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EVALUATION OF THE EFFECT OF TEMPERATURE ON ANTIOXIDANT STATUS OF *PHYLLANTHUS EMBLICA* EXTRACTS USING CELL FREE AND CELL CULTURE SYSTEM

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ABSTRACT

Regular consumption of natural antioxidants rich diets is associated with reduced risk of various diseases like cardiovascular neurodegenerative and other metabolic diseases. Vegetables are mostly consumed in cooked form and this may alter the nature of phytoconstituents, which may result in reduced health benefit to the consumer. In the present study an attempt was made to evaluate the effect of temperature on antioxidant status of the vegetable extracts of amla (PE). Both hot (PEH) and cold (PEC) extracts were prepared for the studies. The extract were studied for its percentage yield, phytochemical screening, total phenolic content, total flavonoid content and the antioxidant potential invitro cell free and cell culture systems. The percentage yield was high in hot extracts (9-15% w/w) compared to cold extracts (5-8% w/w). The total phenol and flavonoid content was found to be high in PE and it was around 400 mg GAE/g of extract and 37- 40 mg QE/g of extract respectively. HPTLC study reveals the presence of quercetin, rutin, and gallic acid in the cold extract. The overall study concludes that PE possess rich antioxidant principles. Among the PE the PEC demonstrated high antioxidant activity in both cell free and cell culture systems. This indicates that the difference in activity may be due to the loss of antioxidant principles during heating process in PEH. Further it indicates that the prolonged cooking process may reduce the health benefit of the vegetables.

KEYWORD: PEC, PEH, Antioxidant, Cell Culture Systems, Cell free Systems.

1. INTRODUCTION

The development of disease is complex in nature and is now being under stood as a leading cause is the imbalance in the basic homeostasis phenomenon in the body. Various measures are being taken up to correct the root cause of this imbalance. Human beings constantly struggle with changing environmental conditions to maintain ideal health and strength throughout life. The human body depends on the continuous interaction between internal and external factors. When this interaction is in a state of equilibrium, Man enjoys health and when it fails, either due to internal deficiency or hostile environmental factors, the balance is disturbed and lead to disharmony and disease.[1]

Oxidative stress is a important etiological factor defined in several chronic human diseases like cancer, hyperlipidemia, diabetes, rheumatic and neurological diseases and also in aging process.[2] Therefore, it is important to increase the body's antioxidant potential for fighting against oxidative stress. For this purpose, it has been widely advised that individuals increase their intake of dietary antioxidants. Regular consumption of fruits and vegetables rich in natural antioxidants is associated with reducing the risk of various diseases.[3]

Most of the diseases are due to free radicals resulting in oxidative stress. Free radicals such as superoxide anion, Hydroxyl radicals and non-radical species such as hydrogen peroxide, singlet oxygen are different form of activated oxygen constituting reactive oxygen species (ROS). Active antioxidant defense system is required to balance the production of free radicals. Antioxidant therapy has gained an enormous importance in the treatment of diseases like diabetes mellitus, cancer and neurodegenerative disease.

Free radicals are continuously produced by our body's use of oxygen such as in respiration and some cell mediated immune function. They are also generated through environmental pollutants, cigarettes smoke, automobile exhaust, radiation, air-pollution, pesticides, etc.[4]

The antioxidant defense system in the body can only protect the body when the amount of the free radicals is within the normal physiological level. But when this balance is shifted towards more of free radicals, increasing their burden in the body either due to environmental conditions or produced within the body, it leads to oxidative stress, which may be result in tissue injury and subsequent diseases.[5]

2. MATERIALS AND METHODS

2.1 Materials

1.1-Diphenyl-2-picrylhydrazyl (DPPH), Dulbecco's modified eagles medium (DMEM), eagles minimum essential medium (EMEM), Trypsin, EDTA, Trichloroacetic acid (TCA), gallic acid and fetal bovine serum (FBS) was procured from Sigma Aldrich, Louis, USA. Quercetin, ammonium molybdate, sulphuric acid, 2 2'-azinobis 3-ethylbenzoline 6-sulfonate, ABTS, Thiobarbituric acid and Folin-ciocalteu's was acquired from HiMedia Laboratory Pvt.Ltd, Mumbai., India. Murine embryonic fibroblasts cell line (NIH3T3) was obtained from national centre for cell sciences (NCCS) Pune, India. Sodium carbonate, ascorbic acid, aluminum chloride, potassium acetate, potassium per sulfate, sodium phosphate was acquired from SRL Pvt. Ltd, Mumbai, India.

