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Cover Page, Introduction, Contents, Call for Papers, All in one file

Contents

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Impacts Of Industrial Effluent On Quality Of Well Water Within Asa Dam Industrial Estate, Ilorin Nigeria

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ABSTRACT

The impact of industrial effluent on the quality of ground water (well) within an Industrial Estate was studied. The quality was assessed in terms of physicochemical parameters and bacteriological parameter. Three wells within the industrial were examined in the course of the study. Results obtained showed that the turbidity varied between 1.5 to 250 NTU and colour ranged from 211 to 2519 Pt- Co. The total, suspended and dissolved solids content were high. The conductivity ranged from 161 to 731 µs, while pH ranged from 6.9 to 7.3.Calcium and Magnesium ions as well as chloride ion content of the water were high. The dissolved oxygen content ranged from 6 to 9mg/l. Bacteriological indices showed that the well water were highly contaminated having high total bacterial counts (1200- 1375 cfu/ml). The well water showed presence of feacal coliform (*E. coli*) and had high coliform counts (1600 - >1800 MPN/100ml). It was observed that the wells were negatively affected by the effluent discharged within the industrial plant. [Nature and Science. 2008;6(3):1-5]. ISSN: 1545-0740.

Key words: Industrial effluent, well, Ground water, Bacterial count.

INTRODUCTION

The importance of water in the control of diseases had long been recognized (Hofkes, 1981; WHO, 1996). Water is a factor of production in virtually all enterprise, including agriculture, industry and the services sector (UNESCO, 2006). The importance of safe drinking water is underlined by the assertion that: "safe drinking water is the birthright of all humankind – as much a birthright as clean air" (TWAS, 2002). It also reported that the majority of the world's population, especially in most parts of Africa and Asia, does not have access to safe drinking water and that as much as 6 million children dies daily as result of waterborne diseases linked to scarcity of safe drinking water or sanitation (TWAS, 2002). WHO (2004) pointed out that diseases related to contamination of drinking-water constitute a major burden on human health: and that interventions to improve the quality of drinking-water provide significant benefits to health.

For most communities the most secure source of safe drinking water is pipe-borne water from municipal water treatment plants. Often, most of water treatment facilities do not deliver or fail to meet the water requirements of the served community; due to corruption, lack of maintenance or increased population. The scarcity of piped water has made communities to find alternative sources of water: ground water sources being a ready source. Wells are a common ground water source readily explored to meet community water requirement or make up the short fall.

Wells are categorized based on the nature of construction: open dug wells are generally considered the worst type of groundwater sources in terms of faecal contamination and bacteriological analysis. Dug wells with windlass or hand pumped or mechanically pumped well are generally regarded to be less prone to contamination (WHO, 2004). WHO (1997) assert that open or poorly covered well heads pose the commonest risk to well-water quality; the possibility of the water being contaminated is further increased by the use of inappropriate water-lifting devices by consumers. The commonest physical defects leading to faecal contamination of dug wells are associated with damage to, or lack of, a concrete plinth, and with breaks in the parapet wall and in the drainage channel (WHO, 1997). The most serious source of pollution of well water is contamination by human waste from latrines and septic tanks resulting in increased levels of microorganisms, including pathogens. Other likely sources of contamination include runoffs, agrochemicals such as pesticides and nitrates used on farm lands and industrial effluents. Contamination of well water due to under seepage has reported in the Niger Delta area of Nigeria (Ibe and Agbamu, 1999). Seepage from effluent bearing surface water would readily contaminate wells located close to the surface water.

Arising from the drive for industrialization, parts of Ilorin town are designated industrial estate/ area to accommodate the industries. One of such industrial estate has the course of River Asa running

through its whole length. The river is flows through Ilorin town almost dividing it into two halves (Olayemi, 1994). This makes it readily prone to abuse as effluent receptacle leading to contamination. Studies have shown that the River's water quality is affected by the discharge of the effluents (Eniola and Olayemi, 1999). This is consistent with the observation of Sangodoyin (1991) that effluents discharge alters the physical, chemical and biological nature of receiving water body. Wells are a vital and common source of water in Ilorin, some of these wells are located along the course of River Asa.

In this study, the effect of the discharge of effluent into river Asa on the quality of water of wells within the immediate catchment of the river was investigated. Water samples from wells within the industrial estate were subjected to physicochemical and bacteriological investigations to ascertain the effect of the effluent on the quality of the well water.

MATERIALS AND METHODS

Open dug well with concrete apron (plinth) around the well head were involved in the study. Water samples from the wells were collected into clean sterile 250ml sampling bottles as described by WHO (1997). The pH, colours (Pt-Co), turbidity, temperature, total Hardness, calcium hardness, magnesium hardness, calcium ion magnesium ion, chloride and conductivity were determined. The suspended, dissolved and total solid contents of the water were determined as described by ASTM (1985). The total heterotrophic bacteria counts were determined using the pour plate method (APHA, 1992). The coliform counts were determined as Most Probable Number (MPN) using the multiple tube fermentation test (APHA, 1992).

RESULTS

The physicochemical characteristics of the well water are shown on Table 1. The bacteriological characteristics are shown on Figure 1. Water from the wells were found to be close to neutral (pH 6.9 to 7.3) with high bacterial count (1200- 1375 cfu/ml). The coliform count was high (1600 - >1800 MPN/100ml) and faecal coliform (*E. coli*) was isolated. The variation in the total suspended and dissolved solids contents of the wells as well as the dissolved oxygen contents of the well water are shown on Figure 2.

Table 1. Physicochemical Characteristics of the water from Wells within Asa Dam Industrial Estate, Ilorin.

Parameters measured	W_1	W_2	W_3
pH	6.9	7.3	6.9
Colour (Pt-Co)	211	2519	240
Turbidity (N.T.U)	1.5	250	4.6
Temperature (^oC)	27	28	28
Total Hardness (mg/l)	149	153	37
Calcium Hardness (mg/l)	102	96	34
Magnesium Hardness (mg/l)	46	57	4
Calcium ion (mg/l)	410	383	135
Magnesium ion(mg/l)	37	46	3
Conductivity (us)	338	731	161
Chloride (mg/l)	155	12	2

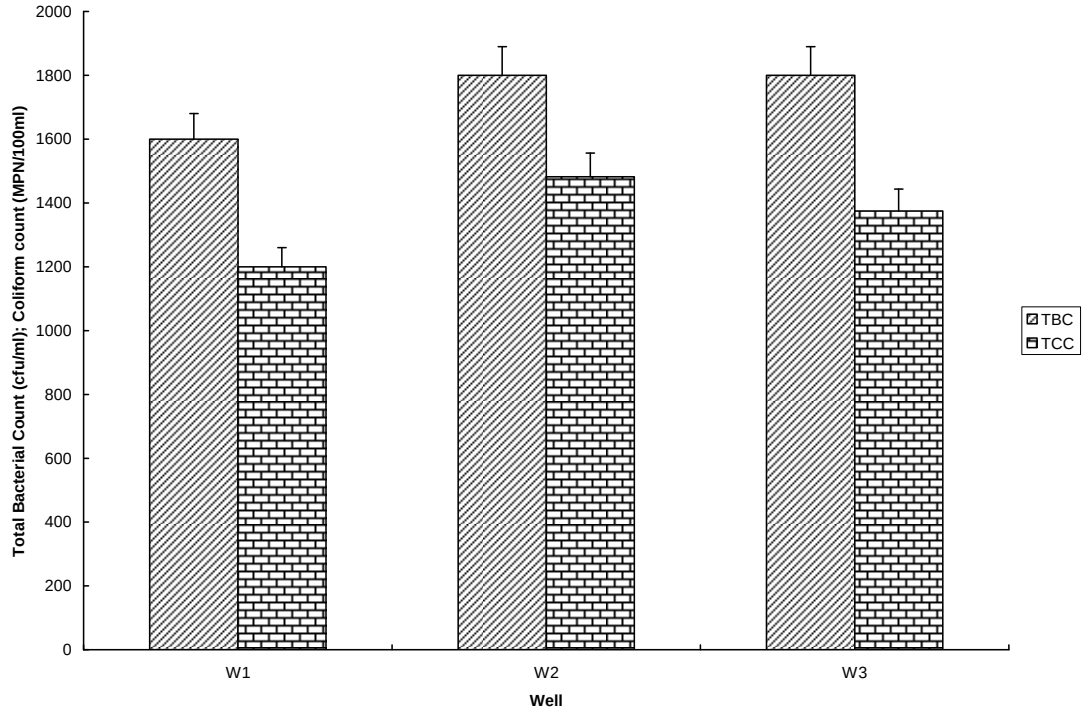

Figure 1: Bacteriological Characteristics of the Water from Wells within Asa Dam Industrial Estate, Ilorin.

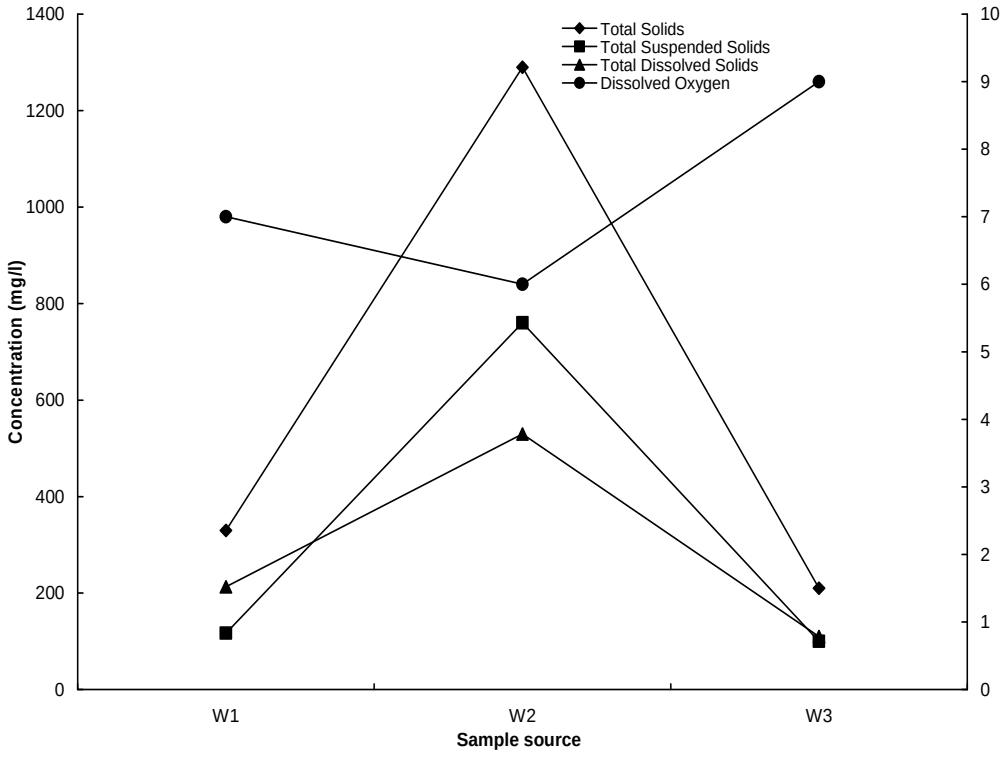

Figure 2. Variation in Total, Suspended and Dissolved Solids Contents of the Wells

Discussion

Water from the wells was observed to be coloured and turbid with the value ranges of 211- 2519 Pt-Co and 1.5 – 250mg/l respectively. Thin films of oil present on the water surface appear to make the value of the colour to be very high. The high turbidity value is as a result of increase in the type and concentration of the suspended matter released by the industry. The content of total solids, suspended and dissolved solids were also high. This is attributable to the industrial waste discharged into the surface water and suggests some of the content of the effluent have found their way into the ground water. Well water containing high total solids, total suspended solids and total dissolved solids are not fit for drinking, laundry work and livestock purpose. The high conductivity values suggest that the dissolved solids are mostly mineral salts. The high chloride is also suggestive of the use of large quantity of Chlorine or its associated compounds in activities within the industrial estate. The high bacterial count is suggestive of presence of organic matter (Gray, 1989, Olayemi, 1994). The values of dissolved oxygen obtained suggest that the water was not overtaxed by the quantity of degradable material in it and also that it was being well re-oxygenated.

Bacteriological speaking water from the wells fall short of the WHO (1997) recommended guideline standard for drinking water. It requires that water intended for drinking should not contain any pathogen or microorganisms indicative of faecal contamination. All the water samples examined contained feacal coliform (*E. coli*) and high population of heterotrophic bacteria, which is consisted with WHO (2004) report that open dug wells are contaminated, with levels of at least 100 faecal coliforms per 100 ml. This is not necessarily a result of the citing of the well along the river course but a reflection of the human activities taking place around the catchment of the wells. The unringed nature of the wells makes contamination by seepage from the soil more likely. The WHO (2004) recommends that wells are ringed and provided with an apron around the head to minimize contamination. The bacteriological quality of the wells requires that they be subjected to treatment if they are to be used for drinking and domestic purpose.

Conclusion

The results obtained showed that the water from the well were not fit for human consumption and their qualities were affected by the presence of the wells within the industrial estate and proximity to river that serves for disposal of industrial effluent.

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June 13, 2008

Dwindling of an endangered orchid *Dactylorhiza hatagirea* (D.Don) Soo: A case study from Tungnath Alpine meadows of Garhwal Himalaya, India

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ABSTRACT: The Central Himalayan region has been rich in biological wealth and would become an uplift resource of socio-economic status of the Himalayan people. Presence of a varied number of medicinal plants indicates its significance. Due to various levels of disturbances, destruction of number of economically important plants in these alpine meadows is continued like declining of *Dactylorhiza hatagirea* (D.Don) Soo, in its natural population. Out of six study sites, only two sites showed its presence, which indicates its declining health from natural population. [Nature and Science. 2008;6(3):6-9]. ISSN: 1545-0740.

Key words: Alpine meadows, study sites, natural population, density, orchid, grazing pressure

INTRODUCTION

The alpine region forms the uppermost catchments of the Himalayan Rivers which supports million of people in the lower hills as well as in plain of north India. Therefore health of the alpine ecosystem has direct bearing on the life-support system, environmental stability, biodiversity and human welfare in the region (Rawat, 2005). The various changes in the Himalayan forests are appearing in their structure, density and composition due to global warming, uncontrolled lopping and felling of trees for fuel wood, fodder and grazing (Gaur 1982, Bargali et al. 1998; Kumar et al. 2004). Changes in climate, exploitation of several plants for medicine and grazing pressure in alpine region of Garhwal Himalaya have led to drastic changes in vegetation composition and population of species during last few decades (Nautiyal et al. 2004). Dactylorhiza hatagirea (D. Don) Soo (Family Orchidaceae), a high value medicinal orchid, is reported to occur in temperate to alpine regions (2500-5000 m) in India, Pakistan and Nepal (Bhatt et al. 2005). Dactylorhiza hatagirea (D. Don) Soo, earlier known Orchis latifolia Hook (Vij et al. 1992). It is a terrestrial orchid and commonly it is known as Salampanja and Hatajari in Garhwal Himalaya. The tubers of this species, commonly sold as 'Salampanja' are known to yield a high quality 'Salep' which is extensively used in local medicine as nervine tonic for its astringent and aphrodisiac properties (Vij et al 1992). It has been categorized as critically endangered (Kala, 2000), rare (Samant et al 2001) and listed under appendix II of CITES (Uniyal et al 2002). This study aims to assess the quantum of availability of a therapeutically important orchid D. hatagirea (D.Don) Soo, in its natural habitats.

MATERIAL AND METHODS

Tungnath (30^0 30$^{'}$ N – 79 ^{0} 15$^{'}$ E and elevation 3300- 4200 m) represent an alpine zone of the Garhwal Himalaya (Sundriyal, 1994). In this region our study area covers an elevation range of 3500 to 4000 m. The rocks around Tungnath alpine meadows are mainly mylonitized gneisses, augengneisses, schist, granite and highly folded having a north west – south west trend (Valdia 1980). The heavy snowfall, frost, drought, low

oxygen and carbon dioxide are the common features of an alpine environment (Billings 1973). The present study deals with a quantitive analysis of herb species in different sites of Tungnath Alpine meadows to asses the quantum of availability of *D. hatagirea* in its natural habitats (Table: 1). Phytosociological data for herbs were quantitatively analyzed in six study sites on northern-west aspect by placing random sampling 40, 1x1 m quadrat. Quadrats data were analyzed for density, frequency and abundance (Muller-Dombois and Ellenberg, 1974).

RESULTS

A total of 24 herbs species were encountered across the study sites. Out of six study sites, only two sites showed presence of *D. hatagirea*. Observable grazing pressure was recorded at all study sites. The maximum density was of *Phleum alpinum* L. (141.52-201.28 ind/m2) followed by *Gaultheria trichophylla* Royle, (14.2-75.0 ind/m2), *Danthonia cachyemyriana* Jaub. and Spach, (8.32-40.32 ind/m2), *Plantago depressa* Willd.(15.0-58.4 ind/m2) and *Ainsliea aptera* DC (2.80- 32.2 ind/m2) and dominant herbs in all study sites. The target species i.e. *D. hatagirea* showed minimum density (0.70- 1.8 ind/m2) in all study sites (Table: 2).

Table: 1 Site description indicating altitudinal range, aspect and dominant herb species

Study sites	Altitudinal range (m)*	Aspect	Dominant herb species
1	3500-3600	NW*	*Phleum alpinum, Gaultheria trichophylla , Plantago depressa*
2	3600-3690	NW	*Phleum alpinum, Ainsliea aptera, Gaultheria trichophylla*
3	3940-4000	NW	*Phleum alpinum, Potentilla peduncularis , Danthonia cachyemyriana*
4	3600-3650	NW	*Phleum alpinum, Plantago depressa Geum elatum*
5	3700-3800	NW	*Phleum alpinum, Plantago depressa, Geum elatum*
6	3550-3600	NW	*Phleum alpinum, Gaultheria trichophylla, Danthonia cachyemyriana*

* m = meter, NW* = north-west

Table: 2 Density of *D. hatagirea* and dominant herbs in different study sites

Sites	Density of *D. hatagirea* (ind/ m2)*	Density of Dominant herb's (ind/m2)*
1	-	*Phleum alpinum (*160.12), *Gaultheria trichophylla (75.00), Plantago depressa(30.32), Danthonia cachyemyriana* (26.72)
2	-	*Phleum alpinum(201.28), Ainsliea aptera (32.20), Gaultheria trichophylla(23.60), Danthonia cachyemyriana* (10.60)
3	1.8	*Phleum alpinum(141.52), Potentilla peduncularis (41.92), Tanacetum longifolium (32.72), Danthonia cachyemyriana* (34.32)
4	-	*Phleum alpinum(190.52), Plantago depressa (36.12), Tanacetum longifolium (16.80), Geum elatum* (17.60)
5	0.7	*Phleum alpinum(174.32), Plantago depressa (58.40), Geum elatum* (17.72)
6	-	*Phleum alpinum(196.72), Plantago depressa (15.0), Gaultheria trichophylla (20.40), Danthonia cachyemyriana* (40.32)

* (ind/m2) = individual per meter square

DISCUSSION

On the basis of field visit, past records and observable grazing pressure, our study sites are fallen within the category of unprotected area. The density of *D. hatagirea* ranged from 0.70- 1.8 ind/m2 in these sites which was comparatively less from the reported density of *D. hatagirea (Orchis latifolia)* i.e. 2.66 ind/m2 in grazed sites and 3.2 ind/m2 in ungrazed sites at Tungnath (Nautiyal et al. 2004). Bhatt et al (2005) also reported 2.02-2.19 ind/m2 density in protected area and 1.13-1.64 ind/m2 in unprotected area in west Himalaya for *D. hatagirea*. These data shows that there is decrease in number of plants of this species with time. Tungnath is one of the famous religious shrines of Hindus where large herds of sheep, goat and buffalo reach every year during May-October for summer grazing (Nautiyal et al. 2004). Therefore the low density in unprotected areas may be due to heavy grazing pressure.

According to local people the Himalayan Monal, *Lophophorus impejanus* also known as the Impeyan Monal or Impeyan Pheasant, destroy its underground part i.e. tubers for food. It was also observed by the first two authors during their field visit. This and other levels of disturbances like grazing pressure because of its palatable nature, over exploitation due to its high medicinal value, and unawareness of the proper procedure of collection and propagation etc. are the major factors for declining of this species from its natural habitats. Chhetri et al. (2005), also reported that, the Sandakphu area in the Singalila range is a natural habitat of precious medicinal plants like *Aconitum, Picrorhiza, Nardostachys, Dactylorhiza*,etc., which are being destroyed by grazing.

Therefore it is a need to promote cultivation, propagation and conservation of this species. Using *in –situ* as well as *ex-situ* conservation efforts we can propagate and conserve this species and would become an ecologically as well as economically important plants of High Altitudes.

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Optimization of flavonoids extraction from the leaves of *Tabernaemontana heyneana Wall.* using L_{16} Orthogonal design

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Abstract: The study has been carried out to investigate the effects of single factors such as temperature, extraction time, concentration of ethanol, material ratio and no. of extractions on the contents of flavonoids present in the leaves of *Tabernaemontana heyneana Wall.* were investigated in this study. On this basis, an L_{16} orthogonal design of experiment was adopted to determine the optimal conditions for the extraction of flavonoids. The amount of flavonoids extracted reached its maxima when extracted at 85ºC for 2hrs by using 75%ethanol with a material ratio of 1:05 and 4 times of extraction. The TLC performed for the optimized extracts showed the presence of rutin, quercetin related compounds. The PTLC of the optimized extracts also proved the presence of flavonoids, especially high levels of rutin related compounds. [Nature and Science. 2008;6(3):10-21]. ISSN: 1545-0740.

Keywords: Flavonoids, orthogonal experiments, Single-factor experiments, *Tabernaemontana heyneana Wall.*

1. INTRODUCTION

Humans have gathered food and medicinal herbs ever since their arrival on earth and were guided then by instinct, followed by experience, and also by rational thought (Havsteen, 2002). For millions of years, mankind has fared quite well using this approach, but after the development of science and technology, people felt that the current state of affairs was quite satisfactory and hence, they failed to support research and education adequately (Harborne, 1988). Therefore, it is time to examine more closely what we are eating, how diseases can be treated more efficiently, and how we can effectively conserve our natural resources. One of our natural resources is the plants in remote forests, some of which may contain compounds of potential medical use. One such compound is flavonoids which appear to play a major role in the successful medical treatments of ancient times and their use has persevered till date (Dixon *et al.,* 1998). Flavonoids are a group of polyphenolic compounds possessing low molecular weight that exhibit a common benzo-γ-pyrone structure. They are categorized into various subclasses including flavones, flavonols, flavanones, isoflavanones, isoflavanoids, anthocyanidins, and catechins (Hodnick *et al.,* 1988; Cook and Samman, 1996). The average human diet contains a considerable amount of flavonoids and the major dietary sources are fruits (i.e., orange, grapefruit, apple, and strawberry), vegetables (i.e. onion, broccoli, green pepper and tomato), soybeans and different herbs.

One of the prominent and medically most useful properties of many flavonoids is their ability to scavenge free radicals (Van Acker *et al.,* 1996). A free radical is molecule containing one or more unpaired electrons in atomic or molecular orbitals that includes super oxide ($O_2^{\cdot -}$), hydroxyl radicals (OH^-) and H_2O_2, collectively known as reactive oxygen species (ROS) (Sathishkumar *et al.,* 2008). These ROS may induce oxidative damage to various macromolecules like polyunsaturated fatty acids in cell membranes, carbohydrates, proteins and DNA which results in homeostatic imbalance. The flavonoids are essential constituents of the cells of all higher plants (Brouillard and Cheminat, 1988). They resemble in their regulatory properties most of the lipid-soluble vitamins, but serve in addition, due to their color and odor, as communicators with the environment (Middleton and Teramura, 1993). The effect of flavonoids on plant growth, which is known, is atleast partly indirect and associated with the action of the auxins. It was reported that flavonoids can improve the blood circulation and lower the blood pressure (Yaqin Xu *et al.,* 2005).

