EVALUATION OF *IN-VITRO* ANTIOXIDANT ACTIVITY OF HUMAN PLACENTAL EXTRACT

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Summary

Human placenta being rich source of therapeutic components has been used for years in management of different diseases. In present work, antioxidant profile of Human Placental Extract (HPE) has been studied by using different *in-vitro* models. Study includes inhibition of oxygen derived free radicals (ODFR) *viz*., hydroxyl radical, reducing power, superoxide anion scavenging assay, nitric oxide scavenging assay and anti-lipid peroxidation. All the antioxidant activities were compared with standard antioxidants such as BHA and α- tocopherol acetate. Extract was found to be a good scavenger of hydroxyl radical, nitric oxide and superoxide anion with an IC_{50} of 1.125 ± 6, 0.981 ± 5, 1.52 ± 4.2 mg/ml respectively. The reducing power was calculated in term of absorbance which represented transfer of ferric ions to ferrous. Anti lipid peroxidation was done in liver and brain homogenate and result showed IC_{50} of 1.42 ± 5.2 and 2.21 ± 3.3 mg/ml respectively. All antioxidant scavenging properties were concentration dependent. The results obtained from the current study suggest that HPE is a potential source of natural antioxidants.

Key words: Lipid Peroxidation, Antioxidant, Superoxide radical, Human Placental Extract.

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Introduction

Living systems have evolved to survive in the presence of molecular oxygen and for most biological systems; life depends upon its presence. The oxidative properties of oxygen play a vital role in diverse biological phenomena, including use of nutrient food, electron transport to produce ATP, and the removal of xenobiotics. However, oxygen has double edged properties, being essential for life; it can also provoke damaging oxidative events within cells[1]. Recent investigations have shown that the antioxidant property could be correlated with oxidative stress defense and different human diseases including cancer, atherosclerosis, aging, inflammation, certain nervous system disorders (like Alzheimer's disease). Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating free catalytic metals and also by acting as oxygen scavengers[2].

Human placenta has been demonstrated to possess various therapeutic activities ranging from wound healing to immunomodulatory. Use of aqueous extract of human placenta in wound healing, ophthalmology, infertility, apoplexy, epilepsy etc has evolved from folk knowledge. Recent research studies reveal that Human Placental Extract (HPE) is the rich resources of various bio-active substances like polydeoxyribonucleotides (PDRN), RNA, DNA, peptides, amino acids, enzymes, trace elements *etc*[3]. In India, HPE is used as a wound healer in burn injuries, chronic wounds and surgical dressings[4]. Placental extract has a beneficial role as a topical agent in the management of chronic non-healing wound[5]. Enzyme-linked immunosorbant assay (ELISA) studies revealed that cytotrophoblasts which expressed interleukin-8, a known mediator of inflammation, was suppressed by glucocorticoid present in human placenta[6]. HPE showed positive results against 5- HT induced acute inflammation; cotton pellet induced sub-acute inflammation and inhibition of platelet aggregation[7]. Aqueous extract shows immuno-modulating potential in rabbit as well as in human[8]. A study showed that the HPE has a peripheral analgesic property, on chemical and thermal nociception in mice, possibly mediated by an opioid mechanism[9].

Considering the various uses of human placenta in management of different disease, the study was undertaken to investigate the antioxidant profile of aqueous extract of human placenta against free radicals using specific *in-vitro* models.

Material and Methods

Test drug: Human placentas weighing between 400-600 g collected at the time of full term spontaneous delivery were immediately placed under ice; the amniotic membrane and umbilical cord were removed, minced into small pieces and washed with cold normal saline. Aqueous extract with these pieces of placenta was prepared, sterilized, and sealed in ampoules (2 ml) under inert condition. The extract 1 ml in the ampoule was derived from 0.1 g of placenta.

