Journal of
Practical Biochemistry and Biophysics

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JPBB

Journal of Practical Biochemistry and Biophysics

July - December 2016

Volume 1, Number 2

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Original Article

Journal of Practical Biochemistry and Biophysics
Volume 1 Number 2, July - December 2016

Effect of Thiamine Hydrochloride on the Lead Induced Acute Toxicity in Albino Rats

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Abstract

The efficacy of thiamine hydrochloride against lead induced acute toxicity was studied in the experimental rats. Hematological parameters such as RBC Count and Heamoglobin content were determined and was found to be decreased throughout the period of study in the lead induced animals. Biochemical parameters such as protein, urea, uric acid levels increased throughout the study when treated with lead acetate. Triglyceride content was also found to be decreased throughout the period of study, in lead induced animals. Biochemical alterations were reversed on treatment with thiamine hydrochloride due to its antagonistic role in lead induced rats.

Keywords: Lead Acetate; Thiamine; Protein; Urea; Uric Acid and RBC Count; Triglyceride.

Introduction

The term heavy metal refers to any metallic element that has a relatively high density and is toxic or poisonous at low concentration. Examples of heavy metals include, cadmium, mercury, arsenic, chromium, titanium and lead. Heavy metals are natural components of the earth crust. They cannot be degraded or destroyed. To a small extent they enter our body via food, drinking water and ambient air. As trace element, some heavy metal (Copper, selenium, zinc) are essential to maintain the metabolism of human body. However at high concentrations they can lead to poisoning. Heavy metal poisoning could result for instance in drinking water emission or intake via food chain.

They are dangerous and tend to bioaccumulate, bioaccumulate means the concentration of metal in the biological system is much higher than normal. Heavy metals can enter a water supply by industrial or consumer waste or even from acidic rain, breaking down soils and release heavy metals into streams or lakes or rivers or ground water. Lead ranks close to cadmium and mercury as a metal of current toxicological concern. Lead is natural element and widespread in the environment. It is an inorganic toxicant of great environment and occupational concern which was classified as a human carcinogen. The concentration of lead in the earth's crust has been estimated at 12.5 ppm, ranking it as the 36th element in abundance (Abbasi & Soni) Lead is a soft, heavy, toxic, malleable and poor metal .

Lead is emitted into the environment through a large number of natural and anthropogenic sources (Soni). Lead acetate is prepared by and treating litharge (Lead monoxide with acetic acid). Gasoline additives, mainly tetraethyl lead, the principal anthropogenic source of lead in the environment, enter the atmosphere as unburned lead alkyl vapours and as lead halides.

This heavy metal is still and added to many products including paints, eye cosmetics, gasoline, water pipes and health care supplies. Among the

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natural sources, the major contribution comes from wind blown dusts, forest fires, volcanic emission and sea salt sprays (Nriagu). Industrial effluents arising from plating units, and from paper, rayon, dye and pigment, chemical fertilizers, Ghee and battery industries and mine drainage (Soni) are the important sources for lead. Thiamine is absorbed from the small intestine, capacity of human intestine to absorb this vitamin is limited to about mg per day. Thiamine undergoes change in skeletal muscle, heart, liver, kidney, and brain. Excess of thiamine is excreted in urine. Closely associated with functioning of nervous system and body musculation, helps in carbohydrate metabolism, production of hydrohloride that facilitates digeston in the stomach, flow of electrolytes in and out nerve and muscle cells and boosting of enzyme activity for proper digestion, absorption of food and nutrients. Thiamine has antioxidant activity, erythrhropoietic property, mood modulating and glucose regulating activities.

Thiamine Tri Phosphate is necessary for the action of pyruvate dehydrogenase and alpha kG in carbohydrate metabolism and for the action of transketolase in HMP shunt. Thiamine protects against lead toxicity by inhibiting lead induced lipid peroxidation (Mamta Dhawan) Thiamine reverses the lead induced inhibition of the activity of blood , 5 –aminolevulinic acid dehydratase. It helps to maintain normal RBC count, improves circulation.

The heavy metal lead in the form of lead acetate is a neurotoxin and a cumulative poison, hence it is used for the present study. Lead is one of the natural components of the environment and it affects metabolic activities of the body. Extrapolation of the rat to human beings is done for risk assessment.

The Vitamin B1 (Thiamine) in the form of thiamine hydrochloride is given as an antidote to the lead intoxicity in albino rats. Thiamine pyrophosphate, the cofactor form of thiamine, plays a significant role in carbohydrate metabolism, lipid peroxidation and has an impact on other metabolic actions on the human body. Vitamin B1 (Thiamine) has got antagonistic effect towards lead intoxication and hence it is used as an antidote for the present study.

Materials and Methods

Adult male Albino rats (weighing 100-160 g), were procured from Agricultural University Extension Centre, Kattupakkam, Chennai, were kept at room temperature (32 ± 2°C) at L:D (12:12) cycles. All studies were conducted in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" [National institute of health 1]. Animals were randomized and separated into four groups (Group I – control), Group II – lead acetate-treated, Group III – lead acetate- and thiamine hydrochloride treated, Group IV – thiamine hydrochloride treated; n = 6 in each group).

The animals were acclimatized to laboratory conditions prior to the experiment following the procedure of Behringer and NIH. Animals were caged in polypropylene cages and bed was prepared for rats with husk and it was replaced alternatively. [CPCSEA No – IAEC 1/2008/02]. Thiamine hydrochloride was purchased from Sisco Research Laboratories Private Limited, Mumbai India. Lead acetate and all other chemicals used in this study were of analytical grade. Group I animals served as controls. Group II animals were administered with lead acetate intraperitoneally (ip) (100 mg/kg) every day for 14 days [Cory et al]. Group III animals were treated with lead acetate as Group II animals along with Thiamine hydrochloride (150 mg/kg) (ip) [Morrison et al]. Group IV animals received Thiamine hydrochloride (150mg/kg) (ip) for 14 days.

Biochemical determinations were performed after14 days of lead acetate and/or thiamine hydrochloride administration. At the end of experimental period (14 days) animals from all the groups were sacrificed by cervical dislocation. Blood samples were collected from each group of rats. Biochemical analyses were performed in blood. Red blood cell count and Haemoglobin content [Drabkin] content was determined in blood. Uric acid [Caraway], protein [Lowry et al], Urea [Marsh], Triglyceride content [Fletcher] and Alkaline phosphatase (Bower) were also determined. Analysis of variance followed by Least Significant Difference test was carried out to detect the significant differences between control and experimental groups.

Results

Total Count of RBC

Red blood cell count in Group I was 6.5 ± 0.18 million. After the administration of lead acetate, the count in Group II animals was decreased to 3.5± 0.02 million on 7th day, which is then followed by a subsequent decrease on 14 th day. After the administration of thiamine hydrochloride to Group

III animal, the count was around 5.0 ± 1.87 million on 7th and 14th day. In the Group IV animals, the RBC count showed a decrease on 14th day when compared to Group I animal, but the count showed an elevation of 4.6 ± 0.11 million when compared with GroupII animal **(Table1 & Figure 1)**.

Hemoglobin Content

Hemoglobin Content count in Group I was 18.5 ± 0.23 gm%. After the administration of lead acetate, the count in Group II animals was decreased to 9.76± 0.04 gm% on 7th day, which is then followed by a decrease till 14 th day. After the administration of thiamine hydrochloride to Group III animal, the hemoglobin content was around 17.5 ± 0.026 gm% on 7th and 14th day. The haemoglobin content in Group IV animals, showed a decrease on 7th day, 15.0±0.018 gm% and a slight increase on 14th day, the value was 16.35± 0.0312 gm% **(Table 2 & Figure 2)**.

Estimation of Urea

The amount of urea in group I was 23.5±2.5 mg/ dl. After the administration of lead acetate, urea level in Group II animal, showed an elevation on 7th day (34.75 ± 1.09 mg), which is followed by a subsequent elevation on 14th day (40.5±1.32). After the administration of thiamine hydrochloride to Group III animal, the amount of urea was (26.2 ± 1.39 mg) on 7th and 14th day. In the Group IV animals, the amount of urea showed a decrease on 7th day (20.5± 1.11mg) and an increase to (30.7 ± 2.86mg) on 14th day **(Table 3 & Figure 3)**.

Estimation of Urea

The amount of urea present in GroupI was 7.5 ± 0.070g/dl .After the administration of lead acetate in Group II animals, the value of urea showed an increase to 10.25 ± 0.1118 on 7th day, and a decrease to 9.7 0.14g on 14th day. In Group III animals, after the administration of thiamine hydrochloride , the value of urea decreased to 8.5 ± 0.19 g on 7th day which is followed by a slight decrease to 7.75± 0.05g on 14th day. and 14th day. In the Group IV animals, the amount of urea showed a decrease on 7th day(20.5± 1.11mg) and an increase to (30.7 ± 2.86mg) on 14th day **(Table 3 & Figure 3)**.

Estimation of Protein

The amount of protein present in GroupI was 7.5 ± 0.070g/dl. After the administration of lead acetate in Group II animals , the value of protein showed an increase to 10.25 ± 0.1118 on 7th day, and a decrease to 9.7 0.14g on 14th day. In Group III animals, after the administration of thiamine hydrochloride , the protein value decreased to 8.5 ± 0.19 g on 7th day which is followed by a slight decrease to 7.75± 0.05g on 14th day. In Group IV animals, the protein content showed a decrease to 6.75 ± 0.070g on 7th day, which is followed by an increase to 7.57±0.072g on 14th day **(Table 4 & Figure 4)**.

Estimation of Uric Acid

The amount of uric acid in group I was 6.0 ± 0.070 mg. After the administration of lead acetate, uric acid level in Group II animal was increased to 6.5 ± 0.25mg on 7th day, which is followed by an increase to 7.1 ± 0.58 on 14th day, elevation on 14th day. In Group III animals, there was a decrease in uric acid level to 5.2 ± 0.2 mg on 7th day and a slight increase to 5.35 ± 0.12 mg on 14th day. In Group IV animals, there was a decrease in uric acid level on 7th day (5.5 ± 0.30 mg) and a moderate increase on 14th day to 6.07± 0.122mg **(Table5 & Figure 5)**.

Estimation of Triglyceride

The amount of Triglyceride in Group I was 11.31 ± 0.07 mg. After the administration of lead acetate, the value of triglyceride in Group II animals decreased to 31± 2.5 mg on 7th day, which is followed by a decrease to 30.7 ±1.45 on 14th day. After the administration of thiamine hydrochloride to Group III animal, the value of triglyceride was 40 ± 1.41mg on 7th day which is followed by a slight increase to 42.2 ± 2.16 mg on 14th day. In Group IV animal, the value showed 43.25 ± 2.0mg on 7th day, and then a significant decrease to 31.5 ± 1.11 mg on 14th day **(Table 6 & Figure 6)**.

Alkaline Phosphatase

The alkaline phosphatase level in GroupI was 11.31 ± 0.07 IU/L. After the administration of lead acetate in Group II animals, the value was 16.15 ± 0.07 IU/L on 7th day, which is then followed by an increase to 20.11 ± 0.007 IU/L on 14th day. In group III animal, the value was to 24.29± 0.25 IU/l on 7th day and a slight increase to 26.2 ± 0.07 IU/L on 14th day. In Group IV animals, the alkaline phosphatase level was 10.07 ± 0.09IU/L on 7th day, which is followed by an decrease to 8.06±0.01 IU/L on 14th day **(Table 7 & Figure 7)**.