2.2 Methods

2.2.1 Collection of fruit

Fresh fruit of Phyllanthus emblica collected from fields of Coimbatore district, Tamilnadu, India and authenticated by taxonomist and the specimen is deposited in the Department of Pharmacognosy KMCH College of Pharmacy, Coimbatore, Tamilnadu. Authentication specimen number is KMCH/P.COG/008/2017.

2.2.2 Extraction Procedure

2.2.2.1 Hot extraction (Soxhlet Apparatus)

The crude PE fruit were reduced to small pieces, dried under shade and were pulverized to coarse crude powder, stored in an air tight container at room temperature till the extraction.

200g of coarsely powdered PE were taken separately in 1000 ml soxhlet apparatus and extracted with 95% methanol for 5 days. The crude methanolic extract were concentrated in rotory evaporator under reduced pressure, the percentage yield was determined and stored in vacuum desiccator until use. The obtained extract named as PEH.

2.2.2.2 Cold extraction (Lyophilization)

Fresh PE fruit were washed thoroughly then juice of whole vegetables were prepare on a juicer and filtered through muslin cloth following whatman filter paper and lyophilized (ALPHA 1-2 LD Plus) by continuous freeze drying. The percentage yield was determined, powder sample stored in air tight container until use. The obtained extract was named as PEC.

2.3 Phytochemical Analysis

2.3.1 Quantitative analysis

2.3.1.1 Estimation of Total Phenol Content

Total Phenolic Content of the extracts was measured by Folin-Ciocalteu (F-C) assay with some modifications.[6] Briefly, 100µl of sample were added to 2ml microcentifuge tube followed by 860µl distilled water, 50µl F-C reagents, mixed and allowed to react for 5 min before adding 100µl 20% Na_2CO_3, 890µl distilled water, mixed and allowed to stand 60 min at room temperature. Absorbance was measured at 725nm. The blank was prepared in similar manner without sample/standard, Calibration curve was plotted using gallic acid as standard. The results were expressed as milligram of gallic acid equivalents (GAE) per gram of extract.

2.3.1.2 Estimation of Total Flavanoid Content

Total flavanoid were estimated by Aluminium chloride colorimetric assay with some modification.[7] An aliquot (1 ml) of diluted sample or standard solution of quercetin (10, 20, 30 and 40µg/ml) was mixed with 50µL of $NaNO_2$ in 2ml microcentifuge tube. After 6 min, 50µL of a 10% AlCl3 solution was added and allowed to stand for 6 min, and then 50µl 1M potassium acetate solution was added to the mixture. Distilled water was added to bring the final volume to 2 ml, and then the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm against prepared blank. Blank was prepared in the same above manner omitting sample/standard. All values were expressed as milligrams of quercetin equivalent per 1g of sample.

2.4 High performance thin layer chromatography

Instrument: - CAMAG Linomat 5

Application Parameter: - Spray gas – Inert gas; Sample solvent type – Methanol

Development Parameter: - Chamber type – Twin Trough Chamber 10^x10cm; Mobile phase- Toluene: Ethyl acetate: Formic acid: Methanol (3:6:1.6:0.4).

Detection parameter: - Detector - CAMAG TLC Scanner; Wave length – 254; Lamp – D2 & W; Measurement type – Remission; Measurement mode – Absorption.

The samples and standard were spotted in form of bands with a Camag microliter syringe on pre-coated silica gel coated aluminum plate 60 F_{254} (10^x10 cm with 0.2 mm thickness) using a camag linomat 5 applicator. The plates were pre-washed with methanol and activated at 60^0c for 5 mts prior to chromatography.

