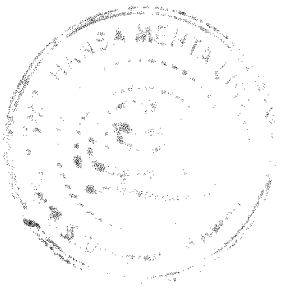
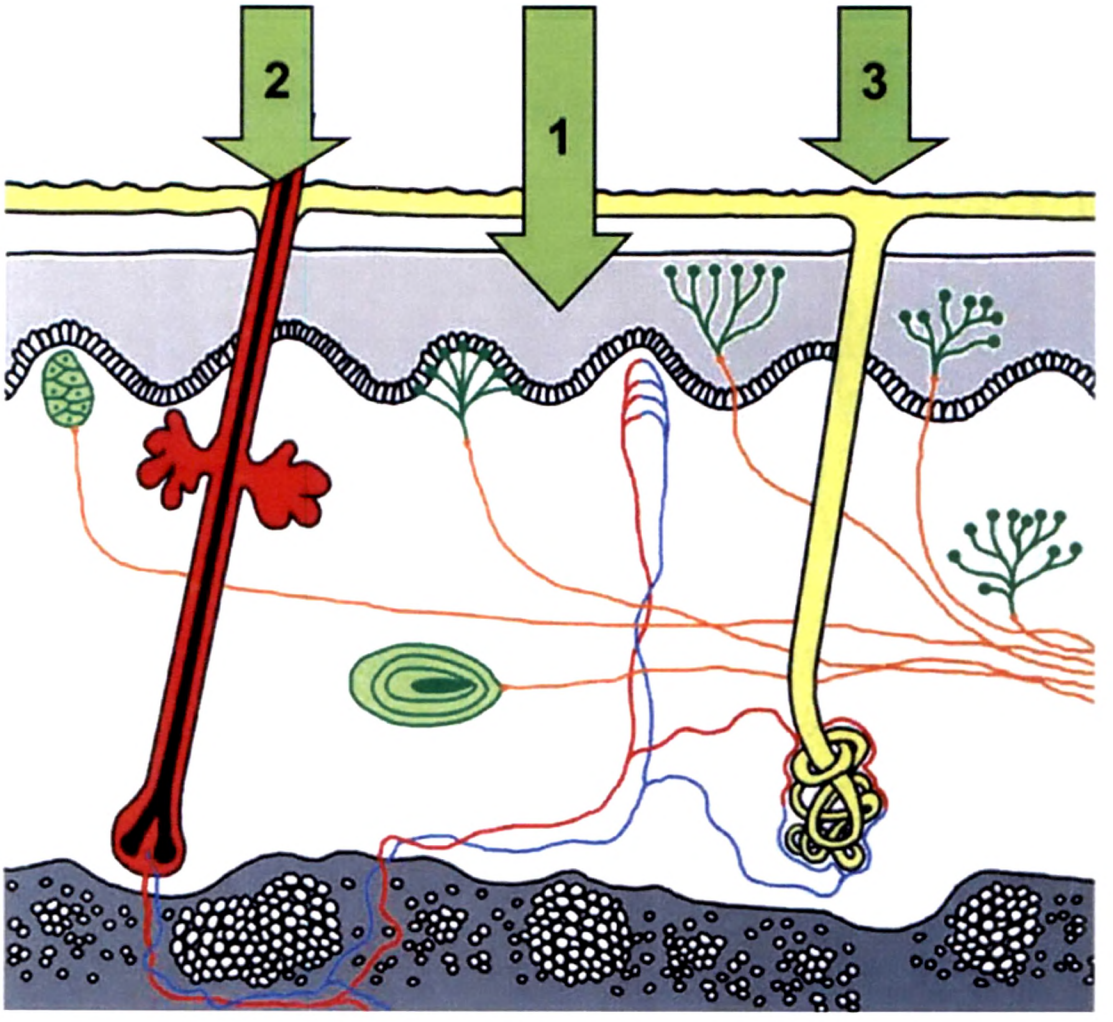
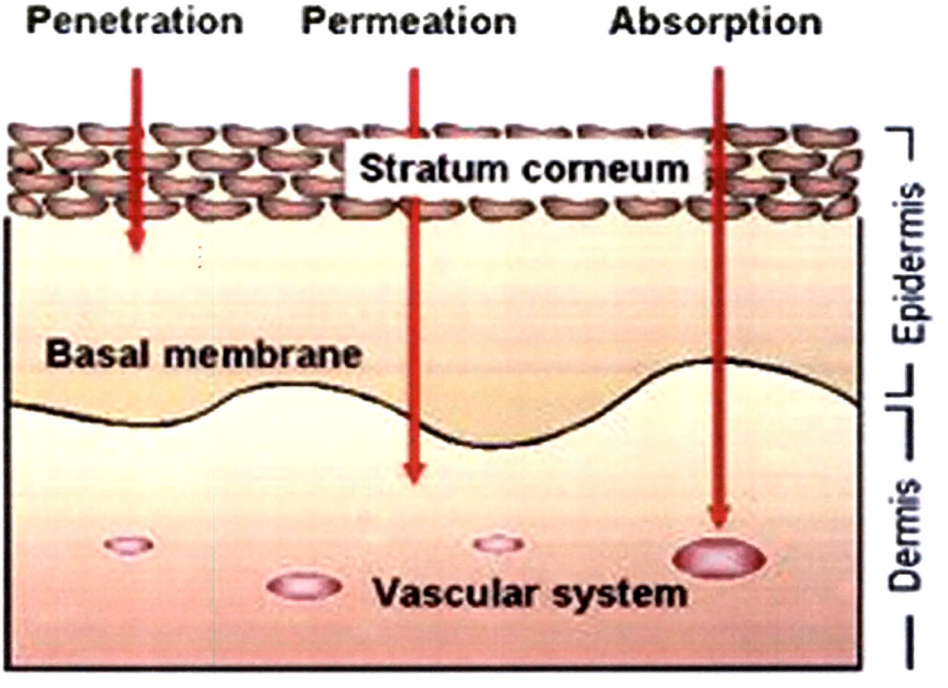