Table 1: Total RBC Count of control and experimental rat (rattus norvegicus) exposed to lead acetate and thiamine hydrochloride

Parameter	Experiment (No. of days)	Control	Lead acetate	Thiamine Hydrochloride	Lead acetate+ Thiamine Hydrochloride
Total RBC count in million/cu.mm.	7	6.52 ± 0.180	3.55 ± 0.022	5.0 ± 1.87	4.62 ± 0.111
	14	6.50 ± 0.132	3.67 ± 0.273	4.87 ± 0.273	4.1 ± 0.180

Values are expressed as Mean ± SD.
Students 't' test
P<0.05, 0.01-significant in all experimental groups.

Table 2: Haemoglobin content of control and experimental animal exposed to lead acetate and thiamine hydrochloride

Parameter	Experimental (No. of days)	Control	Lead acetate	Thiamine Hydrochloride	Lead acetate+ Thiamine Hydrochloride
Haemoglobin content in g%	7	18.55 ± 0.234	9.76 ± 0.040	17.65 ± 0.026	15.02 ± 0.018
	14	18.7 ± 0.187	0.02 ± 0.092	16.56 ± 0.041	16.35 ± 0.0312

Values are expressed as Mean ± SD
Students 't' test
P<0.05, 0.01-significant in all experimental groups.

Table 3: Estimation of uric acid of control and experimental rat exposed to lead acetate and thiamine hydrochloride

Parameter	Experiment (No. of days)	Control	Lead acetate	Thiamine Hydrochloride	Lead acetate + Thiamine Hydrochloride
Uric Acid (mg/dl)	7	6.0 ± 0.070	6.52 ± 0.254	5.22 ± 0.15	5.5 ± 0.308
	14	6.15 ± 0.234	3.67 ± 0.273	5.35 ± 0.122	6.07 ± 0.122

Values are expressed as Mean ± SD
Students 't' test
P<0.05, 0.01-significant in all experimental groups.

Table 4: Estimation of protein of control and experimental rat exposed to lead acetate and thiamine hydrochloride

Parameter	Experiment (No. of days)	Control	Lead acetate	Thiamine Hydrochloride	Lead acetate + Thiamine Hydrochloride
Protein (g/dl)	7	7.5 ± 0.070	10.25 ± 0.1118	8.5 ± 0.192	6.75 ± 0.070
	14	7.25 ± 0.111	9.7 ± 0.147	7.75 ± 0.05	7.57 ± 0.072

Values are expressed as Mean ± SD.
Students 't' test
P<0.05, 0.01-significant in all experimental groups.

Table 5: Estimation of urea or control and experimental animal exposed to lead acetate and thiamine hydrochloride

Parameter	Experiment (No. of days)	Control	Lead acetate	Thiamine Hydrochloride	Lead acetate +Thiamine Hydrochloride
Urea (mg/dl)	7	23.5 ± 2.5	34.75 ± 1.09	26.25 ± 1.39	20.5 ± 1.11
	14	24 ± 1.3	40.5 ± 1.32	26.5 ± 2.69	30.75 ± 2.86

Values are expressed as Mean ± SD.
Students 't' test
P<0.05, 0.01-significant in all experimental groups.

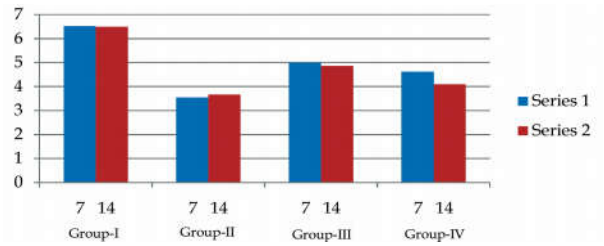
Table 6: Estimation of triglyceride of control and experimental rat (rattus norvegicus) exposed to lead acetate and thiamine hydrochloride

Parameter	Experiment (No. of days)	Control	Lead acetate	Thiamine Hydrochloride	Lead acetate + Thiamine Hydrochloride
Triglyceride (mg/dl)	7	44.25 ± 2.86	31 ± 2.54	40 ± 1.41	43.25 ± 2.046
	14	45.2 ± 1.786	30.75 ± 1.457	42.25 ± 2.165	31.5 ± 1.118

Table 7: Assay of alkaline phosphatase of control and experimental rat exposed to lead acetate and thiamine hydrochloride

Parameter	Experiment (No. of days)	Control	Lead acetate	Thiamine Hydrochloride	Lead acetate + Thiamine Hydrochloride
Alkaline Phosphatase (IU/L)	7	11.31 ± 0.070	16.15 ± 0.070	24.29 ± 0.251	10.07 ± 0.09
	14	10.9 ± 0.050	20.11 ± 0.007	26.2 ± 0.070	8.06 ± 0.0132

Values are expressed as Mean ± SD, Students 't' test, P<0.05,0.01-significant in all experimental groups.



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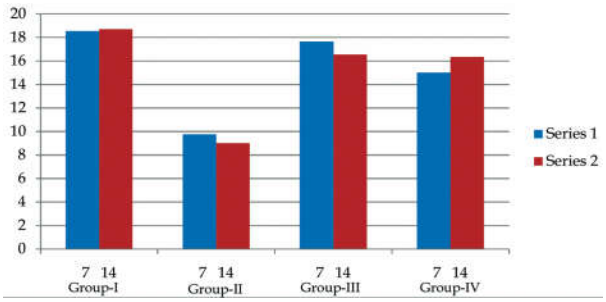

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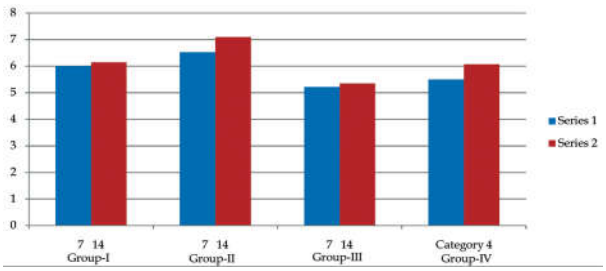

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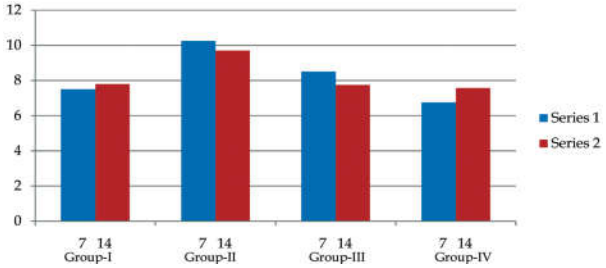

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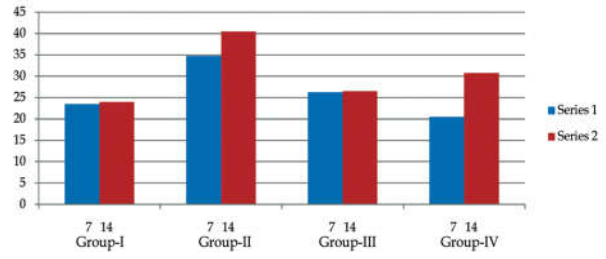

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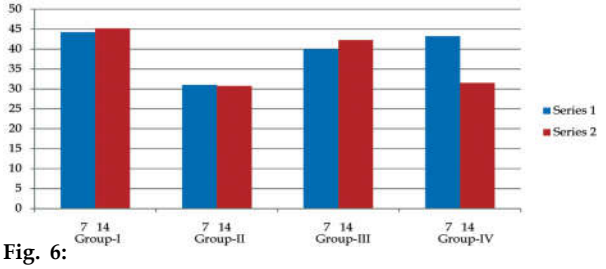

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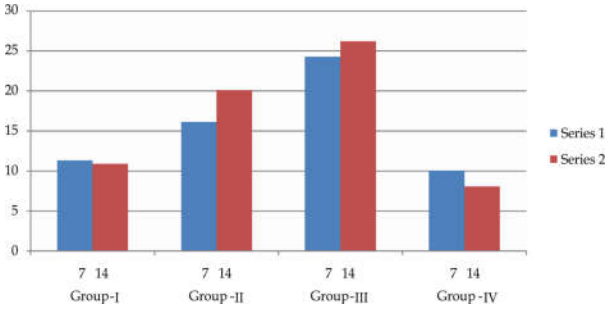

Fig. 6:

Discussion

Lead is a cumulative toxic element of increasing use in industry (Demayo et al). Several reports during past decades have revealed human population at high risk for metal toxicities through environment pollution (Dorn et al., Creason et al ., Lnadrigan et al .).

Industrial and experimental evidence showed lead to be one of the most hazardous metals and increasing use in industry (Goyer, et al).

In the present study with lead acetate, the RBC count was significantly decreased in experimental animals compared to control. Lead has been shown to produce acute haemolytic crisis which results in severe anaemia, haemoglobinuria and stippling of blood cells in mammals (Albahary). The animals which were dosed with thiamine against lead intoxication, maintain the normal RBC count. Thiamine has got erythropoietic activity, helps to maintain normal RBC count and improves circulation.

The haemoglobin content the lead induced rates showed a significant decreases. A fall in the concentration of haemoglobin might be due to depleted rate of haemoglobin synthesis. Depletion in the haemoglobin synthesis rate begins in the polychromatic normoblasts stage in the process of erythropoieses in bone marrow (Lewis). Impaired haemoglobin synthesis might be due to decrease in iron available or reduced iron uptake by developing erythrocytes. Similar results have also been obtained when mice were dosed with lead chromate (Chakravarthy et al).The thiamine antagonistic action lead intoxication, showed an elevation in haemoglobin content.

Lead intoxication also significantly augments the uric acid concentration. Uric acid is the end product of the catabolism of tissue nucleic acid, i.e, purine and pyrimidine bases metabolism (Sharma). The increments in uric acid concentration may be due to

the degradation of purines or to an increase of uric acid levels by either overproduction or inability of excretion. Similar results have been obtained in mice dosed with lead chromate (Chakravarthy et al). Thiamine administration resulted in decrease in uric acid level.

In the lead acetate induced animals, there was an elevated level of protein, followed by a decrease, which clearly depicts the disturbance in liver function depresses the serum protein production and thus results in hypoproteinemia in animals (Thompson et al). Presumably, the effects results have also been obtained in the lead loaded albino rats treatment with chelating agents and natural oils (Binger).

The elevation of urea occurs in the lead acetate induced rats. Urea is the principle product of protein catabolism. Enhanced protein catabolism together with accelerated amino acid deamination for gluconeogenesis is probably an acceptable postulate to interpet the elevated urea. Similar results have also obtained on the lead loaded albino rats and treatment with chelating agents and naturlal oils (Binger).

The triglyceride contents of serum decreased when compared to control in lead induced albino rats. Changes in triglyceride levels are reported in the diseases of liver and biliary tract (Singh et al). They are considered as valuable indicators of drug induced distribution of lipid metabolism, development of fatty liver and impairment biliary secretion (Sharma). In this study, the decreased triglyceride levels might be due to impairment of liver function .

In the present study, the alkaline phosphatase level was increased. It is an intercellular enzyme found primarly in the liver. It is also produced in small amounts by cells lining the intestine, the placenta and the kidney. The primary importance of measuring alkaline phosphatise is to check the possibility of liver damage. When liver and bile duct system are not functioning properly, this enzyme is not excreted through the bile duct as a result ,the enzyme is released into the blood stream. Similar results have also been obtained in mice dosed with lead chromate (Chakarvathy et al). Thiamine hydrochloride has got antagonistic action against lead acetate and hence enzymes level was slightly back to normal as it has got regularly role in all metabolism .

