, Sathishkumar R. *In vitro* antioxidant activity of *Barleria noctiflora* L. f. *Asian Pac J Trop Biomed.* 2012;2:316–322.
[22] Patel DK, Kumar R, Prasad SK, Hemalatha S. *Pedalium murex* Linn (Pedaliaceae) fruits: a comparative antioxidant activity of its different fractions. *Asian Pac J Trop Biomed.* 2011;1(4):316–322.
[23] Locatelli M. Anthraquinones: Analytical techniques as a novel tool to investigate on the triggering of biological targets. *Curr Drug Targets.* 2011;12:366–380
[24] Dutta AK, Gope PR, Banik S, Makhnoon S, Siddiquee MA, Kabir Y. Antioxidant properties of ten high yielding rice varieties of Bangladesh. *Asian Pac J Trop Biomed.* 2012;2:99–103.
[25] Dias MI, Barros L, Sousa MJ, Ferreira ICFR. Systematic comparison of nutraceuticals and antioxidant potential of cultivated, *in vitro* cultured and commercial *Melissa officinalis* samples. *Food Chem Toxicol.*2012;50(6):1866–1873.
[26] Jeong JH, Jung H, Lee S, Lee H, Hwang KT, Kim T. Anti-oxidant, anti-proliferative and anti-inflammatory activities of the extracts from black raspberry fruits and wine. *Food Chem.* 2010;123(2):338–344.
[27] Chandrasekara A, Shahidi F. Antiproliferative potential and DNA scission inhibitory activity of phenolics from whole millet grains. *J Funct Foods.* 2011;3(3):159–170.
[28] Kalt W, MacKinnon S, McDonald J, Vinquist M, Craft C, Howell A. Phenolics of *Vaccinium berries* and other fruit crops. *J Sci Food Agric.* 2007;88:68–76.
[29] Sharififar F, Dehghn-Nudeh G, Mirtajaldini M. Major flavonoids with antioxidant activity from *Teucrium polium* L. *Food Chem.* 2009;112(4):885–888.
[30] Ayoola GA, Coker HAB, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC, et al. et al. Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in Southwestern Nigeria. *Trop J Pharm Res.* 2008;7:1019–1024.
[31] Chen R, Liu Z, Zhao J, Chen R, Meng F, Zhang M, et al. et al. Antioxidant and immunobiological activity of water-soluble polysaccharide fractions purified from *Acanthopanax senticosu*. *Food Chem.* 2011;127(2):434–440.
[32] Oliveira I, Coelho V, Baltasar R, Pereira JA, Baptista P. Scavenging capacity of strawberry tree (*Arbutus unedo* L.) leaves on free radicals. *Food Chem Toxicol.* 2009;47(7):1507–1511.
[33] Jung HA, Woo JJ, Jung MJ, Hwang GS, Choi JS. Kaempferol glycosides with antioxidant activity from*Brassica juncea*. *Arch Pharm Res.* 2009;32:1379–1384.
[34] Kell DB. Towards a unifying, systems biology understanding of large-scale cellular death and destruction caused by poorly liganded iron: Parkinson's, Huntington's, Alzheimer's, prions, bactericides, chemical toxicology and others as examples. *Arch Toxicol.* 2010;84:825–889.
[35] Ng LT, Lin CC, Lu CM. Antioxidative effects of 6-methoxysorigenin and its derivatives from *Rhamnus nakaharai*. *Chem Pharm Bull.* 2007;55:382–384.
[36] Knežević SV, Blažeković B, Štefan MB, Alegro A, Kőszegi T, Petrik J. Antioxidant activities and polyphenolic contents of three selected *Micromeria* species from Croatia. *Molecules.* 2011;16:1454–1470.
[37] Yeh JY, Hsieh LH, Wu KT, Tsai CF. Antioxidant properties and antioxidant compounds of various extracts from the edible basidiomycete *Grifola frondosa* (Maitake) *Molecules.* 2011;16:3197–3211.