CHAPTER-6

IN VITRO DIFFUSION



Skin penetration phases



6.1 In vitro diffusion of elements through human skin

Penetration

The stratum corneum is responsible for the barrier function of the skin. It also behaves as the primary barrier to percutaneous absorption.

The phenomenon of percutaneous absorption (or skin permeation) can be visualized as consisting of a series of steps in sequence: sorption of a penetrant molecule onto the surface layers of stratum corneum, diffusion through it and viable epidermis, and finally , at the papillary layer of the dermis, the molecule is taken up into the microcirculation for subsequent systemic distribution. The viable tissue layers and the capillaries are relatively permeable, and the peripheral circulation is sufficiently rapid (Perl W 1963), so that for the great majority of penetrants, diffusion through the stratum corneum is often the rate-limiting step. The stratum corneum acts as a **passive, but not an inert**, diffusion medium (Tregar RT 1966). **No active transport process has been shown to be involved in skin permeation** (Scheuplein RJ 1965) , Berenson GS 1951). The rate-limiting stratum corneum is composed of dead, keratinized, metabolically inactive horny cells and its thickness is 15um . This layer is partially hydrated with a water content of approximately 20 %.(Yiew Chein 1996).

In vitro studies of the hydration of isolated stratum corneum demonstrated that it is not immediately hydrated upon immersion in water. Fully hydrated stratum corneum was found to absorb water up to five to six times its weight, and the water absorbed was found strongly bound within the intercellular keratin. The hydrated intercellular keratin appears to comprise a stable low – phase system at the macromolecular level; a continuous, water rich polar region intermingled with a network of nonpolar lipids (Scheuplein RJ 1967). Hydration apparently increases the thickness of stratum corneum by several fold.

The rate of diffusion of penetrant molecules across the hydrodynamic diffusion layer on the surface of the stratum corneum and the uptake rate of penetrant molecules by stratum corneum from the applied vehicle are both very rapid compared to diffusion through the rate limiting stratum corneum (Scheuplein R.J. 1971).The

mobility of water molecules in the stratum corneum is around 4.2 x 10^{-10} cm^2/sec. Molecules may diffuse through the skin by three different routes: the intact stratum corneum, the hair follicle region, and the sweat gland ducts.