The haematological and serological findings in the present investigation suggests that lead acetate adversely affects the blood cells of the animals and causes improper functioning of the liver. On the other hand, thiamine acts an antagonistic and almost and reverses the condition .

It is necessary to generate awareness among people regarding the toxic effects of lead acetate, which is classified as a human carcinogen.

Conclusion

Lead, a heavy metal was selected though it is a non- essential element yet a carcinogen and hence it's effect was studied in the Swiss albino rats. The effect of heavy metal and vitamin B1 on the haematological and biochemical parameters was studied. Total count of RBC, haemoglobin content, triglyceride level decreased throughout the period of study, in lead induced animals. Protein, urea, uric acid levels are increased throughout the study when treated with lead acetate. On the contrary, Thiamine hydrochloride acts as an antagonist to the lead acetate and the results were reversed.

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Original Article

Journal of Practical Biochemistry and Biophysics
Volume 1 Number 2, July - December 2016

Histopathological Effects of Melatonin in Hyperammonemia Induced Wistar Rats

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Abstract

Exposure to ammonium acetate induces acute and chronic renal injuries as well as oxidative stress in rats. The aim of this study was to evaluate the effect of exogenous melatonin (MLT) treatment on ammonium acetate induced oxidative stress and hepatotoxicity in Wistarrats using biochemical parameters and histopathology. For histopathological evaluation, liver of all rats were excised and processed for light microscopy. Liver sections of ammonium acetate treated group showed changes in microanatomy. In contrast, these deleterious histopathological alterations resulting from ammonium acetate induced hepatotoxin were absent after MLT treatment in ammonium acetate treatment+MLT treated group of rats. In conclusion, our results demonstrated that MLT through its antioxidant activity effectively ameliorated ammonium acetate induced hepatotoxicity.

Keywords: Melatonin; Ammonium Acetate; Hepatotoxicity; Histopathology; Antioxidants.

Introduction

Ammonia is a catabolic product of protein and nitrogenous compounds that is formed in mammal and humans. At high levels, ammonia is neurotoxic, it affects the functions of the central nervous system, and leads to coma and death [Plum et al 1976]. Hyperammonemia caused by insufficient removal of ammonia in the liver [Meijer et al 1990] or portacaval shunting[Butterworth RF 1987], leads to an increase in ammonia level in the brain [Butterworth RF 1987], which is responsible for development of hepatic encephalopathy [Adams et al 1953, Butterworth 1995]. Ammonia intoxication impairs mitochondrial function [Kosenko et al 1997], which could lead to decreased ATP synthesis and also to increased formation of free radicals [Kosenko et al 1999]. The major toxic effects of ammonia likely involve changes in cellular pH and the depletion of certain citric acid cycle intermediates, in particular á -ketoglutarate. It has been reported that sustained hyperammonemia in mice leads to increased lipid peroxidation in liver and brain, reflecting an oxidative stress condition. Melatonin (N-acetyl-5-methoxy-tryptamine) is the chief secretory product of the pineal gland. It is present in virtually all organisms ranging from bacteria [Manchester et al 1995] to mammals [Poeggeler et al 1991]. Recently, it has been reported that a variety of other tissues including retina [Pang et al 1986], Harderian gland [Menendez et al 1987], ovary [Itoh et al 1997], testes [Tijimes et al 1996] and bone marrow [Conti et al 2000, Tan et al 1999] may also synthesize melatonin. Melatonin is and endogenous free radical scavenger [Tan et al1993] and a broad spectrum antioxidant [Reiter et al 1998]. It detoxifies a variety of free radicals and reactive oxygen intermediates including the hydroxyl radical, peroxy nitrite anion, singlet oxygen and nitric oxide [Reiter et al 1999]. Melatonin, which

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shows extreme diffusibility through membranes, is important for its scavenging action, since it could enter all cells and every subcellular compartment.

Melatonin (MLT) N- acetyl – 5- methoxytryptamine is a hormone found in all living creatures from algae (Caniato et al 2003) to humans, at levels that vary in a diurnal cycle.

MLT is produced by pinealocytes in the pineal gland. Melatonin participates in many important physiological functions, including anti-inflammatory (Cuzzocrea and Reiter 2002), also it is a powerful antioxidant that can easily cross cell membranes and the blood brain barrier (Hardeland 2005). MLT as an antioxidant is effective in protecting membranes lipids, nuclear DNA and protein from oxidative damage both invivo and invitro (Reiter et al, 1998a, Lussardi et al 2000, Altkinson et al 2003).

Melatonin may exert certain biologic effect such as the inhibition of stress – induced immuno-depression by augmenting the immune response (Maestroni ,1993).

Materials and Methods

Adult male Wistar rats (weighing 180–220 g), obtained from National Center for Laboratory Animal Sciences, Hyderabad, were kept at room temperature (32 ± 2°C) at L:D (12:12) cycles. All studies were conducted in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" [18]. Animals were randomized and separated into four groups (Group I – control, Group II – ammonium acetate-treated, Group III – ammonium acetate- and melatonin-treated, Group IV – melatonin-treated; n = 6 in each group). Food pellets (Kamadhenu Agencies, Bangalore, India) and water were available *ad libitum* to animals. Melatonin (salt form) was purchased from Sisco Research Laboratories Private Limited, Mumbai, India. Ammonium acetate and all other chemicals used in this study were of analytical grade. Group I animals served as controls. Group II animals were administered with ammonium acetate intraperitoneally (*ip*) (100 mg/kg) every day for 45 days [Hilgier et al 1990]. Group III animals were treated with ammonium acetate as Group II animals along with melatonin (5 mg/kg) (*ip*) [Liu 2000]. Group IV animals received melatonin (5 mg/kg) (*ip*) for 45 days. Biochemical determinations were performed after 45 days of ammonium acetate and/or melatonin administration. At the end of experimental period (45 days) animals from all the groups were sacrificed by cervical dislocation.

Preparation of Tissue Homogenate

The tissue was rinsed in cold physiological saline, cleaned of gross adventitial tissue, blotted dry and stored in a Biofreezer at -80^{0}C until analysed. Liver tissue was homogenized with Potter Elvejham homogenizer, 10% homogenates, were prepared in 6.7mμ phosphate buffer, pH7.4 and centrifuged at 10,000rpm for 10 min at 4^{0}C and the resultant supernatant was used for measurement of antioxidant enzymes.

Assay of Antioxidant Enzymes

Superoxide Dismutase Activity (SOD)

Liver SOD activity was assayed by the method of Kakkar *et al.,* (1984). Reaction mixture contained 1.2 ml of sodium pyrophosphate buffer (0.052 mM, pH 7.0), 0.1 ml of phenazine methosulphate (PMS) (186 μM), 0.3 ml of nitro blue tetrazolium (NBT) (300 μM). 0.2 ml of the supernatant obtained after centrifugation (1500 x g, 10 min followed by 10,000 x g, 15 min) of 10% liver homogenate was added to reaction mixture. Enzyme reaction was initiated by adding 0.2 ml of NADH (780 μM) and stopped precisely after 1 min by adding 1 ml of glacial acetic acid. Amount of chromogen formed was measured by recording color intensity at 560 nm. Results are expressed as units/mg protein.

Glutathione Peroxidase Activity (GSH-Px)

Glutathione peroxidase (GSH-Px) activity was measured by NADPH oxidation, using a coupled reaction system consisting of glutathione, glutathione reductase, and cumene hydroperoxide (Tappel, 1978). 100 μL of enzyme sample was incubated for five minutes with 1.55 ml stock solution (prepared in 50 mM Tris buffer, pH 7.6 with 0.1 mM EDTA) containing 0.25 mM GSH, 0.12 mM NADPH and 1 unit glutathione reductase. The reaction was initiated by adding 50 μL of cumene hydroperoxide (1 mg/ml), and the rate of disappearance of NADPH with time was determined by monitoring absorbance at 340 nm. One unit of enzyme activity is defined as the amount of enzyme that transforms 1 μmol of NADPH to NADP per minute. Results are expressed as units/mg protein.

Catalase Activity (CAT)

The activity of CAT was measured in the liver

using its perioxidatic function according

to the method of Johansson and Borg, (1988). 50 ìL potassium phosphate buffer (250 mM, pH 7.0) was incubated with 50 µl methanol and 10 µL hydrogen peroxide (0.27%). The reaction was initiated by addition of 100 ìL of enzyme sample with continuous shaking at room temperature (20°C). After 20 minutes, reaction was terminated by addition of 50 µL of 7.8 M potassium hydroxide. 100 µL of purpald (4-Amino-3-hydrazino-5- mercapto-1,2,4-triazole, 34.2 mM in 480 mM HCl) was immediately added, and the mixture was again incubated for 10 minutes at 20°C with continuous shaking. Potassium peroxidate (50 µL 65.2 mM) was added to obtain a colored compound. The absorbance was read at 550 nm in a spectrophotometer. Results are expressed as micromoles of formaldehyde produced/mg protein.

Reduced Glutathione (GSH)

Reduced glutathione (GSH) level in the liver was assayed following the method of Ellman (1959). The homogenate (720 µl) was double diluted and 5% TCA was added to it to precipitate the protein content of the homogenate. After centrifugation (10, 000 x g for 5 minutes) at 4°C the supernatant was taken, 5,5´-dithiolbis-2-nitrobenzoic acid (DTNB) solution (Ellman's reagent) was added to it and the absorbance was measured at 412nm on a spectrophotometer. A standard graph was drawn using different concentrations of standard GSH solution (1 mg/ml). With the help of the standard graph, GSH contents in the homogenates of the experimental animals were calculated.

Thiobarbituric Acid Reactive Substances

(TBARS)

The product of the reaction between malondialdehyde (MDA) and thiobarbituric acid

reactive substances (TBARS) were measured by the method of Ohkawa *et al*., (1997). The reaction mixture consisted of 0.2 ml of 8.1% sodium lauryl sulphate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid was added to 0.2 ml of 10% (w/v) of homogenate. The mixture was brought to 4.0 ml with distilled water and heated at 95°C for 60 minutes. After cooling with tap water, 1.0 ml distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v) was added and centrifuged at 3000 rpm for 10 minutes. The organic layer was taken out and absorbance of the clear upper (n-butanol) layer was measured using Shimadzu UV-1601 (Japan) spectrophotometer at 532 nm. TBARS were quantified using an extinction coefficient of 1.56 x 105 cm-1 M-1 and expressed as nmol of TBARS/ mg tissue protein. Tissue protein was estimated using Biuret method of protein assay and the liver MDA content expressed as nmol of MDA per mg protein.

Procedure for Histopathology

Liver tissues were excised from sacrificed animals, individually weighed, and thin liver slices were cut, fixed in 4% paraformaldehyde and were sequentially embedded in paraffin wax blocks. Tissue sections of 5 ìm thick were cut, and stained with hematoxylin eosin (H-E) and masson's trichrome (Suzuki and Suzuki, 1998) for conventional morphological evaluation, then examined under light microscope (BX50; Olympus, Tokyo). The images were obtained by a digital camera system (Pixcera Co., Osaka, Japan) attached to the microscope. The scoring was done as none (-), mild (+), moderate (++) and severe (+++).

