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In vitro growth inhibition of microbes by human placental extract

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Human placental extract that is used as a wound healer, acts as a stimulating agent for tissue repair. It has an effective inhibitory role on the growth of different microbes like bacteria, e.g. *Escherichia coli, Staphylococcus aureus* and fungi, e.g. *Saccharomyces cerevisiae, Kluyveromyces fragilis* and *Candida albicans*. It also prevents growth of clinically isolated bacteria, e.g. *E. coli* from urine and blood culture and *S. aureus* from pus. Drug-resistant strains such as *E. coli* DH5*a* Pet-16 Amp^R and *Pseudomonus aeruginosa* Cam^R were also significantly inhibited by the extract. The extract has both bacteriostatic and fungistatic activities. Dose-dependent response of the extract was observed. Antimicrobial activity was retained after heating but was lost after dialysis. The MIC of the extract varies between 200 and 8000 mg/l. No antimicrobial activity was observed with human serum and aqueous extract of mouse muscle serving as control. A mixture of polydeoxyribonucleotides appears to be the causative agent. Partial protection of the wound from secondary microbial infection is thus indicated.

USE of placenta as a therapeutic agent has been prevalent for a long time. It is an immunologically privileged organ and has unique pharmacological effects like enhancement of wound-healing, anti-inflammatory action, analgesic effect, etc. A variety of substances with biological and therapeutic activity present in human placenta, have been isolated and identified as hormones, proteins, glycosaminoglycans, nucleic acids, polydeoxyribonucleotides (PDRNs), etc. The composition of placental extract thus depends on the method of its preparation. Consequently, it shows different therapeutic activities[1]. In many countries, intra-muscular and topical use of the extract for burn injuries, chronic wounds and as post-surgical dressing is an age-old practice[2–6]. Under such conditions, an effective tissue-regenerative agent needs to take care of prevention of secondary bacterial or fungal infection. Our objective is to evaluate an extract developed indigenously from human placenta in terms of its functionality and active components. Scientific assessment of such an extract is necessary for its better acceptance in medical practice. Recently, presence of biologically active NADPH[7] and fibronectin type III like peptide[8] in the extract has been demonstrated. Further, different spectroscopic and chromatographic analyses have revealed high degree of consistency among different batches of the extract[9]. Here we report *in vitro* inhibition of growth of different bacterial and fungal strains by this extract.

M/s Albert David Ltd, Kolkata, India, supplied an aqueous extract of human placenta under the trade name 'Placentrex', which is manufactured under proprietary method. In short, fresh placentae stored in ice, were tested for HIV antibody and hepatitis B surface antigen. Single hot and cold aqueous extractions were done at 90 and 6°C respectively, followed by sterilization. It was filtered aseptically, benzyl alcohol was added up to 1.5% as preservative and was sterilized once again. Each millilitre of the extract was derived from 0.1 g of fresh placenta. A single batch was prepared from a pool of several placentae. Overall manufacturing procedure holding confidentiality of the proprietary terms has been described earlier[7]. Dry weight of placental extract was 10 ± 0.50 mg/ml. Benzyl alcohol at the concentration present in the extract has no prolonged effect on microbial growth[10].

Yeast extract, bacto-peptone, bacto-tryptone and agar were from HI-MEDIA Laboratories Pvt Ltd, India and D-glucose was from Qualigen, India. DNAaseI (from bovine pancreas), protease type XIII (from *Aspergillus saitoi*), dialysis tubing (cut off range < 12 kDa), including the benzoylated one (cut-off range < 2 kDa) and hydroxyapetite were from Sigma, USA.

Microbial strains collected were as follows: *Escherichia coli* (DH5*a*); *Saccharomyces cerevisiae* diploid strains derived from *S. cerevisiae* strains 8534-10A (MATa, leu2, ura3, his4) and 6460-8D (MAT*a*, met 3), *Kluyveromyces fragilis* (ATCC No. 10022); ampicillin-resistant *E. coli* DH5*a* Pet-16 (Amp^R); chloramphenicol-resistant *Pseudomonus aeruginosa* (Cam^R) were from different laboratories of our institute. *Staphylococcus aureus* (type strain, MTCC No. 1430) and *Candida albicans* (type strain, MTCC No. 1637) were from Microbial Type Culture Collection,

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IMTECH, Chandigarh, India. Clinical isolates of *E. coli* (from blood and urine) and *S. aureus* (from pus) were from state-owned city hospitals. Respective microbiology divisions of the hospitals identified the strains.