The hydrated stratum corneum has an affinity for both water-soluble and lipid-soluble nonelectrolytes. **The major pathway for the skin permeation of water – soluble molecules is primarily transfollicular, i.e., through cells and cell membranes alike without discrimination** . On the other hand , the pathway for lipid-soluble molecules is not well understood and presumably follows the endogenous lipids within the stratum corneum. These lipids are located both intracellularly and between the keratin filaments within horny cell (Brody I 1960). **Thus, once the soil is wetted with water, the water soluble components of soil dissolve into it. This water also acts as an hydration agent , which increases stratum corneum's permeability . From the measurement of surface free energy and contact angle , Mavon A. (1997) suggested that skin surface lipids, mainly sebum, give the skin surface a hydrophilic character which facilitates diffusion of water soluble components .**

Method

Freshly excised human abdominal skin was obtained from a cosmetic surgery hospital. The skin was made free from fat bodies by cutting with scissors and stored in saline solution at -60°C. The study was completed within 3 days of receipt of the skin.

After removing the skin from the storage, it was kept in distilled water at room temp. to allow it to equilibriate to room temperature . The skin was washed clean and cut into pieces of approximately 3x3 cms square .

Diffusion studies were carried out in Keshrey –Chein diffusion cell having internal diameter 2.0 cms. The receptor compartment was filled with distilled water (20ml) and the donor compartment contained 200mg of soil (120 um size) wetted by 1ml distilled water. Each skin piece, with its stratum corneum side exposed to the air(donor compartment), was mounted horizontally across the upper chamber of the

diffusion cell. The two chambers were held tightly by four springs as shown in the figure no. 45. The temperature of the receptor fluid was maintained at 37°C. and the fluid was stirred by Teflon coated magnetic bar at low speed. The experiment was conducted for 60 minutes and thereafter the receptor fluid was filtered by Whatman filter paper and stored in refrigerator until analysed by ICP-AES for 5 major elements i.e. Ca, Mg, K, Na, P & humic acid. The skin was then removed from the cell, washed with distilled water, wiped dry and weighed accurately. It was then homogenized in a tissue homogenizer with 10 ml of distilled water three times. This extract was then centrifuged, filtered through Whatman filter paper and stored in refrigerator until analysed by ICP-AES for Ca, Mg, K, Na, P and humic acid. Both the samples i.e. receptor liquid and homogenized skin extracted liquid were analysed at Metallurgical Services, Mumbai. The whole set of experiment was conducted in triplicate for each of the 4 muds and Ca, Mg, K, Na, P and humic acid were estimated from it. Blank experiment was run in triplicate in the same manner without the soil. These blank values of element concentration of skin and receptor liquid obtained, were subtracted from the element concentration of skin and receptor liquid respectively, obtained by soil diffusion study.

The pictures of the skin taken by a digital camera (Canon) are shown in fig.no.42 : epidermis, 43:dermis, 44:thickness. Fig. no . 45 shows Keshrey Chein diffusion cell assembly placed in a water bath.

Fig.no. 42 Epidermis of skin

Fig.no.43 Dermis of skin

Fig.no.44 Thickness of skin

Spring for sealing donor and recipient compartment

Fig.no.45 Keshrey Chein diffusion cell assembly

Results & Discussion

Elements play a important role in the pathogenesis of inflammatory skin diseases as second messengers and so monitoring their levels (penetration) would help in understanding in the healing process of the skin. It is also known that elements play a key role as regulatory substances in enzyme metabolism , many scientists have worked on the elemental composition of psoriatic lesions and compared with normal skin by different methods (Forslind B 1997, Forslind B 1986).

Calcium

Kerala Brown and Vadodara showed approx. 45 % of penetration of calcium into the skin (Table no. 34) while Kerala Black, though very rich in calcium (SEM-EDS , AAS and ICP-AES reports) showed only 23% penetration. So was the case with Dwarka . It contained more calcium than Kerala Brown but its penetration was less than Kerala Brown (table no.33 and 34). These results were in correlation with findings of Shani J (1985) who in his findings stated that penetration of ions into skin was more if the concentration of element was less in the fluid. It is also reported that there is an **optimal concentration of ions which governs penetration**. He observed that increasing concentration of Chromium in the solution, increased absorption upto 4% in guinea pig skin but when the concentration was further increased, the absorption decreased to about 1%. This was true for other elements also.