Tabernaemontana heyneana wall. (Apocynaceae) known as kundalam paalai in Tamil, is known to possess antimicrobial activity against skin diseases, venereal diseases, respiratory problems, nervous disorders and various other diseases (Ignacimuthu and Ayyanar, 2005; Ignacimuthu *et al.,* 2006). The stembark decoction is used for cleaning cuts and wounds before dressing them (Chandrashekar *et al.,* 1995). The mixture of leaf and stem powder of this plant along with the stem bark of *Ficus racemosa, Ficus benghalensis, Madhuca longifolia,* is heated with coconut oil and applied externally to cure skin diseases (Ignacimuthu and Ayyanar, 2005; Ignacimuthu *et al.,* 2006). Similarly the same mixture along with the stem bark of *Strychnos nux-vomica* and fruits of *Carica papaya* were taken internally to induce abortion (Ignacimuthu and Ayyanar, 2005; Ignacimuthu *et al.,* 2006).

In many cases, it is difficult to find quickly suitable experimental conditions for a given separation task. Prediction of separation conditions is not yet straightforward. Therefore, good experimental design becomes increasingly important. Orthogonal design which only focuses on the main effects of the factors, allows the number of experiments to be drastically reduced. In separation science, this kind of experimental design has already shown its usefulness in liquid chromatography and capillary electophoresis (Hu Zhide *et al.,* 2002).

At present, there are no scientific reports on the extraction of flavonoids from the leaves of *Tabernamontana heyneana Wall.* In this study, the optimal conditions to extract flavonoids from the leaves of *Tabernamontana heyneana Wall.* were investigated systematically in order to explore a proper process to utilize the *Tabernamontana heyneana Wall.* leaves in the area of healthcare.

2. MATERIALS AND METHODS

2.1 Plant material

The plant was collected from the medicinal garden of Kumaraguru College of Technology, Coimbatore, India and the species was identified, confirmed by Botanical Survey of India (BSI), Southern Circle, Coimbatore, India and a voucher specimen (No. DBT 001) was deposited at Department of Biotechnology, Kumaraguru College of Technology, Coimbatore, India.

2.2 Extraction process

The main factors that affect the extraction of flavonoids like temperature, extraction time, materials ratio (weight of the leaves: volume of the extracting agent), extracting agent (%) and the no. of extraction were studied individually. The optimum extraction conditions were then determined by L_{16} (4^5) orthogonal design of experiments i.e., four levels and five different parameters. A single factor analysis of variance (One way ANOVA) was adopted to investigate the effect of each factor in the extrcation of flavonoids.

2.3 Estimation of total flavonoids (TFC)

TFC was estimated spectrophotometrically (Zhishen *et al.,* 1999) with slight modifications (Beckman DU 530 UV/ Vis spectrophotometer, USA). About 0.1ml of the diluted sample added distilled water to make the volume to 5ml and 0.3 ml 5% $NaNO_2$ was added to this. 3ml of 10% $AlCl_3$ was added 5 minutes later. After 6 minutes, 2 ml of 1 M NaOH was added and the absorbance was measured at 510 nm. Rutin was used as a standard for constructing a calibration curve. Data were reported as mean ± SD for three replicate measurements.

2.4 Identification of flavonoids by thin layer chromatography (TLC)

Chromatographies of the optimized extracts were run one dimensionally in the mobile phase solvent (ethyl acetate - ethanol - water, 5:1:5, v/ v/ v) at room temperature of 20-25ºC. The concentrated extracts were spotted on the lower left of the TLC plate and the diameter of the spot in each chromatogram was

normally about 5mm. Authentic markers of flavonol (quercetin) and flavonoid glycoside (rutin) obtained commercially were co-chromatographed. Identification of the flavonoids in the extracts was identified under UV light after the application of ammonia (Adam *et al.,* 2002; Guorong Fan *et al.,* 2006). A similar preparative thin layer chromatography (PTLC) was also performed to confirm the results of TLC.

3. RESULTS AND DISCUSSIONS

Flavonoids are a broad group of secondary metabolites with varied and important roles in plant physiology as well as they have gained recent interest because of their broad pharmacological activity. Putative therapeutic effects of many traditional medicines may be ascribed to the presence of flavonoids (Saskia Van Acker *et al.,* 1996; Schultz *et al.,* 2008). Flavonoids and other plant phenolics are reported, in addition to their free radical scavenging activity, to have multiple biological activities including vasodilatory, anticarcinogenic, antiinflammatory, antibacterial, immune-stimulating, antiallergic, antiviral, and estrogenic effects, as well as being inhibitors of phospholipase A2, cyclooxygenase, and lipoxygenase (Catherine Rice-Evans *et al.,* 1996). Plant flavonoids usually occur in plants as glycosides, although in some circumstances they may occur as free aglycones. Most glycosides are O-glycosides, with the most common monoglycoside being at the 7-position.

Prevoius reports for the extraction optimization of rutin, a flavonoid glycoside from the leaves of buck wheat revealed that a Solid:liquid ratio of 1:20 for 4 hours at 60ºC was required for higher rutin yield. In this methanol is used as a solvent for extracting the rutin. Huo (Chinese Patent 1217329, 1999) described an extraction of rutin from tartary buckwheat seeds by washing with water, coarse grinding, coarse screening, soaking in water, drying in the air, fine grinding, soaking in edible alcohol, extracting below 60ºC. Balandina *et al.,* (1982) extracted rutin from buckwheat seeds with hot water to remove the desired product and crystallized it. In general, a full evaluation of the effect of five different parameters at four levels on the yield would require 1024 (4^5) experiments. In order to reduce the number of experiments, an L_{16} (4^5) orthogonal design graph was used. In this way, only 16 experiments were necessary to run (Chen *et al.,* 2007).

3.1 Effect of temperature on the extraction of flavonoids

Fig.1 showed the contents of raw flavonoids tended to increase gradually with a rise in the temperature range from 55ºC to 85ºC. The contents of flavonoids gradually increased with a rise in the temperature in a range of 55ºC to 85ºC with a 10ºC temperature interval. It may be probable that the greater speed of the molecule movements in higher temperature so that flavonoids diffused more quickly from cell to extracting agent. But the flavonoids could be oxidized at temperature of surpassing 80° so that the contents of flavonoids extracted started to decrease gradually (Yaqin Xu *et al.,* 2005). Temperature's effect on extraction is dual. On one hand, higher temperature can accelerate the solvent flow and thus increase the flavonoids content and on the other hand, higher temperature can decrease the fluid density that may reduce the extraction efficiency (He Guo-qing *et al.,* 2005). Hence, it was found that 85ºC was the optimum temperature for extracting the raw flavonoids.

3.2 Effect of flavonoids extraction time

The result of Fig.2 showed that the contents of flavonoids extracted for 2h reached maxima and prolonged extraction may not yield an increased content. The contents of flavonoids extracted for 2hrs reached its maxima. Furthermore a decrease in the flavonoids content was noticed for 3hrs extraction and a sudden increase in their content was observed for 4hrs extraction time. This increase in the flavonoids content may be due to the synergistic effect of other parameters involved. A similar report by Chen *et al.,* (2007) revealed that 2hrs was the optimal extraction time for the extraction of a hypotensive drug geneposide from the bark of *Eucommia ulmoides* tree.

3.3 Effect of material ratio on the extraction of flavonoids

Fig.3 showed the contents of raw flavonoids extracted were maxima at 1:05 materials ratio. Further increase in the material ratio leads to a gradual decrease in the flavonoids content revealing a saturated condition. A significant rise in the flavonoids content was observed with the material ratio of 1:05. However, a gradual decrease in the flavonoids content was noticed when there is an increase in the material ratio. This decrease might be due to the fact that when the material ratio reached a certain level, the extract has well dissolved in the solution that may lead the contents of the extract become saturated and prevent further increase (Yaqin Xu *et al.,* 2005).

3.4 Effect of extracting agent (ethanol) on the extraction of flavonoids

The result of Fig.4 revealed that the contents of raw flavonoids extract increases with the concentration of ethanol i.e., 55% to 75%. A decrease in the flavonoids content was noticed further more, i.e., beyond 75%. Considering one of the aims of this work is to propose a suitable solvent for extracting the raw flavonoids. Among various solvents, ethanol was selected as a right choice because it is environmentally benign and relatively safe to human health (He Guo-qing *et al.,* 2005). Ethanol interacts with the flavonoids probably through non-covalent interactions and promotes a rapid diffusion into the solution (Luque de Castro and Tena, 1996). Various concentration of ethanol used exhibited different effect in changing the fluid polarity and thus had diverse effect on the solubility enhancement of the flavonoids (He Guo-qing *et al.,* 2005). The optimal extraction yield may be fulfilled when the polarity of the fluid and its flavonoids are coincident. In this study, the results indicated that the optimal ethanol concentration for extraction flavonoids was found to be 75%.

3.5 Effect of no. of extractions on flavonoids

The contents of raw flavonoids extract increases with the no. of extractions i.e., a gradual rise is noticed from 1 time to 4 times. Obviously, when the no. of extraction times increased the yield of the respective bioactive principle may be increased (Chen *et al.,* 2007). In this investigation, the raw flavonoids content was increased by 4 times of the extraction.

3.6 Optimization of flavonoids extraction using L_{16} orthogonal design

The parameters and the orthogonal design of experiment for the extraction of flavonoids were given in the Table 1 and Table 2. The results were made in the form of range analysis and one way ANOVA by SPSS software. The results were depicted in Table 3 and Table 4. The order of the effect of factors on flavonoids extraction was A>D>E>B>C. The temperature had the greatest effect on the extraction procedure and it was found to be significantly different at 5% level. An equivalent effect was observed in the material ratio change, even though it was not proved to be significant difference at 5% level. The other factors such as solvent (%), extraction duration and no. of extractions did not play a vital role in extracting the flavonoids to a higher yield. The optimum extraction conditions obtained from the statistical analysis were$A_4B_2C_3D_1E_4$. It means that 85°C, 2hrs extraction duration, a material ratio 1:05, 75% ethanol concentration and 4 times of extraction were the optimum conditions for flavonoids recovery.

3.7 TLC and PTLC results

The results of TLC and PTLC revealed the presence of flavonoid glycosides, flavonols and phenolic acids in the optimized extracts (Fig.5 and Fig.6).

Fig.1 Effect of temperature on flavonoids extraction

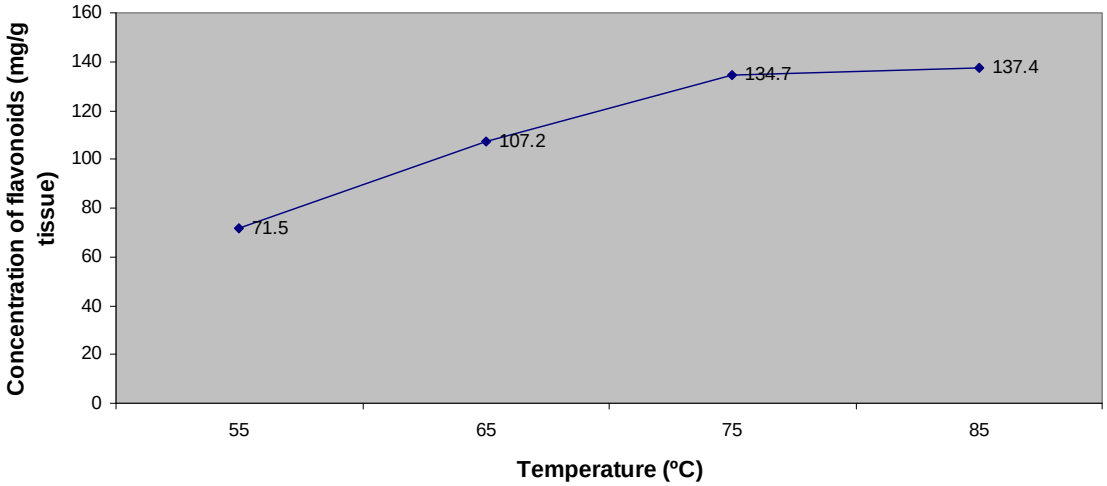


Fig.2 Effect of different extraction time

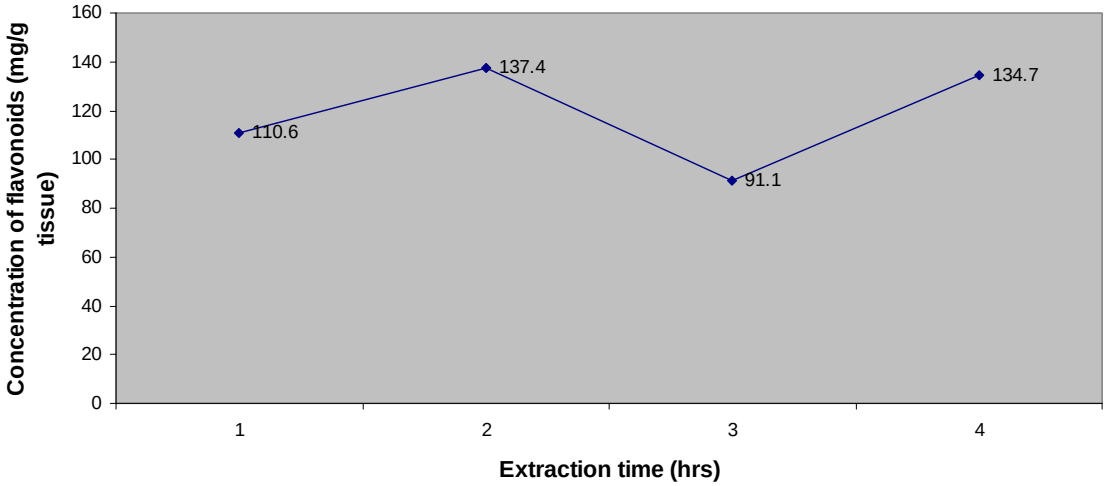


Fig.3 Effect of soild:liquid (w/v) in the extraction of flavonoids

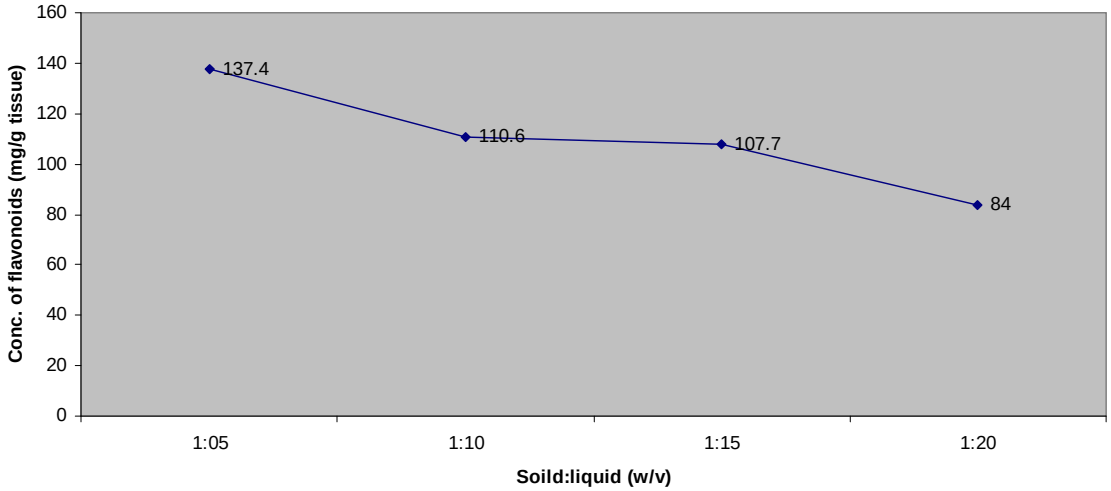


Fig.4 Effect of ethanol on the extraction of flavonoids

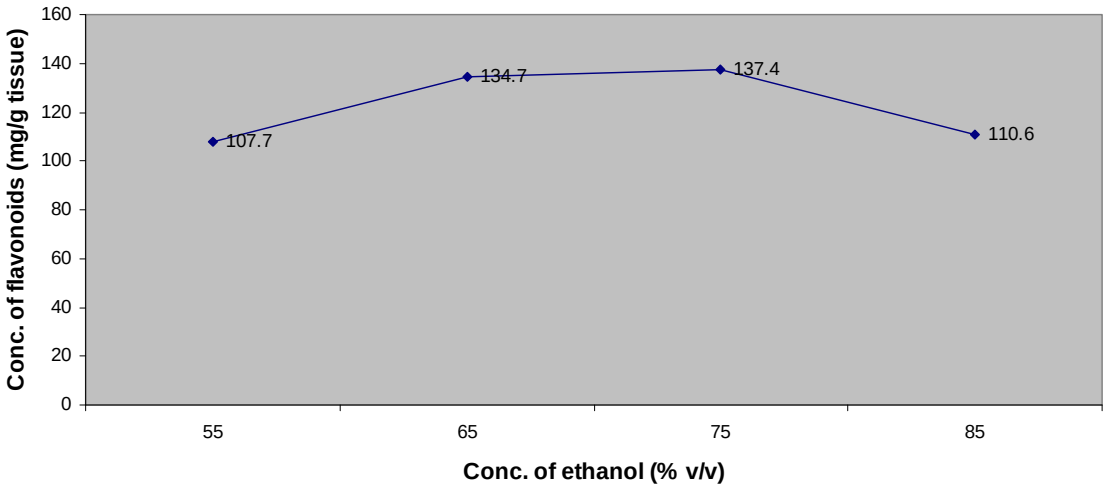
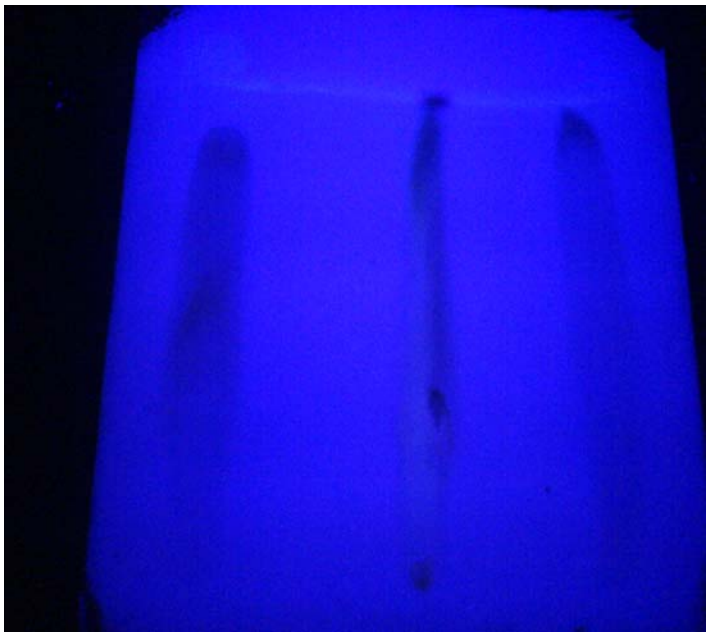


Fig.5 Identification of flavonoids from optimized extract by TLC under UV light



Lane 1 = Quercetin
Lane 2 = Optimized extract
Lane 3 = Rutin

Fig.6 Identification of rutin related compounds by PTLC

Table 1 Factors for the extraction of flavonoids

	A	B	C	D	E
Levels	**Temp. (°C)**	**Ext.tim. (hrs)**	**Solvent (%)**	**Sol : liq (W:V)**	**No.of ext.**
1	55	1	65	1:5	1
2	65	2	75	1:10	2
3	75	3	85	1:15	3
4	85	4	95	1:20	4

Table 2 L_{16} orthogonal design of experiment (Wu et al., 2007)

Exp.	A	B	C	D	E
1	1	1	2	3	4
2	1	2	1	4	3
3	1	3	4	1	2
4	1	4	3	2	1
5	2	1	1	1	1
6	2	2	2	2	2
7	2	3	3	3	3
8	2	4	4	4	4
9	3	1	3	4	2
10	3	2	4	3	1
11	3	3	1	2	4
12	3	4	2	1	3
13	4	1	4	2	3
14	4	2	3	1	4
15	4	3	2	4	1
16	4	4	1	3	2

Table 3 Experimental results and range analysis

Exp.	A	B	C	D	E	Flav. (mg/g)
1	1	1	2	3	4	71.5
2	1	2	1	4	3	32.6
3	1	3	4	1	2	39.4
4	1	4	3	2	1	69.2
5	2	1	1	1	1	107.2
6	2	2	2	2	2	51.2
7	2	3	3	3	3	51.3
8	2	4	4	4	4	43.7
9	3	1	3	4	2	52.9
10	3	2	4	3	1	100.5
11	3	3	1	2	4	91.1
12	3	4	2	1	3	134.7
13	4	1	4	2	3	110.6
14	4	2	3	1	4	137.4
15	4	3	2	4	1	84.0
16	4	4	1	3	2	107.7
$\mathbf{K_1}$	53.2	64.8	84.6	104.7	90.2	
$\mathbf{K_2}$	63.3	80.4	85.3	80.5	62.8	
$\mathbf{K_3}$	94.8	66.4	77.7	82.7	82.3	
$\mathbf{K_4}$	109.9	88.8	73.5	53.3	85.9	
$\mathbf{k_1}$	13.3	16.2	21.2	26.2	22.6	
$\mathbf{k_2}$	15.8	20.1	21.3	20.1	15.7	
$\mathbf{k_3}$	23.7	16.6	19.4	20.7	20.6	
$\mathbf{k_4}$	27.5	22.2	18.4	13.3	21.5	
R	14.2	6.0	2.8	12.9	6.9	

Table 4 One way ANOVA

Levels	Sum of squares	Degrees of freedom	Mean square	F-values
A	8443.84	3	2814.61	4.89
B	1168.31	3	389.44	0.37
C	386.99	3	128.99	0.12
D	5316.78	3	1772.26	2.26
E	1761.59	3	587.2	0.58
		15		

4. CONCLUSION

In conclusion, the extraction conditions for flavonoids were optimized to find that the extraction temperature 85ºC, 2hrs extraction duration, 75%ethanol, 1:05 material ratio and 4 times of extraction were the optimal conditions. Moreover, temperature was found to be a significant factor that affects the extraction procedure. The TLC/ PTLC results of the optimized extracts were found to contained rutin, quercetin related compounds and also certain unknown phenoilc acids. More research on flavonoids biological activity should be done in the future research.

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Molecular recognition of Anions by Novel functionalised Porphyrins,

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ABSTRACT

The design and syntheses of receptors for the molecular recognition of anion is a difficult area in Supramolecular chemistry. This field of research has been receiving increasing attention, considering the significant and indespensable role anions play in nature. Several hosts molecules have been designed, synthesized and anion recognition studies investigated over the years. These include protonated polyamines, quaternary ammonium derived receptors, Lewis acid receptors incorporating Sn, Si, Hg, B, Ca, Ag^+ Lewis acidic centers, positively charged cobalticinium amido, bipyridine amido, calixarenes amido, neutral ferrocenoyl amido, uranyl amido based receptors, calixarenes urea and thiourea hosts amongst others. However, anion recognition by porphyrins is even a more difficult area. Investigations have recently been started and the results obtained so far are very promising. This paper gives an account of the evolutionary trends in Anion recognition by novel functionalised porphyrins. [Nature and Science. 2008;6(3):22-42]. ISSN: 1545-0740.