Chemicals: N-1-naphthylethylenediamine dihydrochloride was purchased from Sigma Chemical Co., potassium ferricyanide & sodium nitrite from Thomas Baker & Co., trichloroacetic acid (TCA), butylated hydroxy anisole (BHA), sodium nitroprusside, ascorbic acid & iron (III) chloride ($FeCl_3$) from E. Merck (I) Ltd., sulphanilamide from SISCO research Labs. Pvt. Ltd.,

butylated hydroxy toluene (BHT), phenazine methosulfate (PMS), Nitro blue tetrazolium (NBT), thiobarbituric acid (TBA) from Hi- Media, α- tocopherol acetate from LOBA Chemie. All other chemicals and reagents used were of analytical grade

Antioxidant Assay

Hydroxyl radical scavenging activity: Hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the test compounds for hydroxyl radicals generated from the Fe^{3+}/ ascorbate/ EDTA/H_2O_2 system. The reaction mixture contained: deoxyribose (2.8 mM) , $FeCl_3$ (0.1 mM) , EDTA (0.1 mM) , H_2O_2 (1 mM) , ascorbate (0.1 mM) , KH_2PO_4- KOH buffer (20 mM, pH 7.4) and different concentration of extract (0.5, 1, 1.5, 2, 2.5, and 3 mg/ml) to a final volume of 2 ml. After incubation for 1 h at 37^{o}C, the deoxyribose degradation was measured as TBARS formation[10].

Reducing power assay: The reducing power of HPE was determined as per the reported method of Oyaizu[11]. Different concentrations of sample extracts (0.5- 3 mg/ml) were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K_3Fe (CN) $_6$] (2.5 ml, 1 %). The reaction mixture was incubated at 50 ^{0}C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10 %) was added to the reaction mixture, which was then centrifuged at 3000 rpm for 10 min. 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 was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power.

Nitric oxide scavenging activity: The assay was performed according to method as described by Sreejayan and Rao[12]. Different concentrations (0.5- 3 mg/ml) of the test sample were added in 100 ml of phosphate buffered saline, to which 0.1489 gm of sodium nitroprusside was added and incubated at room temperature. At different time intervals 5.6 ml of reaction mixture was taken out then 0.2 ml of Griess reagent A (1% sulphanilamide in 5% phosphoric acid) was added, and kept at 30 ^{o}C for 10 min. After incubation, 0.2 ml of Griess reagent B (0.1% N-1-naphthylethylene diamine dihydrochloride) was added and kept at 30 ^{o}C for 20 min. After incubation, absorbance of the chromophore formed was measured at 542 nm against blank. The same reaction without the extract sample but equivalent amount of water serves as control. Concentration of nitric oxide was calculated from standard calibration of sodium nitrite solution of varying concentration.

Superoxide radical scavenging assay: The effect of superoxide radical production was evaluated using Nitro blue tetrazolium reduction method[13]. The reaction mixture consisted of 1ml of NBT solution (156 μM) 1 ml NADH solution (468 μM) and 1 ml of sample solution of HPE at different concentration (0.5-3 mg/ml). The reaction was started by adding 100 μL of phenazine methosulfate solution (60 μM PMS in phosphate buffer, PH 7.4) to the reaction mixture. The reaction mixture was incubated at 25^{o}C for 5 min. and the absorbance at 560 nm was measured against blank. Decreased absorbance of reaction mixture indicates increased superoxide anion scavenging activity.

Anti-lipid peroxidation assay in rat liver and brain homogenate: Anti-lipid peroxidation in rat liver and brain homogenate was evaluated by the TBA method[14, 15]. The reaction mixture containing 0.5 ml (10 %) rat liver homogenate, 1 ml 0.15 M KCl, and 1 ml different concentrations of sample (0.5- 3 mg/ml). Lipid peroxidation was initiated by adding 100 µL of 1mM ferric chloride solution. The reaction mixtures were incubated for 30 min at 37 ^{o}C. After incubation, the reaction was stopped by adding 2 ml ice-cold thiobarbituric acid solution containing 15 % trichloroacetic acid, 0.38 % thiobarbituric acid in 0.25 M HCl and 0.05 % butylated hydroxy toluene. The reaction mixtures were heated for 60 min at 80^{o}C, cooled and centrifuged at 6900 rpm for 15 min. The absorbance of supernatant was measured at 532 nm against a blank, which contained all reagents except liver homogenate and drug. Identical experiments were performed to determine the normal (without drug and $FeCl_3$) and induced (without drug). The percentage of anti-lipid peroxidation effect (ALP %) was calculated by following formula.

$$\text{ALP \%} = \frac{(FeCl_3 \text{ induced}_{\text{Abs.}} - \text{Sample}_{\text{Abs.}}) \times 100}{FeCl_3 \text{ induced}_{\text{Abs.}} - \text{Control}_{\text{Abs.}}}$$

For the experimentation of anti-lipid peroxidation assay of rat liver homogenate, the work was conducted in accordance with standard institutional guidelines, with a prior approval of animal experimentation committee.