Unlike other scientists who have calculated total calcium levels in psoriatic lesions, Menon GK found out that whereas normal human and uninvolved psoriatic epidermis revealed increased calcium-containing precipitates in the uppermost stratum granulosum, in contrast the basal layer of psoriatic lesions contained **less extracellular calcium, a condition that favored enhanced proliferation.** Moreover, all psoriatic suprabasal cell layers displayed heavier than normal concentrations of calcium, indicating loss of the normal calcium gradient that programs **terminal differentiation**. This abnormal profile may account for the differentiation defects that occur In psoriasis. **So penetration of Calcium from mud may restore the imbalance and thus control cell proliferation as well as terminal differentiation**.

Psoriatic lesions displayed retained ionic Ca in intercellular domains of the upper stratum granulosum with absence of normal intercellular bilayers, findings that may underlie the abnormal desquamation and permeability barrier in psoriasis (Menon GK 1991) .This result was also supported by Forslind B (1999) who put forward that the ratio of Ca/Zn in stratum corneum of paralesional psoriatic skin is approximately 8:1 compared to 12:1 in normal skin and 15:1 in atopic skin. This suggests that the differentiation process in paralesional psoriatic skin may actually be an example of disturbed programmed cell death (Forslind B,1999).

Atopic dermatitis also showed imbalance in mineral concentration in the body (Hataguchi Y 2005). Thus penetration of calcium and magnesium from mud into the skin must have helped to restore Ca, Mg levels resulting in improvement in eczematous symptoms.

Magnesium and calcium are regarded as strictly intracellular cations. Calcium plays a major role in regulating epidermal functions, including cell proliferation, terminal differentiation, and cell-to-cell adhesion. Aberrations in calcium regulation have been noted in psoriasis when levels of the calcium binding protein calmodulin are elevated and the normal calcium gradient within the epidermis is altered (Fairley JA 1991). **It is known that part of the intracellular calcium is bound to protein and is thought to be an integral part of the cell periphery or cell 'membrane" and it is established that in psoriatic patients, calcium binding protein calmoludin ,calgranulins A&B,S100 proteins A8&A9 is in large amount and there is altered calcium metabolism** (Plavina T 2008, Benoit S 2006, Karvonen SL 2000, Heilbrun LV 1943). Ca+2 has also been shown to play an important role in apoptosis (Programmed cell death), which is currently a hot subject for the obvious reason that the final differentiation step between the stratum granulosum level and the stratum corneum represents a particular aspect of programmed cell death (Forslind B 1997, Pallon J 1996).

From the above reports it is observed that calcium plays an important role in cell regulation and since 45% penetration of calcium was observed from Kerala Brown, it could be concluded that **the calcium supplemented by mud helps in restoring the**

normal calcium gradient and thus this could explain the improvement in scaling which was the first immediate positive effect on application of mud in psoriasis and starting of healing of skin in eczematous lesion.

Sodium

Sodium is the main extracellular cation and like the chloride concentration, the concentration of sodium is unusually high in the skin as compared with other organs.

It has been recognized that the sweat (extracellular area) excreted from those affected with psoriasis contains twice the amount of sodium and four times the amount of potassium in comparison to those normal individual (Wanjura HJ 1986). Salivary sodium levels were also significantly elevated in psoriasis (Sing G 2006). Though sodium content is found increased in psoriatic cells, no reference is found for its role in the etiology of the disease.

The rule of **optimal concentration was also observed in Sodium** . Kerala Black (38 ug/ml Na) showed 92% penetration while Kerala Brown (93 ug/ml Na) showed 40% penetration. In psoriasis and eczema permeability of cells is increased and extracellular sodium is already high so less penetration of it is preferred and so Kerala Brown would be better in that case.