Dr. Raymond C.Jagessar was born in Guyana, South America. He obtained his BSc degree from the University of Guyana with Honours and was awarded an Oxford scholarship to pursue his PhD in Supramolecular chemistry. At the University of Oxford, he worked on "Anion recognition by novel functionalized porphyrin". After completing his PhD in 1995 he was offered post doctoral research fellowships at Wichita State University and University of South Carolina. At Wichita State University he continued with the Design and syntheses of Porphyrin urea anion receptors for selective recognition and their applications. At the University of South Carolina, he worked on the design and synthesis of "Porphyin based molecular wires and their applications". He is currently a Senior lecturer in the Department of Chemistry, Faculty of Natural Sciences, University of Guyana, South America. Also, currently a member of the American Chemical Society, the Royal Society of Chemistry, the Caribbean Academy of Scientists and a chartered Chemist (CChem) of the Royal Society of Chemistry.

i. Introduction

Anion recognition is an important area of increasing research in Supramolecular chemistry[1]. This originate from many fundamental roles anion play in nature: biologically, chemically, biomedically and environmentally. Biologically, in enzymatic processes, many substrates are anionic in nature. For example, seventy percent of naturally occurring enzymes require an anion, either as a substrate or as a cofactor. The genetic DNA and RNA are polymeric anions. Chemically, anions act as nucleophiles (e.g CN^-, S^{2-}), redox active agents (e.g $S_2O_8^{2-}$), bases (e.g –OR) and as phase transfer agents and catalysts. Environmentally, anions such as nitrate and phosphate constitute a large proportion of current pollutants that cause "eutrophication" of rivers.Considering all these important roles anions fulfill, there is a need for the design and syntheses of anion receptors that can detect and exhibit a high degree of selectivity and strength of binding in highly competitive polar solvents such as DMSO and H_2O. An ideal situation would be a model system that can complex anion selectively in aqueous system considering anions in the environment are found dissolved in water. Medicinally, anions operate in an aqueous system in our body and not in organic solvents such as $CHCl_3$ or CH_3CN. Such receptor would be an excellent candidate to cleanse the aqueous

environment of anionic waste and useful in the field of medicine and biology. Thus, aqueous anion receptors are scarce in constrasts to organic based anion receptors.

The evolution of anion recognition started with the development of positively charged receptors, incorporating ammonium[2] and guanidinium[3] binding sites whose main motif of binding depended exclusively on coulombic or directional electrostatic attractions. Hydrogen bonding sites have also been incorporated to provide additional binding energy as encountered in the binding of Cl^-,F^-,Br^-, N^{3-}, Zwitterion, *w*-aminocarboxylate anions[4] etc. Guandinium[5] hydrogen bonding receptors have also been used for the complexation of carboxylate anions[6]. This was followed by the development of neutral receptors possessing neutral polar hydrogen bonding donor and acceptor groups such as –NHCO- or carboxyl groups that complex anion via anion hydrogen bonding interactions i.e anion----NHCO-.Subsequently, is the development of receptors incorporating Lewis acidic centers such as tin[8], silicon[9], boron, mercury[10] etc. In principle, the complexation is based on the interaction between the Lewis acidic centers and the anion. The binding strength can be further augmented by the incorporation of hydrogen bonding sites as is seen with uranyl amido salophen receptors selectively binding phosphate[11]. To improve the potency of the receptors, positively charged sites, acting cooperatively with hydrogen bond donor and acceptors groups were incorporated in the receptors. This include the syntheses of ferrocene, cobalticinium, bipyridine and calix(n)arene amido[12] and urea receptors[13] etc. Previously, there has been slow progress in the field of anion recognition and coordination. This has been due to the properties of anion. These include variable size, geometry, solvation energies, pH dependence and the charge of the anion[14],[15].

There are several organic host molecules, both acyclic and macrocyclic that have been used recently for anion recognition. These include ferrocene and cobalticinium amides, acyclic and macrocyclic bipyridine amides, calix(4) arene amido and urea macrocyclic receptors[12],[13]. However, there is no report of anion recognition by a naked porphyrin, such as a tetraphenylporphyrin.

Porphyrins are very attractive hosts to be used for anion recognition studies since they are spectrophotoelectroactive, allowing anion complexation to be monitored by a variety of physical methods. Also, porphyrin is an important ingredient of life in the oxygen binding haemoglobin of blood and the sunlight trapping chlorophyll of plant cell. Interestingly, anion recognition by porphyrins has received very little attention until recently [16-19]. The free neutral tetrapyrrolic ligand (1) has no anionic binding power on its own [20,21]. This is due to the small size of the porphyrin cavity which doesn't complex anions via convergent N-H---- anion hydrogen bonding interactions. Also, the rigidity of the porphyrin skeleton and cavity. Hence, this has given birth to the expansion of the porphyrin cavity via the insertion of one or more pyrrolic units in "expanded porphyrins", Fig 1.0. Amongst these are the sapphyrins(3) which complex anion only in its protonated state and exhibit modest anion selectivity due to non-directional coulombic interactions. For example, sapphyrin in its diprotonated state formed a very stable fluoride and phosphate complex in methanol ($K_{assn} = 1 \times 10^5\ M^{-1}$) [22].

It has been shown that a hexaprotonated porphyrin trimer system linked by butadiene linkages (4) complexes large anionic clusters such as $SiW_{12}O_4^{4-}$ and $Os_{10}C(CO)_{24}^{2}$-. However, this review is concerned primarily with Anion recognition by novel functionalised Porphyrins, mainly " Picket Fence" amido and " Picket Fence" urea porphyrins.

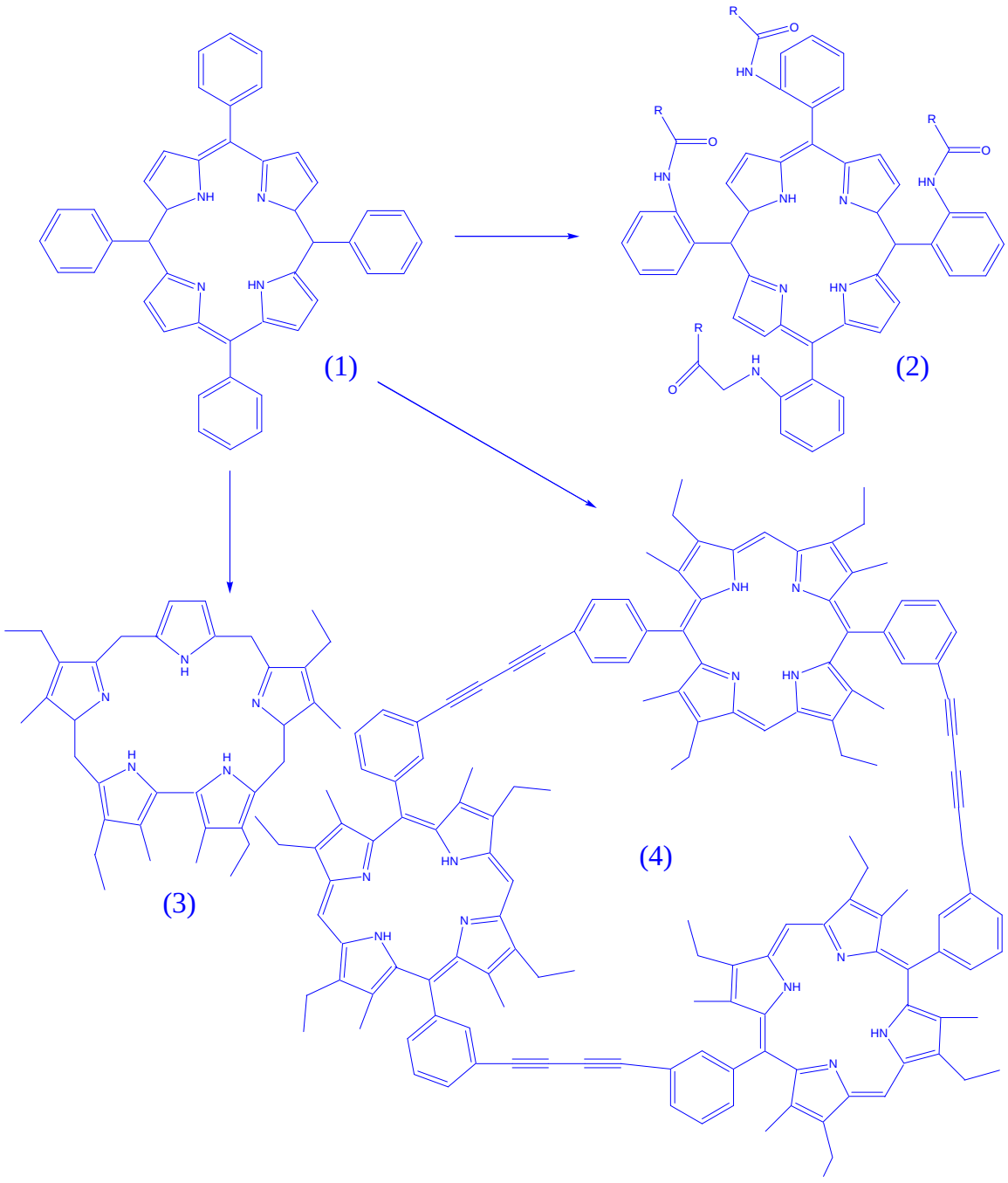


Fig: 1.0: Evolutionary trends in Anion recognition by porphyrins and porphyrinoid compounds.

Anion complexation can be monitored by several means. Below is a description of various approaches used to monitor anion complexation or binding.

ii. Monitoring Anion Complexation

Anion complexation can be monitored using ^{1}H NMR titration studies, UV/Vis, Electrochemical and X-ray studies.

^{1}H nmr studies is conducted by the addition of tetrabutylammonium salts $Bu_4N^+X^-$ (X= Cl^-, Br^-, NO_3^- or HSO_4^-) to deuterated CD_2Cl_2, $CDCl_3$ or d_6-DMSO solutions of the receptor and monitoring the shifts in the host (porphyrin) protons. The concentration of the receptor is usually 0.01 $moldm^{-3}$. The initial ^{1}H nmr spectrum of the porphyrin receptor is recorded.This is followed by the addition of stoichiometric amount of anion. After each addition, the ^{1}H nmr profile is recorded and changes in the chemical shifts of diagnostic protons observed. Also, overall changes in the spectrum recorded. This include the appearance and disappearance of peaks. Host protons monitored are amide –NHCO-, aromatic ArH and substituent protons etc. The amide protons are labile and are the best to study since they are the most sensitive to the negatively charged electrostatic anion field via anion hydrogen bonding interactions viz anion----NHCO. From the ^{1}Hnmr titration studies, the displacement of chemical shifts in the host respective proton is plotted versus the number of equivalents of anion added to yield titration curves from which the receptor anion stoichiometry can be obtained i.e whether a 1:1 or 2:1 complexation via mole ratio method, EQNMR and Job's plot. From the shifts in the host protons, the computer program EQNMR can also be used to estimate the stability or association constants of binding.

The magnitude of the association constants gives an indication of the strength, degree and selectivity of anion binding. Also, it can indicate the mode of anion binding.

Anion complexation can also be monitored by UV/Vis spectral titration studies. This works well for hosts that posses strong and distinct UV/Visible spectroscopic bands or chromophore i.e richly photoactive resulting from the conjugated nature of the molecule. The porphyrin host molecule is a good UV/Visible host as it possess an intense Soret band of high extinction coefficient and four visible satellite bands of much lesser intensity (S_1,S_2,S_3 and S_4). In a metalloporphyrin, two of these are not evident as a result of a change in the symmetry of the porphyrin host from d_{4h} to d_{2h}. Also, the intensity of these bands will differ pending the type of "Picket fence".

UV/Vis spectroscopic complexation involve the addition of stoichiometric amounts of anions usually as their tetrabutyl ammonium salts: $Bu_4N^+X^-$, X= Cl^-, Br^-, NO_3^- or HSO_4^- to a dilute solution of the receptor of appropriate concentration in a cuvette. The initial spectrum is also recorded. This is followed by the addition of stoichiometric amount of anion and recording the spectrum. Changes in absorbance of diagnostic bands are observed. Titration curves are generated by a plot of change in absorbance versus the equivalents of anion added. The association constants are found using specific computer programs such as EQNMR[24].

The porphyrins is electro or redox active, having defined electrochemical redox waves, p/p^+, p^+/p^{2+} p/p^- and p^-/p^{2-}. The incorporation of redox active moieties further augments the redox properties of the porphyrins. As with other studies, the electrochemical nature of the free base porphyrin is recorded in a suitable solvent (CH_2Cl_2/CH_3CN) in the presence of conducting electrolyte. This is followed by the addition of stoichiometric amounts of anion. After each addition, the electrochemical nature of the host is recorded and compared with the initial electrochemical wave. Specific computer programs are then used to calculate the association constants of binding.

The uncomplexed functionalised porphyrin anion receptor is characterized by a spectral and electrochemical property, Spr and Epr. Upon complexation with substrate such as an anion, it emits a different spectral signal and electrochemical waves. This porphyrin superanionmolecule is characterized by a spectral and electrochemical property, Spr2 and Epr(2). If the binding is reversible, the porphyrin supramolecule can release the bound anion and return to its initial spectral and electrochemical state, Spr and Epr, Fig 2.0. Hence, performing the function of a spectral and electrochemical sensor and also as a transport mimic. Binding is not always reversible. The porphyrin supramolecule can retain the complex anion or if it does release it, its spectral and electrochemical properties changes. To investigate these hypotheses, the design and syntheses of ferrocene and cobalticinium atropisomeric " picket fence" porphyrins based anion receptors were initiated.

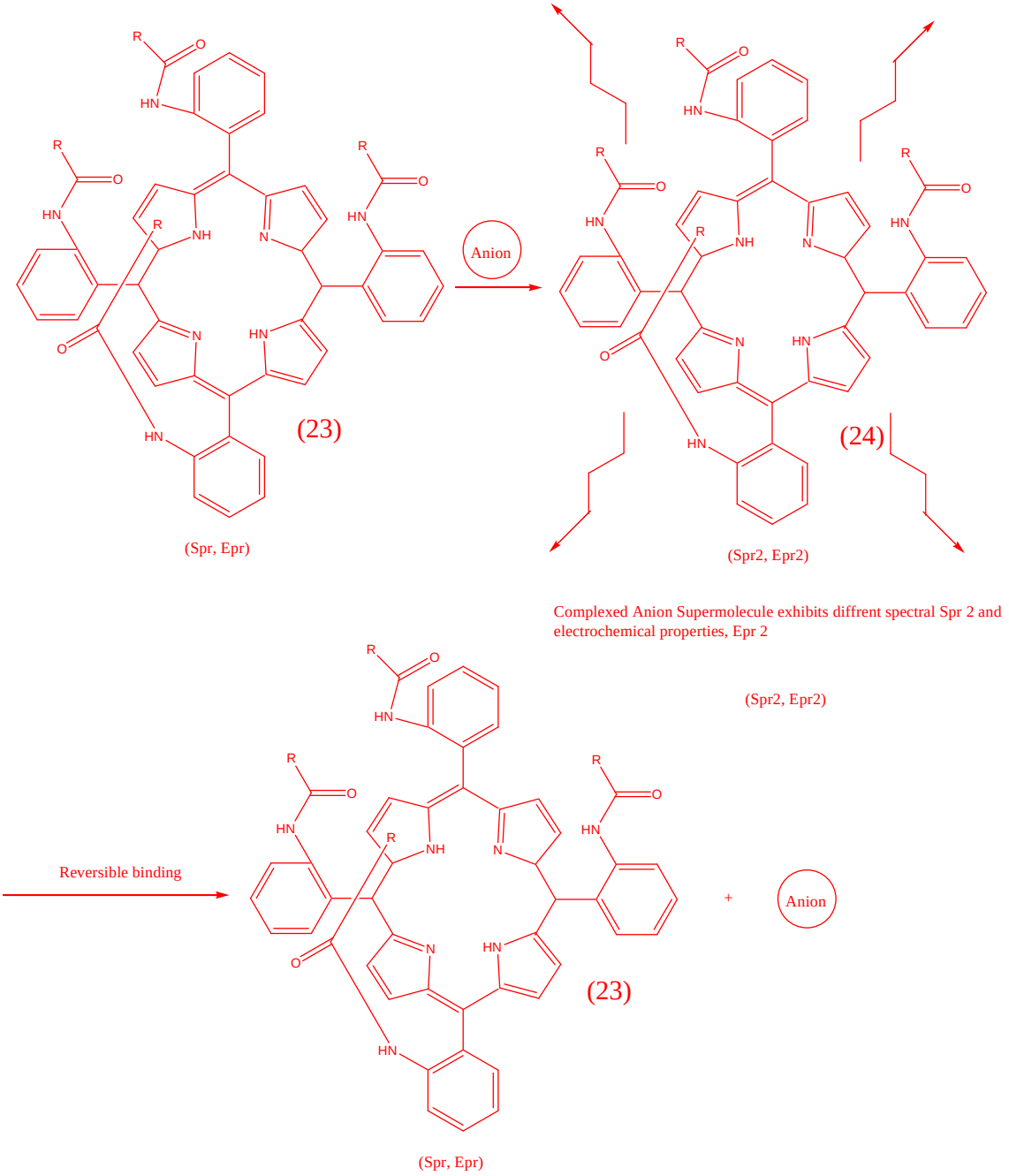
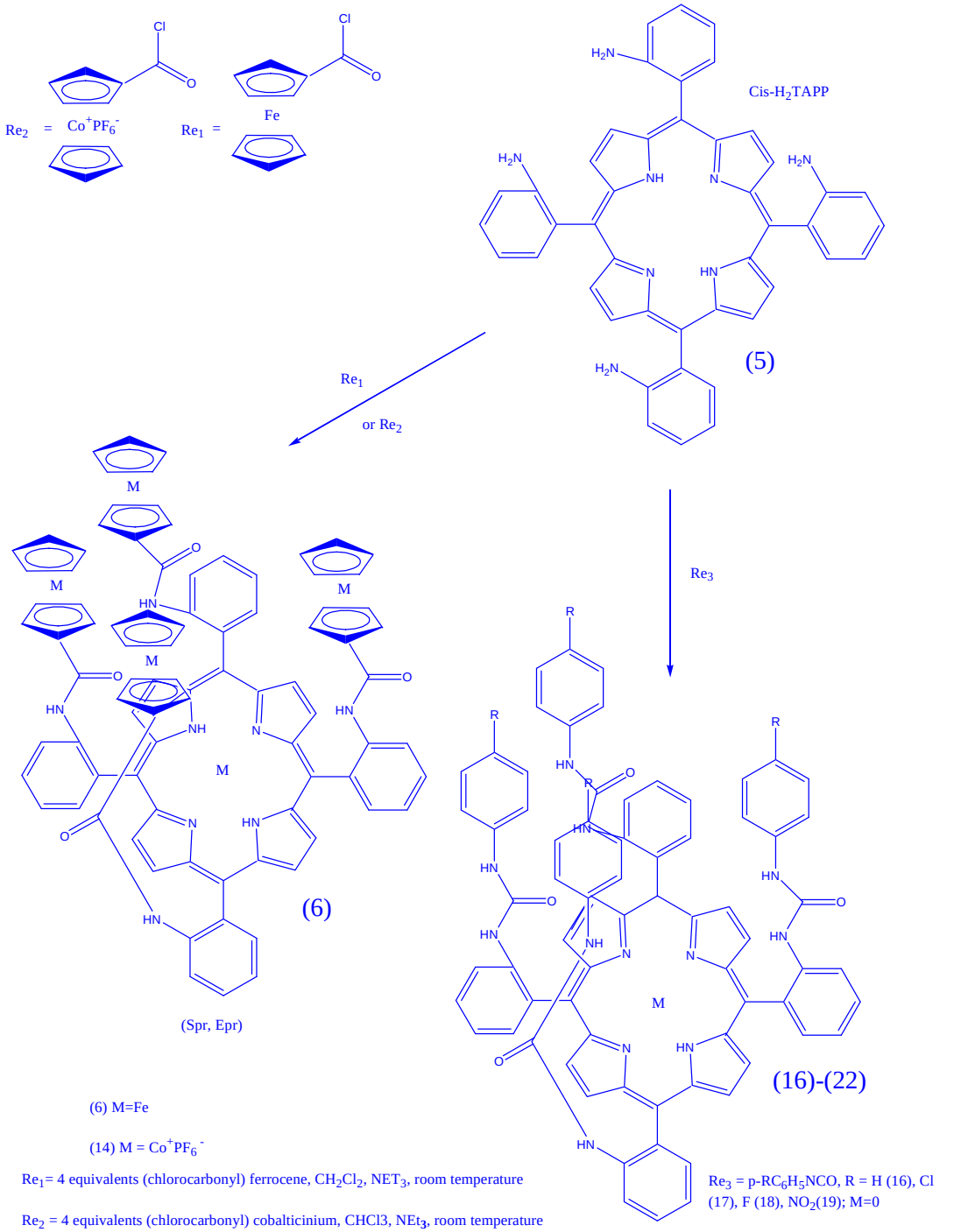


Fig 2.0

iii. <u>Porphyrin Syntheses</u>

Before further discussion can be made, the syntheses of these novel porphyrins anion receptors need to be discussed. The syntheses of these porphyrin have already been mentioned [26,27,28]. The porphyrins were synthesized under a dry and inert atmosphere of N_2 for 24 hours. For receptor (6)-(9), this involves the condensation of the respective atropisomer of 5, 10, 15, 20-*meso* tetrakis(2-amino) phenyl porphyrin, H_2TAPP(5) with an excess amount of four equivalents of chlorocarbonyl ferrocene in dry dichloromethane, using triethylamine as the base. For receptor (14) and (15), this is achieved by the condensation of four and two equivalents of cobalticinium acid chloride respectively with *cis*-H_2TAPP in CH_3CN and subsequent purification via sephadex LH-20. For receptor (16)-(19), this involves the addition of four equivalents of the requisite isocyanate to the *cis*- isomer of H_2TAPP in $CHCl_3$. Receptors (16)-(19) and (20)-(22) were purified via silica gel chromatography using dichloromethane-ethylacetate (4:1, v/v) and dichloromethane-ethylacetate (8:1, v/v) respectively. This yielded purple microcrystalline solids in yields of 70-85% for receptor (16)-(19). The Zn complexes (20)-(22) were synthesized by stirring the free base porphyrins with excess $Zn(OAc)_2.2H_2O$ in CH_2Cl_2/MeOH (2:1, v/v) and were obtained in 90-95% yield following purification by silica gel chromatography using dichloromethane/ethylacetate (8:1, v/v) respectively.