Statistical analysis: Data were analyzed by Student's *t*-test. Experimental results were mean ± SD of five parallel measurements. *P* values < 0.05 were considered as statistically significant.

Results and Discussion

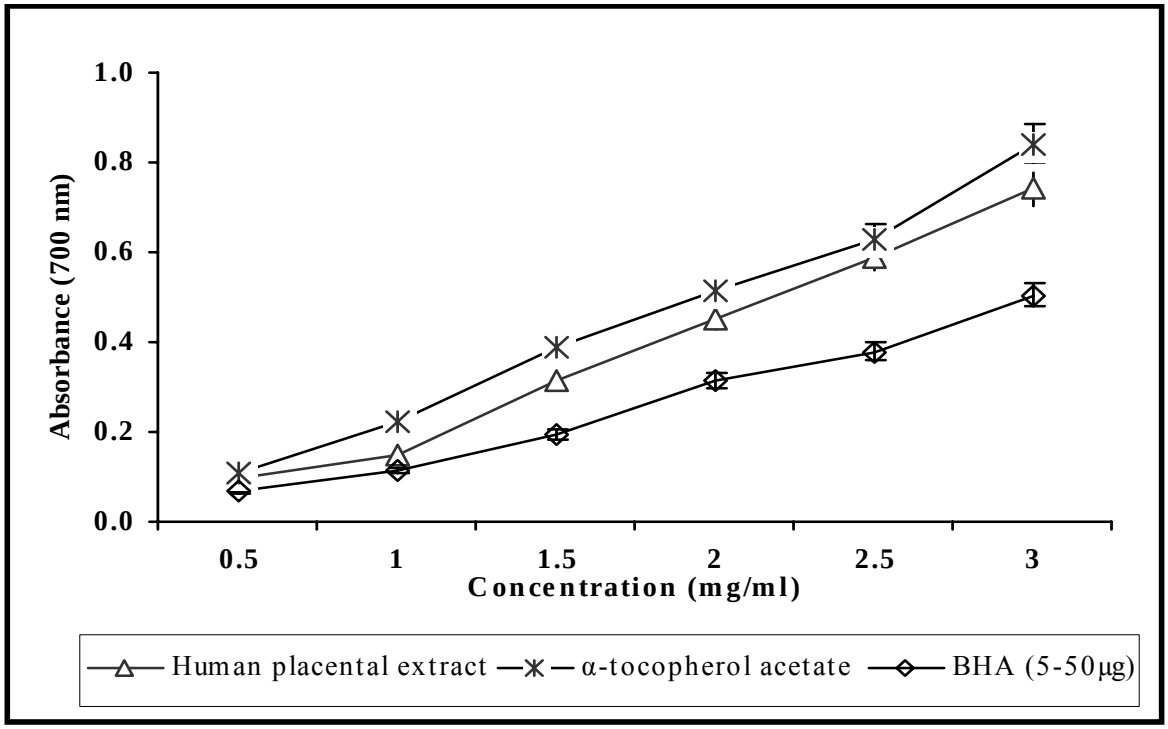
Free radicals, highly reactive species having an unpaired electron in their atomic or molecular orbit, are generated under physiological condition during aerobic metabolism. As free radicals are potentially toxic, they are usually inactivated or scavenged by human body complex antioxidant enzyme defense system or non enzymatic antioxidant component before they can inflict damage to lipids, proteins or nucleic acids[16]. However, when free radicals are generated in excess or when the cellular antioxidant defense system is defective, they can stimulate chain reactions by interacting with protein, lipid and nucleic acids causing cellular dysfunction and even cell death[17].

The free radical scavenging activity was demonstrated by hydroxyl radical and nitric oxide scavenging assay. HPE inhibited hydroxyl radicals generated by Fe^{3+}/ ascorbate/ EDTA/ H_2O_2 system with IC_{50} of 1.125 ± 6 mg/ml in comparison with α- tocopherol acetate and BHA with IC_{50} of 677.45 ± 2.5 µg/ml and 42.95 ± 3 µg/ml respectively (Table-1). Nitric oxide scavenging activity was done by the method described by Sreejayan and Rao. The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which were measured by Griess reaction. Scavenger of nitric oxide competes with oxygen, leading to reduce production

of nitrite ions. The NO scavenging property of the human placental extract showed significant results with an IC_{50} of 0.981 ± 5 mg/ml (Table-1).