Phosphorus

Scanning electron-microscopy in combination with secondary electron imaging and x-ray energy spectrometry (electron probe micro analysis) on the stratum corneum of psoriatic skin indicated significant, elemental alterations including an **increase in phosphorus and calcium levels** (Burkhart CG 1983) . **In a 31P Magnetic resonance spectra of psoriatic skin, it was observed that there was elevations in phosphomonoester concentrations and phosphomonoester/phosphodiester ratio and in conjunction with chromatographic analysis, it showed a defect in phosphometabolism in psoriatic skin (Zemtsov A 1994, Heng MC, 1994). Vitamin D analogs have found to be very useful in treating psoriasis, eczema and acne and it is established that Vit .D controls Calcium and Phosphorus metabolism and itching (Goetz DW 2010) , thus we can conclude that penetration of phosphorus of soil**

into skin must be responsible for the relief of itching observed, as the immediate effect of mud application in the clinical study. There was considerable amount of penetration of phosphorus into the skin from all the four muds. Kerala Black (0.26 ug/ml P) showed 84% penetration , Kerala Brown (0.163 ug/ml P) showed 73% penetration , Dwarka (0.332ug/ml P) showed 45% ,while Vadodara (0.501ug/ml P) showed only 24% of penetration (Rule of optimal concentration). But in the case of Kerala Black, though phosphorus penetration was 84%, sometimes itching complaint persisted in some patients. This may be due to the fact that the pH of Kerala Black was slightly acidic (5.89) which may have caused irritation induced itching.

Magnesium

Magnesium ions suppressed Langerhans cells function when added to epidermal cell suspensions in vitro. The reduced antigen –presenting capacity of Langerhans cells after treatment with $MgCl_2$ was associated with a reduced expression by Langerhans cells of HLA-DR and costimulatory B7 molecules, and with a suppression of the constitutive tumor necrosis factor –alpha production by epidermal cells in vitro (Schempp CM 2000, Jerome MB 1900). These findings demonstrate that magnesium ions specifically inhibit the antigen presenting capacity of Langerhans cells and **may thus contribute to the efficacy of any mud containing penetrable magnesium in the treatment of inflammatory skin diseases**. In a research conducted to study the effect of elements on tissue culture of psoriatic skin and normal skin , the number of cells and their cyclic AMP content were used as parameters for cell division and for proving the selective involvement of magnesium salts in the antiproliferative effect . The results showed that **the inhibitory effects of magnesium bromide and magnesium chloride on cell growth were significantly stronger than those of their corresponding potassium salts or of sodium chloride** (Levi-Schaffer F 1996).

Kerala Black (45.61 ug/ml Mg) showed penetration of 10%, Kerala Brown (10.37 ug/ml Mg) showed 83%, Dwarka (5.94 ug/ml Mg) showed 40% while Vadodara (2.79 ug/ml Mg) showed 40% penetration (rule of optimal concentration). It seemed that mud should have 10-12 ug/ml concentration of Mg for maximum penetration.

This data of penetration coincided with clinical observation described later, of reduction of plaque thickness in psoriasis and decrease in intensity and number in comedones, (proliferation) on application of mud on psoriasis and acne patients. In eczema it was observed that the skin healed faster.

Potassium

Elemental distribution of potassium in psoriatic skin varies with the functional state of the keratinocytes, e.g.,electrolytes influence cell metabolism and cell proliferation, and trace elements play a crucial role in a great number of enzymes. As high K levels prevent the Ca-induced differentiation of keratinocytes, high K levels may be the cause of the high cell differentiation in psoriatic skin (Kurz K 1987). In chronic lesions of psoriasis, if all layers of the lesion were analyzed in total, the potassium content was found to be greatly increased from an average of 259.5mg to an average of 491.5 mg. in 100 gm dry tissue (Dorffel 1930, 1931). This change might be caused simply by the acanthosis: the cellular content of psoriasis lesions is much greater than normal, and epithelial cells are rich in potassium. The main cations inside the cell are potassium and magnesium. The relative amount of potassium is much higher, possibly because of the high potassium content of the epidermis and other cellular elements of the skin, such as follicular epithelium, sweat gland cells, sebaceous gland cells, endothelial cells, and fibroblasts.

However, Role of potassium in the etiology of eczema and acne is not well established.

Kerala Black showed 81% penetration (3.16ug/ml K), Kerala Brown , 9.7% (10.5 ug/ml K) , Dwarka ,10.5% (5.7ug/ml K), and Vadodara showed 27.7% (2.16 ug/ml K) of penetration of potassium into the skin in in vitro studies (rule of optimal concentration). But since potassium levels are already high in psoriatic skin and penetration follows simple diffusion law, Kerala Brown whose permeation is very low , may be favored in treating psoriasis.

