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Evaluation of antioxidant activity of aqueous fraction of *Prunus persica* L leaf aqueous extract

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ABSTRACT

An antioxidant is a chemical that prevent the oxidation of other chemicals. They protect key cell components by neutralizing the damaging effect of free radicals, which are natural by production of cell metabolism free radicals are form when oxygen is metabolized. In the body oxygen is essential for the survival of aerobic cell but it has long been known to be toxic to them when supplied at concentration greater than those in normal air. The biochemical mechanism responsible for oxygen toxicity include lipid per oxidation and the generation of H_2O_2 the super oxide radicals, O_2. These free radicals can inhibit or propagate the process of lipid per oxidation. In the present study the reducing power, and DPPH radical scavenging activity was exhibited by aqueous fraction of Prunus persica L leaf aqueous extract. But the products were produced less effect than rutin that used as standard antioxidant. It is evident from our study that the aqueous fraction has shown dose dependent increase in free radical scavenging activity.

INTRODUCTION

Free radicals are atoms or molecules with singlet, i.e. unpaired electron which makes them highly reactive. Oxidative free radicals are generated by metabolic reactions create a chain reaction leading to membrane and other lipid peroxidation, DNA damage, etc. This has been implicated in atherosclorosis (oxidated LDL is more atherogenic), cancers, neurodegenerative and inflammatory bowel diseases[1].

Many endogenous and dietary compounds like superoxide dismutase, ferritin, trasferrin, reruloplasmin, tocopherol, carotene and ascorbic acid have anti oxidant and free radical scavenging properties[1].Small amounts of reactive oxygen species are continually formed in the body in the cell membrane and close to the cells organelles. They act where they are generated. Hence, they can damage most cell structures including membrane lipids, proteins, enzymes and nuclic acids.

The body has mechanisms to mop up the small amounts of oxidants normally formed during metabolic reaction[2].Reactive species such as are formed in controlled amounts by neutrophil lencocytes on exposure to microbes are beneficial to the body in that they participate in destroying the microbes. Excess of oxidants, however, can be harmful to the body[2].Liver is also under constant threat of oxidants and some of the free radical especially H_2O_2. lipid peroxidation has been demostred as one of the important feature after exposure to hepato-toxic substances and also is a measure of extent of hepatic damage[3].Several herbs and herbal formulations are available for the scavenging activity. In addition to this there is a global trend to revive the traditional systems of medicines and renewed interest in the natural remedies for treating human ailments.

With this background we thought of finding a remedy available at a hand's stretch for the treatment and management of several elements due to oxidative stress. In this regard we concentrate on certain herbs which were find out upon through literature survey of various natural product, it was found that a native practitioner has claimed that leaves of *Prunus persica* L possess protective activity. Therefore in the present study was undertaken with a view to establish a scientific basis for the claim that the *Prunus persica* L leaves possess anti-oxidant activity.

METERIALS AND METHODS

1. Plant Material

Prunus persica L leaves were collected from the garden of IBSD, Imphal. The plant was identified and authenticated by Dr. Biseswhori Thongam, Scientist – C (Plant Taxonomy), IBSD, Takyelpat, Imphal, Manipur where a voucher specimen were deposited for reference to Plant Taxonomy and conservation Lab, IBSD, Takyelpat, Imphal. The leaves were shade dried at room temperature. The powder obtained was subjected to soxhlet extraction with the water as solvent. Aqueous extract divided in two equal volumes. One portion concentrated in vacuum evaporator and dried in desiccators and other portion was mixed with equal quantity of petroleum ether and vigorously shack in separating funnel to separate aqueous and petroleum ether portions. Same aqueous portion was again mixed with Chloroform, n- butanol and ethyl-acetate one after another and separated respective portion in similar manner to get the Chloroform, n-butanol, ethyl-acetate and aqueous fractions respectively.

The aqueous fraction of *P. persica* aqueous extract was used for *in- vitro* antioxidant studies. The products were concentrated under reduced pressure and stored in refrigerator 8 ± 2° C.

In-vitro **antioxidant activity:**

Reducing power

Different doses of aqueous fraction of of *Prunus persica* L. leaves aqueous extract were mixed in 1 ml of distilled water so as to get 10 g, 20 g , 40 g, 80 g and 100g concentration. This was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 50 C for 20 minutes. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance (OD) was measured at 700nm.