For the measurement of the reductive ability**,** "Fe^{3+}- Fe^{2+} transformation" in the presence of test sample was found out and compared with α- tocopherol acetate and BHA. The reductive capability of HPE was found to increase with rising concentrations (Fig.1).

Fig. 1 Reducing power of aqueous extract of human placenta, α- tocopherol acetate and BHA (butylated hydroxy anisole) by spectrophotometric detection of Fe^{+3}- Fe^{+2} transformations. Results are mean ± S.D of five parallel measurements



The activity of HPE against superoxide radical was of significance because superoxide can decrease the activity of other antioxidant defence enzymes such as catalase and glutathione peroxidase as well as it can be cytotoxic by generating more reactive species like peroxy nitrite[18]. Superoxide radicals are more detrimental due to their role as second messenger in fibroblast proliferation in inflammation and mediators of tissue destruction[19]. In the PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen, by PMS-NADH coupling reaction, reduces NBT (yellow dye) to blue coloured product called formazon. Drug possessing superoxide scavenging activity decreases the reduction of NBT, which is a measure of superoxide anion scavenging activity that is indicated by reduction in absorbance at 560 nm. HPE was found to scavenge the superoxide generated with an IC_{50} of 1.52 ± 4.1 mg/ml while α-tocopherol acetate and BHA showed IC_{50} of 577.45 ± 2.6 µg/ml and 43.95 ± 3.1 µg/ml (Table-1).

Table 1: Effect of aqueous extract of human placenta on oxygen derived free radical generation *in- vitro*

Scavenging Activity	Tested samples	IC_{50} (mg/ml)[a]	
	Human Placental Extract	α-tocopherol acetate	BHA (μg/ml)[a]
Hydroxyl radical	1.125 ± 6	0.677 ± 2.50	42.93 ± 3.0
Nitric oxide	0.981 ± 5	0.621 ± 2.67	32.00 ± 3.9
Superoxide anion	1.52 ± 4.1	0.577 ± 2.60	43.95 ± 3.1
Lipid peroxide (Liver)	1.42 ± 5.2	0.840 ± 3.52	41.50 ± 3.9
Lipid peroxide (Brain)	2.21 ± 6.3	0.770 ± 5.24	58.43 ± 3.3

[a]Values are mean ± S.D (n = 5). $P < 0.05$ vs. control, Student's *t*-test.

Acute intraperitoneal administration of the human placental extract showed hepatoprotection in elevation of serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, serum lactate dehydrogenase and blood methemoglobin. Activities of catalase, glutathione peroxidase and glutathione reductase were represented in a dose-responsive way by treatment with HPE which also increased the activity of glutathione S-transferase[20]. L-Trp is one of the main bioactive peptide present in human placenta. L-Trp effect is based on the suppression of lipid peroxidation in the oxidative stress status. Study suggests that L-Trp isolated from human placental extract showed higher inhibitory activity than mannitol and DMSO on the Fenton reaction-induced degradation of 2-deoxy-D-ribose and on cytochrome P-450-dependent lipid peroxidation than uracil. On the other hand, the inhibitory effect of L-Trp on the Fenton reaction-induced protein oxidation was smaller than that of uracil[21].

It is known that cleavage products of lipid peroxidation accumulate in nervous system, cardiac, and muscle fibres[22.] Prevention of lipid peroxidation in rat liver and brain homogenate confirmed that it is active against effects of free radicals on biological membrane. The anti-lipid peroxidation effect of HPE was observed in $FeCl_3$- induced lipid peroxidation in rat liver and brain homogenate against control. The anti-lipid peroxidation effects of HPE were shown in Table-1. A concentration range 0.5- 003 mg/ml in liver homogenate showed IC_{50} of 1.42 ± 5.2 mg/ml where in brain homogenate showed IC_{50} of 2.21 ± 6.3 mg/ml. The result represents that inhibition of TBARS formation in rat liver and brain homogenate increased by increasing concentration. HPE being a rich source of amino acid, enzyme and trace element showed good result in the inhibition of various free radicals generated in the system. The data collected from the present project conclude that HPE has pronounced antioxidant effects.

Conclusion

The results from the present study clearly suggest that the use of human placental extract in various pathological conditions is justified and it give protection against various free radicals by inhibiting hydroxyl radical, superoxide anion, nitric oxide and decreasing the lipid peroxidation level. Different models used to evaluate the antioxidant activity suggest that HPE is a good source of natural antioxidants. For further study, this work also necessitates *in-vivo* evaluation of antioxidant potential of human placenta.

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