Looking at the concentrations of elements in soils and their penetration , a rough idea of value of **optimum concentration of constituents needed for maximum penetration** could be derived from our study.

P – 0.26 - 0.28 ug/100mg soil

Mg – 10-12 ug/100mg soil

Na - 38-40 ug/100mg soil

K - 3-4 ug/100mg soil

Ca – 5-9 ug/100mg soil

This conclusion has been drawn from in vitro studies. Its correlation with invivo studies in normal and diseased individuals needs to be done before giving any final conclusion because in psoriasis and eczema permeability of skin is increased (Shani J 1985,Grice KA 1967,Cerimele D 1978) and so there are chances that optimal concentration may change.

One of the aim of our study was to correlate physicochemical properties of soil with its efficacy and this data helped us to conclude that penetration of Ca and Mg, which are two important elements in cell regulation in any inflammatory disease, was highest in Kerala Brown and so it must be having good clinical efficacy against psoriasis, eczema and acne. This expectation was confirmed by results of preliminary clinical studies data described in chapter 7 of the text.

Table no: 33 Concentration of elements in mud as measured by ICP-AES

	Conc. in ug/ml			
Elements	Kerala Black	Kerala Brown	Dwarka	Vadodara
Phosphorus	0.26	0.163	0.332	0.501
Silicon	1.14	36.98	1.36	5.38
Magnesium	45.61	10.37	5.94	2.79
Sodium	38.64	93.5	36.73	33.92
Potassium	3.16	10.5	5.71	2.16
Calcium	27.88	4.81	10.59	6.94

Table No: 34 % Penetration of elements of soil into human skin

	% elemental penetration			
Elements	Kerala Black	Kerala Brown	Dwarka	Vadodara
Phosphorus	84.61±0.06	73.61±0.44	45.18±0.24	24.75±0.02
Silicon	8.77±0.8	1.05±0.8	4.41±0.11	0.557±0.67
Magnesium	10.89±4.62	83.22±12.03	40.06±0.11	40.86±0.56
Sodium	92.13±9.02	40.98±5.62	61.2±4.52	38.26±2.32
Potassium	81.32±0.46	9.71±0.5	10.85±0.93	27.77±0.47
Calcium	23.09±0.65	45.53±0.48	30.78±0.6	44.66±0.84