Results

Statistical significance was evaluated using ANOVA followed by Least Significant Difference (LSD) test. Group II is compared with Group I (^{a} $p < 0.001$), Group III is compared with Group II ($p < 0.001$), Group IV is compared with Group I; ns not significant. The units for TBARS is n moles/10g tissue, GSH is mg/g tissue, SOD is 50% inhibition of NBT redn /min/mg/protein and CAT is µmoles of H_2O_2 consumed/min/mg/protein. GPx µg of GPx consumed/ min /mg/protein.

Table 1: Changes in the levels of TBARS and antioxidants in liver

	TBARS	GSH	SOD	CAT	GPx
Group I	1.77 ± 0.08	23.62 ± 1.97	4.94 ± 0.49	77.13 ± 7.43	12.03 ± 1.24
Group II	3.73 ± 0.32^{xxx}	11.30 ± 0.91^{xxx}	2.22 ± 0.09^{xxx}	34.28 ± 2.35^{xxx}	4.71 ± 0.25^{xxx}
Group III	2.42 ± 0.16^{xxx,a}	18.23 ± 0.79^{xxx,a}	4.40 ± 0.49^{xxx,a}	68.62 ± 3.32^{xxx,a}	8.78 ± 0.64^{xxx,a}
Group IV	1.73 ± 0.10^{ns}	23.14 ± 1.61^{ns}	5.03 ±0.51^{ns}	74.80 ± 6.06^{ns}	12.00 ± 1.16^{ns}

Table 2: Histopathological changes in liver

Microscopic Observation	Group I (Control)	Group II (Ammonium acetate treated)	Group III (Ammonium acetate& melatonin treated)	Group IV (Melatonin treated)
Hepatic damage	A	+++	A	A
Inflammatory collections	A	+++	A	A
Sinusoidal dilatation	A	+++	++	+

A - Absent
\+ - Present
++ - Moderately present
+++ - Highly present

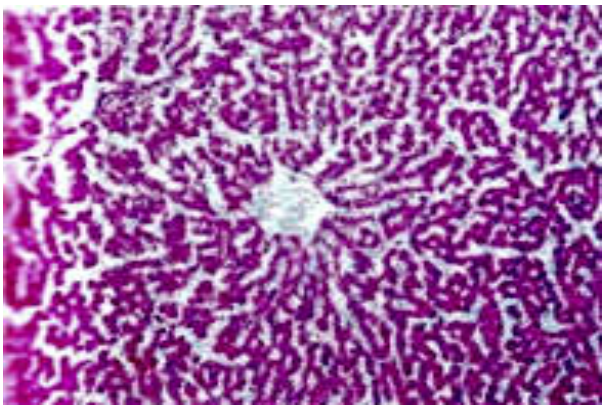

Fig. 1: Normal architecture of liver (control) H&EX 20

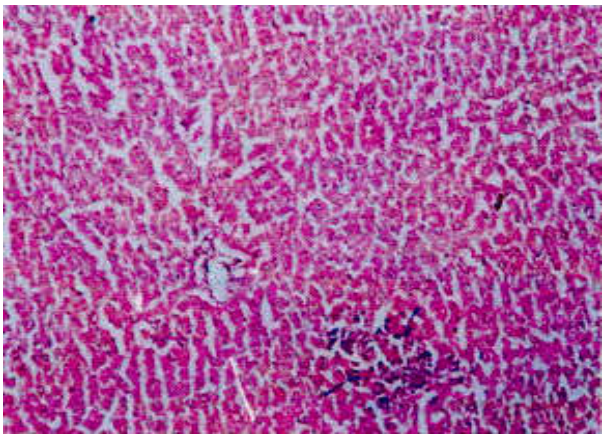

Fig. 2: Liver section showing focal hepatic damage with inflammatory collections (ammonium acetate treated) H&EX20

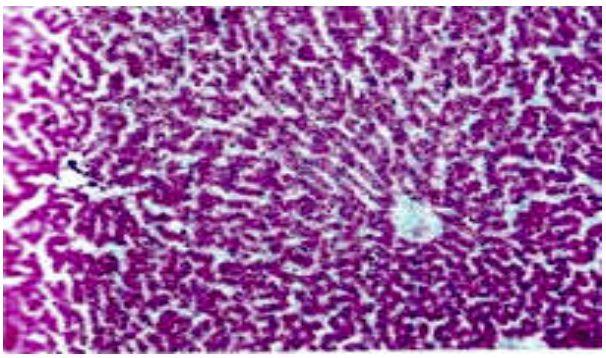

Fig. 3: Liver section showing marked reduction in inflammatory collection (ammonium acetate & melatonin treated) H&EX20

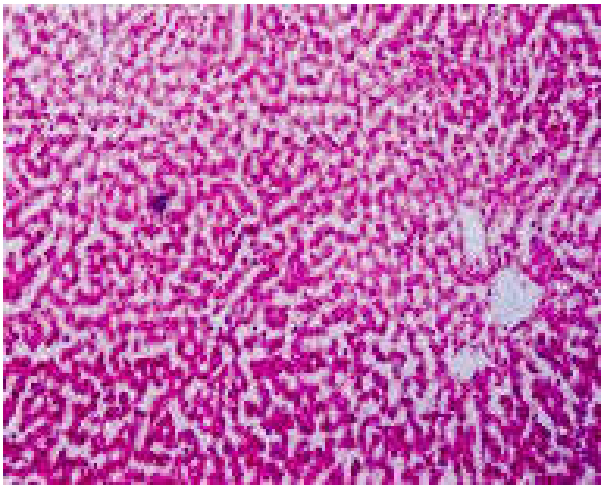

Fig. 4: Liver section of melatonin treated rat showing normal architecture

Discussion

Reports have shown that excess ammonia induces nitric oxide synthase which leads to enhanced production of nitric oxide, leading to oxidative stress and liver damage [Kosenko et al 1999, Schliess et al 2002]. The decrease in urea and ammonia in melatonin-treated rats maybe due to the antioxidant potential of melatonin. Melatonin has been proved to be an effective free radical scavenger [Reiter et al 1998, Tan et al 1993], by inhibiting the pro-oxidant enzyme nitric oxide synthase [Pozo et al1994].

Ammonia intoxication enhances lipid peroxidation and leads to the formation of free radicals [Kosenko et al 1997, Vidhya et al 2003]. This might account for the increased levels of TBARS (which is a measure of lipid peroxidation and an index of membrane oxidative damage) and decreased vitamin C and E levels in ammonium acetate-treated rats. Ammonia intoxication depletes the level of glutathione (GSH) [Kosenko et al 2000]. The protective capability of antioxidants against free radical-induced damage is increased if the scavenging molecule can be recycled. Mahal et al. (1999) reported regeneration of melatonin from the one-electron oxidized melatonyl radical by both ascorbate and urate. Melatonin directly scavenges hydrogen peroxide to form N1-acetyl-N2-formyl-

5methoxykynuramine, which, by the action of CAT forms N1-acetyl-5-methoxykynuramine [Tan et al 2000]. These biogenic amines could also scavenge hydroxyl radical and reduce lipid peroxidation.

In our study, the decreased activities of antioxidant enzymes (SOD and CAT, GPx and GSH) in ammonium acetate treated group may be due to the inhibition of these enzymes by nitric oxide. It is known that ammonia-induced inhibition of antioxidant enzymes is mediated by the activation of NMDA receptors of nitric oxide synthase and formation of nitric oxide which inhibits the activities of antioxidant enzymes [Kosenko et al 1999]. The indole moiety of the melatonin molecule is the reactive center of interaction with oxidants due to its high resonance stability and low activation energy barrier towards the free radical reactions. The methoxy and amide side chains also contribute significantly to melatonin's antioxidant capacity. The methoxy group in C5 appears to keep melatonin from exhibiting pro-oxidative capacity [Tan et al 2002].

Control Rats

The liver of control rats of all experimental groups examined by the light microscope showed that same normal hepatic structure. The structural unit of liver is the hepatic lobule, which is made up of cords or strands of cells forming a network around a central vein. The liver strands are alternating with narrow sinusoids. These sinusoids have irregular boundaries composed of a single layer of fenestrated endothelial cells and large irregularly phagocytic cells called as Kupffer cells. The hepatocytes are polyhedral in shape with relatively large size and granular cytoplasm. Each cell has a centrally located nucleus and binucleate hepatocytes were observed. Outside the hepatic lobules, collagen fibres are distributed and branches of portal vein, hepatic artery and bile duct are present.

Ammonium Acetate Treated Rats

Ammonium acetate treatment in rats results in loss of normal architecture of the liver with marked cytoplasmic vacuolation and sinusoidal dilatation was also observed. Microscopic foci of hepatic injury were observed with small hepatocytes admixed with macrophages, Cirrhosis and necrosis was also observed. Acute inflammation in blood vessels was detected.

Ammonium Acetate and Melatonin Treated Rats

The histological examination of liver of ammonium acetate and melatonin treated group showed less necrosis and less inflammation. It exhibited some histological regeneration and the reversed cirrhosis condition.

Melatonin Treated Rats

These rats showed no inflammation but a mild sinusoidal dilatation was observed in them. These rats showed no changes and were similar to that of control rats.

Conclusion

Melatonin is a very potent endogenous free radical scavenger. Receptor- dependent actions of melatonin, e.g. an antioxidative enzyme induction, oxide synthase which generates nitric oxide (ii) directly scavenging a variety of radicals and reactive oxygen species, (iii) inducing antioxidative enzymes which reduce steady state levels of reactive oxygen species. It reacts with highly toxic radicals and provides protection against oxidative damage. Melatonin acts as a primary non enzymatic antioxidative defense against the devastating action of free radicals (Abdel Wahab 1997). Melatonin has been proved to be an effective free radical scavenger [Reiter et al 1998, Tan et al 1993], by inhibiting the pro-oxidant enzyme nitric oxide synthase [Pozo et al 2000].

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Review Article

Journal of Practical Biochemistry and Biophysics
Volume 1 Number 2, July - December 2016

Herbal Medicinal Plant for Disease Including Tuberculosis: A Critique

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Abstract

Medicinal plant including herbal medicine/material contains more active analogues or active principles also called as natural products such as alkaloids, flavonoids, terpenoids, essential oils, flower absolutes. Herbal medicines have a strong traditional or conceptual base and the potential to be useful as drugs in terms of safety and effectiveness leads for treating Tuberculosis. World Health Organization (WHO) has made an attempt to identify all medicinal plants used globally and listed more than 20,000 species. According to the WHO more than 80% of the world's population realize on traditional herbal medicine for their primary health care. Although herbal medicine has existed since the dawn of time, our knowledge of how plants actually affect human physiology remains largely unexplored. Numbers of plants are claiming various medicinal uses and a lot of research is going for the treatment of tuberculosis. The potential of herbal medicinal plants to combat with the disease is increasing more and more attention as they have lesser side effects as compare to the routine medicines used. Plants like *Abutilon indicum*, *Allium cepa*, *Andrographis peniculata* are largely studied for Tuberculosis treatment and management. This innovative effort has been made for the enrichment and indirect contribution in Science and Technology.

Keywords: Medicinal Plant; Secondary Metabolites; Tuberculosis; Health and Disease.

Introduction

The traditional practice of herbal drugs comprise of medicinal plants, minerals, and organic matter; etc. Herbal drugs constitute only those traditional medicines, which primarily use plant preparations for therapy. The earliest recorded evidence of their use in Indians, Chinese, Egyptians, Greek, Roman and Syrian texts dates back to about 5000 years. The herbal/traditional medicine had been devised from rich traditions of ancient civilization and scientific heritage. Plant based active principles can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, and the others.