S. cerevisiae and *K. fragilis* were plated in the medium containing 1% yeast extract, 1% bacto-peptone, 2% D-glucose and 2% agar. *E. coli* and *S. aureus* were inoculated in the medium containing 1% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar. The pH of the medium was 7.4 and growth temperature was 37°C. *Candida albicans* was plated in the media containing yeast extract 3 g, bacto-peptone 10 g, D-glucose 20 g, agar 1.5 g in 1 l of water. The pH was 7.2 and temperature was 30°C. After growth on solid culture, the organisms were transferred to appropriate broth solution. They were grown at a specific temperature with continuous shaking for 16 h to prepare the preinoculum.

Following 16 h growth, 1% (v/v) of the preinoculum of each organism was inoculated into fresh broth (inoculum size 10^6 cfu/ml) and incubated at specified temperature. For estimating growth inhibition, the medium was prepared by replacing water with the extract or by adding lyophilized form of the extract, whereby pH of the medium remained unaltered. Growth of the microbes was measured from turbidity of the medium at 650 nm[11]. In every case, the organisms were allowed to grow for 6 h because at that point, the stationary phase began. Dose-dependent inhibitory effect of the extract, was measured after 6 h of growth and MIC was determined for each strain. Growth was measured spectrophotometrically by measuring turbidity at 650 nm. After 6 h of growth, the turbidity of the broth for each organism was different because it is directly proportional to cell size provided the number of cells remains constant[12]. In addition, growth inhibition was checked after 12, 24, 48 and 72 h incubation of the organism in the presence of the extract to determine its efficacy as an antimicrobial agent. The organisms were separated from the medium containing placental extract after mild centrifugation and were allowed to grow in the recommended medium. Time-dependent growth for each microbe was followed. DNAase and protease treatments were done by incubating approximately 0.1 mg of enzyme with 1 ml of the extract containing 0.1 ml of 0.1 (M) Tris-HCl, pH 7.5 at 37°C for 16 h.

While testing the inhibitory property, microbes were classified into four categories: laboratory strains, e.g. *S. cerevisiae*, *K. fragilis* and *E. coli* (DH5*a*); type strains, e.g. *S. aureus* and *C. albicans*; clinical isolates, e.g. *E. coli* and *S. aureus* and drug-resistant strains, e.g. *E. coli* DH5*a* pet-16 Amp^R, *P. aeruginosa* Cam^R. Time kinetics of growth of *C. albicans* in the presence and absence of the extract is shown in Figure 1 *a*. Complete inhibition of growth was observed in the presence of the extract. All other strains have shown similar complete inhibition of growth in presence of the extract differing only in kinetic parameters compared to controls, i.e. growth in the absence of the extract (results not shown). In all cases complete inhibition was observed up to an extended period of seven days of incubation in the presence of the extract. Growth inhibition of microbes was checked using human serum and aqueous extract of mouse gastrocnemius muscle as biological control. Human serum was diluted with PBS to 1 mg/ml, of which 0–500 µl was applied to 2-ml culture of *S. cerevisiae*. Mouse muscle in small pieces (2 mg/5 ml of PBS) was incubated for 48 h at 4°C and the extract of the same volume as the serum was applied to *S. aureus* culture. The growth of the microbe in either case was monitored as stated earlier. No inhibition was observed in both the cases.

After separation of the microbes from media where their growths were prevented by the placental extract, they grew normally in their culture medium and maintained their normal growth kinetics (control set, Figure 1 *a*). Thus, the growth inhibitory factor had both bacteriostatic and fungistatic activities. The placental extract after treatment with DNAase or protease, or after heating at 100°C for 20 min retained full efficacy in terms of inhibiting bacterial growth.