2.5 In Vitro Pharmacological Studies

2.5.1 Antioxidant activity in cell free system

2.5.1.1 Estimation of Total Antioxidant Capacity (TAC)

TAC of extracts was determined by phosphomolybdenum assay. Briefly 0.2 ml of various concentrations of extract and 2 ml phosphomolybdenum reagent (28mM sodium phosphate and 4mM ammonium molybdate in 0.6 M sulphuric acid) were mixed in capped test tubes and were incubated at 95^0C for 90 min on a water bath. Test tubes were removed from the water bath, cooled at room temperature and absorbance of reaction mixture was measured at 695nm. Similarly, series of reaction mixture were made using different concentrations of ascorbic acid (AA). The blank was prepared in the similar manner without extract. TAC of extract was calculated from the graph and expressed as milligrams of ascorbic acid equivalent (AAE) per gram of dry weight of extract.

2.5.1.2 DPPH radical scavenging assay

The free radical scavenging activities of the samples were measured using the stable DPPH radical assay method with some modifications.[8] Sample extracts of different concentrations were added to 1ml of 0.1mM methanolic DPPH solution. The mixture were shaken vigorously and allowed to stand for 20 min in the dark at room temperature and the absorbance was monitored at 517 nm. DPPH solution without samples served as the control. Ascorbic acid at a concentration range of 1-5 µg/ml was considered as standard. DPPH radical scavenging activity percentage was calculated for the samples and the standard using following formula;

% scavenging activity = $\{(Ab_C - Ab_S) \div Ab_C\} \times 100$

Where, Ab_C is Absorbance of control and Ab_S is absorbance of sample.

2.5.1.3 ABTS radical scavenging assay

This assay is based on the inhibition of the absorbance of the radical cation 2, 2'-azinobis (3-ethylbenzothiazoline 6-sulfonate; ABTS.[9] ABTS was dissolved in water to a 7mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 Mm potassium persulfate (final concentration) an allowing the mixture to stand in the dark at room temperature for 24 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature.

For the study $ABTS^{\bullet+}$ solution was diluted with phosphate buffer saline (PBS) (pH 7.4) to an absorbance of 0.70 (±0.04) at 734 nm and equilibrated at 30^0C. After addition of 1.8 mL of diluted $ABTS^{\bullet+}$ solution to 20µL of various concentrations of sample extracts or series concentration of Ascorbic acid standard, the reaction mixture was incubated for 6 min in a microcentrifuge tube at 30^0C. The decrease in absorbance at 734 nm was determined exactly at 6 min after initial mixing for all samples. The absorbance of the $ABTS^{\bullet+}$ without sample/standard, i.e. the control, was measured daily. All measurements were performed in triplicate. The percentage inhibition of $ABTS^{\bullet+}$ by the samples were calculated according to formula:

$$\% \text{ Inhibition} = (\{A_{C(0)}-A_{A(t)}\}/A_{A(0)})\times 100$$

Where $A_{C(0)}$ is the absorbance of control at t=0 min; and $A_{A(t)}$ is the absorbance of the antioxidant (sample extracts or Ascorbic acid standard) at t=6 min.

2.5.2 Antioxidant activity in animal cell culture model

2.5.2.1. Animal cell culture

NIH3T3 were maintained in Dulbeccos's modified eagle's eagle's medium (DMEM) supplement with 10% v/v fetal bovine serum. Cells were maintained at 37 C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passage weekly, and the culture medium was changed twice a week.

2.5.2.2 Induction of oxidative stress

The Cells (NIH3T3) were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using hemocytometer. Cells were seeded into 24 well plates at the cell density of 4×10^4 cells per well and incubated at 37^0C, 5% CO_2, 95% air and 100% relative humidity for 24 h to assay the capacity of extracts to protect the NIH3T3 cells from ROS-mediated oxidative injury, cells were pre incubated for 48 h in the presence of different concentrations of extracts (5 and 10µg/ml). At the end of the pre incubation time, the medium was changed before the addition of the oxidative stress inducing agents such as 100µM Fe_2SO_4 for lipid peroxidation studies and 100mM of H_2O_2 to evaluate the superoxide dismutase (SOD) and catalase activities. The oxidation was performed in phosphate buffered saline (PBS). Ascorbic acid was used as standard antioxidant at 100µM concentration.