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India is one among the 25 hotspots of the richest and highly endangered eco-regions of the world. Plants have been the major source of drugs in Indian system of medicine and other ancient systems in the world. The Indian tradition has an ancient heritage of traditional medicine. Indian traditional medicines based on various systems including Ayurveda, Siddha, Unani and Homeopathy. With the emerging worldwide interest in adopting and studying traditional systems and exploiting their potential based on different health care systems, the evaluation of the rich heritage of traditional medicine is essential (Saha R, *et al.* 2011). Development of traditional herbal medicines into a modern drug of great therapeutic value is exemplified by the discovery of reserpine, a hypotensive drug from the roots of *rauwolfia serpentina* (Trease and Evans 1989). In year 2001, Iqbal *et. al* worked on Antimicrobial and phyto-chemical studies on 45 Indian medicinal plants

against multi-drug resistant human pathogens. There are list of plants which were traditional in therapeutic uses and medically important.

Table 1: Showing List of plants tested for anti tuberculosis action on Lowenstein Jensen Medium (adopted from Renu Gupta *et al.* 2010)

Botanical Name/Family	Common Name	Part used	Extracts
Acalypha indica L.*(Euphorbiacea)*	Acalypha	Leaf	Water Extract
Adhatoda vasica Nees. (Acanthaseae)	Vasaka	Leaf	Water Extract
Allium cepa L. (*Alliaceae)*	Onion	Bulb	Water Extract
Allium sativum L. (*Alliaceae)*	Garlic	Clove	Water Extract
Aloe vera L. (*Aloaceae*)	Aloevera	Leaf	Pure gel

Biological Properties of Medicinal Plants

Abutilon Indicum

Traditional potential medicinal usage

Tonic

Ancient Indian doctors including Charak and Sushruta made use of extract of the entire plant to promote vitality to their patients. They considered it a revitalizing nerving tonic and an aphrodisiac. The root was considered the better part for this purpose. In southern India the root is used for neurological disorders including treatment of hemiplagia, Bell's palsy, sciatica and debility (C.P. Khare, 2004).

Gastrointestinal Diseases

The demulcent property of extracts of *A. indicum* is made use in the treatment of various gastrointestinal disorders. The extract together with clarified butter treats diarrhoea and biliousness (C.P. Khare, 2004) (K.M.Nadkarni, 1976).

The high fiber content of the leaves and the mucilaginous property is taken advantage in the treatment of haemorrhoids. A decoction of the leaves is a good mouth wash for toothaches and gum ailments. Finely powdered seeds are given as a laxative.

Respiratory Diseases

The powdered flowers of *A. indicum* are a remedy for cough as recommended by the Ayurvedic and Unani systems. In the west it is the roots that is used to protect the respiratory system. A decoction of it is given to treat bronchitis and chest pains.

Genito-urinary Diseases

Western herbal medicine believed *A. indicum* has protective properties towards the urinary system. The roots are considered diuretic and its decoction is given for all types of dysurias. It is the medicine prescribed by Unani practitioners for strangury, polyuria and haematuria. The seeds on the other hand were given to treat impotency and spermatorrhoea. The seeds are distinctly useful in gonorrhoea, gleet and chronic cystitis. The leaves and roots too could be used to treat gonorrhoea and other forms of urethritis.

Other uses

The powdered herb was used for meno-metrorrhagia. The leaves are used to treat various skin diseases including wounds and ulcers while the seeds are used for leprosy.

Pharmacological Study

Antidiabetic Activity

Seetharam *et al.* (2002) found that both the aqueous and alcoholic extracts of the leaves of *A. indicum* had significant hypoglycaemic effects in normal rats 4 hours after administration. Adisakwattan *et al.* (2009) further looked into the alcoholic (methanol) leaf extract's hypoglycaemic activity and observed that the extract was able to suppress the postprandial hyperglycaemia by inhibiting a-glucosidase and sucrase activities in the intestine. The effects of aqueous leaf extract showed that in addition to inhibition of β-glucosidase and sucrase, this extract was found to stimulate the insulin production by the b-cells of the pancreas (Krisanapun C, *et al.* 2009) .

Hepatoprotective activity

The aqueous extract of *A. indicum* showed significant hepatoprotective activity when it was found the it was able to reduce biochemical parameter changes as a result of exposure of experimental rats to CCl_4 and Paracetamol (Porchezhian E, 2005).

Analgesic Activity

Eugenol was isolated from *A. indicum* using

bioactivity guided isolation. Eugenol was found to possess the ability to inhibit acetic acid induced writhing in mice and prolonged tail flicking time (Ahmed M, *et al.*2000) this indicates that the extract posses significant analgesic activity via both peripheral and central mechanism.

Immunomodulatory Activity

Aqueous and ethanol extracts of the leaves of *A. indicum* were assessed for the immunomodulatory activity using various animal models. The results showed that there was significant increase in the production of circulating antibody titre in response to sheep red blood cells, increase in both primary and secondary haemagglutination antibody, a significant potentiation of delayed type hypersensitivity reaction.

There was also a significant increase in percentage of neutrophils adhesion to nylon fibre and phagocytic activity. The results indicate that the extracts were able to trigger both specific and non-specific immunological activity and that this could be attributed to the flavonoids content (Dashputre N. L. *et al.* 2010).

Antimicrobial Activity

Methanolic extract of the leaves of *A. indicum* was found to have remarkable antifungal activity against *Trichophyton rubrum.* This activity may be due to the presence of flavonoids in the extract especially Quercetin (Rajalakshmi Padma Vairavasundaram, 2009).

Antioxidant Activity

Yasmin *et al.* (2010) studied the antioxidant activity of organic solvent extracts of aerial parts and roots of *A. indicum*. They found that all the extracts contain both slow reacting and fast reacting antioxidant.

Chemical Constituents of the Plant

Gallic acid, asparagine, fructose, galactose, glucose, beta-sitosterone, vanillic acid, p-coumaric acid, p-hydroxybenzoic acid, caffeic acid, fumaric acid, p-beta-D-glycosyloxybenzoic acid, leucine, histidine, threonine, serine, glutamic acid, aspartic acid and galacturonic acid, alantolactone, isoalantolactone, threonine, glutamine, serine, proline, glycine, alanine, cycteine, methionine, isoleucine, valine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine.

Allium Cepa

Traditional Medicinal Application

Due to the anti-inflammatory agents in onions they help reduce the severity of symptoms associated with conditions such as the pain and swelling of the osteo- and rheumatoid arthritis, the allergic inflammatory response of asthma, and the respiratory congestion associated with common colds. The onions anti-inflammatory effects are not only due to their vitamin C and quercetin, but other active components called isothiocyanates have made onions a good ingredient for soups and stews during cold and flu season. WHO recommends the use of fresh onion extracts for treating coughs, colds, asthma, bronchitis and also relieving hoarseness. The World Health Organization also supports the use of onions for the treatment of appetite loss and preventing atherosclerosis.

Pharmacological Study

The fleshy bulb that grows below the ground is used medicinally as well as for food. Onion is believed to have a positive effect on the circulatory system. It has been used as a diuretic to reduce swelling. It is also thought to help reduce arteriosclerosis by lowering blood cholesterol levels and preventing the formation of blood clots. Onion has been used to treat diabetes and is reputed to lower blood sugar levels. Externally; fresh onion juice is used to prevent bacterial and fungal infections. It can be applied to wounds and stings on the skin, used to remove warts, used to stimulate hair growth, and even used to reduce unwanted skin blemishes. Warm onion juice dropped in the ear is said to help relieve earache. Baked onion is used to draw pus from abscesses. The onion also may be of benefit in cardiovascular disease, as it possesses hypo-lipidemic effects and has anti-platelet actions, retarding thrombosis. But certain lipid-reducing and blood pressure-lowering effects in humans have not yet been clinically proven. Some studies have been performed concerning diabetes treatment by onion with promising results in animal experimentation. Although more research is needed on the use of onion as a treatment for diabetes in humans, many articles describe onion's benefits in improving glucose levels. The onion also is a proven antioxidant and may be helpful in treating certain cancers. Green Onion as Alternative Medicine. It contains vitamin A and C. The white part of it has calcium, too. It is a good appetizer (K. P. Sampath Kumar *et al.* 2010).

Health benefits of Onion include substantial relief from number of diseases such as common cold,

asthma, bacterial infections, respiratory problems, angina, and cough. Onions are also known to repel blood thirsty insects. In other words, you may say that onions are godsend gifts. Onions are known to possess curative value from ancient time.

Hepatoprotective Activity

It was reported that *A. cepa* leaf extract also can significantly restored the elevated AST, ALT and ALP enzyme levels to the normal levels (S.F. Ige, *et al.* 2011). Recently, Riyaz Shaik *et al.* (2012) demonstrated that *A. cepa* leaves protected hepatocytes by preventing the release of these 3 enzymes. The study of B.Ogunlade *et al.*(2012) demonstrated that administration of *A. cepa* by rabbits with alcohol abuse remarkably reduced serum levels of liver biomarker enzymes. In the study, performed by K. Eswar Kumar *et al.* 2013, demonstrated that *A. cepa* aqueous bulb extract had reduced levels of AST, ALT and ALP which were elevated by ethanol administration.

Anti Tuberculosis and Antimicrobial Activity

In 2009 Chitra Shenoy *et al.* described about the preliminary phytochemical investigation and wound healing activity of *Allium cepa* Linn (Lilaceae) Renu Gupta *et al.* 2010 described that exhibited activity against MDR isolates of *M. tuberculosis* in her research entitled Anti-tuberculosis activity of selected medicinal plants against multi-drug resistant *Mycobacterium tuberculosis* isolates. Jain RC, 1993 and Ratnakar P, Murthy PS,1996 in their study confirms that the *Allium cepa* has the anti-tubercle activity. *Allium cepa* have the anti tubercle activity as studied by Sivakumar A and Jayaraman G in 2011.

In the year 1999 a monograph was published by WHO on selected medicinal plants VOLUME 1 in which *A. cepa* is considered to have antibacterial property against tuberculosis. N. Benkeblia (2004) discussed on Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). Ivan A. Ross (2005) in his book Medicinal Plants *of the* World Volume 3 *Chemical Constituents, Traditional and Modern Medicinal Uses* describe the medicinal property of *A. cepa* for tuberculosis.Vikrant Arya (2011) in his "A Review on Anti-Tubercular Plants" describe the antibacterial property for tuberculosis, later on in 2013 Soundhari C and Rajarajan S worked *in vitro* screening of lyophilized extracts of alpinia galangal L. and *Oldenlandia umbellata L.* for anti-mycobacterial activity and described Water and ethanolic extracts of selected medicinal plants (*A. sativum* -bulb, *A. cepa* -tissue, *S. aromaticum* –flowerbud, *C. verum* -bark) observed to have anti-TB activity for *M. tuberculosis* H37Ra by Microtiter Alamar Blue assay and confirmed to (Sivakumar A and Jayaraman G 2011). M. Muthuswamy (2013) described that *Allium cepa* and *Aloe vera* were selected to test their activity further against MDR trains of *M. tuberculosis*, while selection of *Acalypha indica* was based on its ethno-medicinal uses in respiratory disorder. *A. cepa* was selected on the basis of knowledge that *A. sativum* has anti-tuberculosis activity; therefore other species of *Allium* might also have anti- tuberculosis activity in his study of screening of anti-tubercular activity of some medicinal plants from Western Ghats of India. Water and ethanolic extracts of selected medicinal plants (*A. sativum* -bulb, *A. cepa* -tissue, *S. aromaticum* –flowerbud, *C. verum* -bark) observed to have anti-TB activity for *M. tuberculosis* H37Ra by Microtiter Alamar Blue assay (Sivakumar A and Jayaraman G 2011). Onion has the property of anti-cancer and anti-oxidant because of presence of phenols and flavonoids. It is rich in proteins, carbohydrates, sodium, potassium and phosphorous. It has been reported to be an anti-bacterial, antiviral, anti-parasitic, anti-fungal and has hypoglycemic, antithrombotic, anti-hyperlipidemic, anti-inflammatory and antioxidant activity (Parmar Namita and Rawat Mukesh, 2012).