[38] Ganie SA, Zargar MA, Masood A, Haq E. *In vitro* and *in vivo* evaluation of free radical scavenging potential of ethanolic extract of *Podophyllum hexandrum*. *Afr J Biochem Res.* 2010;4(7):191–195.
[39] Dandlen SA, Lima AS, Mendes MD, Miguel MG, Faleiro ML, Sousa MJ, et al. et al. Antioxidant activity of six Portuguese thyme species essential oils. *Flavour Frag J.* 2010;25:150–155.
[40] Choi C, Cho H, Park J, Cho C, Song Y. Suppressive effects of genistein on oxidative stress and NF kappa B activation in RAW 264.7 macrophages. *Biosci Biotechnol Biochem.* 2003;67(9):1916–1922.
[41] Park CM, Park JY, Noh KH, Shin JH, Song YS. *Taraxacum officinale* Weber extracts inhibit LPS-induced oxidative stress and nitric oxide production via the NF-kappaB modulation in RAW 264.7 cells. *J Ethnopharmacol.* 2011;133(2):834–842.
[42] Tiwari M, Dwivedi UN, Kakkar P. Suppression of oxidative stress and pro-inflammatory mediators by*Cymbopogon citratus* D. Stapf extract in lipopolysaccharide stimulated murine alveolar macrophages. *Food Chem Toxicol.* 2010;48(10):2913–2919.
[43] Esmaeili MA, Sonboli A. Antioxidant, free radical scavenging activities of *Salvia brachyantha* and its protective effect against oxidative cardiac cell injury. *Food Chem Toxicol.* 2010;48(3):846–853.
[44] Lefer DJ, Granger DN. Oxidative stress and cardiac disease. *Am J Med.* 2000;109:315–323.
[45] Yoo KM, Lee CH, Lee H, Moon BK, Lee CY. Relative antioxidant and cytoprotective activities of common herbs. *Food Chem.* 2008;106(3):929–936.
[46] Burdulis D, Sarkinas A, Jasutiene I, Stackevicene E, Nikolajevas L, Janulis V. Comparative study of anthocyanin composition, antimicrobial and antioxidant activity in bilberry (*Vaccinium myrtillus* L.) and blueberry (*Vaccinium corymbosum* L.) fruits. *Acta Pol Pharm.* 2009;66:399–408.
[47] Ramos AA, Azqueta A, Pereira Wilson C, Collins AR. Polyphenolic compounds from *Salvia* species protect cellular DNA from oxidation and stimulate DNA repair in cultured human cells. *J Agric Food Chem.*2010;58(12):7465–7471.
[48] Kalim MD, Bhattacharyya D, Banerjee A, Chattopadhyay S. Oxidative DNA damage preventive activity and antioxidant potential of plants used in Unani system of medicine. *BMC Complement Altern Med.* 2010;10:77–87.
[49] Kurek A, Grudniak AM, Kraczkiewicz-Dowjat A, Wolska KI. New antibacterial therapeutics and strategies.*Pol J Microbiol.* 2011;60(1):3–12.
[50] Baydar NG, Özkan G, Sağdiç O. Total phenolic contents and antibacterial activities of grape (*Vitis vinifera*L.) *extracts*. *Food Control.* 2004;15(5):335–339.
[51] Nohynek LJ, Alakomi HL, Kahkonen M, Heinone M, Helander IM, Oksman-Caldentey KM, et al. et al. Berry phenolics: antimicrobial properties and mechanisms ofaction against severe human pathogens. *Nutr Cancer.*2006;54:18–32.
[52] Puupponen-Pimia R, Nohynek L, Meier C, Kahkonen M, Heinonen M, Hopia A, et al. et al. Antimicrobial properties of phenolic compounds from berries. *J Appl Microbial.* 2001;90:494–507.
[53] Sener A, Dulger B. Antimicrobial activity of the leaves of *Verbascum sinuatum* L. on microorganisms isolated from urinary tract infection. *Afr J Microbiol Res.* 2009;3(11):778–781