(6) M=Fe

(14) M = $Co^{+}PF_6^{-}$

Re_1= 4 equivalents (chlorocarbonyl) ferrocene, CH_2Cl_2, NET_3, room temperature

Re_2 = 4 equivalents (chlorocarbonyl) cobalticinium, CHCl3, NEt_3, room temperature

Re_3 = 4 equivalents, p-RC_6H_4NCO, $CHCl_3$, room temperature

Re_3 = p-RC_6H_5NCO, R = H (16), Cl (17), F (18), NO_2(19); M=0

M = Zn, R=H (20), R=Cl (21), R=F (22)

Scheme 1.0

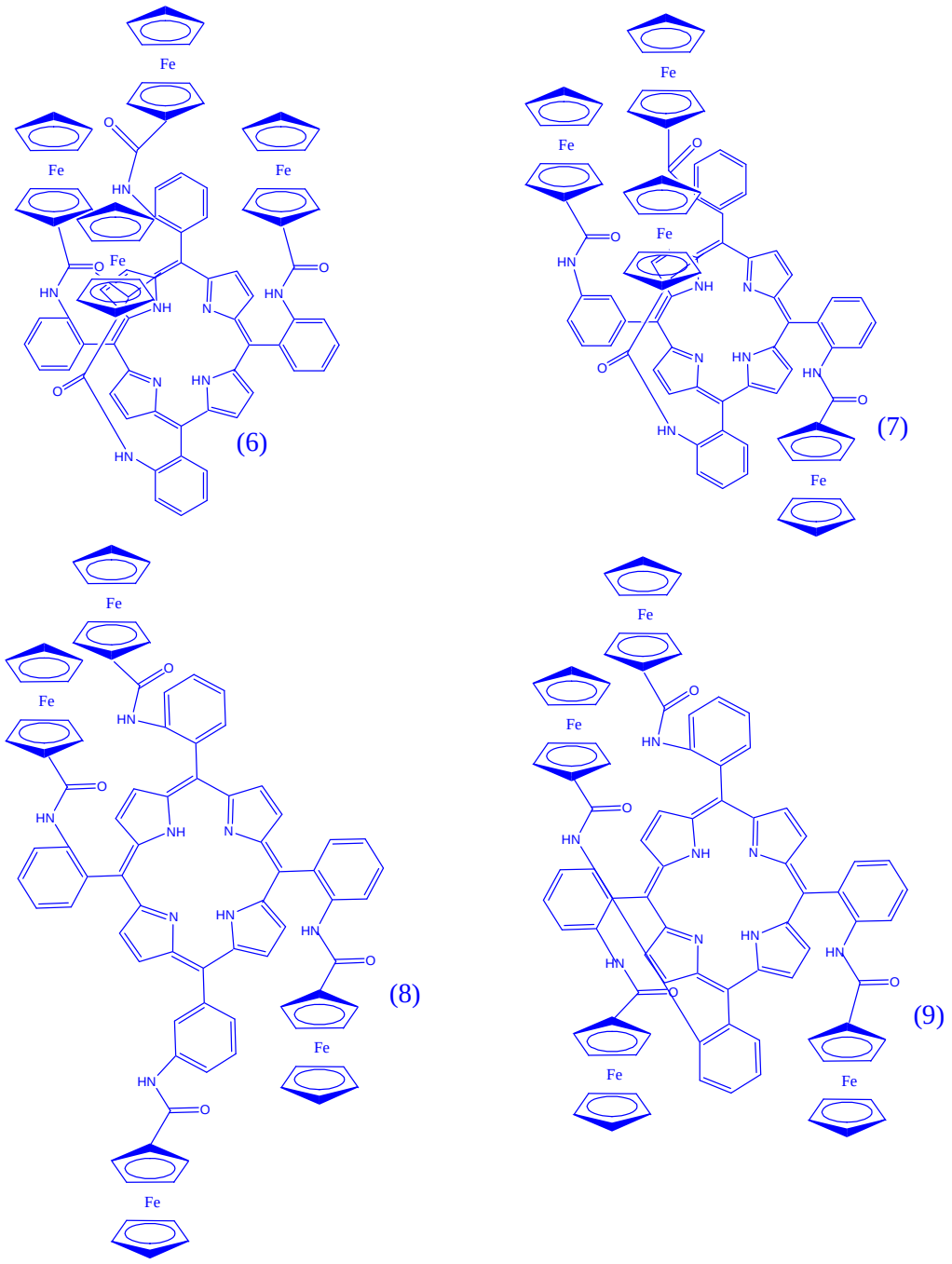
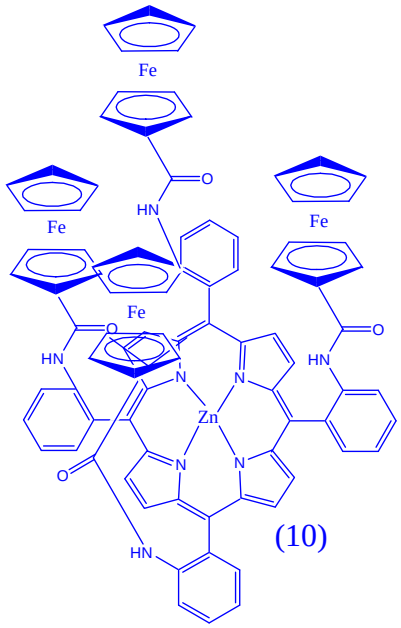
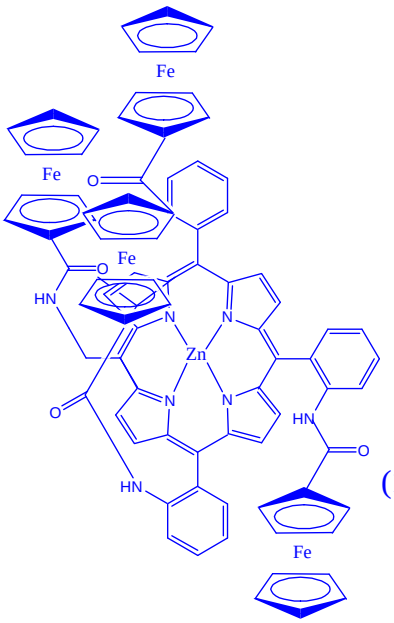


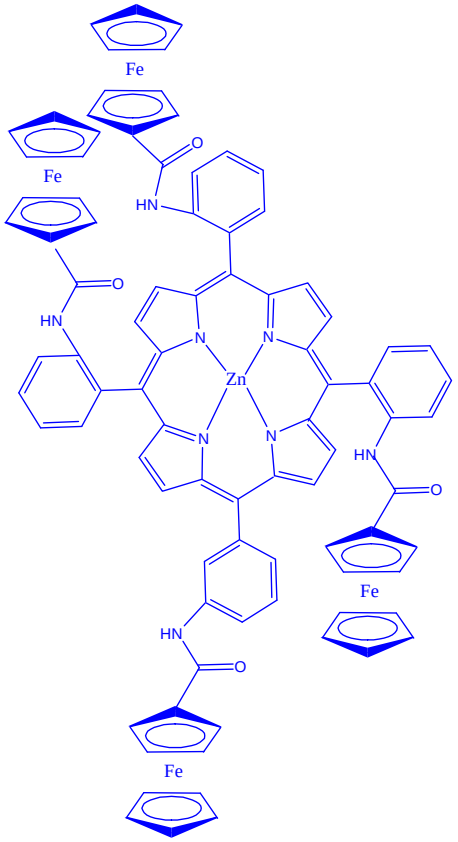
Fig 3.0



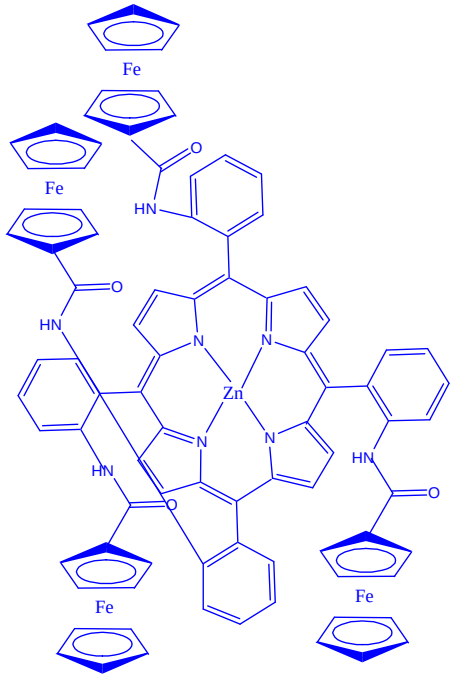
(10)



(11)



(12)



(13)

Fig 4.0:

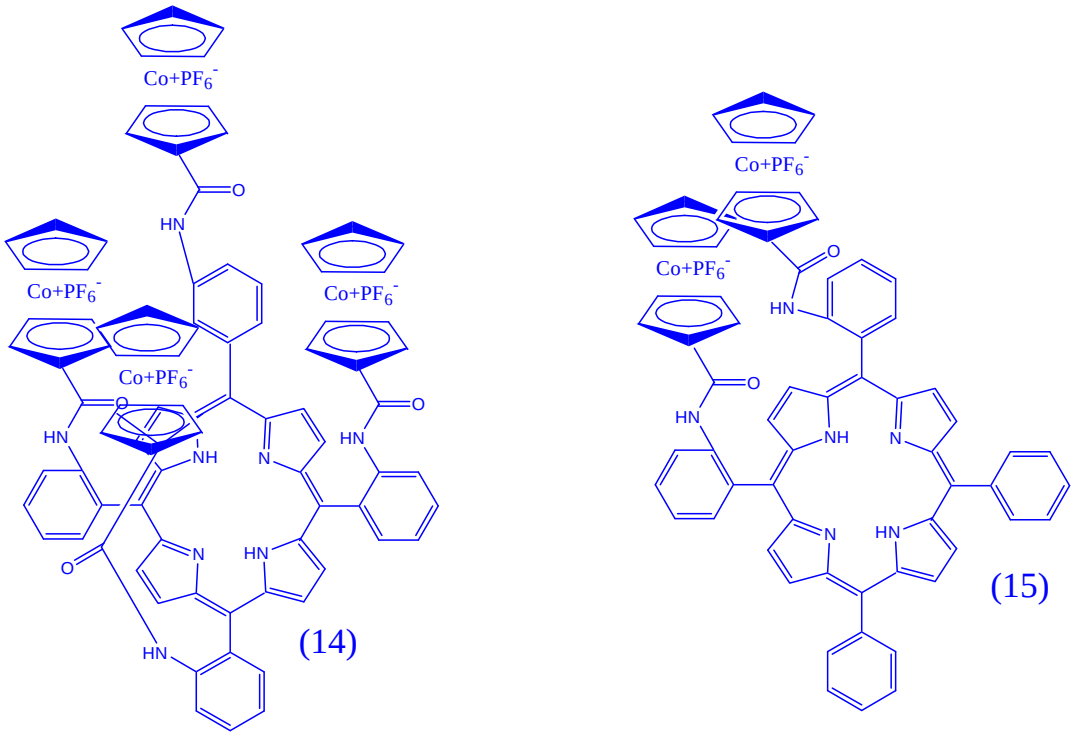


Fig 5.0: 5,10,15,20-*meso tetrakis* atropisomeric cobalticinium porphyrin receptors

iv: Design of Porphyrin Anion Receptors:

Following the work on expanded porphyrins, is the development of functionalised porphyrin based anion receptors. It was envisaged that neutral functionalised amido porphyrin receptors and those that have a positively charged binding sites in close proximity to amide hydrogen bonding environment should complex anion via virtue of an electrostatic interaction of the anion sphere and the positively charge center and favourable anion----NHCO---- hydrogen bonding interactions. In addition, amides are thermodynamically stable, pH insensitive and hydrolysis resistant.

Before an anion receptor can be synthesized, its design is necessary and of utmost importance. The design must make the compound synthetically accessible in large yields so that extensive physical studies can be carried out and also for application in technological science studies.

In the design, the porphyrin basal template used are the atropisomers of 5,10,15,20 – *meso tetrakis* tetraphenylporphyrins.Attached to the planar porphyrins are the "Picket fence" amide or urea arms which delineate a spherical cavity. The purpose of the cavity or pocket is to shield the bound anion or guest from the solvation forces of the environment i.e to act as an hydrophobic cavaity. There will be competition between the solvent molecule and anion guest for the host binding site. Thus, complexation to neutral host may involve solvent desolvation. Also, the host must be preorganised for substrate binding. It is anticipated that the four amide or eight urea NHs groups are pointing in the cavity, a position that allows them to converge on the anion. Also, another factor to take into consideration is the accumulation of H-bond donor sites in close vicinity to each other. This allows for a maximum number of hydrogen bonding contacts for the anionic guests, resulting in improve binding. Also, the urea –NHCONH- binding units must be placed at such a distance that there is no intermolecular hydrogen bonding. What about the ring current effect of the porphyrins? The porphyrin's internal β-pyrrole protons resonate far upfield at negative ppm as a result of the diamagnetic shielding effect of the ring current, whereas the outer *meso* proton deshielded by the aromatic ring current resonate far downfield resulting from the paramagnetic effect of the ring current, Fig 7.0. It is expected that the bound anion would in one region of the porphyrin augments the ring current and in another region depreciate it. Thus, the ring current can affect the association constants. It is left to the imaginative Supramolecular chemist to design a porphyrin receptor whose ring current should increase the overall association constant.

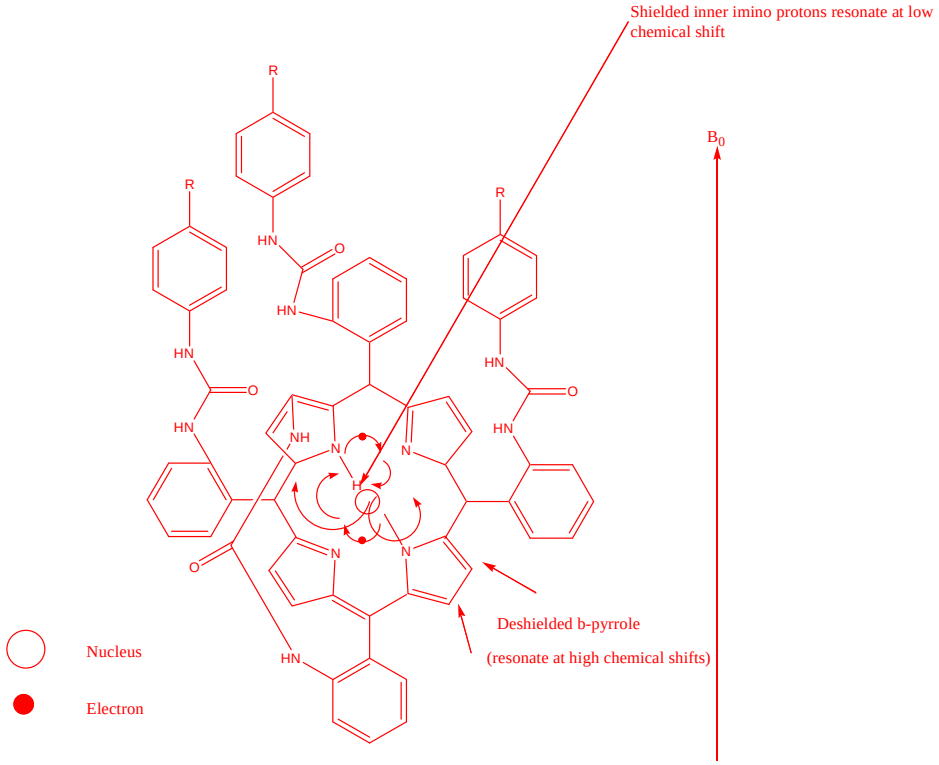


Fig 6.0: Ring current effect of Porphyrin

Recently, several types of porphyrin based anion receptors have been synthesized. These are as follows:

(v) Novel Ferrocene, Cobalticinium functionalized amido and Urea porphyrins anion Receptors.

(a) Novel Ferrocene Amido Porphyrins Receptors

The first set of receptors synthesized, characterized and anion binding studies investigated are a series of neutral ferrocenoyl amido atropisomeric porphyrins receptors as shown in Fig 3.0. In nature, the selective binding for anion is dependent on the positional alignment of the anion binding sites. These receptors are design so that the positional alignment of the hydrogen bond groups and cavity dimensions varies.

It was found that all neutral ferrocene atropisomeric receptors don't complex anions. This is shown by negligible shifts in the host protons $\Delta < 0.05$ ppm, unperturbed Soret and Q bands and unperturbed electrochemical diagnostic redox waves. This was surprising, considering neutral acyclic and macrocyclic ferrocene amides complex anions[30]. Maybe, the ferrocene amide protons in the "Picket fence" arrangements are not acidic enough for anion complexation. It was thought that a combination of a Lewis acidic site in close proximity to amido linkages should do so. Thus, in the presence of a Lewis acidic centre, Zn^{2+} incorporated in the porphyrin core for (10)-(13) and the rich surrounding convergent amido linkages "switch" on anion binding, both spectrally and electrochemically for (10)-(13). For example, the addition of tetrabutylammonium salts: $Bu_4N^+X^-$ (X = Cl^-, Br^-, NO_3^- and HSO_4^-) to deuterated CD_2Cl_2 solutions of compounds (10)-(13) resulted in pertinent significant shifts in the host protons. Porphyrin amide proton perturbation of δ = 0.40 ppm and 0.64 ppm were observed for (10) and (13) after the addition of one equivalent of nitrate[26] and bromide.

(b) Novel Ferrocenoyl Amido Zinc Porphyrins Receptors

It was envisaged that all metallated amido porphyrins should complex anions using the metal Lewis acidic center as the primary recognition element and the surrounding amido linkages as the secondary recognition element, Fig 7.0, the second hypothetical mode of anion binding. It is anticipated that these atropisomeric zinc metallated porphyrins should be selective for anion binding, since in nature, the selective binding for anion is achieved by the positional alignment or directive hydrogen bonding[31]. For example, sulphate and phosphate binding proteins complex anions exclusively via directive hydrogen bonds.

Association constant (K/dm^3mol^{-1}) for receptors (10)-(13) in dichloromethane at room temperature.

Anions	Receptor (10)	Receptor (11)	Receptor (12)	Receptor (13)
Br^-	6200	3200	5600	5800
NO_3^-	2300	5000	1600	1300
HSO_4-	2100	2000	900	600
Cl^-	-----	------	1000	------

Errors were typically <5 to 10%.

[1] H nmr titration studies revealed that receptor (10) displayed a preference or selectivity for the complexation of spherical anions (Cl^-,Br^-) over non spherical anions (HSO_4^- and NO_3^-), indicating that a complementary spherical host hydrogen bonding amide environment exists for the complexation of anion with all four or two hydrogen bonds acting cooperatively in complexation for the spherical anion. This may also be reflective of the higher charge to radius ratio polarisabilities of the anion. Also, receptor (10) showed the highest for halide as compared with the $\alpha,\alpha,\beta,\beta$ or $\alpha,\beta,\alpha,\beta$ atropisomer, since four cooperative convergent –NHCO- hydrogen sites are involved in the anion receognition process as compared with two for (12) and (13). Also,

the magnitude of the halide association constant is greater for the α,β,α,β atropisomer as compared with the α,α,β,β atropisomer. This may be due to the cooperative amide hydrogen bonding functionality converging in an anti manner for (13) as opposed to (12). Receptor (13) may be described as a cleft type receptor. Interestingly, the α,α,α,β isomer showed a preference of binding for NO_3^- over halides (Cl^-, Br^-) and hydrogen sulphate i.e the selectivity trend of $NO_3^- > Cl^- > Br^- > HSO_4^-$. This indicates that a trigonal shaped hydrogen bonding environment is prevalent for the complementary NO_3^-ions.

In all cases, the mode of anion complexation involves an electronic interaction of the anion electrostatic coordination sites with the primary recognition zinc lewis acid centre and a secondary interaction with the amide hydrogen bonding environments as shown in Fig 7.0.

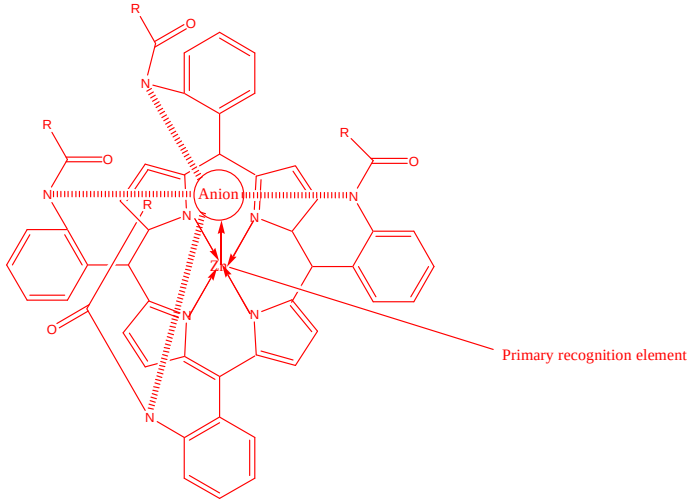


Fig 7.0: Illustration of mode of anion binding for compound (10).

(b) **Novel Cobalticinium Amido Porphyrin Receptors**:

To investigate the third hypothetical mode of anion binding, a *cis meso tetrakis* cobalticinium amido porphyrin has been synthesized and characterized. Here, the combination of a positively charge cobalticinium centre in close proximity to the amide hydrogen bonding should complex and switch on anion binding for this porphyrin amide system. This has been found to be so, spectrally and electrochemically. Unlike a zinc metallated " Picket fence porphyrins, a *cis meso tetrakis* cobalticinium amido porphyrin has been shown to complex anions with small anion selectivity between halides, nitrate and sulphate in CD_3CN. The selectivity been: $Cl^- > Br^- >> NO_3^- > HSO_4^-$. A high degree of selectivity for spherical chloride anions suggests that anion binding recognition site delineates a spherical cavity and all four amide hydrogen bonds act cooperatively in complexing the anion. The association constants for compound (14) are shown in Table 2.0

Table 2.0: The association constants in CD_3CN for compound (14).

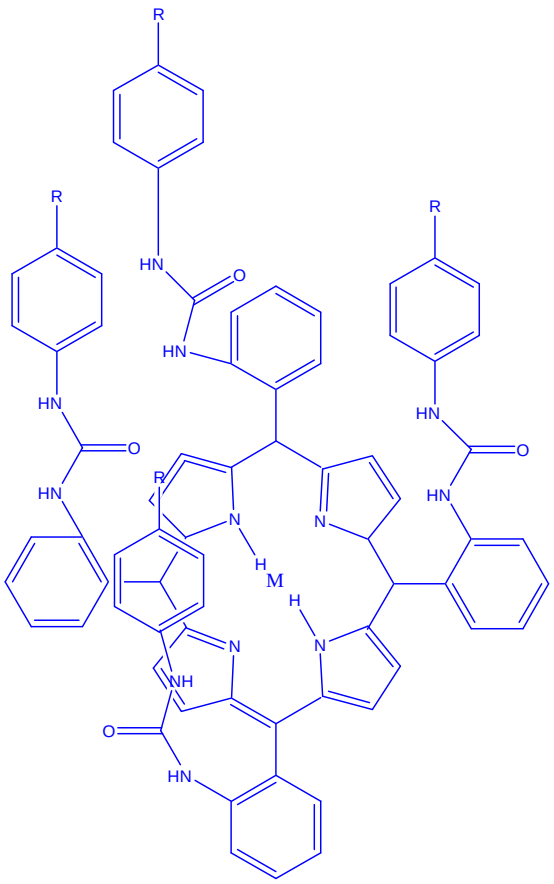
Receptor (14)	Anion	K/dm^3mol^{-1}
	Cl-	1000
	Br-	824
	NO_3-	450
	HSO_4-	420

By varying the geometrical arrangement of binding sites, different selectivity can be sought. For example, the *bis* amido *α,α,ββ-meso tetrakis* cobalticinium amido porphyrins have shown a high degree of selectivity for nitrate anions over chloride, thus exhibiting the rare selectivity trends: NO_3- > Br^- > Cl^-. This indicates that a complementary trigonal host cavity exists for nitrate[30].

(c) Novel Urea Functionalised Porphyrin Anion receptor:

So far it has been shown that neutral porphyrin ferrocene amides don't complex anion. Also, even though cobalticinium amides are good anion binders, the preparation of cobalticinium amide porphyrin receptors are arduous. Besides, cobalticinium amide porphyrin receptors are virtually chromatographically immobile on silica gel, making them difficult to purify and hence relatively scare. Hence, this has provided the impetus for the search of other porphyrin anion receptors with a high affinity for anion.

To broaden the scope of porphyrin based anion recognition, it would be interesting to prepare neutral porphyrin urea based anion receptors. Neutral receptors have certain advantages. They are more selective in comparison to positively charged hosts. Neutral receptors are scare relative to positively charged ones. Also, in nature the selective binding for anion is achieved by the positional alignment of hydrogen bond donor groups. For example, sulfate and phosphate binding proteins complex anion exclusively via hydrogen bonding with a selectivity factor of 10^5 [31]. In positively charged host, selectivity is modest due to non directional coulombic interactions. Neutral Lewis acidic host have limited synthetic flexibility for optimizing binding selectivity. In addition, Lewis acidic hosts suffers from the problem of solvent competition with the guest species. Most organic solvents are Lewis bases and exceed molar concentration of a guest anion by several orders of magnitude. Solvation design is more difficult, the smaller and more Lewis basic the solvent molecules are. The syntheses of neutral anion receptors that are highly selective and exhibits a strong degree of binding is also relatively scarce. Neutral receptors use hydrogen bonding motif exclusively to complex anion guest species. They are electroneutral. In addition, several hydrogen bonding sites can be incorporated in the host in different geometrical arrangements. Also, the molecular framework can be be further elaborated. There can be self assembly of neutral receptors. A further advantage is that electroneutrality is important in the application of membrane transport or potentiometric ion sensing. Neutral receptors reported to date bind strongly to phosphate and carboxylate anion. There is a need to broaden the scope of neutral receptors selectivity and applicability. Thus, a series of neutral urea appended free base porphyrin receptors were prepared [27,28] , Fig. 9.0.