Antioxidant Activity

Phenolic compounds are the major group contributing to the antioxidant activity of vegetables, fruit, cereals and other plant-based materials. The antioxidant activity of the compounds is partly due to one electron reduction potential that is the ability to act as hydrogen or electron donors (Chan *et al.* 2007).

The findings of the study by Siti Fairuz Che Othman *et al.*(2011) have shown that red onion (*A. cepa L.*) possesses higher Total phenolic content (TPC) than garlic (*A. sativum L.*).

Chemical Constituents of the Plant

Onions not only provide flavor, they also provide important nutrients and health-promoting phytochemicals. High in vitamin C, onions are a good source of dietary fiber, and folic acid. They also contain calcium, iron, and have a high protein quality (ratio of mg amino acid/gram protein). Onions are low in sodium and contain no fat. Onions contain quercetin, a flavonoid (one category of antioxidant compounds). Antioxidants are compounds that help delay or slow the oxidative damage to cells and tissue

of the body. Studies have indicated that quercetin helps to eliminate free radicals in the body, to inhibit low-density lipoprotein oxidation (an important reaction in the atherosclerosis and coronary heart disease), to protect and regenerate vitamin E (a powerful antioxidant), and to inactivate the harmful effects of chelate metal ions. (http://onions-usa.org/all-about-onions/onion-health-research[online] accessed on 20-05-2014)

Pheonix Dectylefera (Date palm)

Traditional Medicinal Application

The different parts of this plant is majorly used in conventional medicine for treatment of various disorders like memory instability, fever, pain, stammering, nervous disorders (Nadkarni K.M. 1976). Fatmah H (2013) discussed on Effect of Tempeh Dates Biscuits on Nutritional Status of Preschool Children with Tuberculosis.

Pharmacological Study

Antifungal Activity

Antifungal activity of water, acetone and methanol extracts of leaves and pits of *phoenix dectylifera* Linn. were evaluated against several pathogenic fungi by Bokhari NA *et al.* in 2012. Except water extract acetone and methanol extracts showed varying degree of growth inhibitors against *Fusarium oxysporum, Fusarium species and Fusarium solani.*

Anti-hyperlipedemic Activity

Coronary heart disease is related to decrease in the concentrations of high density lipoprotein cholesterol(HDL) and increase of low density lipoprotein cholesterol (LDL). Salah and Al miaman (2013) have reported that feedeing of defatted date seed flour containing diet at 1.5%, 2.5%, and 5.2% to rats reduced the plasma triglycerides, total cholesterol and low density liporoteins.

Anti-Ulcer Activity

Pre-treatment with date fruit ethanolic and aqueous extracts at a dose of 4 ml/kg for 14 days markedly ameliorated the ulcer index, histological indices such as necrosis, haemorrhage, congestion and oedema in stomach sections and biochemical levels of some enzymes such as gastrin in plasma and mucin and histamine in gastric mucosa of ethanol-induced gastric ulceration in rats (Al-Qarawi A, *et al.*2005). This support to the local folk medicinal claim that dates may be useful to humans with ulcers.

Anticancer Activity

The polysaccharides (glucans) prepared from the date fruits exhibited a dose dependant anticancer activity with an optimum activity at a dose of 1 mg/kg in tumour induced by subcutaneously transplanting allogenic solid Sarcoma-180 tumor cells into the right side of female CD1 mice. (Ishurda O, *et al.* 2005) This research validated the traditional claim of date fruits to be used against various kinds of tumors.

Anti-Diarrhoeal Activity

Aqueous extract of *Phoenix dactylifera L* at doses of 3, 6 and 12 mg/kg produced a statistically significant reduction in both castor oil induced intestinal transit and frequency of diarrhoea in rat (Abdulla Y, Al – Taher, 2008). These properties may explain the rational for the effective use of the plant as an anti-diarrhoeal agent in traditional medicine.

Effect on Gastrointestinal Transit

Water and ethanolic extracts from date flesh and date pits at doses of 0.01, 0.02 and 0.04 ml/kg showed a dose dependant increase in the gastrointestinal transit time. While water extract from dialyzed date flesh extract induced a dose-dependent decrease the gastrointestinal transit time (Al-Qarawi AA**,** *et al.* 2003). The possible reason for this may be the method based extraction of pirticular component which could be valuable towards respective clinical conditions.

Effect on Reproductive System

Oral administration of date palm fruit suspensions at doses of 120 and 240 mg/kg improved the sperm count, motility, morphology, and DNA quality with a concomitant increase in the weights of testis and epididymis (Bahmanpour S, *et al.* 2006). Moreover, date extracts have been shown to increase sperm count in guinea pigs and to enhance spermatogenesis and increase the concentration of testosterone, follicle stimulating hormone (FSH) and luteininzing hormone (LH) in rats (Elgasim EA *et al.* 1995). This study suggests its usefulness in solving infertility problems in males. El-Desoky and his co-workers (1995) looked into the effect of date palm pollen grains (*Phoenix dactylifera*) on sexual hormonal balance, cholesterol, total lipids, total protein, albumin, globulin, and liver functions in control male and female rats, and castrated and ovariectomized rats. Their findings showed a decline in serum

testostrerone level; in control male rats, but a slight increase was detected in the castrated rats. Similarly, serum estradiol content was elevated in both control and ovariectomised rats. Progesterone level; however, decreased in control female rats, and was slightly increased in ovariectomized rats, with slight increase of serum FSH and LH in both normal and ovariectomised female rats. Pollen grains significantly increased serum globulin, total protein and total lipids in ovariectomized rats. Also serum ALP activity was increased in normal male rats. There was an increase in serum plasma glutamate pyruvate (GPT) activity in normal male, ovariectomized female and castrated rats, and similarly, glutamate oxaloacetate transaminase (GOT) activity was also increased in overiectomized female and normal male rats. All these GPT and GOT values were still within the normal range in rats (Reshod A, Al-Shagrawi, 1998).

Nephroprotective Activity

Al-Qarawi (2008) *et al*. studied the effect of the extracts of the flesh and pits of Phoenix dactylifera on gentamicin induced nephrotoxicity in rats was investigated in which the significantly reduced the increase in plasma creatinine and urea concentrations induced by gentamycin nephrotoxicity and ameliorated the proximal tubular damage. Antioxidant components in the date (e.g., melatonin, vitamin E, and ascorbic acid) were suggested to be the basis of the nephroprotection. **(**http://ispub.com/IJPHARM/7/1/8733[online]accessed on 24-05-2014**)**

Hepatoprotective Activity

Pre and post treatment with aqueous extract of date flesh or pits significantly reduced CCl4 induced elevation in plasma activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), Alkaline phosphatase (ALP) enzymes and bilirubin concentration and ameliorated morphological and histological liver damage in rats. This study suggests that CCl4-induced liver damage in rats can be reversed by treatment of extracts from date flesh or pits. Moreover it can also be used prophylactically as a dynamic liver support GT (Al-Qarawi AA, *et al.* 2004), enzymes and plasma concentration of bilirubin but also exhibited an enormous increase in the reduced serum levels of testosterone, alpha fetoprotein (AFP) and glucose in the thioacetamide induced chirrotic rats. The extracts also showed significant reduction in oxidative stress evidenced by significant rise in the hepatic malonaldehyde (MDA) levels and decline in hepatic glutathione levels by normalising them. In another study the date flesh or pit extracts not only normalised the elevated plasma activities of AST, ALT, ALP, lactate dehydrogenase (LDH).

H.A. Abdelrahman *et al.* in 2012 also studied the protective effect of dates (*Phoenix dectylifera*) on carbon tetrachloride induced hepatotoxicity in Dogs.

Antioxidant Activity

Phytochemicals from fruits have been shown to possess significant antioxidant capacities that may be associated with lower incidence and lower mortality rates of degenerative diseases in human (Javanmardi J, *et al.* 2008).

Various *in vitro* and *in vivo* antioxidant activites have been carried out on various extracts of different parts of *Phoenix dectylefera*. Studies conducted on antioxidant activity and phenolic content of various fruits of *Phoenix dactylifera* cultivated in Iran, Algeria and Bahrain demonstrated a linear relationship between antioxidant activity and the total phenolic content (TPC) of date fruit extract (Allaith, Abdul AA, 2005). Aqueous date extract was found to inhibit significantly the lipid peroxidation and protein oxidation and also exhibited a potent superoxide and hydroxyl radical scavenging activity in a dose-dependent manner in an in vitro study (Dammak I *et al.* 2007). Methanolic extract of Phoenix dectylefera seeds showed a significant increase in plasma levels of vitamin C, E and A, β-carotene and significant decrease in the elevated MDA levels due to the lipid peroxidation in adjuvant arthritis in rats. (Mohamed DA *et al.* 2004). These findings suggests its possible use in diseases such as scurvy, ataxia and night blindness caused due to the deficiency of vitamins C, E and A respectively. Date seed oil was found to limit oxidative injuries induced by hydrogen peroxide in human skin organ culture which confirmed the potent free radical scavenging activity of the plant (Dammak I *et al.* 2007).

Studies indicate that the aqueous extracts of dates have potent antioxidant activity (Mansouri *et al.* 2005). The antioxidant activity is attributed to the wide range of phenolic compounds in dates including p-coumaric, ferulic and sinapic acids, flavonoids and procyanidins (Gu *et al.* 2003 and Al-Farsi *et al.* 2005).

Antimicrobial Activity

The anti bacterial study on *Pheonix dectylefera* carried out by Ramesa Shafi Bhat *et al.* and Saleh FA, *et al.* observed the extracts of fruit showed the

antibacterial activity against the human pathogen such as *S.aureus, S. pyogenes, B.subtillis*, *E. coli* and *P. aeruginosa* and *Staphylococcus saprophyticus* (Ramesa Shafi Bhat *et al.* 2012, Saleh FA).

Chemical Constituents of the Plant

The fruits of *Phoenix dactylifera* contain different chemical compounds such as saturated and unsaturated fatty acids, Zinc (Zn), Cadmium (Cd), Calcium (Ca), and potassium (K). Saturated fatty acids include stearic and palmitic acid and unsaturated fatty Acids contain linoleic and oleic acids which could inhibit 5-α reeducates enzyme (Shariati *et al.* 2008). Also, dates contain at least six vitamins including a small amount of vitamin C, and vitamins B1 (thiamine), B2 (riboflavin), nicotinic acid (niacin) and vitamin A (Al-Shahib and Marshall, 1993). Dates contain a high percentage of carbohydrate (total sugars, 44-88%), protein (2.3-5.6%), fat (0.2-9.3%), essential salts and minerals, vitamins and an elevated proportion of dietary fiber (6.4-11.5%) (El Hadrami *et al.* 2009). They also contain oil in the flesh (0.2-0.5%) and the seed (7.7-9.7%). The seed represents 5.6-14.2% of the entire fruit weight. Dates are very rich in vitamins, especially β-carotene (vitamin A), thiamine (B1), riboflavin (B2), niacin, ascorbic acid (C) and folic acid (folacin) (El Hadrami, 2009). Some of these vitamins provide 10-50% of the daily recommended intake of an adult. Ripe fruits were reported to contain a substantial amount of carotenoids including lutein and various forms of β-carotene and minor carotenoids. The contents vary with the cultivar and stage of ripeness, with the total content of carotenoids decreasing towards the final ripening stages and in storage.