Increased absorbance of the reaction mixture indicates increase in reducing power. The % Reducing power was calculated by using the formula:

% reducing power = (Test OD – Control OD/Test OD) X 100

DPPH Free radical scavenging activity

The free radical scavenging activity of aqueous fraction of *Prunus persica* L leaves aqueous extract were measured by 1,1 – diphynyl -2 picryl – hydrazil (DPPH). Where, 0.1mM solution of DPPH (Himedia, Munbai) in ethanol was prepared. Then, 1 ml of this solution was added to 3ml of solution of AECc, AFCc, nBFCc, EtFCc and ChFCc at different doses (10 g, 20 g, 40 g, 80 g and 100 g). The mixture was shaken vigorously and allowed to stand at room temperature (25 ± 2o C) for 30 minutes. The absorbance was measured at 517 nm in UV – Visible spectrophotometer (Shimadzu UV – 1700 Pharma Spec) against 60% ethanol solution after 30 minutes where lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The DPPH radical concentration was calculated using the following equation:

DPPH scavenging effect (%) = 100 – (A0 –A1/A0 X 100)

Where A0 was the absorbance of the control (DPPH solution & water without any test sample) reaction and A1 was the absorbance in the presence of test sample. DPPH scavenging effect of test samples were also expressed as an effective concentration at 50% (EC50) i.e. the concentration of the test sample required to give 50% decrease in the absorbance compared to that of control reading. EC50 was calculated from the different graph for different samples (DPPH scavenging effect (%) vs Concentration of samples) showing 50% inhibition. The results are compiled in Table -2

Methods for evaluating antioxidant activity

The various methods used for the assessment of antioxidant activity are assay of lipid, peroxydation, and reduced glutathione assay, DPPH free -radical scavenging activity, nitric oxide scavenging, super oxide scavenging and chemiluminescence.

A. Invitro antioxidant activity:

The following in-vitro models are use to evaluate antioxidant activity.

1. In vitro CCl_4 - induced lipid peroxidation.
2. Reducing power.

3. Superoxide anion scavenging activity.
4. Hydroxyl radical scavenging activity.

1. In vitro Carbon tetrachloride (CCl_4) – induced lipid peroxidation[6]:

The process of lipid peroxidation proceed to form a conjugated diene, 'malonaldehyde like' which has λ_{max} 553nm. Thus, the concentration of MDA determines the extent of lipid peroxidation. A red colour complex is formed out of reaction of MDA with thiobarbituric acid, which is read colorimetrically at 553nm.

Procedure :

A solution of 30% (w/v) rat liver homogenate in ice cold KCl (0.15 M) was prepared by using a homogenizer and 0.5 ml of the homogenate was transferred to small conical flasks. The flasks containing homogenates were added with 10 l of CCl_4. Then 1.5 ml of 0.15M KCl and 0.5 ml of sample in different dose respectively. To the control flask only 0.5 ml of vehicle (Phosphate buffer) was added. Flasks were then incubated at 37 C in a constant shaker bath (150 cycles/min) for 45 minutes. After incubation, the reaction was stopped by addition of 4.0 ml of 10% w/v trichloroacetic acid. The mixtures were centrifused, 2ml of thiobarbituric acid (0.68% w/v) was added (containing 2 ml supernatant) prior to heating in a water bath for 15 minutes. The colour was stabilised with KOH and the optical density (OD) was measured at 543 nm.

Increased absorbance indicates greater MDA concentration. Conversely, reduction in absorbance indicates lesser concentration of MDA and indirectly, less extent of lipid peroxidation. The % inhibition of lipid peroxidation upon addition of varying doses of extracts was calculated by using the following formula

$$\%\ \text{inhibition in lipid peroxidation} = \frac{\text{Control OD} - \text{TestOD} \times 100}{\text{Control OD}}$$

2. Reducing power[7]:

The reducing power can determined according to the method of Oyaizu (Oyaizu, 1986):

Procedure:

Different doses of sample are mix in 1 ml of distilled water so as to get 10 g, 25 g and 50 g concentration. This was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 50 C for 20 minutes. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifused at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance (OD) was measured at 700nm.