R = H, (16)

R = Cl, (17)

R = F, (18)

R = NO_2 (19)

M = Zn

R =H (20), R=Cl (21), R = F (22)

Fig 9.0: Urea Appended Free base Porphyrins

These receptors bind exceptionally strong to Cl^- (K ass > 10^5 dm^{-3} mol^{-1}) in $(CD_3)_2SO$ and in DMSO/H_2O, highly competitive solvent medium and exhibit significant selectivity for Cl^- over NO_3^- and $H_2PO_4^-$ since they complex with Cl^- to a much greater extent 1000:1 compared with the trigonal NO_3^- and 280: 1, compared with the tetrahedral $H_2PO_4^-$ anions in DMSO. They displayed a higher degree of selectivity and strength of binding for anions as compared with protonated expanded porphyrins such as (3), positively charged functionalized cobalticinium amido porphyrins (14) and (15) and metallated zinc ferrocene atropisomeric porphyrin receptors (10)-(13). Neutral ferrocenoyl atropisomeric receptors don't complex anions unless metallated. Both cobalticium and metallated zinc ferrocene atropisomeric receptor systems showed little anion selectivity (a 4:1 binding selectivity between halides, nitrate and sulfate ions in acetonitrile and dichloromethane respectively.

Expanded porphyrin receptors can complex anions only in the mono or diprotonated state and selectivity is modest. For example, sapphyrin in its diprotonated state complexes $H_2PO_4^-$ and F^- ions with similar association constants in methanol. (K assn = 1 x 10^5 M^{-1}). Unlike, expanded porphyrins these receptors don't need to be protonated. Receptors (16)-(18) are indeed the first examples of a neutral class of free base functionalized porphyrins anion receptors that are remarkably selective and binds exceptionally strong for Cl^- in a highly competitive solvent solvent system such as DMSO and DMSO/H_2O. Table 3.0 gives the association constants for receptors (16)-(21) with various anionic substrates.

Association constants (K/dm^{-3} mol^{-1}) for receptor porphyrins (16)-(21) with anions: Cl^-, Br^-, NO_3^- and $H_2PO_4^-$ in $(CD_3)_2SO$.

Table 3.0

Receptor Porphyrin	Cl^-	Br^-	NO_3^-	HSO_4^-	$H_2PO_4^-$
(16)	> 10^5	1.01 x 10^4	90	115	400
(17)	> 10^5	1.0 x 10^4	60	137	300
(18)	> 10^5	9.99 x 10^3	55	147	1400
(19)	> 10^5	1.00 x 10^4	163	226	9.6 x 10^3
(20)	9.5 x 10^3	1.51 x 10^3	23	d	49
(22)	9.82 x 10^3	1.1 x 10^3	d	d	489

Association constants were determined using EQNMR and the errors were in the range: 5 to 10%.

d: impossible to determine the association constant due to broadness in the host proton.

These receptors display a high affinity and selectivity for Cl^-. This is due to the complementary nature of the urea binding site for the spherically shaped anion and the strength of the urea anion binding interaction. Infact, the association constants for the binding of Cl^- are very much larger than those reported for urea and non urea functionalized anion receptors [1,32].

It is anticipated that the attachment of electronegative substituents such as F, Cl and Br to the phenyl ring would intensify the acidity of the urea NH protons via an inductive effect and thus strengthen their hydrogen bonding propensity for anions, leading to a stronger degree of complexation. Electronic tuning via the attachment of electronegative substituents resulted in a small degree of selectivity between the p-fluorophenylurea porphyrin and other porphyrin receptors (16)-(22) in their affinity for $H_2PO_4^-$. However, modification of the phenyl ring with the NO_2 substituent resulted in the anion binding selectivity changing dramatically with the association constants increasing by a factor of 24 i.e from 400 for (16) to 9600 for (19). Infact, the *para* substituted nitro porphyrins exhibits the largest binding constants for anions and the same order of selectivity as for the other receptors. With nitrate and sulphate, there is apparently not much degree of selectivity.

To investigate selectivity for Cl^-, anion complexation was done in a more competitive solvent system such as DMSO/H_2O (88:12%, v/v). The results revealed that the p-fluorophenyl (18), 9.73 x 10^3 is more selective than (16), 1.36 x 10^3 for the Cl^-. The higher affinity for Cl^- is due to the complementary nature of the urea binding cavity and hydrogen bonding sites for the spherically shaped anion. The selectivity trend: Cl^- > Br^->$H_2PO_4^{2-}$->HSO_4^->NO_3^- displayed by these receptors is novel for any urea anion binding receptors.

The zinc complexes showed a smaller degree of binding and selectivity for the various anions in DMSO. This is due to an increase in rigidity or decrease in flexibility of the porphyrin core as a result of the insertion of the zinc. This is shown in Table 3.0. In order to complex a substrate, there must be a balance between rigidity and flexibility of the receptor active site. However, for those receptors (20)-(22), the selectivity trend of Cl^- > Br^- > $H_2PO_4^{2-}$-> NO_3^- was the same.The stoichiometry of anion binding was found to be 1:1 for all urea porphyrins receptors using Job's method of continous variation and EQNMR. In all cases, where anion binding was evident, shifts in the porphyrin protons were observed. For example, shifts were observed for the receptor porphyrin, β- pyrroles, porphyrin NH, urea NHs, urea phenyl protons and *meso*-phenyl. Also, diagnostic shifts were observed. For example, upfield shifts were

always observed for the β-pyrrole whereas downfield shifts were observed for the amide and urea NH protons, indicating NH----anion and urea NH----anion hydrogen bonding interactions.

(vi) **X-ray crystallography**

X-ray crystallography both in solid and solution states is also a useful tool to study anion complexation. An X-ray crystal structure of the zinc complex of (10) was isolated. Here a bound methanol solvent is coordinated to the zinc atom. The zinc atom is 0.27Å above the plane of the four nitrogen atom and is towards the coordinated methanol oxygen atom. An important feature is the positioning of one carbonyl oxygen atom in the cavity and the other three outside of the cavity.

A step was taken further to isolate the first coordination complex of an anion (chloride, bromide) bound by a neutral free base porphyrins, α,α,α,α-5,10,15,20-*meso-tetrakis* (2-(4-chlorophenylurea) phenyl porphyrin[28], a great achievment!!. For compounds (16)-(22), crystal structures obtained are (21).5DMSO, (17) + TBABr.5DMSO, (17) + TBABr.3DMSO and (17) + TBACl.5DMSO.

X-ray crystallography of free base porphyrin (17) + TBABr.5DMSO shows that the single bound anion is deeply buried in the pocket of the porphyrin and is positioned over one pyrrole. The anion is kept intact via four NH hydrogen bonds resulting from two adjacent urea groups. In addition, in (17) + TBABr.5DMSO, a DMSO molecule is positioned in the center of the cavity and provides further stabilization via a coulombic interaction between the electron deficient sulfur and the chloride or bromide anion. The crystal structure supports the 1:1 stoichiometry of binding. The high selectivity for the halides is due to the complementary nature of spherical cavity created by four urea groups together with the inclusion of the ordered solvent DMSO between the two arms of the "urea pickets". There is also no self association of urea groups.

The urea free base porphyrins exhibit a high degree of binding for the halides at a factor of 10^5. It is also interesting to note that sulfate binding protein exhibit a $K_{assn}=10^6M^{-1}$ with discrimination against hydrogen phosphate by a factor of 10^5 and *vice versa*. Thus, the "Picket fence" urea *meso tetrakis* porphyrin design and synthesized above have indeed been able to match the selectivity of binding, reminiscent of nature's sulphate and phosphate binding proteins. Also, X-ray crystallography shows that the anion is deeply buried in the interior of the protein with the help of seven hydrogen bonds in contact with the guest. In addition, there is no functional groups present in the binding cavity that would perform the role of an hydrogen bond acceptor as is required for the complexation of the $H_2PO_4^2$-. In the above mentioned, X-ray crystallography also shows that the anion is deeply buried in the pocket of the porphyrin urea receptor and is positioned over one pyrrole and is bound to four hydrogen bonds.

(vii) **UV/Vis Spectral Anion Recognition Studies:**

The porphyrin is highly spectrophotoactive. Its an 18 π electron conjugated systems resulting in a longer wavelength of the absorption maximum. The chromophore is characterized by an intense Soret band around 420-425 nm and four visible satellite Q bands in the range 450-700 nm. These transitions are π-π*. It is anticipated that the complexed anion should perturb these π-π* bands. This was found to be so.

UV/Visible spectral anion studies revealed perturbation of the Soret and the visible (Q) bands of receptors (16)-(22) following the addition of anions Cl^-, NO_3^-, HSO_4^- and $H_2PO_4^-$ as their NBu_4^+ salts. Each anion induced almost specific or diagnostic changes in the spectrum, indicating selective behaviour. The fact that these anions produced diagnostic shifts in the Soret and Q bands with specific anions means that these receptor can act as a good UV/Vis sensors or spectral probe. A comparison can be drawn for receptors (14) and (16)-(19) for illustrative purposes.

Anions	Ligand (14)	Ligand (16)-(19)
Cl^-	Hypsochromatic shift of the Soret	Hypsochromic shift with concomitant decrease in intensity of the Soret band
Br^-	Decrease in intensity, Hypochrome effect	Hypsochromic shift with concomitant decrease in intensity of the Soret band

NO_3^-	Decrease in intensity, Hypochrome effect	Decrease in absorbance, Hypochromic effect
HSO_4^-	Broadening and a split Soret Band	Hypochromic effect, gradual broadening and splitting of the Soret
$H_2PO_4^-$	Bathochromatic shift of Soret and Q bands	Hypsochromic followed by bathochromic shift and an increase in absorbance of Soret and Q bands. For (17) and (18), a broadened Soret band with a shoulder was observed.

The fact that these changes are distinct meant that the anions are bound within the "Picket fence" cavity or anion binding cavity of the porphyrin.

(viii). Electrochemical Studies:

Electrochemistry is used as a means of characterizing and studying the anion binding properties of porphyrins receptors. First, the electrochemical nature of the host had to be characterized. The electrochemical nature of receptor (6)-(9) and (10)-(13) were investigated by Cyclic and Square wave voltammetry. Receptors (6)-(13) exhibited the typical "Picket Fence" porphyrin redox properties. A single two one electron porphyrin oxidation wave at positive potential range 0.67 to 0.93V and two one electron reduction waves at negative potential range –1.08 to -1.79V. It is interesting to note that the four ferrocene moieties of (6), (8), (9) and (10), (12) and (13) display a single four electron oxidation wave, indicating that the ferrocene redox centers are electronically equivalent and undergo independent reversible one electron transfer at the same potential. However, receptor (7) and (11) exhibit two oxidation waves for the ferrocene moieties. Receptor (14) displayed a reversible reduction wave for the cobalticinium/cobaltocene redox wave in the region –1.25 to –1.75V. The p/p^{2+} occurred at 0.75V whereas the reversible p/p^- and p/p^{2-} occurred in the region –1.2 to –1.27 V respectively.

Cyclic voltammograms were then recorded after the progressive addition of stoichiometric equivalents of anion guests to the electrochemical solutions of receptor (6)-(9), (10)-(13), (14) and the results are shown in Table 4.0. It is seen that significant one wave cathodic shifts of the respective porphyrin oxidation waves and the ferrocene redox couples were observed for the porphyrin oxidation and cobalticinium/cobaltocene redox waves for receptors (14). This results from the complexed anion in close proximity effectively stabilizing the respective oxidized redox couple of (10)-(13) and the porphyrin p/p^{2+} cation radical making each harder to reduce. The magnitude of the anion cathodic perturbation of the porphyrin oxidation wave is larger than those of the ferrocene or cobalticinum redox couple, indicating that the bound anion is closer to the porphyrin skeleton than the ferrocene or cobalticinium redox centers.

The magnitude of the cathodic shift is dictated by the polarizing power i.e the charge to radius ratio of the anionic guest species and follows the sequence: $HSO_4^- > Cl^->Br^->NO_3^-$ for receptor (10)-(13) and $HPO_4^- > HSO_4^- > Cl^->Br^- > NO_3^-$ for receptor (14). It is interesting to note that in all cases negligible shifts of $\Delta E < 5$ mV were observed for the p/p^{2-} redox couple with all anionic guest species.

Table 4.0: Eletrochemical behaviour of receptor **(10)-(13)** in the presence of anions.

Receptor	(10)	(10)	(11)	(11)
ΔE, Anion, mV	Porphyrin Oxidation	Ferrocene Oxidation	Porphyrin Oxidation	Ferrocene Oxidation
Cl-	115	30	90	25
Br-	85	20	75	20
NO_3^-	110	25	100	20
HSO_4^-	100	60	125	50

Receptor	(12)	(12)	(13)	(13)
ΔE, Anion, mV	Porphyrin Oxidation	Ferrocene Oxidation	Porphyrin Oxidation	Ferrocene Oxidation
Cl-	70	20	90	20
Br-	65	20	75	20
NO_3^-	60	60	100	15
HSO_4^-	150	105	125	105

The above data were obtained in dichloromethane/acetonitrile solution (3:2, v/v) containing 0.2 moldm^{-3} NBu_4BF_4 as supporting electrolyte. Solutions were 5 x 10^{-4} moldm-^{3} and compound potentials were referenced with respect to Ag/Ag$^+$ electrode.
H

Electrochemical behaviour of receptor (14) in the presence of anions.

ΔE, Anion, mV	ΔEpa(p/p^{2+})	$E_{1/2}$(Co/Co$^+$)	ΔEpa(p/p^2-)
$H_2PO_4^-$	75	225	40
HSO_4^-	50	75	30
Cl^-	15	40	25
Br^-	10	35	10
NO_3^-	5	5	5

Obtained in acetonitrile solution containing 0.1 moldm^{-3}($NBu^n{}_4BF_4$) as the supporting electrolyte.Solutions were 1.0 x 10^{-4}M in compound and potentials were determined with reference to a Ag/AgCl electrode at 21 + 1C, 50 mVs-1 scan rate, E_{pa} and E_{pc} represent the anodic and cathodic current peak potentials of the cobaltocene/cobaltocenium redox couple of the free ligand.

In conclusion, a series of neutral and metallated 5, 10, 15, 20- *meso tetrakis*(*ortho* metallocene) amido phenyl porphyrins and 5, 10, 15, 20- *meso tetrakis(ortho* urea phenyl porphyrins anion receptors were synthesized, characterized and anion binding studies investigated in polar solvents. The 5,10,15,20- *meso tetrakis*(*ortho* ferrocenoylcarbonylamidophenyl porphyrin receptors do not complex anion whereas the corresponding zinc metallated 5,10,15,20- *meso tetrakis*(*ortho*-ferrocenoylcarbonylamidophenyl-substituted) atropisomeric *meso tetrakis* zinc metalloporphyrin have been shown to do so with very good association constants. In constrasts, both neutral and zinc metallated *meso tetrakis*(*ortho* urea phenyl porphyrins receptors complex anions with excellent selectivity in highly competitive solvents such as DMSO and DMSO/H_2O(88:12, v/v). Some of these receptors have found direct application as ion selective electrodes. For examples, receptors (16)-(19) have been tested and incorporated in ion selective electrode for acetate and is thus a good probe to measure the acetic acid content in Vinegar. This is the first example of a porphyrin ionophore without a metal center been used for an anion selective electrode.

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Effect Of The *Cucumis sativus* Extract In Labeling OF Blood Elements With Technetium-99m.

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Abstract: Human beings have been widely used natural products as medicines. However, sometimes the biological effects of these products are not fully known. The cucumber (*Cucumis sativus*) is a plant which is indicated as anti-inflammatory and anti-anginous. It is concerned that many natural remedies may contain potentially toxic ingredients and contaminants such as heavy metals. Red blood cells (RBC) and plasma proteins labeled with technetium-99m (99mTc) have several clinical applications and it has been reported that some natural products are capable of reducing the efficiency of this radiolabeling. The aim of this work was to assess the effect of an extract of a cucumber extract on the labeling of blood elements with 99mTc. In the preparation of the extracts it was used 50g of cucumber diluted in 500mL of saline solution (NaCl 0.9%). Samples (0.5mL) of blood from Wistar rats were incubated with 0.1 mL of the extracts during 1 hour. After that, the samples were incubated with stannous chloride ($SnCl_2$) and 99mTc. The blood was centrifuged and plasma (P) and RBC were isolated. P and RBC were also precipitated with trichloroacetic acid and soluble (S) and insoluble (I) fraction (F) were determined. The results have shown that the extract has not altered the radiolabeling . It was described that some extracts as *Fucus vesiculosus*, *Paullinia cupana*, *Mentha crispa L* were able to alter the radiolabeling of blood elements. In the light of the results obtained we suggest that the referred extract has a antioxidant properties. [Nature and Science. 2008;6(3):43-52]. ISSN: 1545-0740.

Keywords: cucumber, technetium-99m, red blood cells, antioxidant.

Introduction

The discovery of the ionizing radiations and composites endowed with natural radioactivity soon interested biology and medical sciences. In principle, for the damages that they caused in the alive structures, but later, for its value as half to assist the diagnosis and the treatment of the illnesses. During the last few years science learned to produce, to manipulate and to control radioactive substances, allowing that the involved processes in its production, its storage and its use if became safe more. Some existing chemical elements in the nature already are radioactive. Others can be generated in the nuclear reactors or the particle accelerators. Therefore, the radioisotopes can generically be classified in natural and artificial. The radioactive emission deeply modifies the atomic structure of the emitting element; therefore it modifies

the composition and the energy rocking of its nucleus. The unstable nuclei waste the energy excess that they possess emitting radiations. As the origin of the phenomenon is radioactive nuclear, the isotopes that emit radiation are more properly called radionuclide (Garcia, 1998).

The applications of radioisotopes in nuclear medicine are many and can be: as sources of irradiation and as tracers. In the first case, the biological material receives only the radiation emitted by the radionuclide used, in the second; the very radioisotope is incorporated into the biological environment that you want to study (Knapp & Merzadeth, 1994).

The first step in the preparation of radiopharmaceuticals is the production of a radionuclide appropriate. There are two main sources for the production of radionuclides which are used in procedures in nuclear medicine. There are primary and secondary sources. The primary source involves the direct production of radionuclides from nuclear reactor until the particle accelerator. The method involves a secondary source of indirect production of a radionuclide of a system known as a producer of radionuclides. In a nuclear reactor at the heart of a stable chemical component is bombarded with neutrons of low energy (thermal neutrons). For the absorption of neutrons the nucleus of the atom bombed rearranged is to become so unstable (radioactive). This instability is followed by the emission of particles, gamma rays or fission. There are two types of particle accelerators from: the linear and cyclotron. The core component of a stable chemical is bombarded with different particles such as electrons, protons, and particles. In the linear accelerator, the particles are accelerated α deuterons bombardments along a linear path, while in a cyclotron; the particles are accelerated along a circular path using an electrical current and a magnetic field. These particles are provided with sufficient energy to overcome the barrier of potential generated by the core (the Coulomb barrier). The secondary source is an indirect method to produce radionuclides using a generator system which is constructed in a manner that is easy to chemically separate the radionuclide son of radionuclide father in local hospitals or central radio pharmacy(Owunwanne et al, 1995).

A generator is built on the basis of the relationship between the decay of radionuclide father (half-long life) and the increase in child radionuclide (half-life short). The chemical properties of nuclide father and son radionuclide should be different, so they can be easily separated. The importance of generating radionuclide lies in the fact that they are easily transported and serve as sources of radionuclides in institutions far from the site of the installation of the reactor or cyclotron (Early & Sodee, 1995; Saha, 1998).

The generators are built with Mo-99/Tc-99m of alumina (Al_2O_3) conditional on a column of plastic or glass. The Mo-99 is absorbed in alumina in the chemical form of MoO_4^{-2} (molybdate). In preparing the column is routinely flushed with isotonic saline solution to remove the radioactivity undesirable. The amount of alumina is used by around 5 to 10, depending on the activity of the total Mo-99. The columns of generators are protected with lead. Usually the generators Mo-99/Tc-99m, are produced with Mo-99 obtained by fission of U-235(Hladik III et al, 1987; Taskaev et al, 1995).

The range of scintillation camera or camera is the main instrument used to obtain scintigraphic images. The modern range cameras have crystal detector that can be rectangular with more than 90 valves photo multiplication. The collimator is important in the formation of image and especially in reducing the amount in the spread of radiation. The collimators are commonly referred to as low energy (140 keV), average energy (150-300 keV) and high-energy (300-400 keV). The scintillations based on the ownership of certain crystals that, after being excited by a radiation incident, on their return to their basic level of energy, emit photons of visible light that can be detected and recorded by electronic circuits (Perkins & Frier, 1999).

The vast majority of radiopharmaceuticals used in nuclear medicine for diagnosis are marked with Tc-99m, since this has a great facility to train with chelating complex and diverse molecules, encouraging the taking of several radiopharmaceuticals (Hladik III, 1987; Srivastava & Straub, 1990; Srivastava et al, 1996). Similarly, the Tc-99m also has been used in the marking of various anatomical structures, in order to diagnosis, can also be used in procedures for biological research (Baum, 1987; Bernardo-Filho, 1988).

While presenting many advantages over other radionuclides used in medical sciences, the contact of Tc-99m with the cells can cause various types of injuries, among which are particularly important where they could change the deoxyribonucleic acid (DNA), with obvious risks. This radionuclide has its decay associated with the emission of electrons Auger (EA) (15-21 keV) and electron internal conversion (JIS) (128-138 keV) (Saha, 1998; Silva et al, 1998, Bernardo-Filho, 1999).

The process of marking of cells and molecules with Tc-99m almost always requires the use of a reducing agent, since the eluate obtained in the event, as ion pertechnetate, is not easily connect to other

chemical species. Therefore, it is necessary to reduce this radionuclide of Valencia +7 abilities to lower (+3, +4, +5) (Saha, 2005).

On a practical level, the use of ion stannous was the key to the development of many radiopharmaceuticals. None reducing agent has, to date, marking an efficiency of the radioactive tracer greater than that achieved with the use of chloride stannous thus justify its preference, not only in nuclear medicine, but also the marking of various structures of interest biomedical (Rao et al, 1986; Bernardo-Filho, 1999; Saha, 2005).

The reduction of ion pertechnetate can be obtained through various chemical agents, and the chloride stannous ($SnCl_2$) is the reducing agent most often used for this purpose (Dewanjee et al, 1990; Srivastava & Straub, 1990; Harbert et al, 1996), and allows the labeling of many molecules, and different cell types, with, typically, an additional step of purification. Although the $SnCl_2$ be employed in minimum concentrations in the marking of a number of structures, some harmful effects of the substance have been described (Bernardo-Filho et al, 1994; Dantas et al, 1996). It is suggested that during reactions of oxy-reduction, the $SnCl_2$ could be generating reactive oxygen species. This is explained by the fact $SnCl_2$ act as a reducing agent and having a great affinity for oxygen, which in turn is an excellent oxidizing agent. One of the features of biological importance of $SnCl_2$ is its ability to form cationic organometallic compounds of high lipid solubility, enabling them to cross biological membranes and exert their toxic effects within the cells (Dantas et al, 1996).

A radiopharmaceutical is a radioactive compound used for diagnosis and treatment. In nuclear medicine, approximately 95% of radiopharmaceuticals are used for diagnostic purposes. The primary applications of radionuclides in the health science are as a source of radiation or radioactive tracers. The tracers that have well-defined characteristics for employment in humans are called radiopharmaceuticals. Thus, a radiopharmaceutical can be defined simply as a substance or cell containing a radioactive atom in its structure and which by their pharmaceutical form, quantity and quality of radiation, its administration becomes suitable for use in humans with end of diagnosis or treatment of diseases, whatever the route of administration. Usually the radiopharmaceuticals have no pharmacological effect because in most cases are used in minimum quantities. In these cases show no dose-response relationship, and then differ from conventional drugs. Because they are administered in humans, are sterile, a toxics and free to pyroxenes and should be subject to all measures required for quality control of a conventional drug (Saha, 2005). Advances in nuclear medicine, with several studies are being targeted regarding research and development of several new radiopharmaceuticals (Saha, 2005).

In the nuclear medicine radiopharmaceuticals are used (a) to obtain images, as a radiopharmaceutical with selectivity by an organ and / or system is administered and the radiation emitted is externally captured, processed or recorded on paper, film or video monitors, (b) Study of functions in vivo, measuring the function of an organ or system, in particular based on absorption, dilution, concentration or excretion of radioactivity after administration of radiopharmaceutical, (c) therapeutic procedures, where a specific organ or tissue is selected and, through the issuance of the beta radiation tissue is destroyed (Baum, 1987).