2.5.2.3 Lipid peroxidation

The extend of Lipid peroxidation was estimated by the levels of malondialdehyde measured using thiobarbituric acid-reactive species (TBARS) assy. Adherent cells were detached using trypsin/EDTA solution and centrifuged at 3000 rpm for 10 min. The pellet was re suspended in 500µl of deionized water and lysed by 5 cycles of sonication during 20 s at 35%. 1ml of TBA solution (15% trichloroiacetic acid, 0.8% thiobarbituric acid, 0.25 HCl) was added. The mixture was heated at 95^0C for 15 min to from MDA-TBA adduct. Optical density (OD) was measured by a spectrophotometer at 532nm.values were reported to a calibration curve of 1, 1, 3, 3-tetramethoxypropane (1.1.3.3.TMP).

2.5.2.4 Determination of SOD activity

The SOD activity was determined by spectrphotometry (420 nm) using the pyrogallol assay.[10] with certain modified as follows: The rate of autoxidation of pyrogallol in Trios-cacodylic acid diethylnetriaminepentaacetic acid (DTPA) buffer (pH 8-8.2) was determined (A1). The autoxidation of pyrogallol was evaluated under the same conditions after addition of 25µl of cells lysate (A2). The percentage inhibition of pyrogallol oxidation was determined using the formula,

% inhibition = {(A1-A2)/A1} ×100

3. RESULTS AND DISCUSSION

3.1 Extraction

The hot and cold extraction methods were used to prepare the extracts of fruit of *Phyllanthus emblica* L (PE). Hot extractions (PEH) were carried out by soxhlet apparatus using 95% methanol and cold extraction (PEC) by freeze drying of aqueous juices by using lyophilizer and the obtained extracts were stored in desiccator until further use. He hot extract was appeared as brownish, whereas the cold extract were found to be greenish in colour. It was found that the percentage yield was high for PEH than PEC.

Table 1: Percentage yield and consistency.

S. No.	Extract	Colour	Consistency	% Yield
1	PEH	Brownish	Semisolid	15.55
2	PEC	Greenish Yellow	Powder	8.41

3.2 Total phenolic and flavonoid content

Plant phenolic and flavonoids are major group of plant constituents acts as powerful antioxidants to scavenge free radicals responsible for many ailments.

TPC and TFC were found high in cold extraction method when compared to hot extraction method.

Table 2: Total Flavonoid and Phenol content.

S.No	Sample	Total phenolic content in mg GAE/g	Total flavonoid content in mg GAE/g
1	PEH	409.5	37.2
2	PEC	413	40.2

3.3 High performance thin layer chromatography

HPTLC was performed to study the presence of phenolic constituents by comparing with standard markers quercetin, rutin and gallic acid. Cold extract showed the presence of all the three markerse while hot extract contains rutin and gallic acid.

Table 3: HPTLC analysis of Standard Phenolic markers.

Sample	Quercetin	Rutin	Gallic acid
PEH	-	+	+
PEC	+	+	+

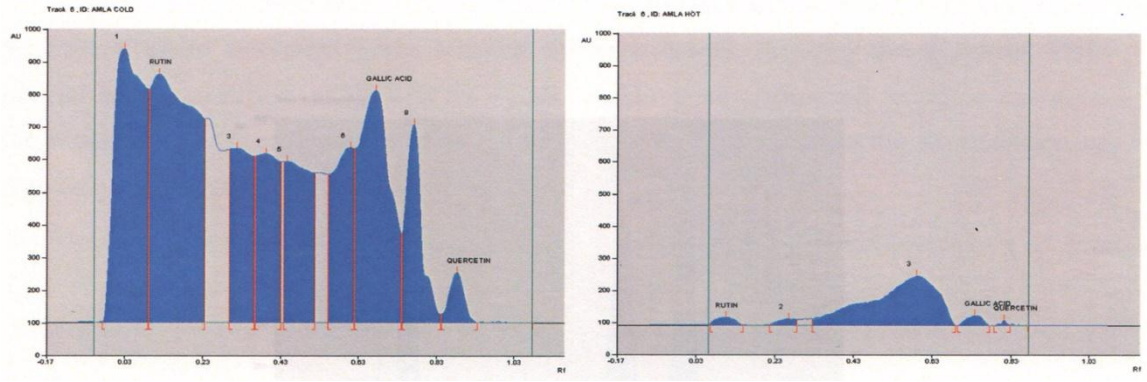

Fig. 1: Chromatogram of PEC. Fig. 2: Chromatogram of PEH.