Andrographis Peniculata

Traditional Medicinal Application

Andrographis paniculata is a plant that has been effectively used in traditional Asian medicines for centuries. Its perceived "blood purifying" property results in its use in diseases where blood "abnormalities" are considered causes of disease, such as skin eruptions, boils, scabies, and chronic undetermined fevers. Controlled clinical trials report its safe and effective use for reducing symptoms of uncomplicated upper respiratory tract infections. Since many of the disease conditions commonly treated with *A. paniculata* in traditional medical systems are considered self-limiting, its purported benefits need critical evaluation. (*A. paniculata* http://altmedrev.com/publications/16/1/66.pdf[online] accessed on 24-05-2014)

A. paniculata contains diterpenes, lactones, and flavonoids. Flavonoids mainly exist in the root, but have also been isolated from the leaves. "The aerial parts contain alkanes, ketones, and aldehydes. Although, it was initially thought that the bitter substance in the leaves was the lactone andrographolide, later investigations showed that the leaves contained two bitter principles–andrographolide and a compound named kalmeghin. Four lactones – chuanxinlian A (deoxyandrographolide), B (andrographolide), C (neoandrographolide) and D (14-deoxy-11, 12-didehydroandrographolide) – were isolated from the aerial parts in China. *A. Peniculata* contains diterpene glucoside (deoxyandrographolide- 19beta-D-glucoside) has been detected in the leaves (Weiming C, 1982) and six diterpenoids of the ent-labdane type, two diterpene glucosides and four diterpene dimers (bis-andrographolides A, B, C, and D) have been isolated from aerial parts (Matsuda T,1994).

A. paniculata is extensively used as a hepatostimulant and hepatoprotective agent in Indian systems of medicine (Trivedi NP, *et al.* 2001). *A. paniculata* is also an ingredient in several polyherbal preparations used as hepatoprotectants in India, (Ram VJ, 2001) one of which has been reported as efficacious in chronic hepatitis B virus infection (Rajkumar JS, 2007). S.K. Mitra *et al.* (1998) described Protective effect of HD-03, a herbal formulation, against various hepatotoxic agents in rats. Rakshamani Tripathi *et al.* (2005) worked on Modulation of oxidative damage by natural products. Shahid Akbar, MD, PhD (2011) reviewed the *Andrographis paniculata* in his monograph about the Pharmacological Activities and Clinical Effects. G. Shivaprakash (2011) described the Evaluation of *Andrographis paniculata* leaves extract for analgesic activity. Anil Kumar (2012) worked on *Andrographis paniculata* and review on king of bitter (Kalmegh with the result *Andrographis paniculata* nees (Acanthaceae) is a medicinal plant traditionally used for the treatment of anti-inflammatory, antibacterial, antioxidant, antiparasitic, antispasmodic, antidiabatic, anti-carcinogenic, antipyretic, antidiarrhoeal, hepatoprotective, nematocidal.

Pharmacological Study

Andrographis paniculata has been reported as having antibacterial, antifungal, antiviral, choleretic, hypoglycemic, hypocholesterolemic, adaptogenic, anti-inflammatory, emollient, astringent, diuretic, carminative, anthelmintic, antipyretic, gastric and liver tonic. It is also recommended for use in cases of leprosy, gonorrhea, scabies, boils, skin eruptions,

and chronic and seasonal fevers. Juice or an infusion of fresh leaves is given to infants to relieve griping, irregular bowel habits, and loss of appetite. Leaves and root are also used in general debility, during convalescence after fevers, for dyspepsia associated with gaseous distension, and in advanced stages of dysentery. In China, the herb derived from the leaves or aerial parts of Andrographis *paniculata* is known as Chuanxinlian, Yijianxi or Lanhelian. It is described as bitter and cold, is considered to be antipyretic, detoxicant, anti-inflammatory, and detumescent, and is thought to remove "pathogenic heat" from the blood. *Andrographis paniculata* is used for the treatment of pharyngolaryngitis, diarrhea, dysentery, and cough with thick sputum, carbuncle, sores, and snake bites. Various preparations and compound formulas of the herb have been used to treat infectious and non-infectious diseases, with significant effective rates reported for conditions such as epidemic encephalitis B, suppurative otitis media, neonatal subcutaneous annular ulcer, vaginitis, cervical erosion, pelvic inflammation, herpes zoster, chicken pox, mumps, neurodermatitis, eczema, and burns. A primary modern use of *Andrographis paniculata* is for the prevention and treatment of the common cold. It appears to have antithrombotic actions, suggesting a possible benefit in cardiovascular disease. Pharmacological and clinical studies suggest the potential for beneficial effects in diseases like cancer and HIV infections.

Hepatoprotective Activity

Soumendra Darbar *et al.* (2009) described the antioxidant and hepatoprotective action of *Andrographis paniculata* by induction of hepatotoxicity in rats using single doe of Diclofan (DIC). The results of the study concluded that the hepatoprotective effect of aqueous ethanol extract of *Andrographis paniculata* against DIC –induced acute toxicity is mediated either by preventing the decline of hepatic antioxidant status or due to its direct radical scavenging capacity.

Antimicrobial Activity

Andrographis paniculata has been extensively shows antimicrobial and antiparasitic activities such as bacteria, viruses, and parasites. Singha *et al.* (2007) reported significant antibacterial activity of an aqueous extract and attributed it to the combined effect of andrographolides and arabinogalactan proteins. A similar conclusion was reached by Zaidan *et al.* (2005) found crude aqueous extract of leaves exhibit significant antimicrobial activity against gram positive *S. aureus*, methicillin-resistant *S. aureus* and gram-negative *Pseudomonas aeruginosa*, but had no activity against *Escherichia coli* or *Klebsiella pneumoniae*. Andrographolide, neoandrographolide, and 14-deoxy-11, 12-didehydroandrographolide are reported to be viricidal against herpes simplex virus 1 (HSV-1) without having any significant cytotoxicity at viricidal concentrations. Alcoholic extract of the rhizome was reported to possess significant *in vitro* activity against *Ascaris lumbricoides* and chloroform extract completely inhibited malarial parasitic growth within 24 hours of incubation at a concentration of 0.05 mg/mL. Same inhibition was achieved in 48 hours with methanol extract at a concentration of 2.5 mg/mL. (Anil Kumar *et al.* 2012)

Antioxidant Activity

The main and most interesting biological constituent of *A. paniculata* herb (aerial part) is a group of diterpene lactones belonging to the ent-labdane class, present in both free and glycosidic forms, and named andrographolides (Lim, J. C, *et al.*2012). Andrographolide is the bitter principle, a colourless, neutral crystalline substance, was first isolated by Boorsma from different parts of *Andrographis paniculata*. In 1911 Gorter proved that it is structurally a lactone and named it andrographolide (in the Chinese literature it is sometimes cited as andrographis B). (María A *et al.* 2013).

The second diterpene isolated from *A. paniculata* was the minor non-bitter constituent neoandrographolide, which was first described by Kleipool in 1952. The structure of neoandrographolide was described as a diterpene glucoside and its amount in the plant is around 0.5-1%. The main preclinical effects are anti-inflammatory (Parichatikanond *et al.* 2010, Batkhuu *et al.* 2002, Liu, J *et al.*2007), chemosensitizer, anti-herpes-simplex virus and antioxidant.

Chemical Constituents of the Plant

The plant is widely used in ayurvedic and homeopathic systems of medicines. The medicinal value of this plant is due to the presence of active ingredients *viz* andrographolide and neoandrographolide which are derivatives of diterpenoids. It prevents oxidative damage and inhibits binding to toxic metabolites to DNA.

Conclusion

Worldwide, Research and innovation are now on fast track for new bioactive compounds from herbal medicinal plants. Now the scientists targeting on specific disease like tuberculosis so as to eradicate all cases. In this direction, medicinal plants proffer a great expect to complete these desires and have been used for curing diseases for many centuries. These have been used extensively as pure compounds or as a crude material. Only a few plant species have been thoroughly investigated for their medicinal properties. India is one of the few countries in the world which has unique wealth of medicinal plants and vast traditional knowledge of use of herbal medicine for cure of various diseases. So far, few plants have been tested against mycobacteria and a few plants which showed anti-TB activity were Salvia hypargeia, Euclea natalensis, etc. The increasing incidence of MDR- and XDR-TB worldwide highlight the urgent need to search for newer anti-tuberculosis compounds/ drugs for the welfare of society and mankind.

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Review Article

Journal of Practical Biochemistry and Biophysics
Volume 1 Number 2, July - December 2016

Protein Folding, Denaturation and Stability: A Brief Introduction

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Abstract

Protein folding refers to the process and path by which a nascent polypeptide obtains its 3-D or native state. Over the years the field of protein folding has evolved, as are the questions pertaining with this perplexing field. Levinthal, was first to propose that the folding code for a polypeptide to fold, is its primary structure or sequence of amino acids. But, still in present era, a major challenge is to predict the native structure of a protein solely from its sequence. This has become more challenging with ever increasing sequence data being accumulated with each day. Thus, protein folding problem continues to be a complex problem to be solved and has perplexed scientists over the decades. In this article, main focus will be to sum up the broad aspects of protein folding, protein denaturation and discuss in brief the methods to estimate protein stability.

Keywords: Protein Folding; Denaturation; Stability; Equilibrium Methods; Differential Scanning Calorimetry.

Introduction

It has long been established through the works of Anfinsen and his colleagues that the primary sequence holds the key for protein folding process, i.e., it has the code for folding a nascent polypeptide chain to the functional native state in a given milieu [1, 2]. Anfinsen's work on ribonuclease A (RNase A) clearly showed that the compact, three-dimensional structure (native state) could be reached from the primary amino acid sequence (denatured state) through purely physicochemical processes without any need of molecular chaperones [3, 4]. Folding into the native conformation is a very fast process with time scale ranging in milliseconds to microseconds. Thus, Cyrus Levinthal pointed out that it is impossible for a real unfolded protein to achieve native fold on the biological timescale if it goes randomly searching all the possible structural conformations [4, 5] . He suggested that this folding must occur according to specified pathways. Currently, it is believed that the folded native state of a protein is the main determinant of the folding process and ultimately provides the stability needed for the native protein to be functional [6, 7]. The complex process of protein folding has perplexed scientists for past several decades and different models have been suggested for the folding process.

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Folding Models

Various models of protein folding have been

proposed to describe the possible pathways through which a protein's primary sequence achieves the native fold. First, the "framework model" describes folding as a stepwise mechanism involving a hierarchical assembly of local elements of secondary structure from the primary sequence but independent from tertiary structure. This greatly reduces the conformational search and tertiary structure is attained by diffusion and collision of the local elements of secondary structure whereby favorable amalgamation occurs [8-10]. This model does not place emphasis on the formation of native tertiary contacts directly from the primary structure; thus, secondary structure formation is independent of how the final folded tertiary protein should look. Second, the "nucleation-condensation" model is a modified form of the "nucleation" model and describes folding from more of a helix-coil perspective. The model describes folding via the formation of a loosely packed (extended) nucleus, derived from initial helix or sheet "seeding" of native secondary structural elements, which becomes more compact in the transition state and is directly responsible for the formation (condensation) of higher order tertiary structure [11-14]. Third, the "hydrophobic collapse" model hypothesizes that the native protein fold is formed from a "molten globule" as a result of the polypeptide chain having a concentrated region of hydrophobic side chains [14-17]. The molten globule then quickly rearranges due to the narrowed conformational search leading up to the native fold.