After intravenous administration, the $99mTcO_4Na$ is distributed in the vascular compartment. About 70 to 80% of ions pertechnetate linking up initially to plasma proteins, and this connection is reversible (Nickel, 1995). The plasma elimination is very fast and balance between the compartment vascular and interstitial fluid is completed in a short time, between 2 to 3 minutes. The half-life of elimination from the plasma is approximately 30 minutes, and 30% of the administered dose, is excreted in the first 24 hours. The total urinary excretion of fecal and activity of Tc-99m, is about 50% in 3 days and 70% in 8 days (HladiK III et al, 1987). The dose varies with the type of study to be conducted and is used about 10 to 20 mCi for brain imaging, from 1 to 5 mCi for thyroid, and 20 to 25 mCi for the marking of red cells in vivo. The studies of thyroid and brain are performed after 20 to 30 minutes of the administered dose. When the blood-brain barrier (BHE) is changed, because the presence of abnormal cells per occurrence of tumors, stroke encephalic, abscesses, and other diseases, the accumulation of radiotracer occurs in brain tissue. A normal brain scan shows no distribution of activity on the grounds of protecting the blood-brain barrier that is responsible for the exclusion of sodium pertechnetate (Nickel, 1995). The pertechnetate despite captured, is not up taken by the thyroid gland. For this reason is mainly used for studies of morphological and location of the thyroid. The image of the thyroid with 99mTcO4Na, is also employed in conditions that the normal thyroid cells modified, resulting in areas of lack of tracer. In these diseases are included benign tumors, malignant tumors, cysts, inflammation and bleeding. For detection of tissue para thyroidal hyper functioning shall be used in conjunction with the pertechnetate the thallium-201 (AI-201), for viewing on

the basis para thyroidal anomalies such as tumors and hyperplasia (Bergenfeltz et al, 1992). The image of the salivary glands with 99mTcO4Na, is used to detect some tumors may result in an increase or decrease the capture gland. The gastric mucosa is usually displayed after administration of sodium pertechnetate. It is used to detect mucosal areas located on the outside of the stomach (ectopic mucosa), the Meckel's diverticulum in the intestine, and the region of the distal esophagus. It is also used in the evaluation of gastric resection in surgical procedures (Fernandez-Ulloa et al, 1992).

Natural products are widely used as food or food additives, or as a substance in medicinal treatment for humans. Medicinal plants are widely used worldwide for the treatment of many diseases. Sometimes the toxic and/or genotoxic effects of these products are not fully known. Practically all Countries utilize radioisotopes in medicine, industry, agriculture and research. Technetium-99m (99mTc) has been the most utilized radionuclide in nuclear medicine procedures and it has also been used in basic research. Natural drugs can alter the labeling of red blood cells with technetium-99m (^{99m}Tc) (Early & Sodee, 1995; Braga et al., 2000; Saha, 2005;). When a radionuclide has its capability to bind to blood elements altered by natural and therapy drugs, the process of labeled red blood cells may be repeated, resulting in an additional radiation dose to the patient (Hesslewood & Leung, 1994; Sampson, 1996).

The cucumber is a herbal plant rusticate with gavinhas of stem and acute branches and aspires, leaves of wolves triangular acute, unisexual flowers, yellow, solitary (male) or in bunches, it originating in Southeast Asia. As active principle stand out carbohydrates, proteins and fats, salts of potassium, phosphorus, calcium, magnesium and sodium iron, vitamins A, B1, B2 and C. The cucumber gives effect anti-angiogenesis and anti-tumor. The part used is the fruit. In popular medicine is given as a sedative and diuretic, anti-rheumatic and somniferous has tonifying action of the liver and kidneys. Excellent due to its properties which tonifying hair, nails and skin, and anti-inflammation of the eyes. It is a good stimulant appetite when used before meal. Vouldoukis et al (2004) reported the antithrombotic effect of a glicosaminoglican extracted from the seeds of cucumber.

Then, we have evaluated the influence of a cucumber extract on the labeling of RBC and plasma proteins with ^{99m}Tc using *in vitro* study.

Material and Methods

Radiolabeling process: Samples of heparinized blood (0.5 mL) withdraw from *Wistar* rats were incubated with 100 μL of a preparation (100%v/v) of Cucumber extract (0.1g.mL^{-1}) during 1h at room temperature. After that, it was added 0.5 mL of stannous chloride (1.2 μg. mL^{-1}), as $SnCl_2.2H_2O$, for 1h at room temperature. After this period of time, ^{99m}Tc (0.1 mL), as sodium pertechnetate, was added and the incubation continued for another 10 min. These samples were centrifuged and plasma (P) and blood cells (BC) were separated. Samples (20 μL) of P and BC were precipitated with 1 mL of trichloroacetic acid (TCA) 5% and soluble (SF) and insoluble fractions (IF) were separated. The radioactivity were determined in a well counter. After that, the % of radioactivity (%ATI) was calculated, as previously reported (Bernardo-Filho et al., 1994).

Enzymatic activity (AchE activity) examination: to the watery phase 0.5mL of the enzymatic preparation of the Kit had been added and the residue of the total evaporation of the solvent was dissolved in 0.25 mL of the same enzymatic preparation diluted 2 times. After incubation of 120 min 37°C, 50μL had been removed of the incubation mixture and it was added 0.5 mL of reagent of color and 0.5 mL of substratum. The reaction of formation of the product was mediated in 412 nm during 5 min. The enzymatic activity was express in average of addition of absorvance per minute. This value determined for the control (distilled water extract) corresponds the 100% of the enzymatic activity. The results of percentage of inhibition of the samples had been interpolated in the express curve metil paration standard and results in ppm of metil paration equivalents. The limit of detention of the method is of 0.2 ppm in metil paration equivalents.

Results

The table 1 has shown the effect of the cucumber extract on the labeling of blood elements with 99mTc. Related to the results obtained the extract was not capable of altering the pattern of radiolabeling of blood elements.

Samples blood were incubated with the extract. Saline solution (NaCl 0.9%) was used as control. Then, stannous chloride (1.2 μg. mL^{-1}) and 99mTc, as sodium pertechnetate were added. These samples were centrifuged and (P) and (BC) were separated. Blood samples were precipitated with TCA and SF and IF were separated. The radioactivity in P, BC, SF-BC , IF-BC, SF-P and IF-P was determined in a well

counter and the % of radioactivity (% ATI) was calculated. A statistical analysis (Kruskal Wallis test, n= 5) was used to compare the results.

Table 1- Effect of cucumber extract on the radiolabeling of blood elements

Concentrations of the extract	C	IF-C	IF-P
Control	96.32 ± 3.02	89.65 ± 2.90	77.82 ± 3.96
6.25 %	95.45 ± 3.81	88.23 ± 3.38	81.17 ± 2.72
12.5 %	97.91 ± 0.71	88.15 ± 3.17	74.33 ± 7.14
25 %	97.30 ± 0.93	87.77 ± 2.09	72.08 ± 6.63
50 %	97.72 ± 1.56	86.33 ± 3.08	74.91 ± 4.86
100 %	97.84 ± 1.23	89.04 ± 3.96	77.58 ± 3.50

Discussion

The use of natural products has grown over the years. Generally demand through the use of these products, and among them, in the herbal cure for many diseases and palliative in nature less toxic. So it is of extreme importance of a more scientific evaluation of the biological effects of natural products. For the natural product chosen, the cucumber, few properties in relation to their use are described as a diuretic action, hypotensive, anti-angiogenic and anti-tumor and anti-carcinogenic (Vouldoukis et al, 2004). On the other hand, has a high level of consumption in popular medicine.

Kam & Liew (2002), reported that despite the medicinal herbs used in Traditional Chinese Medicine be relevant at the level of therapy may have in their constitutions components that trigger side effects.

As it was described as extracts of plants could change the labeling of blood elements with Tc-99m (Oliveira et al, 1997; Vidal et al, 1998; Oliveira et al, 2000; Oliveira et al, 2002; Capriles et al, 2002 ; Oliveira et al, 2003; Moreno et al, 2004), we decided to assess whether the extracts of cucumber would also be able to interfere in the process of radiolabelling. The labeling of the blood products with Tc-99m, as sodium pertechnetate, depends on the presence of a reducing agent and stannous chloride is widely used. The determination of optimal concentration of chloride stannous is a predominant factor in the technique of labeling with Tc-99m (Rao et al, 1986; Hladik III et al, 1987; Kelly et al, 1992). In the case of red blood cells, they capture the ion stannous half of extra cell. When the ion concentration is low, the same shall be incorporated by virtually all red blood cells. Thus, in maximum concentration of reducing agent, the red blood cells have the highest percentages of marking, possibly due to "completion" of up to link the sites of molecules of hemoglobin. After the treatment of red blood cells with high concentrations of chloride stannous, the system that controls the flow of this ion is saturated and the same is not able to capture this ion so undefined, which causes the increase of extracellular agent in the middle. To be added the Tc-99m, as ion pertechnetate, it would have to cross the barrier of reducing agent, which would prevent the achievement of red blood cells, thus causing a low efficiency of marking (Bernardo-Filho, 1988).

The mechanism of transport of ions for the intracellular environment has not yet been fully established, but the evidence suggests that the chloride stannous cross the plasma membrane channels by selective calcium (Gutfilen et al, 1992; Sampson, 1996) and ion pertechnetate by the system transport "anion banda-3" (Callahan et al, 1990; Sampson, 1996).

The presence of certain drugs in the blood could change% of the radioactivity of Tc-99m linked to blood elements because they could act: (a) competing with $SnCl_2$ or with the Tc-99m (b) changing the permeability of cell membranes or favoring blocking mechanism of transport of these elements, (c)

occupying sites of binding of Tc-99m or preventing the $SnCl_2$ they occupy the (d) facilitating the connection Tc-99m to plasma proteins or (e) as a reducing agent or oxidizing agent modifying the valence of ions stannous and / or pertechnetate (Hladik et al, 1987; Santos et al, 1995).

Although other plants, as *Thuya occidentalis* (Oliveira et al, 1997), *Nicotiana Tabacum* (Vidal et al, 1998); *Maytenus ilicifolia* (Oliveira et al, 2000); *Syzygium jambolanum* (Santos et al, 2002); *Mentha crispa L*. (Santos-Filho et al, 2002); *Ginkgo biloba* (Moreno et al, 2002; Moreno et al, 2004); *Stryphnodendron adstringens*(Costa et al, 2002); *Solanum melongena*(Capriles et al, 2002); *Fucus vesiculosus* (Oliveira et al, 2003); *Coffea arabia* (Oliveira et al, 2003) have changed the efficiency of labeling of red blood cells with Tc-99m, concentrations of the extracts of chayote studied by Diré et al, 2002 , when administered *in vitro*, did not interfere significantly in the mechanisms of binding of Tc-99m to blood elements. Similar results were found with cucumber extract, as well as with other results reported in studies with extracts of *Peumus boldus* (Reiniger et al, 1999) and Piper methysticum (Santos-Filho et al, 2002) which not induced change in the marking of blood elements with Tc-99m. Lima et al, 2001, reported that an extract of cauliflower (*Brassica oleracea*) to be administered during the same period of time *in vivo*, was unable to change the labeling of blood constituents with Tc-99m. Depending on the action suggested anti-oxidant of cucumber, one can speculate that this natural product prevents oxidation of ion stannous and consequent reduction of blood labeling the elements. Regarding the anti-carcinogenic action of this natural product, its purported anti-oxidant action could justify the potential use of this natural product in the anti-oncogenic and anti-inflammatory. The globulins are proteins that have lower molecular weight when compared to that of albumin. Neither is related to the transport of substances (Villee et al, 1996; Guyton & Hall, 2006), the change in its quantity and the generation of free radicals that could compete and / or change the sites of the link Tc-99m to plasma proteins, this effect could reduce the determination of Tc-99m to the insoluble fraction of the plasma. With respect to the treatment with the cucumber, was not found radiolabelling change in the efficiency of the plasma proteins.

As described for other drugs (Hladik et al, 1987; Hesslewood & Leung, 1994; Gomes et al, 1998; Mattos et al, 2000; Gomes et al, 2002; Amorim, 2003), the extract of cucumber was unable to change the biodistribution of radiopharmaceutical pertechnetate sodium in blood compartments in *in vitro* testing. Lima et al, 2001, reported that an extract of cauliflower (*Brassica oleracea*) was not able to change the biodistribution of radiopharmaceutical sodium pertechnetate, however Capriles et al, 2002, found that an extract of eggplant (*Solanum melongena*) changed the biodistribution of the radiopharmaceutical, similar results were described by Moreno et al, 2002, in studies with an extract of *Ginkgo biloba* and with an extract of *Punica granatum* (Amorim et al, 2003).

The red blood cell (RBC) is one of the most studied biological structures. We know much more about the membrane of RBC than on any other membrane of eukaryotic cells. The easy availability and easy of storage of red blood cells make them ideal object to search for anyone who can make use of a microscope of good quality (Alberts et al, 1996; Stryer, 1996).

The normal form of red blood cells is only one among the many that they can take when environmental conditions change. It should therefore consider how representing a balance between the properties of the cell and the physical forces that act on it. When any of these forces is changing, for example by changing the molecules adsorbed to the surface membrane or alteration of the membrane, is changing the way promptly. Different types of changes can occur in red blood cells in abnormal physiological conditions. The study of these forms can be an indispensable tool in the diagnosis of different diseases (Oliveira-Lima et al, 1992; Ross et al, 1997; Junqueira & Carneiro, 2004). The main method that reveals the cell types of peripheral blood is the distension of the blood. This method differs from conventional forms of preparation seen in histology laboratories because the sample is not included in paraffin. The careful study of distensions of blood diagnostic data provides very important. It is said that 90% of the conclusions which take the examination cytological are provided by the study of distensions stained. This is therefore the best measure of morphological study of the elements, as well as provide a rough idea of the concentration of hemoglobin and the number of red blood cells, white blood cells and platelets (Oliveira-Lima et al, 1992).

A change of the morphology of RBC by extract of medicinal plant (Oliveira et al, 1997; Vidal et al, 1998; Oliveira et al, 2000; Braga et al, 2000; Diré et al, 2001; Oliveira et al, 2002; Moreno et al, 2002; Oliveira et al, 2003) and possible consequent amendment of the transport of ions stannous and pertechnetate into the RBC could lead to a decrease in the labeling of this structure with Tc-99m. There are many evidences that have shown that the shape of the cell depends on the structural organization of the membrane proteins and proteins adsorbed on its surface (Stryer, 2004). Thus, one could suggest that the

extracts of cucumber here have tested the effect of not change the morphology of red blood cells. Furthermore, *in vitro* studies to extract the smoke (Braga et al, 2000), *Thuya occidentalis* (Braga et al, 2000), *Maytenus ilicifolia*(Oliveira et al, 2000), *Paullinia cupana* (Oliveira et al, 2002), *Ginkgo biloba* (Moreno et al, 2002), Fucus (Oliveira et al, 2003), *Coffea arabica* (Oliveira et al, 2003) have shown a relationship between the change in labeling of red blood cells with Tc-99m and changes in quality in the morphology of red blood cells. In *in vitro* studies conducted with mint (*Mentha crispa*) (Santos-Filho et al, 2002) there was a relationship between the change of labeling red blood cells with Tc-99m and quantitative changes in level of red blood cells in morphology. In quantitative studies conducted *in vitro* with an extract of Kava Kava (*Piper methysticum*) (Santos-Filho et al, 2002) described that despite the morphological changes in red blood cells induced by that statement, there was no change in the labeling of red blood cells with Tc - 99m. Diré et al (2001), in a qualitative study, observed morphological changes in red blood cells of animals treated with chayote for 15 days. These findings could justify the reduction of the labeling of red blood cells with chayote, when used blood samples from animals treated with the extracts of this plant, which was also found morphological change of red blood cells. These findings reinforce the idea that metabolites are generated when the extracts of chayote are administrated to animals instead of water. The analysis of the results which were obtained in an *in vitro* study shows that the biological effects of the extracts of cucumber would be associated with anti-oxidants present in the extract natural product that does not thereby altering the labeling of red blood cells and plasma and cellular proteins with Tc - 99m.

Conclusion

The results allow us to assess that the extract of cucumber presents an anti-oxidant action by not change the radiolabelling *in vitro* of red blood cells and plasma proteins and blood cells isolated from rats and is feasible to suggest that the biological effects attributed to this plant reported in level of popular medicine, supposedly, are related to molecules which are present *in nature* in the constitution of that phytochemical fruit.

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Pretreatment effects of the Micro-polluted Water Supply in the Reservoirs by Subsurface Constructed Wetland

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Abstract: In order to improve water quality of the source of drinking water and mitigate load of drinking water treatment plant，A pilot test was conducted with integrated horizontal flow constructed wetland to pre-treat the Water Supply in the reservoirs of the Yellow Rive. Experiment was carried on in Yuqing Lake Reservoir in Ji'nan city and the water of it comes from Reservoirs of the Yellow River。Results show that under hydraulic loading rate a load of 2 $m^3 \cdot d^{-1}$, the average removal rates of chemical oxygen demand(COD), total nitrogen(TN), ammoniacal nitrogen(NH_4^+-N), nitrate Nitrogen (NO_3^--N) , and Total phosphorus (TP) in the horizontal flow constructed wetland were 49.21%%,52.04%, 47.20%、53.65% and 49.09%, respectively. This show horizontal flow constructed wetland may effectively pretreated with the micro-polluted Water Supply in the Reservoirs. [Nature and Science. 2008;6(2):53-58]. ISSN: 1545-0740.

Key words: Constructed wetland; micro-polluted Water Supply in the Reservoirs; Pretreatment

Yuqing Lake Reservoir, which takes raw water from Yellow River, is the most important project for water supply of Ji'nan city. After pretreatment to remove sand and other solid particles due to a long stay in the reservoir, water was transported to Yuqing drinking water treatment plant for further purify. With more and more discharge of domestic sewage and industrial wastewater into Yellow River, the water quality is becoming more and more deteriorated in recent years. The raw water was mainly micro- polluted by organism, nitrogen and phosphorus, with COD around 40 mg/L, TN around 4 mg/L and TP around 0.05 mg/L.

Constructed wetlands, as a lower cost, lower energy, lower technical-demanding sewage treatment method, have aroused more interests around the world. Constructed wetlands have been applied for various wastewater treatments, such as sewage wastewater(Winthrop et al.,2002), industrial wastewater(Ji G D,et al.,2002), rainstorm runoff in cities(Scholes L et al.,1998), wastewater out of farms(Kem J et al.,1999), lake pollution(Sakadevan K et al.,1999). But with our best knowledge, constructed wetland is rarely used for the pretreatment of micro-polluted Water Supply in the Reservoirs, either inland or outland.

This study therefore intends to investigate the feasibility of pretreatment of micro-polluted Water Supply in the Reservoirs by constructed wetland and also to provide the operation parameters for a full scale constructed wetlands to improve the quality of influent water to Yuqing Lake Reservoir.

1. Materials and Methods

1.1 Description of the constructed wetland system

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The subsurface constructed wetland system for the experiment is built in No.2 pumping station of Yuqing Lake Reservoir. The system is a reed beds with hydraulic characteristics of a horizontal flow. Dimension of unit is 6m×1.5 m×0.6m (L×W×D) and base slope is 1% .The wetlands beds are filled orderly with bigger gravels (average diameter 52mm, height 300mm), smaller gravels (average diameter 20mm, height 300mm) and local soils (height 150mm). The local common bulrush were planted in the wetland with the density of 20 plants/m².The wetland bottoms are plastered by concrete ， with brick built up in layers and mortar plastered. The water level of effluent is adjustable. Bottom of catchment area install a porous catchment pipe which was connected with a exterior vertical pipe. Vertical pipe was installed outlets to regulate water level in different height with 4m，8m，12m，16m. The regulation of the water level of effluent water can boost the growth of the roots of the plants and domesticate the super microorganism of the different depths.

1.2 Running of the system

The system began to run at the middle of May, 2005, with a load of 2 $m^3 \cdot d^{-1}$ and the hydraulic retention time of 1.35 days.

1.3 Sampling and testing

Water samples were collected both from the influent and effluent at regular, short intervals. Temperature ,pH, chemical oxygen demand (COD), total nitrogen(TN), total phosphorus (TP), ammonium-nitrogen (NH_4^+-N), nitrate–nitrogen (NO_3^--N) and nitrite–nitrogen（NO_2^--N） were measured according to the methods of the EPA of China (1993). COD, TN, TP , NH_4^+-N , NO_3^--N ,pH, NO_2^--N, and Temperature were measured 6-8 per month From May3, 2006 to November 27, 2006.

COD was determined by titrimetric method. Determination of NH_4^+-N, NO_3^--N, NO_2^-N,TN and TP were performed using a segmented flow analysis (Skalar San^{++} Automated Wet Chemistry Analyzer, the Netherlands). The physico-chemical water parameters, such as water temperature, redox-potential (Eh), pH, and dissolved oxygen (DO) were measured in situ. DO was assayed using an Orion Dissolved Oxygen Probe (Model 862Aplus, USA). Water temperature and Eh were recorded with an Orion 250Aplus ORP Field Kit, and water pH with an Orion Portable pH Meter (Model 250Aplus, USA). All the parameters mentioned above were determined according to the method as described in the Standard Method for Examination of Water and Wastewater (Standard Method for the Examination of Water and Wastewater Editorial Board, 1993) and all analyses were completed within 24 h of sample collection.

2. Results and discussion

2.1 Removal of COD

Fig.1 show the variations of influent and effluent COD concentrations and COD removal rate for the raw water treatment by constructed wetlands. Concentrations in those figures are the testing results for month average value of samples. Under the operation conditions with a inflow COD of 19~35mg/L and the load of 2 $m^3 \cdot d^{-1}$, the removal rate of COD in raw water by horizontal flow constructed wetland is between 40%~60%,. The average COD concentrations of the effluent are 14.90 mg/L. According to other literatures, the removal rate of organic contaminations in sewage and other wastewater treatment by constructed water is normally 80%~90%. But in our current research, the organic contamination in the raw water is more

hardly to be removed due to the lower concentration. Also, the hydraulic retention time of this system is only 1.35 days, shorter than the common HRT of 5~30 days, resulting a little low COD removal. But the effluent COD is less than 20mg/L, which can meet the China standard for surface water resources (GB3838-2002), i.e. 20mg/L for water resource of drinking water treatment plant.

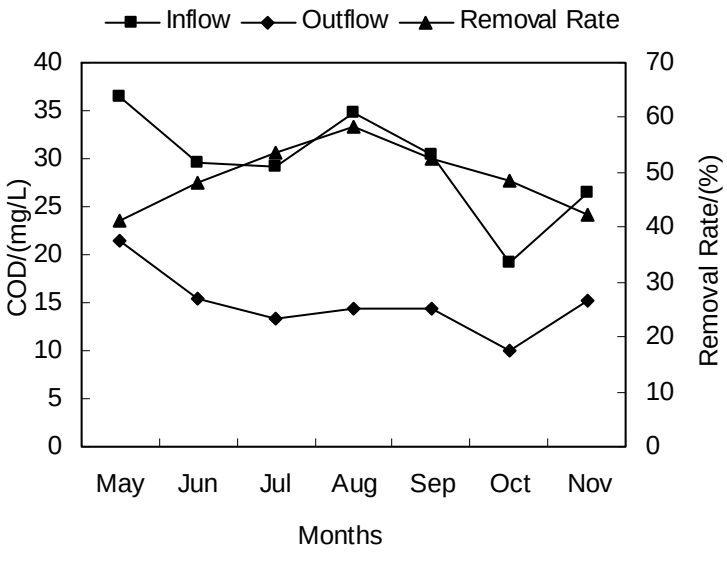

Fig.1The removal efficiency of COD by horizontal flow constructed wetland

2.2 Removal of TN

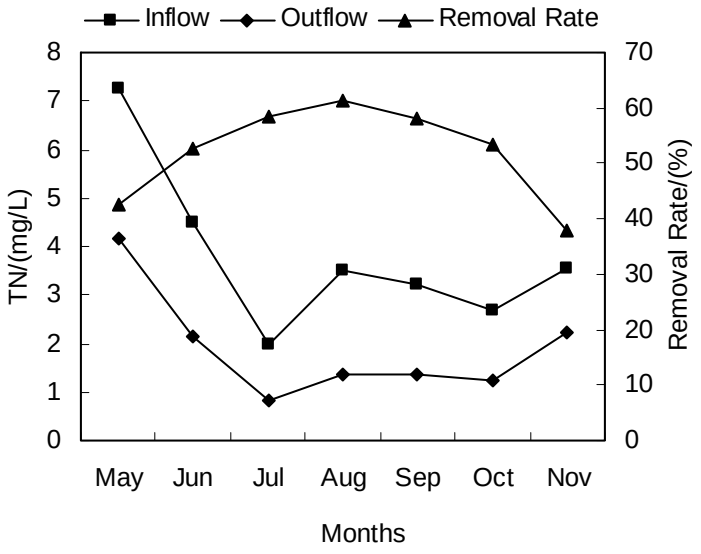

Fig.2 The removal efficiency of TN by horizontal flow constructed wetland

Fig.2 illustrate the variations of influent and effluent TN concentrations of the constructed wetlands. The figures show The removal rates of TN in the horizontal flow constructed wetland were 52.04%. According to the China standard for surface water resources (GB3838-2002), Mean influent TN were Grade Ⅲ, inferior Grade V. After the disposal of the constructed surface wetlands. Mean effluent TN can nearly reach Grade V.