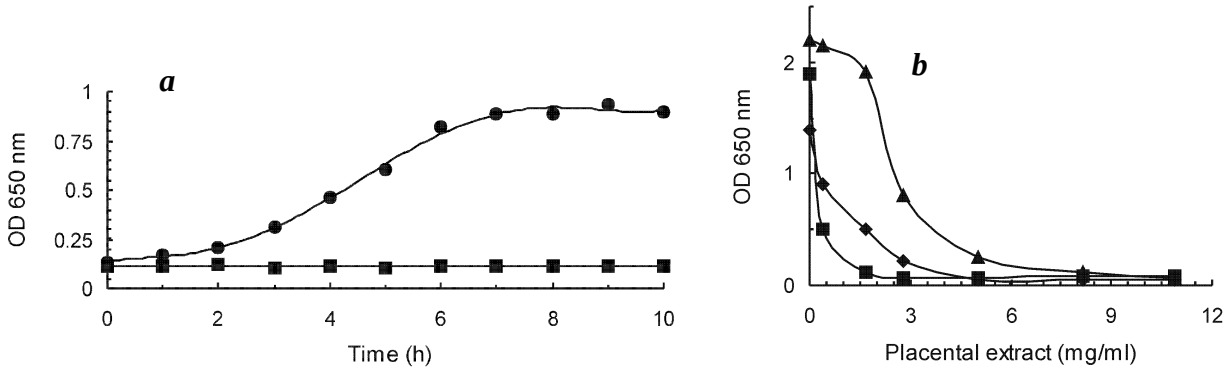

Figure 1. ***a***, Effect of placental extract on growth of *Candida albicans.* Growth in absence (as control) and in presence of placental extract is presented by (●) and (■) respectively. Placental extract concentration was 4000 mg/l. Growth was measured from turbidity at 650 nm. Result presented is an average of 4–5 sets of experiments, where variations were ± 5%. ***b***, Dose-dependent effect of human placental extract on (■) *Saccharomyces cerevisiae*, (◆) *E. coli* (isolated from urine) and (▲) *Staphylococcus aureus* (isolated from pus). For each strain, the growth was measured after 6 h of incubation. Result presented is an average of 4–5 sets of experiments where variation is ± 5%.

However dialysis against water completely removed the inhibitory factor, indicating that its size was less than 12 kDa.

Placental extract has a significant dose-dependent effect on microbial growth. This has been studied between 0.1 and 12 mg/ml of the extract for each strain (Table 1). The results for *S. cerevisiae*, *E. coli* (isolated from urine) and *S. aureus* (isolated from pus) are shown in Figure 1 *b*. *C. albicans* and *S. aureus* are the two strains which can survive beyond a dose of 3 mg/ml, but are completely inhibited at 4.5 and 8 mg/ml of the extract respectively. Drug-resistant strains such as *E. coli* DH5*a* Pet-16 Amp^R and *P. aeruginosa* Cam^R were also significantly inhibited by the extract. MIC_{50} and MIC_{90} of the strains tested are shown in Table 1. The tolerance of an extract in the blood for a host should be higher than the MIC_{90} of the microbe. For clinical purpose this is a good target, since there is a high chance that the extract will inhibit growth of the organism (bacteriostatic effect) and enable the immune system of the host to destroy it. As the placental extract is being applied on humans since long, even at a dose of 4 ml/day, which is much higher than the MIC_{90} obtained for tested organisms, the extract appears to be an effective antimicrobial *in vivo* too. Human placenta contains different tissue-regenerative components like nucleic acids, growth factors, laminin, etc.[13,14]. It is also known that human placenta contains antibacterial peptide[15] and antiviral factor[16]. After extraction from the placenta, whether the antimicrobial activity is retained or not, has not yet been reported. 'Placentrex', a tissue repair-stimulating agent, obtained from human placenta has been tested for this purpose. The pathogens, which mainly affect wound, e.g. *S. aureus*, *E. coli*, *C. albicans*, etc. are inhibited by placental extract in their culture medium. The extract is also capable of preventing the growth of non-pathogenic fungi like *S. cerevisiae* and *K. fragilis*. The growth of pathogens like *E. coil*, *S. aureus* and their clinical isolates as well as the more virulent form such as ampicillin-resistant strains of *E. coli* DH5*a*, Pet-16 and chloramphenicol-resistant *P. aruginosa* were significantly inhibited by the placental extract. The MIC of this drug varies between 200 and 8000 mg/l (Table 1). Such a value is consistent with its topical use as biological dressing in burn injuries and surgical wounds[17]. Thus local or intramuscular application of placental extract on wound seems to be beneficial in terms of preventing infection of pathogens.

The growth inhibitory property of the extract was retained after treatment with DNAase and protease. Hence large nucleic acids and proteins present in the extract are not probably responsible for this property. However, the role of small peptides or nucleotides escaping degradation or organic molecules remains uncertain. Retention of activity after heating but abolition after dialysis, further suggests this speculation. The activity was retained by 25% after dialysis using benzyolated membrane (cut-off < 2 kDa).