Increased absorbance of the reaction mixture indicates increase in reducing power. The % increase of reducing power was calculated by using the formula mentioned in the estimation of lipid peroxidation in the earlier pages.

3. Hydroxyl radical scavenging activity[8]:

In biochemical systems, superoxide radical and H_2O_2 react together to form the hydroxy radical, OH*, which can attack and destroy almost all known biochemical.

Phenylhydrazine when added to erythrocyte ghosts cause peroxidation of endogeneous lipids and alteration of membrane fluidity. This peroxidation damage to erythrocytes is probably initiated by active oxygen species like O_2*, OH* and H_2O_2 which are generated in solution from auto-oxidation of phenylhydrazine. This forms the basis of this experiment.

Procedure:

Hydroxyl radical generation by phenylhydrazine has been measured by the 2-deoxyribose degradation, assay of Hathwell and Gutteridge[7] in 50 mM phosphte buffer (pH 7.4) containing 1 mM deoxyribose, 0.2 mM phenylhydrazine hydrochloride and other additions as necessary in a total volume of 1.6ml. incubation was terminated after 1 hour or 4 hour and 1 ml each of 2.8% TCA and 1%(w/v) thiobarbituric acid were added to the reaction mixture and heated for 10 minutes in a boiling water bath. The tubes were cooled and absorbance taken at 532 nm.Decrease in absorbance is indicating the increase in the hydroxyl free radical scavenging activity. The % reduction in the OD is calculated.

4. Superoxide anion scavenging activity[9]:

Oxygen is essential for the survival of aerobic cells, but it has long been known to be toxic to them when supplied at concentration greater than those in normal air. The biochemical mechanisms responsible for oxygen toxicity include lipid peroxidation and the generation of $H_2O_2^+$ the superoxide radical, O_2*. This superoxide radical can inhibit or propagate the process of lipid peroxidation. Measurement of superoxide anion scavenging activity can done by using the method explained by Nishimiki (Nishimiki et al., 1972) and modified by Ilhami et al.

Procedure :

About 1 ml of nitroblue tetrazolium (NBT) solution (156 m NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (468 m in 100 mm phosphate buffer, pH 7.4) and 0.1 ml of sample solution mix. The reaction was started by adding 100 1 of Phenazine methosulphate (PMS) solution (60 PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction was mixture was incubated at 25 C for 5 minutes, and the absorbance at 560 nm was measured against blank.

Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. % inhibition of OD was calculated by using the formula mentioned earlier.

5. Super oxide radical scavenging activity[10]

In-vitro super oxide radical scavenging activity is measured by riboflavin/light/NBT (Nitro blue tetrazolium) reduction. Reduction of NBT is the most popular method. The method is based on generation of super oxide radical by auto oxidation of riboflavin in presence of light. The super oxide radical reduces NBT to a blue colored formazon that can be measured at 560nm. The capacity of extracts to inhibit the colour to 50% is measured in terms of EC50. Antioxidant activity of Ailanthus, flavanoids and triphala has been reported in terms of super oxide radical scavenging activity. The super oxide radicle can also be detected by oxidation of hydroxylamine, yielding nitrite which is measured colorimetric reaction.

Preparation of reagents used

Phosphate buffer: 200ml of phosphate buffer of pH 7.6 was prepared according to IP.

Riboflavin solution: 5mg riboflavin was dissolved in 25ml phosphate buffer.
EDTA solution: 402mg EDTA was dissolved in 10 ml phosphate buffer. **NBT solution:** 5mg NBT was dissolved in 5ml phosphate buffer.

Procedure -

100 l riboflavin solution 200 l EDTA solution, 200 l ethanol and 100 l NBT solution was mixed in a test tube and the reaction mixture was diluted up to 3 ml with phosphate buffer. The absorbance of solution was measured at 590 nm using phosphate buffer as blank after illumination for 15 minutes. This was taken as controlled reading.

Screening of test sample of different concentration: 100 l test sample, 100 l riboflavin, 200 l EDTA, 200 l ethanol and 100 l NBT solution was mixed in a test tube and the reaction mixture was diluted upto 3ml with phosphate buffer. The absorbance of solution was measured after illumination for 15 minutes at 590 nm.