The figures also show that, from July to the first half of October, TN concentration in the effluent is kept at a lower level, normally around 1mg/L, while the air temperature was high and the system ran steadily. It implies that the constructed wetland is a natural, biological treatment system, which entirely depends on natural energy inputs such as sunlight and wind. Temperature has a major impact on micro-biological process rates and obviously on plant growth as well, resulting in an effect on the higher removal rate of contaminations. The removal of nitrogen in wetlands depends on nitrification and denitrification of micro-organisms, the absorption of foliages and fillings and the volatilization of NH_4^+-N. Normally, the nitrification and denitrification of micro-organisms are important in the constructed wetlands. If there are lots of nitrobacteria, denitrobacteria, and also compatible situations, most of nitrogen will be removed.

2.2.1 Nitrogen species

The average concentrations of TN、NH_4^+-N、NO_3^--N、NO_2^--N from influents and effluents are showed in Table 1.

It is showed in Table 1 that most nitrogen (above 80%) from influents and effluents is in the form of NO_3^--N due to its stability. Concentrations of NH_4^+-N and NO_2^--N in the effluents can meet the nearly second standard of GB3838-2002. little NH_4^+-N and NO_2^--N be removed. The first reason is that the hydraulic retention time is short and the second is that the concentrations of contaminations are very low.

Table 1. The average concentration of nitrogen species in influents and effluents

	TN/（mg/L）	NH_4^+-N/（mg/L）	NO_3^--N/（mg/L）	NO_2^--N/（mg/L）
Influents	3.8087	0.4514	2.9474	0.03725
effluents	1.8979	0.2407	1.4017	0.01581

2.2.2 Removal of NO_3^--N

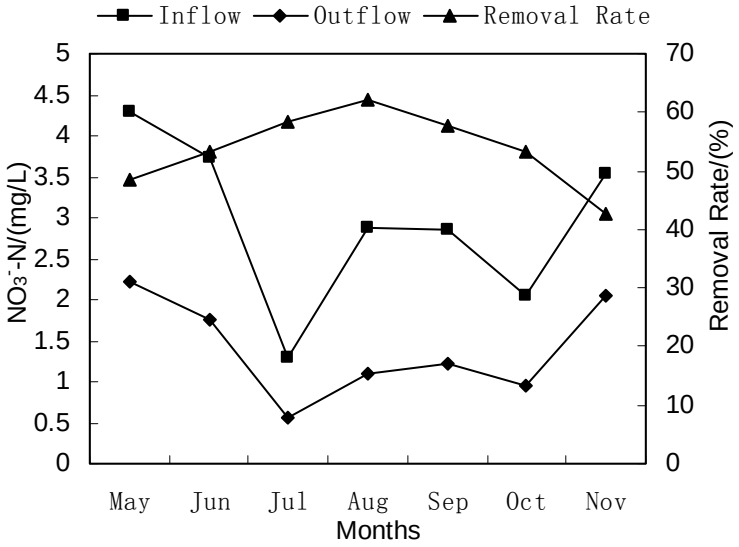

Fig.3 The removal efficiency of NO_3^--N by horizontal flow constructed wetland

Fig.3 show the removal efficiency of NO_3^--N from the raw water by constructed wetlands. It's so similar to that of TN, due to that most nitrogen is in the form of NO_3^--N as mentioned above.

2.3 Removal of TP

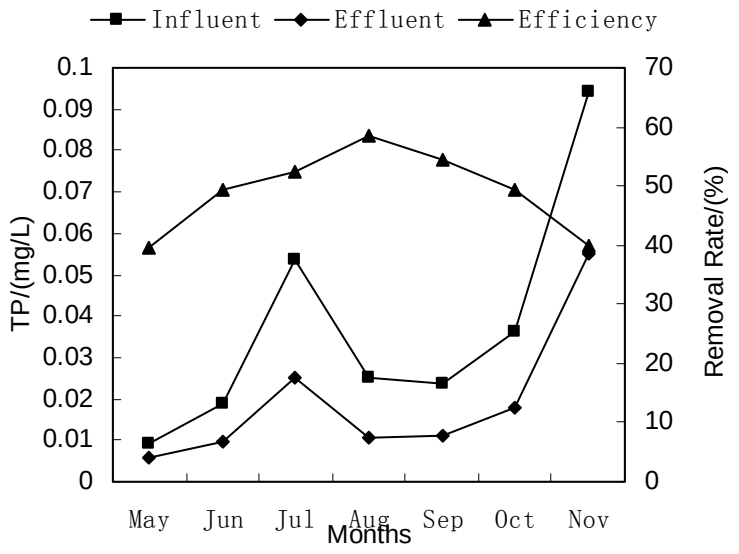

Fig.4 The removal efficiency of TP by horizontal flow constructed wetland

Fig.4 show the removal efficiency of TP from the raw water by horizontal flow constructed wetland. Usually, the removal of TP in wetlands depends on adsorption, replacement and sedimentation(Verhoeven J T A et al.,1999) The main removal mechanisms are adsorption to the filter and/or soil particles, adsorption to the detritus layer and precipitation with certain metals such as Fe, Al, Ca and Mg. When there is a

saturation of sorption sites or the depletion of complication ligands, According to the China standard for surface water resources (GB3838-2002).By treatment function of the system Mean effluent TP from Grade Ⅱ into close to Grade Ⅰ .Data finally suggest a substantial effect of temperature, with better removal efficiencies during the growing season. The most probable explanations are plant uptake on the one hand and P-leaching from decaying detritus on the other hand.

3. Conclusions

The results show horizontal flow constructed wetland effectively pretreated with the micro-polluted Water Supply in the Reservoirs. , the average removal rates of chemical oxygen demand(COD), total nitrogen(TN), ammoniacal nitrogen(NH_4^+-N), nitrate Nitrogen (NO_3^--N) , and Total phosphorus (TP) in the horizontal flow constructed wetland were 49.21%%,52.04%, 47.20%、53.65% and 49.09%, respectively. Temperature has a positive effect on the contamination removal. A higher removal rate can be obtained when the water temperature is higher.

Because of the low feeding load, the wiped function of system can't be fully exerted. Therefore, future experiment should enhance the feeding load so that the effects of system can be adequately exhibited.

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Diversity Of Water Borne Conidial Fungi As Root Endophytes In Temperate Forest Plants Of Western Himalaya

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ABSTRACT*:* Diversity of water borne conidial fungi occurring as root endophytes was studied. Twenty one species were recorded as root endophytes in healthy roots of forest plants of Western Himalaya. Three species viz., *Camposporium pellucidum, Diplocladiella scalaroides* and *Helicomyces roseus* are being reported for the first time as root endophytes whereas 8 species viz., *Acaulopage tetraceros, Alatospora acuminata, Anguillospora longissima, Campylospora purvula, Cylindrocarpon aquaticum, Heliscus lugdunensis, Tetracladium marchalianum* and *Tetracladium setigerum* are recorded with their new host record. Maximum host diversity was represented by *Cylindrocarpon aquaticum* and *Tetracladium setigerum* as the former endophyte was isolated from 14 host plants followed by latter which was isolated from 12 host plants. [Nature and Science. 2008;6(3):59-65]. ISSN: 1545-0740.

Key words: Diversity, Water borne conidial fungi, Endophyte

INTRODUCTION

Microbes living with interior tissues of healthy plants without causing disease symptoms are called endophytes. Wide occurrence of endophytic fungi on many plants including bryophytes and *Botrychium* have been explored from all parts of the world (Clay, 1989; Petrini, 1986; Petrini et. al. 1992; Carroll and Carroll, 1978 and Sati and Belwal, 2005). The role of endophytic hyphomycetes has now been suggested by various workers as promoters of plant growth (Bills and Polishok, 1992; Petrini, 1991and Dreyfuss and Chapela 1992).

The water borne conidial fungi were described previously by Ingold (1942) as these complete their life cycle on submerged substrate in well-aerated waters but Waid (1954) reported some of these water borne conidial fungi from root surface also. The observation of Waid (1954) provided a pavement to various workers who further reported some more water borne conidial fungi from aquatic and terrestrial roots **(**Gourley, 1969; Nemec, 1969 and Watanabe, 1975). Fisher et al (1986) recognized a separate group of water borne conidial fungi as endophytic hyphomycetes and later Fisher et al (1991) confirmed their occurrence on plant roots of aquatic habitats through experimental basis by examining the bark and xylem of aquatic roots of *Alnus gluitinosa*. Similar reports have been made by other mycologists on endophytic aquatic hyphomycetes (Marvanova and Fisher, 1991; Marvanova et. al., 1992 and Sridhar and Barlocher, 1992a).

Endophytic fungi are also known to be a rich source of antibiotic, secondary metabolites and it has been postulated that endophytic fungi may be beneficial to their host plant by antagonizing pathogens and / or by inducing plant defense responses. Webber (1981) was the pioneer to report plant protection by an endophytic fungus *Phomopsis oblonga* protected elm trees against the beetle. This was confirmed by Claydon *et al* (1985) who showed that endophytic fungi synthesize secondary metabolites in host which affect the beetle larvae.

The present study provides an assessment of species diversity of endophytic hyphomycetes associated with the roots of temperate plants growing nearby areas of water bodies of Western Himalaya.

METHODOLOGY

'Three step sterilization' method of Fisher and Petrini (1989) was followed for the systematic study of root endophytic fungi. Living roots of different tree plant species including herbs and shrubs growing in the ravine and wet areas located at Nainital and Almora districts in Western Himalaya were collected in 3 replicates of each. Nearly 10-15 cm long roots were cut off with a sharp knife and washed with sterile water. These root samples were then keep in sterile polythene bags, brought to the lab and processed within 4-5 hours after collection. Root samples were washed under running tap water for about 10 minute to remove extraneous adhering soil particles and cut into 3-4 cm size segments. These were then rinsed with sterile water after surface sterilization with 90% alcohol for 2-3 minutes. The segments were incubated at 20 ± 2^0C for 5-20 days in sterile petri-dishes containing 30 ml of sterile water. Incubated dishes were observed periodically to detect the conidia of endophytic fungi under low power of microscope.

Simultaneously, some of the surface sterilized root segments were placed in 2% Malt Extract Agar, supplemented with streptopenicillin or tetramycin solution (250mg/l) and incubated for a few days depending upon the growth of emerging fungi. Fungal mycelia growing on agar blocks were transferred into another petri dishes containing sterile water for sporulation and identification.

RESULTS

The incubated root segments of different host plants revealed a total of 21 species of water borne conidial fungi belonging to 15 genera as root endophytes (Table 1 and Fig. 1). A perusal of available literature indicates that *Camposporium pellucidum, Diplocladiella scalaroides* and *Helicomyces roseus* are being reported for the first time as root endophytes.

As seen in table 1, *Alatospora acuminata, Campylospora purvula, Cylindrocarpon aquaticum, Heliscus lugdunensis, Tetracladium marchalianum* and *Tetracladium setigerum* were the most commonly occurring endophytes while four species viz., *Alatospora pulchella, Campylospora chaetocladia, Camposporium pellucidum* and *Diplocladiella scalaroides* occurred in only one host species. Similarly all the reported species of *Lemonniera*, as root endophytes, showed their occurrence restricted to *Lyonia ovalifolia*. The maximum host diversity was reported in *Cylindrocarpon aquaticum* as it was colonized on 14 host plants followed by *Tetracladium setigerum* (13), *T. marchalianum* (9), *Heliscus lugdunensis* (8), *Campylospora purvula* (8), *Alatospora acuminata* (6) and *Anguillospora longissima* (6). The remaining species viz., *Acaulopage tetraceros, Anguillospora crassa, Campylospora chaetocladia, Clavariopsis aquatica, Camposporium pellucidum, Diplocladiella scalaroides, Helicomyces roseus, Lemonniera cornuta, L. pseudofloscula, L. terrestris, Lunulospora curvula, Pestalotiopsis submerses* and *Tetrachaetum elegans* showed little host diversity.

Among the 24 host plants studied, the roots of *Lyonia obvalifolia* were colonized by maximum number of endophytes, followed by *Botrychium* (8) and *Machillus duthiei* (6) while *Artemisia vulgaris, Rosa moschata, Debregeasia* species, *Aesculus indica, Valeriana wallichei, Myrseine semiserrata, Nepeta leucophyla* and *Salix tetrasperma* were colonized by less number of root endophytes. As evident from Table-2, the relative contribution to diversity of fungal endophytes in different host plant, maximum relative contribution was found to *Lyonia ovalifolia* i.e. 47.6% and minimum relative contribution of fungal root endophytes was confined to *Aesculus indica, Geranium nepalenses, Murraya koengii* and *Salix tetraspera* (4.7%each).

DISCUSSION

Of all the water borne conidial fungi recorded in this study, 8 species were also reported by earlier mycologists. Two species namely, *Anguillospora longissima* and *Tetracladium marchalianum*, reported earlier by Nemec (1969) from Strawberry plant roots, were recorded here on roots of an Unidentified grass, *Botrychium* sp., *Geranium nepalense, Barberis* sp., *Machilus duthiei, Symplocos chinensis, Eupatorium adenophyllum, Equisetum* sp., *Lyonia ovalifolia, Quercus floribunda, Salix tetrasperma*, unidentified fern and *Viburnum mullaha*. *Campylospora purvula* earlier reported by Fisher and Petrini (1989, 1990) was isolated from *Botrychium* sp., *Lyonia ovalifolia, Barberis* sp., *Acer pictum, Machilus duthiei, Symplocos chinensis, Vibrunum mullah* and *Strobilanthus dalhousianus, . Heliscus lugdunensis* was isolated from *Acer pictum, Barberis* sp., *Botrychium* sp., *Delbergeasia* sp, *Lyonia ovalifolia, Machilus duthiei, Rosa moschata* and *Symplocos chinensis*. It was also reported by Fisher et al (1991) and Sridhar and Barlocher (1992) from the roots of species of *Alnus, Acer* and *Betula*. *Lunulospora curvula*, earlier reported by Sridhar and Barlocher (1992a) on *Alnus* species, in present study it was isolated from *Eupatorium adenophyllum* and

Botrychium sp. *Tetracladium setigerum* recorded earlier from strawberry roots by Watanbe (1975) was isolated from *Alnus nepalensis, Eupatorium adenophyllum, Lyonia ovalifolia***,** *Murraya koenegii,* unidentified fern**,** *Equisetum* sp.**,** *Machilus duthiei, Botrychium* sp.**,** *Quercus floribunda, Artemisia vulgaris, Valeriana wallichii* and *Delbergeasia* sp. In the present study, *Clavariopsis aquatica* was isolated from *Botrychium* sp. and *Quercus floribunda***,** whereas, Sridhar and Barlocher **(**1992a) reported it from roots of *Alnus* and *Picea* sp. Fisher et al (1991) have also found this species as an endophyte. *Cylindrocarpon aquaticum,* found as a most frequently colonizing fungus, was collected from plant roots of *Aesculus indica, Acer pictum, Barberis* sp., *Artemisia vulgaris, Strobilanthus dalhousianus, Valeriana wallichii***,** *Geranium nepalense, Nepta leucophylla, Rosa moschata***,** *Vibrunum mullah***,** *Alnus nepalensis* and *Delbergeasia* sp. It was also earlier reported by Sridhar and Barlocher (1992a, 1992b) from maple and spruce roots.

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Western Himalaya

S. No	Endophyte	Host
1.	*Acaulopage tetraceros* Derchsler	Mk / Lo / Ea
2.	*Alatospora acuminata* Ingold	UG / E / Md / Vm / Id / An
3.	*A. pulchella* Marvanová	UG
4.	*Anguillospora crassa* Ingold	E / B
5.	*A. longissima*(Sacc.and Therry) Ingold	UG / Bm / Gn / B / Md / Sc
6.	*Camposporium pellucidum*(Grove) Hughes	Sd
7.	*Campylospora chaetocladia* Ranzoni	Mk
8.	*C. purvula* Kuzuha	Bm / Lo / B / Ap / Md / Sc / Vm / Sd
9.	*Clavariopsis aquatica* de Wildeman	Bm / Qf
10.	*Cylindrocarpon aquaticum*(Nils.) Marvanova and Descals	Ai / Ea / Md / Ap / B / Av / Sd / Vw / Gn/ Nl / Rm / Vm / An / D
11.	*Diplocladiella scalaroides* Arnaud	Gn
12.	*Helicomyces roseus* Link	Qf / Ap
13.	*Heliscus lugdunensis* Ingold	Ap / B / Bm / D / Lo / Md / Rm / Sc
14.	*Lemonniera cornuta* Ranzoni	Lo
15.	*L. pseudofloscula* Dyko	Lo
16.	*L. terrestris* Tubaki	Lo
17.	*Lunulospora curvula* Ingold	Ea / Bm
18.	*Pestalotiopsis submersus* Sati and Tiwari	E / F/ Lo
19.	*Tetrachaetum elegans* Ingold	Lo / Bm
20.	*Tetracladium marchalianum* de Wildeman	Ea / E / Lo / Ms / Bm / Qf / St / UG / F
21.	*T. setigerum*(Grove) Ingold	An/Ea/LO/ Mk/ F / E / Md / Bm / Qf / Av / Vw / D

Mk = *Murraya koenegii*, **Lo** = *Lyonia ovalifolia*, **UG** = Unidentified grass, **E** = *Equisetum* sp., **B** = *Barberis* sp. , **Bm =** *Botrychium sp.*, **Ai=** *Aesculus indica*, **Ea** = *Eupatorium adenophyllum*, **F** = Unidentified *Fern*, **Md =** *Machilus duthiei*, **Vm** = *Vibrunum mullah*, **Id** = *Ilex* diphyrena, **An** = *Alnus nepalensis*, **Gn** = *Geranium nepalense*, **Sc** = *Symplocos chinensis*, **S** = *Strobilanthes* sp., **Ap** = *Acer pictum*, **Qf** = *Quercus floribunda*, **Av** = *Artemisia vulgaris*, **Vw** = *Valeriana wallichii*, **Nl** = *Nepta leucophylla*, **Rm** = *Rosa moschata*, **D** = *Debregeasia* sp., **St** =*Salix tetrasperma*

* Calculated against 24 studied host species (Table 2)

Table 2: Colonization of endophytes on roots of different host plants

S. No.	Host	Percent contribution to diversity - of fungal endophyte(%) *
1.	*Acer pictum* Thunb	19.0
2.	*Aesculus indica C*olebr	4.7
3.	*Alnus nepalensis* D. Dori	14.3
4.	*Artemisia vulgaris* Linn	9.5
5.	*Berberis* sp. Roxb.	23.8
6.	*Debregeasia* sp Gaud.	14.3
7.	*Equisetum* sp. Linn.	23.8
8.	*Eupatorium haterophyllum* Linn.	23.8
9.	Unidentified *Fern*	14.3
10.	*Geranium nepalense* Sw.	19.0
11.	*Ilex diphyrena* all.	4.7
12.	*Lyonia ovalifolia* Wall	47.6
13.	*Machilus duthiei* King	28.5
14.	*Murraya koenegii* Spreng.	14.3
15.	*Nepeta leucophylla* Benth.	4.7
16.	*Botrychium* sp.	38.1
17.	*Quercus floribunda* Wall.	19.0
18.	*Rosa moschata* J. Herrm.	9.5
19.	*Salix tetrasperma* Roxb.	4.7
20.	*Strobilanthes* sp Blume.	19.0
21.	*Symplocos chinensis* ochinchinewis(Lour)	14.3
22.	Unidentified grass	19.0
23.	*Valeriana wallichii* . DC.	9.5
24.	*Viburnum mullaha* Buch- Ham. Ex . D. Don	14.3

* Calculated against 21root endophytes altogether recorded (Table 1)

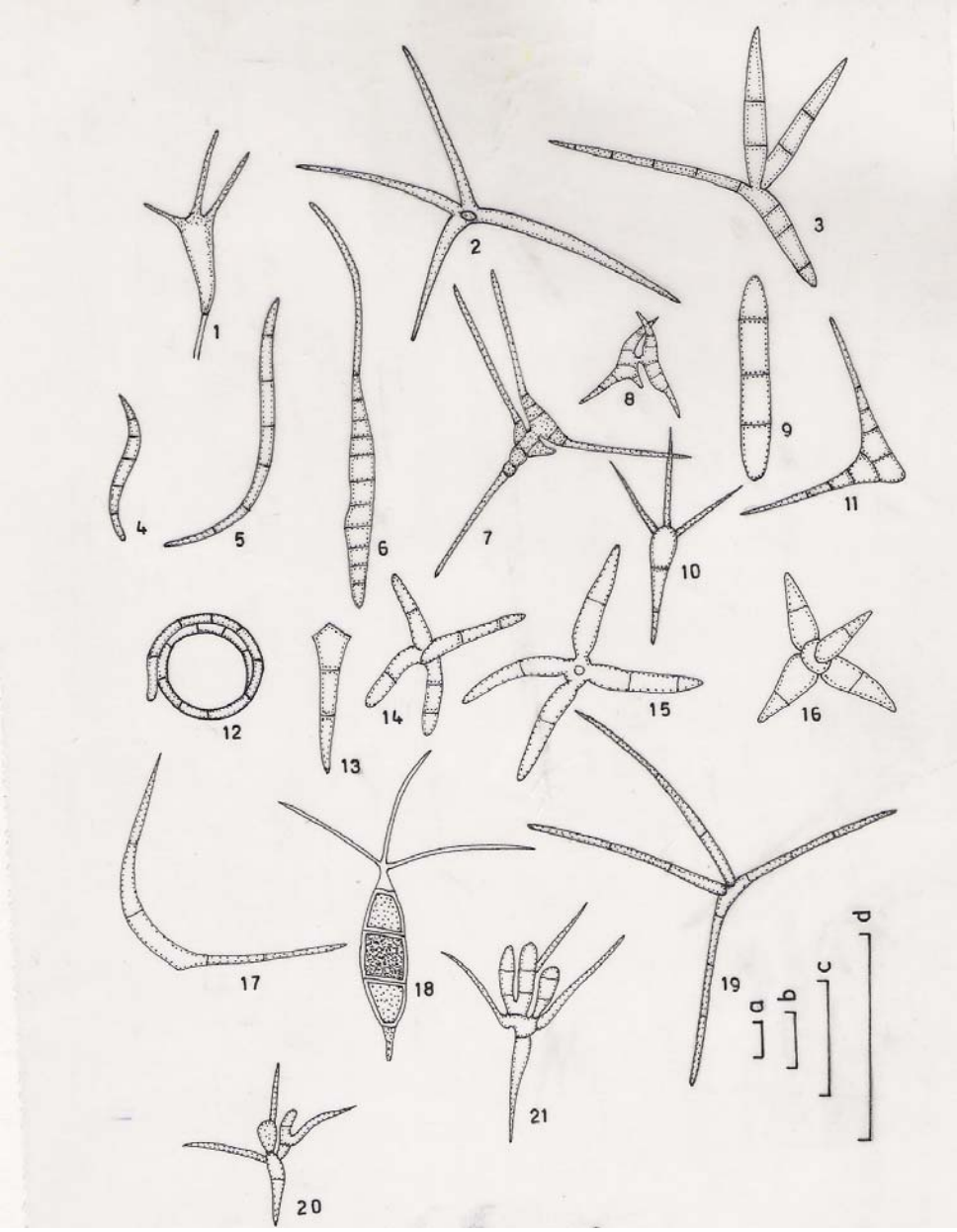

Fig.1

Figs. 1,7, 8, 9,11,13,14-18, 20 and **21** in **scale c**; **4-5** in **scale a**; **6, 10, 12** and **19** in **scale b** and **2-3** in **scale d**. ***(Fig. Nos. are corresponding to Table No. 1)***

29-05-08

RNA, DNA and protein isolation using TRIzol reagent

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Abstract: TRIZOL Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi. During sample homogenization or lysis, TRIZOL® Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. After removal of the aqueous phase, the DNA and proteins in the sample can be recovered by sequential precipitation. Precipitation with ethanol yields DNA from the interphase, and an additional precipitation with isopropyl alcohol yields proteins from the organic phase. Beside the RNA isolation using TRIzol, TRIzol also can be used to isolate DNA and protein after the RNA isolation. This review and technical article just give a description of the TRIzol usage to help the reader to TRIzol reagent better. [Nature and Science. 2008;6(3):66-75]. ISSN: 1545-0740.