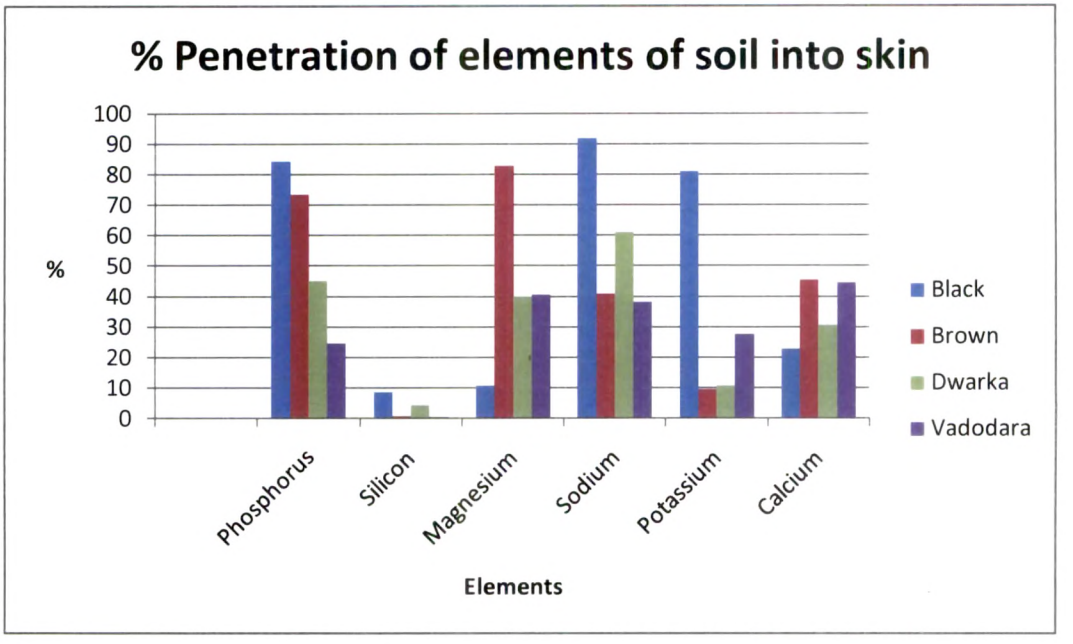


Fig. no. 46 % Penetration of elements of soil into human skin

6.2 In vitro diffusion of Humic acid through human skin

Introduction

Natural Humic acid is found to induce apoptosis in human premyelocytic leukemia HL60cells in the concentration range of 50-400ug/ml. Humic acid is found to stimulate NO (Nitric Oxide) production in cells and and it appears that psoriasis is a disease which results from a dysfunction of mechanism which regulates wound healing via production of nitric oxide and thus probably soil (which contains humic acid) helps in healing psoriasis patches.(Morhenn VB 1997, Yang HL 2004, Hseu YC 2002). Humic acid exhibits an antiproliferative effect by inducing apoptosis that is associated with cytochrome c translocation, caspase 3 activation, degradation of PARP (poly (ADP-ribose) polymerase) and dysregulation of Bcl-2 and Bax protein in HL -60 cells. (Yang HL 2004).

It is observed that psoriatic lesions contain less extracellular calcium, a condition which favors proliferation(Menon GK 1991) and interaction of HA with epidermal cells directly increase their Ca^{2+} permeability, thus overstimulating the Ca2+ second messenger system through activation of protein kinase and /or other Ca2+dependent enzymes thereby regulating proliferation and differentiation of keratinocytes (Yang HL 1994,1998). **Reduction in plaque elevation observed during clinical trials of mud on psoriasis patients could be due to humic acid.** This is also supported by the study conducted at Dead Sea by Emmilia Hodak et al(Emmilia Hodak 2002).

Our immune system, too, reaps the benefits of humic acid. Humates can increase polysaccharide sugars in our body which bind to Killer T Cells (immune killer cells) and facilitate communication between the Killer T Cells and other body cells. The function of Killer T cells is then modulated by the polysaccharides. Excessive Killer T Cell function in the body is part of what facilitates auto-immune diseases like rheumatoid arthritis and psoriasis (Lubitskaia, NS. 1999). Apart from these there are other effects of Humic acid on living organisms which are tabulated in table no. 35.

TableNo: 35 Principal biological effects of HA on living organisms.

SR.No	Observed effects	references
01	Stimulation of biomass accumulation	Gorovaya, A.I., Orlov, D.S. and Shcherbenko, O.V. (1995) *Humic substances: structure, functions,mode of action, protective properties, role in the environment*, Naukova dumka, Kiev., Zhorina L. V. and Stepchenko L. M. (1991) The content of free amino acids in the tissues of broiler chicks administered sodium humate in the ration, *Nauchnye Dokl. Vyss. Shkoly Biol. Nauki* **10,** 147-150..
02	Immunomodulating activity	Gorovaya, A.I., Orlov, D.S. and Shcherbenko, O.V. (1995) *Humic substances: structure, functions,mode of action, protective properties, role in the environment*, Naukova dumka, Kiev, Lange, N., Golbs, S. and Kuhnert, M. (1987) Grundlagenuntersuchungen zu immunologishen Reaktionen an der Laboratoriumstratte unter dem Einflus von Huminsauren, *Arch. Exper. Veter.-Med.* **41**, 140-146.
03	Desmutagenic activity	Sato, T., Ose, Y. and Nagase, H. (1986) Desmutagenic effect of humic acid, *Mutat. Res.* **162**, 173-178, Sato, T., Ose, Y., Nagase, H. and Hayase, K. (1987) Mechanism of desmutagenic effect of humic acid, *Mutat. Res.* **176**, 199-204. Cozzi, R., Nicolai, M., Perticone, P., De Salvia, R. and Spuntarelli, F. (1993) Desmutagenic activity of natural humic acids: inhibition of mitomycin C and