Percentage reduction was calculated and this activity was expressed as an effective concentration at 50% (EC 50) i.e. the concentration of the test sample required to give 50% decrease in the absorbance compared to that of control reading. EC 50 was calculated from the graph showing 50% inhibition.

$$\% \text{ Reduction} = \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \times 100$$

Table-1Anti Oxidant activity (Reducing power) of aqueous fraction of *P. persica* L leaf aqueous extract

Group	Concentration	Absorbance	inhibition (%)	EC_{50} (g)
Control		**0.018**		
Control + Standard (Rutin)	1 g	0.029	37.93	**1.550**
Control + Standard (Rutin)	2 g	0.034	47.06	
Control + Standard (Rutin)	4 g	0.040	55.00	
Control + Standard (Rutin)	8 g	0.064	71.87	
Control		**0.005**		
Control + Standard (Rutin)	10 g,	0.049	89.79	
Control + Standard (Rutin)	20 g ,	0.058	91.37	
Control + Standard (Rutin)	40 g	0.082	93.90	
Control + Standard (Rutin)	80 g	0.118	95.76	
Control + Standard (Rutin)	100 g	0.134	96.26	
Control		**0.023**		
Control + Aqueous Fraction	10 g, ,	0.030	23.33	60.796
Control + Aqueous Fraction	20 g ,	0.039	41.02	
Control + Aqueous Fraction	40 g	0.042	45.24	
Control + Aqueous Fraction	80 g	0.052	55.77	
Control + Aqueous Fraction	100 g	0.061	62.29	

Standard Drug: Rutin

Table-2 Anti Oxidant activity (DPPH radical Scavenging) of aqueous fraction of *P. persica* leaf extract

Group	Concentration	Absorbance	Inhibition(%)	EC_{50} (g)
Control		**0.372**		
Control + Standard (Rutin)	1 g	0.233	37.36	21.498
Control + Standard (Rutin)	2 g	0.217	41.67	
Control + Standard (Rutin)	4 g	0.199	46.50	
Control + Standard (Rutin)	8 g	0.186	50.00	
Control		**0.467**		
Control + Standard (Rutin)	10 g	0.224	52.03	
Control + Standard (Rutin)	20 g	0.193	58.67	
Control + Standard (Rutin)	40 g	0.188	59.74	
Control + Standard (Rutin)	80 g	0.177	62.09	
Control + Standard (Rutin)	100 g	0.167	64.24	
Control + Aqueous Fraction	10 g	0.405	13.28	72.79
Control + Aqueous Fraction	20 g	0.371	20.56	
Control + Aqueous Fraction	40 g	0.278	40.47	
Control + Aqueous Fraction	80 g	0.207	55.67	
Control + Aqueous Fraction	100 g	0.195	58.24	

Standard Drug: Ascorbic acid

Table-3 Anti Oxidant activity (Super-oxide Scavenging) of aqueous fraction of *P. persica* leaf extract

Group	Concentration	Absorbance	Inhibition(%)
Control		**0.021**	
Control + Standard (Rutin)	1 g,	0.022	04.54
Control + Standard (Rutin)	2 g,	0.024	12.50
Control + Standard (Rutin)	4 g,	0.055	61.81
Control + Standard (Rutin)	8 g,	0.67	68.65
Control		**0.006**	
Control + Standard (Rutin)	10 g	0.022	72.73
Control + Standard (Rutin)	20 g	0.032	81.25
Control + Standard (Rutin)	40 g	0.040	85.00
Control + Standard (Rutin)	80 g	0.047	87.23
Control + Standard (Rutin)	100 g	0.050	88.00
Control		**0.013**	
Control + Aqueous Fraction	10 g	0.027	51.85
Control + Aqueous Fraction	20 g	0.029	55.17
Control + Aqueous Fraction	40 g	0.031	58.06
Control + Aqueous Fraction	80 g	0.141	90.78
Control + Aqueous Fraction	100 g	0.144	90.97

Standard Drug: Rutin

CONCLUSION

The aqueous Fraction of *Prunus persica* L leaf aqueous extract demonstrate dose dependent free radical scavenging activity in *in-vitro* models. However standard rutin has shown more antioxidant activity than test products. On the basis of such information, further studies can be done on isolation, characterization of phytochemical anti-oxidant present in aqueous and extract and chloroform fraction using different *in-vitro* and *in-vivo* model that may give protection to the mankind for various dreaded disease in future.

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