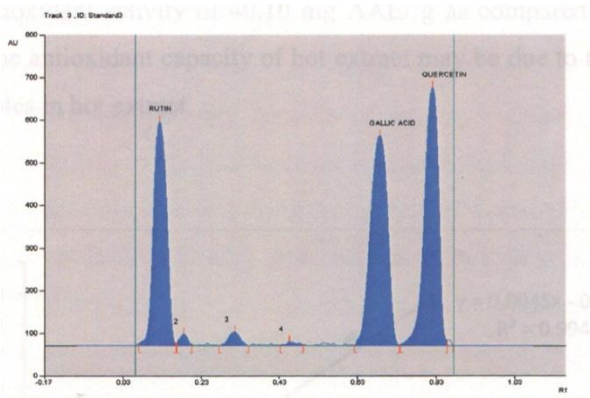

Fig. 3: Chromatogram of standard (rutin,quercetin,gallic acid).

3.4 Invitro Pharmacological Studies

3.4.1 Antioxidant activity in cell free system

3.4.1.1 Total antioxidant capacity

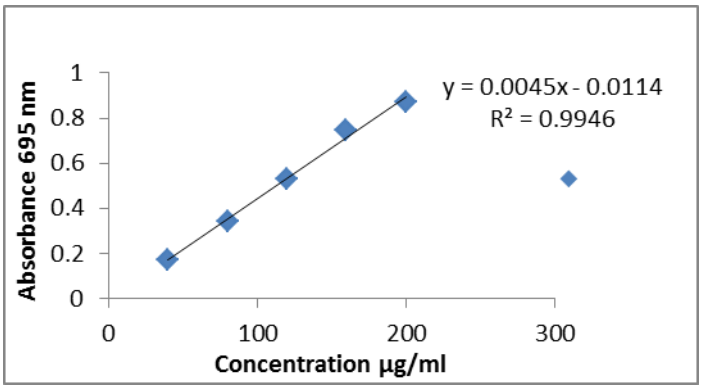

Fig. 4: Standard graph of ascorbic acid.

Table 4: Total antioxidant capacity.

Si No.	Sample	Total antioxidant capacity (Ascorbic acid mg/g equivalent)
1	PEH	25.15
2	PEC	4010

3.4.1.2 DPPH radical scavenging activity

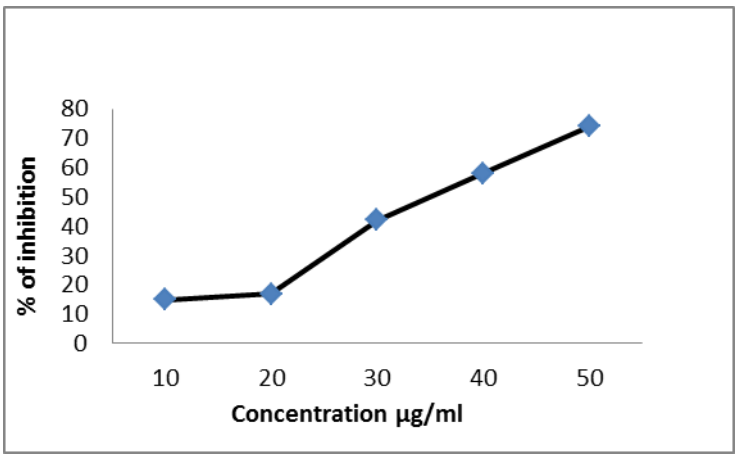

Fig. 5: Standard graph of ascorbic acid.

Table 4: DPPH radical scavenging activity of extracts.

Si No.	Sample	IC 50 Value (µg/ml)
1	Standard	1.45
2	PEH	35
3	PEC	23

3.4.1.3 ABTS radical scavenging activity

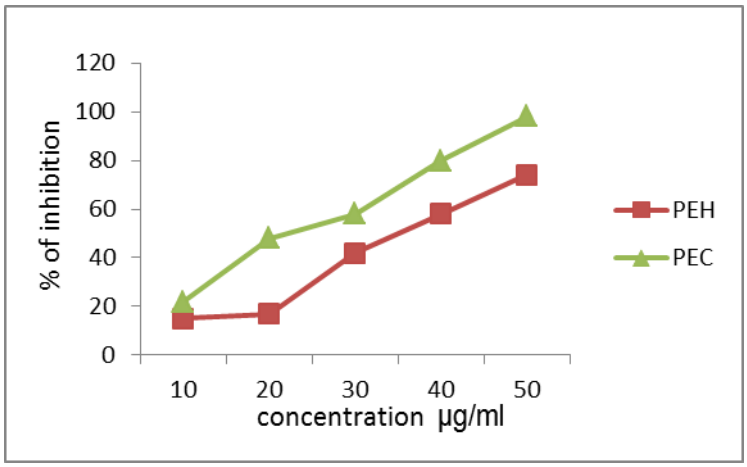

Fig. 6: Absorbance of PEH and PEC.