		maleic hydrazide mutagenicity, *Mutat. Res.* **299**, 37-44.
04	Detoxifying ability	Carlberg, G.E., Martinsen, K., Kringstad, A., Gjessing, E., Grande, M., Källqvist, T. and Skare, J.U. (1986) Influence of aquatic humus on the bioavailability of chlorinated micropollutants in Atlantic salmon, *Arch. Environ. Contam. Toxicol.* **15**, 543-548., *Humic acids and their sodium salts* (1999) Summary report, February, The European Agency for the evaluation of medical products, Committee for veterinary medical products, (EMEA/MRL) 554/99- FINAL,,, Leversee, G.J., Landrum, P.F., Giesy, J.P. and Fannin, T. (1983) Humic acids reduce bioaccumulation of some polycyclic aromatic hydrocarbons, *Can. J. Fish. Aquat. Sci.* **40**, 63-69. Day, K.E. (1991) Effects of dissolved organic carbon on accumulation and acute toxicity of fenvalerate, deltamethrin and cyhalothrin to *Daphnia Magna* (Straus), *Environ. Toxicol. Chem.* **10**, 91-101.
05	Toxicity	Ribas, G., Carbonell, E., Creus, A., Xamena, N. and Marcos, R. (1997) Genotoxicity of humic acids in cultured human lymphocytes and its interaction with the herbicides alachlor and maleic hydrazide, *Environ. Mol. Mutagen.* **29**, 272-276.,,,,,,, Gau, R.J., Yang, H.L., Chow, S.N., Suen, J.L. and Lu F.J. (2000) Humic acid suppresses the LPS induced expression of cell-surface adhesion proteins through the inhibition of NF-kappa B activation, *Toxicol. Appl. Pharmacol.* **166**, 59-67.
06	Antioxidant activity	Westerhoff, P., Aiken, G., Amy, G. and Debroux, J. (1999) Relatioships between the structure of natural organic matter and its reactivity towards molecular ozone and hydroxyl radicals, *Wat. Res.* **33**, 2265-2276.

Method

Diffusion studies of humic acid was conducted in the same manner as diffusion of soil constituents through human skin . 200mg of soil was taken in the donor compartment. The receptor compartment's liquid (d. water) was centrifuged , filtered through Whatman filter paper, and absorbance was measured at 224nm on UV spectrophotometer (Shimadzu 1601). The skin was homogenized, extracted with d. water, centrifuged, filtered and absorbance was measured at 224nm on UV spectrophotometer. Blank, receptor solution and skin extract was o btained in the same manner without using the soil.

Results and discussion

Humic acid could not be detected in the receptor compartment fluid in all samples.

Table No. 36 Humic acid retained in the skin

Sr.No.	Soil	Humic acid ug/100mgsoil sq.cm. skin	**% HA** retained per cm^2 skin
01	Kerala Black	3.457±0.03	1.899
02	Kerala Brown	1.997±0.02	1.636
03	Dwarka	Not detectable	-
04	Vadodara	Not detectable	-

It was observed that out of 0.182**mg**/100mg(soil) of humic acid (table no.29) in Kerala Black soil, only 3.457**ug** penetrated and retained into $1cm^2$ area of human skin. This meant that only 1.899% of humic acid retained in 1 sq.cm of the skin . Similarly 1.6% of humic acid of Kerala Brown soil was retained in the skin. Such low % of penetration of humic acid may be due to its high molecular weight, nevertheless, this concentration must be enough to elicit pharmacological action because as seen earlier natural humic acid was found to induce apoptosis in human premyelocytic leukemia in the concentration range of 50-400 ug/ml. Initial concentration of Humic acid in Dwarka and Vadodara was already less(0.086 and 0.076 % w/w of soil) and so its penetration into skin was beyond detection limits of our instrument.

Conclusion

Thus it can be concluded that elements permeate through the skin by passive diffusion and can play a important role in the healing of skin disorders. Since psoriasis, eczema and acne are epidermal diseases, concentrations of these elements in skin might play an important role in their treatment. Moreover, it has been established by Flusser D (2002) that minerals are the active constituents of mud and mineral depleted mud does not give good pharmacological actions

against osteoarthritis . Humic acid, which also is responsible for the efficacy of mud, was found to be retained in the skin from Kerala samples which had higher humic acid content. This may be one of the reasons for the therapeutic efficacy of these muds which are widely used by Nature Cure Centers at Kerala.

Note: The references of this chapter are given behind chapter 7