There are reports of separation of components of aqueous extracts of placenta using HPLC[1]. Using a similar protocol, an attempt has been made to identify the specific component/s responsible for the antimicrobial property. The extract was passed through Waters reversed phase C_{18} *m*-Bondapak analytical column (7.8×300 mm; 125 Å, 10 µm), equilibrated and eluted isocratically with 10 mM Na-phosphate, pH 7.2 at a flow rate of 1 ml/min (Figure 2). Elution was monitored at 220 nm. Three peaks of very low abundance were eluted around 10 min (retention time, $R_t = 7.47 \pm 0.02$ min, relative abundance, RA = 0.10 ± 0.02%; $R_t = 8.51 \pm 0.02$ min, RA = 0.20 ± 0.03%; $R_t = 12.33 \pm 0.03$ min, RA = 0.45 ± 0.04%) ($n = 4$). The major component appeared at $R_t = 32.56 \pm 0.52$ min, having abundance of 99.1 ± 0.20%. The fractions were pooled separately, concentrated and their antimicrobial activity was checked. It was only the major component/s that exhibited antimicrobial property to the same extent as that of the original extract.

To identify the component/s present in that fraction, its absorption spectrum was scanned between 220 and 300 nm, showing, maxima at 260 nm and insignificant absorption at 280 nm. Incidentally, the absorption maximum of the supplied extract was also at 260 nm between the said spectral zone, with a small but significant absorption at 280 nm[9].

Table 1. MICs of human placental extract for different microbes

Organism	MIC_{50} (mg/l)	MIC_{90} (mg/l)
Saccharomyces cerevisiae ($n = 1$)	200 ± 10	300 ± 50
Kluveromyces fragilis ($n = 1$)	200 ± 10	400 ± 50
Escherichia coli (DH5*a*) ($n = 1$)	200 ± 50	500 ± 50
E. coli isolated from urine ($n = 5$)	500 ± 100	2000 ± 800
E. coli isolated from blood ($n = 5$)	1500 ± 200	2500 ± 500
Staphylococcus aureus isolated from pus ($n = 5$)	2500 ± 200	7500 ± 300
S. aureus (type strain) ($n = 1$)	1000 ± 100	3000 ± 200
Candida albicans (type strain) ($n = 1$)	2000 ± 200	4000 ± 200
E. coli DH5*a* Pet-16 Amp^R ($n = 1$)	1500 ± 250	2500 ± 450
Pseudomonus aeruginosa Cam^R ($n = 1$)	2500 ± 300	5500 ± 200

n, Number of isolates tested; Maximum variation was expressed out of 4–5 experiments for each set.
MIC_{50}, Concentration of an antimicrobial that will inhibit 50% growth of an organism.
MIC_{90}, Concentration of an antimicrobial that will inhibit 90% growth of an organism.

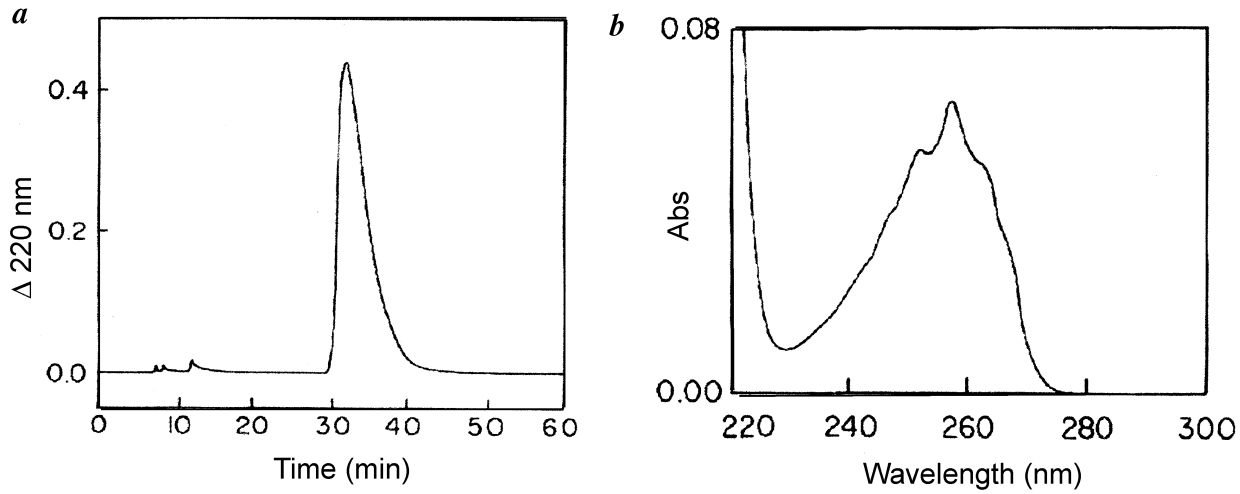

Figure 2. ***a***, C_{18} reverse phase HPLC profile of placental extract under isocratic elution in the presence of 10 mM Na-phosphate, pH 7.2 at a flow rate of 1 ml/min and monitored at 220 nm. A volume of l5 µl of the extract was applied. ***b***, Absorption spectra between 220 and 300 nm of major peak of R_t = 32.56 min of (***a***).