Table 5: ABTS radical scavenging activity of extracts.

Si No.	Sample	IC 50 Value (µg/ml)
1	Standard	215
2	PEH	355
3	PEC	230

3.4.2 Antioxidant activity in animal cell culture model

Among the extracts, PE demonstrated higher antioxidant potential in all in vitro cell fee method. However there was a difference in antioxidant potential of PEH and PEC and the later proved to be higher when compared to PEH. Further to confirm the antioxidant status, animal cell culture method was performed using murine embryonic fibroblast (NIH 3T3) Cell line.

3.4.2.1 Lipid peroxidation

Cells were cultured with or without addition of extract for 24 hours in an 24 well plates. There were two different concentrations, which did not show any toxic effects such as 5 and 10 µg/ml were used. Oxidative stress was induced by adding 100 µmol Fe2+ solution (as Fe2SO4) in PBS for one hour. Malondialdehyde, a lipid peroxidation marker was evaluated. The oxidative treatment with 100 µmol Fe2+ resulted in the increase of MDA level due to enhancement of lipid peroxidation reaction. The oxidative treatment resulted in at least 8 fold increase in TBARS concentration (24.2±3.5 µmol) compared with controlled cells (3.0±0.6 µmol). A significant protection against ROS inducing damage was obtained for both PEH and PEC at different concentrations. PEC exhibited higher peroxidation against ROS when compared with PEH.

Table 6: MDA levels of PEH and PEC treated NIH 3T3 cell line after induction of oxidative stress by 100 µM of Fe_2SO_4.

Si No.	Sample	Malondialdehyde content (µmol)
1	Cells	3.0 ± 0.6
2	Cells + Fe^{2+}	24.2± 3.5
3	Ascorbic acid	8.3 ± 0.8
4	PEH 5 µg	17.3 ± 0.5
5	PEH 10 µg	16.0 ± 0.9
6	PEC 5 µg	12.2 ± 0.4
7	PEC 10 µg	10.9 ± 0.5

3.4.2.2 Antioxidant enzyme activities

Bio activity of the extract on SOD as antioxidant enzyme was measured in NIH 3T3 cells. The induction of oxidative stress with H_2O_2 leads to an increase in the SOD activity which can be explained by an adaptation of the enzymatic anti-oxidant system of the cell to the ROS production. The standard substrate of SOD is the superoxide anion which was dismuted to H_2O_2. However, it was recently reported that high level of H_2O_2, this enzyme, especially Mn SOD, enhanced the reverse reaction yielding to superoxide anion[11] which can explain the increased SOD activity after the treatment with H_2O_2. The treatment of the cells with both PEH and PEC extracts at different concentrations induce a significant decrease in SOD activities. This result could be explained by the reestablishment of the oxidant/antioxidant balance in the cell line and confirm the anti-oxidant property of the extracts. This antioxidant activity could be explained by high poly phenol content.

Table 7: Effect of pretreatment of NIH3T3 Cell line with hot and cold extracts of Amla on superoxide dismutase activity.

Si No.	Sample	SOD (% Inhibition)
1	Cells	22.3 ± 2.2
2	Cells + H_2O_2	93.5 ± 5.1
3	Ascorbic acid	25.7 ± 1.9
4	PEH 5 µg	45 ± 0.7
5	PEH 10 µg	42 ± 1.7
6	PEC 5 µg	33 ± 2.1
7	PEC 10 µg	29 ± 2.6

4. CONCLUSION

Our studies clearly bring out that among the PE the PEC demonstrated high antioxidant activity in both cell free and cell culture systems. This indicates that the difference in activity

may be due to the loss of antioxidant principles during heating process in PEH. Further it indicates that the prolonged cooking process may reduce the health benefit of the vegetables.

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