Keywords: RNA; TRIzol; isolation; gene; DNA

Introduction

RNA isolation and purification is one of the key factors for the RT-PCR assays and other related molecular biology detections. TRIzol Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (Gauthier, Madison and Michel, 1997). During sample homogenization or lysis, TRIZOL® Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. After removal of the aqueous phase, the DNA and proteins in the sample can be recovered by sequential precipitation. Precipitation with ethanol yields DNA from the interphase, and an additional precipitation with isopropyl alcohol yields proteins from the organic phase (Chomczynski, 1993).

Beside the RNA isolation using TRIzol, TRIzol also can be used to isolate DNA and protein after the RNA isolation. TRIzol is a very useful reagent in RNA, DNA and protein isolation. This review and technical article just give a description of the TRIzol usage to help the readers to TRIzol reagent better, and to improve the development of the molecular biology researches.

Brief prescriptions

TRIZOL® Reagent, Cat. No. 15596-018 by Invitrogent Company, can be stored at 4-23^{o}C up to 12 months.

Copurification of the DNA may be useful for normalizing RNA yields from sample to sample (Eichler and Eales, 1985). This technique performs well with small quantities of tissue (50-100 mg) and cells (5 × 106), and large quantities of tissue (≥1 g) and cells (>107), of human, animal, plant, or bacterial origin. The simplicity of the TRIZOL® Reagent method allows simultaneous processing of a large number of samples (Ahmann et al., 2008). The entire procedure can be completed in one hour. Total RNA isolated by TRIZOL® Reagent is free of protein and DNA contamination. It can be used for Northern blot analysis, dot blot hybridization, poly (A)+ selection, in vitro translation, RNase protection assay, and molecular cloning. For use in the polymerase chain reaction (PCR*), treatment of the isolated RNA with amplification grade DNase I (Cat. No. 18068) is recommended when the two primers lie within a single exon.

TRIZOL® Reagent facilitates isolation of a variety of RNA species of large or small molecular size (Ahmann et al., 2008). For example, RNA isolated from rat liver, electrophoresed on an agarose gel, and stained with ethidium bromide, shows discrete bands of high molecular weight RNA between 7 kb and 15 kb in size, (composed of mRNA's and hnRNA's) two predominant ribosomal RNA bands at ~5 kb (28S) and at ~2 kb (18S), and low molecular weight RNA between 0.1 and 0.3 kb (tRNA, 5S). The isolated RNA has an A260/A280 ratio ≥1.8 when diluted into TE.

Brief steps for the TRIzol usage on RNA isolation:

1. Add Trizol TRIzol platelet pellet (5-10 x 10^6 cells/1 ml).
2. Incubate at room temperature for 5 min.
3. Add 0.2 ml chloroform/1 ml TRIzol.
4. Shake tubes vigorously by hand for 15 seconds.
5. Incubate at room tempearture for 3 min.
6. Centrifuge 15 min at 4^{o}C at less than 12,000 g (10000 rpm, r=65 mm).
7. RNA is in top (aqueous phase, about 60% volume).
8. Transfer the RNA aqueous phase (top) to a fresh tube.
9. Save organic phase for DNA and protein isolation.
10. Pricipitate RNA from aqueous phase with isopropanol (isopropyl alcohol, C_3H_8O).
11. Add 0.5 ml isopropanol/ml TRIzol.
12. Incubate 10 min at room temperature.
13. Centrifuge 10 minutes at 4^{o}C by less than 12000 g (10000 rom, r=65 mm).
14. RNA is in bottom as gel-like pellet.
15. Remove and dispose supernatant.
16. Wash RNA pellet once with 75% ethanol: Add 75% ethanol 1 ml/ml TRIzol.
17. Vortex and centrifuge 5 min at 4^{o}C by less that 7500 g (8000 rpm, r=65 mm).
18. Save RNA pellet (air-dry 5-10 min).
19. Dissolve RNA in 0.03–0.06 ml RNase-free water or 0.5% SDS solution.
20. Mix with pipett.
21. Incubate 10 min at 55-60^{o}C.
22. Redissolved in 100% formamide (deionized) and stored at -70^{o}C.
23. De-frozen and for PCR usage.

Precautions for Preventing RNase Contamination:

RNases can be introduced accidentally into the RNA preparation at any point in the isolation procedure through improper technique. Because RNase activity is difficult to inhibit, it is essential to prevent its introduction. The following guidelines should be observed when working with RNA.

1. Always wear disposable gloves. Skin often contains bacteria and molds that can contaminate an RNA preparation and be a source of RNases. Practice good microbiological technique to prevent microbial contamination.
2. Use sterile, disposable plasticware and automatic pipettes reserved for RNA work to prevent cross-contamination with RNases from shared equipment. For example, a laboratory that is using RNA probes will likely be using RNase A or T1 to reduce background on filters, and any nondisposable items (such as automatic pipettes) can be rich sources of RNases.
3. In the presence of TRIZOL® Reagent, RNA is protected from RNase contamination. Downstream sample handling requires that nondisposable glassware or plasticware be RNase-free. Glass items can be baked at 150°C for 4 hours, and plastic items can be soaked for 10 minutes in 0.5 M NaOH, rinsed thoroughly with water, and autoclaved.

Other Precautions:

1. Use of disposable tubes made of clear polypropylene is recommended when working with less than 2-ml volumes of TRIZOL® Reagent.

2. For larger volumes, use glass (Corex) or polypropylene tubes, and test to be sure that the tubes can withstand 12,000 × *g* with TRIZOL® Reagent and chloroform. Do not use tubes that leak or crack.
3. Carefully equilibrate the weights of the tubes prior to centrifugation.
4. Glass tubes must be sealed with parafilm topped with a layer of foil, and polypropylene tubes must be capped before centrifugation.

INSTRUCTIONS FOR RNA ISOLATION:

When working with TRIZOL® Reagent use gloves and eye protection (shield, safety goggles). Avoid contact with skin or clothing. Use in a chemical fume hood. Avoid breathing vapor. *Unless otherwise stated, the procedure is carried out at 15 to 30°C, and reagents are at 15 to 30°C.*

Reagents required, but not supplied:

1. Chloroform
2. Isopropyl alcohol
3. 75% Ethanol (in DEPC-treated water)
4. RNase-free water or 0.5% SDS solution [To prepare RNase-free water, draw water into RNase-free glass bottles. Add diethylpyrocarbonate (DEPC) to 0.01% (v/v). Let stand overnight and autoclave. The SDS solution must be prepared using DEPC-treated, autoclaved water.]

Practical protocol for the RNA isolation using TRIzol

1. HOMOGENIZATION

a. ***Tissues***

Homogenize tissue samples in 1 ml of TRIZOL® Reagent per 50-100 mg of tissue using a glass-Teflon® or power homogenizer (Polytron, or Tekmar's TISSUMIZER® or equivalent). The sample volume should not exceed 10% of the volume of TRIZOL®.

b. ***Cells Grown in Monolayer***

Lyse cells directly in a culture dish by adding 1 ml of TRIZOL® Reagent to a 3.5 cm diameter dish, and passing the cell lysate several times through a pipette. The amount of TRIZOL® Reagent added is based on the area of the culture dish (1 ml per 10 cm^2) and not on the number of cells present. An insufficient amount of TRIZOL®. Reagent may result in contamination of the isolated RNA with DNA.

c. ***Cells Grown in Suspension***

Pellet cells by centrifugation. Lyse cells in TRIZOL® Reagent by repetitive pipetting. Use 1 ml of the reagent per 5-10 × 10^6 of animal, plant or yeast cells, or per 1 × 10^7 bacterial cells. Washing cells before addition of TRIZOL® Reagent should be avoided as this increases the possibility of mRNA degradation. Disruption of some yeast and bacterial cells may require the use of a homogenizer.

OPTIONAL: An additional isolation step may be required for samples with high content of proteins, fat, polysaccharides or extracellular material such as muscles, fat tissue, and tuberous parts of plants. Following homogenization, remove insoluble material from the homogenate by centrifugation at 12,000 × *g* for 10 minutes at 2 to 8°C. The resulting pellet contains extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernatant contains RNA. In samples from fat tissue, an excess of fat collects as a top layer which should be removed. In each case, transfer the cleared homogenate solution to a fresh tube and proceed with chloroform addition and phase separation as described.

2. PHASE SEPARATION

Incubate the homogenized samples for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 1 ml of TRIZOL® Reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 seconds and incubate them at 15 to 30°C for 2 to 3 minutes. Centrifuge the samples at no more than 12,000 × *g* for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIZOL® Reagent used for homogenization.

3. RNA PRECIPITATION

Transfer the aqueous phase to a fresh tube, and save the organic phase if isolation of DNA or protein is desired. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL® Reagent used for the initial homogenization. Incubate samples at 15 to 30°C for 10 minutes and centrifuge at no more than 12,000 × *g* for 10 minutes at 2 to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

4. RNA WASH

Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL® Reagent used for the initial homogenization. Mix the sample by vortexing and centrifuge at no more than 7,500 × *g* for 5 minutes at 2 to 8°C.

5. REDISSOLVING THE RNA

At the end of the procedure, briefly dry the RNA pellet (air-dry or vacuum-dry for 5-10 minutes). Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an A260/280 ratio < 1.6. Dissolve RNA in RNase-free water or 0.5% SDS solution by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 to 60°C. (Avoid SDS when RNA will be used in subsequent enzymatic reactions.) RNA can also be redissolved in 100% formamide (deionized) and stored at -70°C (5).

RNA Isolation Notes:

1. Isolation of RNA from small quantities of tissue (1 to 10 mg) or Cell (102 to 104) Samples: Add 800 µl of TRIZOL® to the tissue or cells. Following sample lysis, add chloroform and proceed with the phase separation as described in step 2. Prior to precipitating the RNA with isopropyl alcohol, add 5-10 µg RNase-free glycogen (Cat. No 10814) as carrier to the aqueous phase. To reduce viscosity, shear the genomic DNA with 2 passes through a 26 gauge needle prior to chloroform addition. The glycogen remains in the aqueous phase and is co-precipitated with the RNA. It does not inhibit first-strand synthesis at concentrations up to 4 mg/ml and does not inhibit PCR.

2. After homogenization and before addition of chloroform, samples can be stored at -60 to -70°C for at least one month. The RNA precipitate (step 4, RNA WASH) can be stored in 75% ethanol at 2 to 8°C for at least one week, or at least one year at –5 to -20°C.

3. Table-top centrifuges that can attain a maximum of 2,600 × *g* are suitable for use in these protocols if the centrifugation time is increased to 30-60 minutes in steps 2 and 3.

INSTRUCTIONS FOR DNA ISOLATION:

After complete removal of the aqueous phase, as described in the RNA isolation protocol, the DNA in the interphase and phenol phase from the initial homogenate may be isolated. Following precipitation and a series of washes, the DNA is solubilized in 8 mM NaOH. Full recovery of DNA from tissues and culture cells permits the use of TRIZOL® Reagent for the determination of the DNA content in analyzed samples. Simultaneous extraction of genomic DNA allows for normalization of the results of Northern analysis per genomic DNA instead of the more variable total RNA or tissue weight. (Depending on the source, the DNA pellet obtained may require additional purification (e.g., phenol extraction) prior to other applications.

Reagents required, but not supplied:

1. Ethanol
2. 0.1 M Sodium citrate in 10% ethanol
3. 75% Ethanol
4. 8 mM NaOH

Unless otherwise stated, the procedure is carried out at 15 to 30°C.

1. DNA PRECIPITATION

Remove the remaining aqueous phase overlying the interphase, and precipitate the DNA from the interphase and organic phase with ethanol. Add 0.3 ml of 100% ethanol per 1 ml of TRIZOL® Reagent used for the initial homogenization, and mix samples by inversion. Next, store the samples at 15 to 30°C for 2-3 minutes and sediment DNA by centrifugation at no more than 2,000 × *g* for 5 minutes at 2 to 8°C.

Careful removal of the aqueous phase is critical for the quality of the isolated DNA.

2. DNA WASH

Remove the phenol-ethanol supernatant, and if desired, save it for protein isolation. Wash the DNA pellet twice in a solution containing 0.1 M sodium citrate in 10% ethanol. Use 1 ml of the solution per 1 ml of TRIZOL® Reagent used for the initial homogenization. At each wash, store the DNA pellet in the washing solution for 30 minutes at 15 to 30°C (with periodic mixing) and centrifuge at 2,000 × *g* for 5 minutes at 2 to 8°C. Following these two washes, suspend the DNA pellet in 75% ethanol (1.5-2 ml of 75% ethanol per 1 ml TRIZOL® Reagent), store for 10-20 minutes at 15 to 30°C (with periodic mixing) and centrifuge at 2,000 × *g* for 5 minutes at 2 to 8°C.

An additional wash in 0.1 M sodium citrate-10% ethanol solution is required for large pellets containing > 200 μg DNA or large amounts of a non-DNA material.

3. REDISSOLVING THE DNA

Air dry the DNA 5 to 15 minutes in an open tube. (DO NOT DRY UNDER CENTRIFUGATION; it will be more difficult to dissolve.) Dissolve DNA in 8 mM NaOH such that the concentration of DNA is 0.2 – 0.3 μg/μl. Typically add 300 – 600 μl of 8 mM NaOH to DNA isolated from 107 cells or 50 – 70 mg of tissue. Resuspending in weak base is HIGHLY recommended since isolated DNA does not resuspend well in water or in Tris buffers. The pH of the 8 mM NaOH is only ~9 and should be easily adjusted with TE or HEPES once the DNA is in solution. At this stage, the DNA preparations (especially from tissues) may contain insoluble gel-like material (fragments of membranes, etc.) Remove the insoluble material by centrifugation at >12,000 × *g* for 10 minutes. Transfer the supernatant containing the DNA to a new tube. DNA solubilized in 8 mM NaOH can be stored overnight at 4°C; for prolonged storage, samples should be adjusted with HEPES to pH 7-8 (see table) and supplemented with 1 mM EDTA. Once the pH is adjusted, DNA can be stored at 4°C or –20°C.

Quantitation and Expected Yields of DNA

Take an aliquot of the DNA preparation solubilized in 8 mM NaOH, mix it with water and measure the A260 of the resulting solution. Calculate the DNA content using the A260 value for double-stranded DNA. One A260 unit equals 50 μg of double-stranded DNA/ml. For calculation of cell number in analyzed samples, assume that the amount of DNA per 1 × 106 diploid cells of human, rat, and mouse origin equals: 7.1 μg, 6.5 μg, and 5.8 μg, respectively (3).

Applications:

Amplification of DNA by PCR:

After redissolving the DNA in 8 mM NaOH, adjust the pH to 8.4 with 0.1 M HEPES (see table). Add 0.1 to 1.0 μg of the DNA sample to your PCR reaction mixture and perform the standard PCR protocol.

Restriction endonuclease reactions:

Adjust the pH of the DNA solution to a required value using HEPES (see table). Alternatively, samples may be dialyzed against 1 mM EDTA, pH 7 to pH 8.0. Use 3-5 units of enzyme per microgram of DNA. Use the conditions recommended by the manufacturer for the particular enzyme, and allow the reaction to proceed for 3 to 24 h. In a typical assay, 80-90% of the DNA is digestible.

pH Adjustment of DNA Samples Dissolved in 8 mM NaOH:

For 1 ml of 8 mM NaOH use the following amounts of 0.1 M or 1 M HEPES, free acid. Final pH 0.1 M HEPES (μl) Final pH 1 M HEPES (μl).

DNA Isolation Notes:

1. The phenol phase and interphase can be stored at 2 to 8°C overnight.
2. Samples suspended in 75% ethanol can be stored at 2 to 8°C for months.
3. Samples dissolved in 8 mM NaOH can be stored overnight at 2 to 8°C. For long-term storage, adjust the pH to 7-8, and adjust the EDTA concentration to 1 mM.

INSTRUCTIONS FOR PROTEIN ISOLATION:

Proteins are isolated from the phenol-ethanol supernatant obtained after precipitation of DNA with ethanol (step 1, DNA PRECIPITATION). The resulting preparation can be analyzed for the presence of specific proteins by Western blotting (2).

Reagents required, but not supplied:

1. Isopropyl alcohol
2. 0.3 M Guanidine hydrochloride in 95% ethanol
3. Ethanol
4. 1% SDS

1. PROTEIN PRECIPITATION

Precipitate proteins from the phenol-ethanol supernatant (approximate volume 0.8 ml per 1 ml TRIZOL® Reagent) with isopropyl alcohol. Add 1.5 ml of isopropanol per 1 ml TRIZOL® Reagent used for the initial homogenization. Store samples for 10 minutes at 15 to 30°C, and sediment the protein precipitate at 12,000 × *g* for 10 minutes at 2 to 8°C.

2. PROTEIN WASH

Remove the supernatant and wash the protein pellet 3 times in a solution containing 0.3 M guanidine hydrochloride in 95% ethanol. Add 2 ml of wash solution per 1 ml of TRIZOL® Reagent used for the initial homogenization. During each wash cycle, store the protein pellet in the wash solution for 20 minutes at 15 to 30°C and centrifuge at 7,500 × *g* for 5 minutes at 2 to 8°C. After the final wash, vortex the protein pellet in 2 ml of ethanol. Store the protein pellet in ethanol for 20 minutes at 15 to 30°C and centrifuge at 7,500 × *g* for 5 minutes at 2 to 8°C.

3. REDISSOLVING THE PROTEIN PELLET

Vacuum dry the protein pellet for 5-10 minutes. Dissolve it in 1% SDS by pipetting. Complete dissolution of the protein pellet may require incubating the sample at 50°C. Sediment any insoluble material by centrifugation at 10,000 × *g* for 10 minutes at 2 to 8°C, and transfer the supernatant to a fresh tube. The sample is ready for use in Western blotting or may be stored at -5 to -20°C for future use.

Protein Isolation Notes:

1. The protein pellet suspended in 0.3 M guanidine hydrochloride-95% ethanol or in ethanol can be stored for at least one month at 2 to 8°C, or for at least one year at -5 to -20°C.
2. The following protocol is an alternative approach that allows for more efficient recovery of proteins. Dialyze the phenol-ethanol supernatant against three changes of 0.1% SDS at 2 to 8°C. Centrifuge the dialyzed material at 10,000 × *g* for 10 minutes. Use the clear supernatant for Western blotting.
3. Proteins may be quantified by the Bradford method as long as the concentration of SDS is low enough (<0.1%) so that it will not interfere. Methods that do not have detergentinterface problems, and that do not rely on A260/A280 measurements may be used (traces of phenol may cause overestimation of protein concentrations).

For RNA ISOLATION, Expected yields of RNA per mg of tissue or 1 × 106 cultured cells:

1. Liver and spleen, 6-10 µg
2. Kidney, 3-4 µg
3. Skeletal muscles and brain, 1-1.5 µg
4. Placenta, 1-4 µg
5. Epithelial cells (1 × 106 cultured cells), 8-15 µg
6. Fibroblasts, (1 × 106 cultured cells) 5-7 µg

For DNA ISOLATION, Expected yields of DNA per mg of tissue or 1 × 106 cultured cells:

1. Liver and kidney, 3-4 µg
2. Skeletal muscles, brain, and placenta 2-3 µg
3. Cultured human, rat, and mouse cells (1 × 106), 5-7 µg
4. Fibroblasts, 5-7 µg

Figure 1 gives a brief steps for the TRIzol usage on RNA isolation, as an example of RNA isolation from human platelet with the scheme description, for readers to sue it as a reference.

An example of RNA isolation from human platelet:

Platelet pellet

↓

Trizol 5-10X10^6 cells/1 ml TRIzol

↓

5 minutes incubate at room temperature

↓

0.2 ml chloroform/1 ml TRIzol

↓

Shake tubes vigorously by hand for 15 seconds

↓

Incubate at room tempearture for 3 minutes

↓

Centrifuge 15 minutes at 4^{o}C at less than 12,000 g (10000 rpm, r=65 mm)

↓

RNA is in top (aqueous phase, about 60% volume)

↓

Transfer the aqueous phase to a fresh tube

↓

Save organic phase for DNA and protein isolation

↓

Pricipitate RNA from aqueous phase with isopropanol (isopropyl alcohol, C_3H_8O)

↓

Add 0.5 ml isopropanol/ml TRIzol

↓

Incubate 10 minutes at room temperature

↓

Centrifuge 10 minutes at 4°C by less than 12000 g (10000 rom, r=65 mm)

↓

RNA is in bottom as gel-like pellet

↓

Remove and dispose supernatant

↓

Wash RNA pellet once with 75% ethanol

↓

Add 75% ethanol 1 ml/ml TRIzol

↓

Vortex and centrifuge 5 minutes at 4°C by less that 7500 g (8000 rpm, r=65 mm)

↓

Save RNA pellet (air-dry 5- 10 minutes)

↓

Dissolve RNA in 0.03–0.06 ml RNase-free water or 0.5% SDS solution

↓

Mix with pipett

↓

Incubate 10 minutes at 55-60°C

↓

Redissolved in 100% formamide (deionized) and stored at -70°C

De-frozen and for PCR usage

Figure 1. TRIzol steps for RNA isolation

To get better treatment of human blood, the following is platelet isolation by Ficoll-Pague method:

1. Human bloods were collected in 2.7-ml tubes containing 2.7 ml of 3.2% (0.109 M) Na citrate.
2. About 2.5 ml of blood was collected in each tube and 8 tubes for each drawing, and the total blood for each draw was about 20 ml.
3. Each blood drawing was done by pre-dialysis and post-dialysis separately (about 6 hours for the dialysis), and each patient was drawn blood four times totally (one pre-dialysis and one post-dialysis for first drwaing, and another one pre-dialysis and one post-dialysis for second drawing).
4. Centrifuge at 800 rpm for 15 min, at room temperature.
5. Collect platelet-rich-plasma (PRP) (supernatant).
6. Put PRP into the 14-ml Corning centrifuge tube that has 3 ml of Ficoll-Pague Premium solution (slowly along side).
7. Centrifuge at 1350 rpm for 35 min, at room temperature.
8. Collect PRP in the top of the tube.
9. Centrifuge at 4000 rpm for 30 min, at room temperature.
10. Collect platelet (pellet) and freeze the supernatant for protein measurement.
11. Wash platelet with 5 ml of physiological buffered solution (PBS).
12. Centrifuge at 4000 rpm for 30 min, at room temperature.
13. Keep platelet (pellet) at freezer (-70°C) until RNA isolation.

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