The protein/peptide components of the fraction appeared to be separated as the earlier separating components. The retention time and absorption spectrum of the active fraction were similar to those of well-characterized PDRN fraction of human placental extract as separated by HPLC[1]. In addition, since small DNA fragments bind to hydroxyapetitite[18], as supportive evidence, this fraction was stirred with 1 : 1 (v/v) slurry of the resin at 25°C for 15 min and the supernatant was collected after mild centrifugation. As a result, absorbance at 260 nm was reduced by 70%. This was accompanied by reduction of antimicrobial activity against clinical isolates of *E. coli* and *S. aureus* by 87 and 85% respectively. Further, it has been reported that PDRN constitutes more than 80% of the aqueous extract of human placenta[13] that shows anti-inflammatory and anti-platelet aggregatory activity[19]. It is also responsible for wound-healing of the ulcerative lesions of lower limbs[20], curing of second-degree burns[13], healing of gastric ulceration[21], treatment of acute radiodermatitis[22], enhancement of growth of human knee fibroblast[13], etc. All these observations collectively indicated that PDRN was responsible for the antimicrobial property of the extract.

The crude extract, when subjected to 20% PAGE analysis, revealed smear of small sized PDRN of 6–100 bp with respect to DNA molecular weight markers (12, 20 and 50 bp oligonucleotide, Gibco BRL), when viewed under UV-transilluminator. This was similar to earlier reports[1]. Similar photographic quality of gels could not be reproduced from the HPLC-resolved fraction. Possibly the fraction being too dilute, a major part of the PDRN remained adhered to the glass surface or elsewhere, leading to DNA gels of low smearing intensity.

Clinical evaluation of the effect of dressing with placental extract on infected wounds of patients was reported earlier[23]. Clinical trials of the extract have established healing of chronic, non-healing, infected wounds[24]. Here we report the effect of human placental extract on *in vitro* bacterial and fungal growth. The extract has both bacteriostatic and fungistatic activity. PDRN is possibly playing a key role in growth inhibition of microbes. Though the mechanism of such growth-inhibitory activities has not been studied, it is predicted that the PDRNs present in the extract enter the microbes and interfere with their replication machinery[25]. This antimicrobial property of the extract is expected to act against secondary infections, particularly during long-term healing from burn injuries, surgical, chronic and infected wounds.

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An altered G + C% region within potential filamentous hemagglutinin open reading frames of *Ralstonia solanacearum*

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Analysis of hemagglutinin open reading frames (ORFs) of *Ralstonia solanacearum* using FramePlot exhibited an altered G + C% (altered GC regions) at the 3′ regions of most large ORFs. The average G + C% values of these hemagglutinin ORFs and the altered GC regions are found to be 66.51 and 51.37 respectively. The amino acid usage of this altered GC region is similar to the whole protein, while the codon usage pattern is different. We reason that this altered GC region is an evolutionary adaptation rather than acquisition by horizontal gene transfer. The codon usage in the altered GC region could have a regulatory effect in the rate of translation of these large surface proteins.

FILAMENTOUS hemagglutinin genes were first discovered in the human pathogenic bacterium *Bordetella pertussis* and play an important role in the attachment of this bacterium to host cells[1]. The genome sequence of *Xylella fastidiosa* (the first phytopathogenic bacterium whose genome was completely sequenced) revealed the occurrence of three filamentous hemagglutinin genes in this plant pathogen[2]. Genome sequences of plant and animal pathogenic bacteria have revealed the wide occurrence of these genes among pathogenic bacteria[3]. *Ralstonia solanacearum* is a Gram-negative bacterium that causes a lethal wilt disease in more than 200 plants. One of the striking features of the *R. solanacearum* genome sequence is the presence of twenty-seven hemagglutinin genes, of which thirteen are called probable hemagglutinins as they exhibit homology with the filamentous hemagglutinin (FhaB) of *B. pertussis*, and the HMW1A/HMW2A adhesins of *Haemophilus influenzae*[4]. The remaining fourteen are called hemagglutinin-like proteins because these open reading frames (ORFs) contain variable internal repeats that are structurally related to filamentous hemagglutinins[4]. Here we discuss the probable hemagglutinins as potential filamentous hemagglutinins. Filamentous hemagglutinins are surface-localized adhesins encoded by large ORFs. After their synthesis in the cytosol, the huge polypeptides are translocated to the surface across the cytoplasmic membrane using the sec-protein apparatus[5]. This is followed by translocation across the outer membrane using the two-partner secretion system[6]. It has been speculated that during transport, the protein remains in an

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