Recent research in protein folding has lead to a more modern and general view of protein folding. The two main competing models have been proposed. The "predetermined pathway – optional error (PPOE)" model claims that all of a protein population folds by essentially the same stepwise pathway. This single pathway is defined by predetermined cooperative native-like foldon units (intermediates) and how those foldon units interconnect in the final native fold [18] . This model predicts the transition state as a single obligatory step having a few well-defined structures that all protein molecules need to pass through. The intermediates are all downhill from the transition state, thus are "hidden" and are only seen when there is an error in folding (misfold); thus, proteins behave as two-state or multi-state folders, depending on the spectroscopic probe being used [18-20]. This model is mainly derived from hydrogen exchange data. The main idea of this model is presented in **Figure 1**.

Second model, the more popular "folding funnel" model, is derived from statistical mechanics and concepts of polymer physics, rather than those of classical chemical dynamics – hence, is called the "new view" [21, 22]. This model represents the energy landscape of the protein folding pathway as an energy funnel as depicted in **Figure 2** [23]. At the top (rim) of the funnel is a heterogeneous mixture of rapidly-exchanging, high enthalpy, high entropy, polypeptide conformations in the unfolded denatured state ensemble (DSE). Polypeptides can explore funnel shaped potential energy surfaces downhill towards native state along several paths. [3]. Since the funnel is not smooth, the rugged nature of the funnel may lead to transiently populated various intermediate states [24]. Partially folded or misfolded states have tendency to aggregate because of exposed hydrophobic residues which are otherwise buried in native state [25]. The formation of these aggregates is prevented by the chaperone machinery in the cells but these highly ordered and thermodynamically stable aggregates accumulate under stress or when protein quality control fails [26]. Narrow bottom of the folding funnel indicates that there are few low-energy native like conformations and many more open unfolded structures [27,28]. This model has been widely agreed since it accommodates many of the ideas from previously mentioned models and also provides reasonable explanations for protein behavior both in vitro and in vivo.

Protein Denaturation

The conformation of a protein in which it is functional or active is called the "native state" of the protein. After a protein is synthesized on ribosome, a protein achieves its "native" conformation (out of millions & trillions possible conformation) on biological time-scale of few milliseconds. This is quite amazing and is difficult to explain how a protein achieves its native conformation at such a fast rate. Since starting from the primary structure of a protein is quite impractical given the number of possible conformations, protein folding is usually studied by how a protein unfolds or denatures from its native state. A protein has a certain pH, temperature, pressure and ionic concentration range where it remains in its native conformation. Any alteration in any of these parameters leads to loss of this native conformation. The first theory of protein denaturation was proposed by Hsien Wu in 1931 [29]. According to this, denatured state is viewed, as alteration of a highly compact and ordered structure into more or less open structure. The denaturation involves no change in the primary structure i.e. no effect on the covalent bonds linking the amino acids . The unfolding or denaturation is characterized by a

polypeptide chain becoming less compact, highly solvated and more flexible leading to increased intrinsic viscosity and partial or complete loss of secondary structural elements [30].

Protein denaturation can be brought about in different ways and the products of denaturation have been characterized by various techniques. Main modes of denaturation are (1) heat denaturation (3) organic solvents and solutes (4) inorganic salts, e.g., lithium chloride, lithium perchlorate, lithium bromide, calcium chloride, potassium thiocyanide and sodium bromide (5) detergents and (6) guanidinium chloride and urea. Though the denatured state of many proteins has been characterized using various biophysical techniques, the mechanism and the details of the chemistry underlying the mode of denaturation are still not clearly understood. Some methods employed for protein denaturation are discussed as under;

- ***Thermal Denaturation***

Temperature occupies a central and unique role as a perturbant of the equilibrium between different conformational species in macromolecules. Thermal denaturation or heat-induced denaturation is brought by heating a protein solution and following the change in observable physiochemical property as a function of temperature. Thermal denaturation may be reversible or irreversible. Reversibility is checked by regaining the native state if initial conditions are restored. Irreversibility is mainly due to aggregation or precipitation of protein. In some proteins, high temperature results in disulfide rupture or disulfide interchange (particularly at alkaline pH) [30], but usually only non-covalent interactions are affected by heat .

- ***Guanidinium Chloride (GdmCl) and Urea Denaturation***

Guanidinium chloride (GdmCl) and urea are the widely used protein denaturants and give the extensively unfolded state [30-32]. It is remarkable that a mechanistic understanding of how they affect protein structure is still elusive. The main difficulty arises from the fact that GdmCl and urea are weakly interacting molecules, and concentrations in the molar range are usually required to destabilize proteins [33,34]. It is still not clear whether the denaturant molecules modulate solution properties or interact with the protein directly by binding at specific sites which are otherwise occupied by H2O molecules [35]. If the latter is correct, it is still not clear what type of interaction (be it polar, hydrophobic, or van der Waals) is the driving force for denaturation, and whether the effect of the cosolvent on the polypeptide backbone or on the amino acid side chains is more important [36]. The molar concentrations at which chemical denaturants tend to operate make it certain that many cosolvent molecules will find themselves in close proximity to the protein chain. Thus, in the context in which chemical denaturation generally takes place, interaction between cosolvent and protein is a fact that must be accounted in the discussion of the process of denaturation [37]. It is also possible that some of these questions ultimately will have different explanations for urea than for GdmCl. With the advent of computer technology, molecular dynamics (MD) simulations have been carried out for both urea and GdmCl solutions. O'Brien et al. simulated both urea and GdmCl solutions with a model peptide and focused on the relative frequency of direct contacts between cosolvent molecules and polar atoms on the protein. They observed strong, direct associations between the guanidinium cation and charged or polar groups in both protein side chains and backbone, but did not observe similar associations for urea, and thus argued that direct electrostatic interaction is a driving force for denaturation mediated by GdmCl more strongly than for urea [38].

A long debate is going on the nature of the denatured states obtained by heat and guanidinium chloride (GdmCl) or urea denaturation. Work by Tanford and coworkers suggested that thermally denatured proteins have more residual structure than GdmCl-denatured proteins [30,39]. Cooperative loss of structure from thermally-denatured lysozyme, chymotrypsin and RNase A upon addition of GdmCl provides strong evidence that thermally denatured states contain residual structure [40]. NMR studies of thermally denatured barnase showed that some fraction of denatured ensembles contain residual, non random structure [41]. Singh et al. also have shown that the heat-denatured state is less unfolded than the GdmCl (or urea)-denatured state in case of lysozyme, RNase-A, α-lactalbumin, and α-chymotrypsinogen A [42]. Very recently, heat denatured state and GdmCl-danatured were also shown to be different in case of yeast iso-1-cytochrome c [43].

- ***Other Salt Denaturations***

Salts are known to affect the protein solubility, stability and biological activity[44]. Salts mainly affect the electrostatic and hydrophobic interactions. The effect of a salt depends on the concentration: salting in at lower concentration and salting out at

higher concentration. Salts mainly affect the electrostatic and hydrophobic interactions [45]. Salting out phenomena i.e. decreased solubility and aggregation of the protein at high salt concentration is mainly due to the effect of salt on the hydrophobic interactions [46]. Based on their ability to salt in or salt out proteins Hofmeister arranged them in a series known as Hofmesiter series [47,48]. Anions: SO_4^{2-} > HPO_4^{2-} > acetate > Cl^- > NO_3^- > ClO_3^- > I^- > ClO_4^- > SCN^-; Cations: NH_4^+ > K^+ > Na^+ > Mg^{2+} > Ca^{2+} > Gdn+. The mechanism of the Hofmeister series is not entirely clear. It has been argued that denaturation does not seem to result from changes in general water structure, instead more specific interactions between ions and proteins and ions and the water molecules directly contacting the proteins are important [49]. Inorganic salts induce conformational changes in proteins at room temperature. Kugimiya and Bigelow found that LiCl and $LiClO_4$ denatured states of lysozyme do possess some residual structures [50]. LiCl-denaturation in case of horse L94G mutant cytochrome and yeast iso-1-cytochrome c induces pre-molten globule formation [51, 52].

- ***pH Denaturation***

The dependence of protein stability on pH has been the focus of researchers for a long time. Linderstrom- Lang [53] was the first to explain the lower stability of proteins at extremes of pH. Extremes of pH far away from the isoelectric point favor denaturation because sensitive areas of the protein molecule acquire more charge causing internal repulsion or perhaps lose charges, which were previously involved in attractive forces holding protein together. In the native state, a number of acidic and basic amino acids are placed in environments that modify the pKa's of their side chains. In the denatured state the pKa,s of some or all of these residues return to values typical free amino acids. As a result, additional protons bind to the acidic denatured state and additional protons are lost from the alkaline denatured state. These protons must be lost (or retrieved) in order for the protein to refold and the free energy cost of doing so exceeds the intrinsic free energy stabilizing the native state when the pH is sufficiently low (or high).

Both activity and stability are pH dependent and very often pH optimum of activity is correlated with the pH optimum of stability and changes in pH are not unexpected for cellular function [54]. This suggests that other factors in addition to overall charge are important in determining the contribution of ionizable groups to the overall folding energy of globular proteins. Lysozyme and RNase A have been found to be stable at even pH 2.0 without any conformational change [55]. Acid or alkaline denaturation of proteins is mainly due to instability of the buried groups [56]. pH denaturation can induce a minor conformational change to nearly random coil conformation (at low ionic concentration) [57].

- ***Denaturation by Organic Solvents***

Organic solvents are known to perturb the protein structure. The effects of the water-miscible straight chain and branched alcohols and glycols on the native conformation of sperm whale myoglobin, cytochrome c, and a-chymotrypsinogen have been investigated by spectral, difference spectral, and circular dichroism methods [58-60]. Based on the midpoints of the denaturation transitions, it is concluded that the effectiveness of the alcohols as protein denaturants increases with increasing chain length. The glycols are found to be less effective than the corresponding alcohols [61]. The action of organic solvents on proteins is a function of their proton acceptor and proton donor tendency. Solvents such as dioxin, acetonitrile, dimethylformamide, pyridine and dimethylsulphoxide, which are good proton acceptors but weak proton donors, have a very weak tendency to disturb peptide hydrogen bonds [62]. The ability of alcohols to induce a-helical conformations in proteins was first noted in optical rotatory dispersion experiments by Tanford et al. on a-lactoglobulin [63]. Tamburro et al. [64] studied the effects of trifluoroethanol (TFE) on the conformations of the ribonuclease S-peptide; TFE was found to stabilize the small peptide in the same (a-helical) conformation that it adopts in native protein. Since then, alcohols have been used widely to examine the conformational (particularly helical) propensities of peptides [65-68] and to induce conformational changes in intact proteins [69-72]. TFE has also been shown to induce to induce β-turns, β hairpins and also β-strands[73]. It can also promote switching between different secondary structures, generally from a β-sheet to an a-helical structure [74].

Methods for Determination of Protein Stability

Protein stability is quantitatively described by the standard Gibbs energy change, ΔG_D^o, involved in unfolding the unique, three dimensional structure to randomly coiled polypeptide chains (N↔D).

This is also referred to as conformational stability of a protein. Studying of conformational stability can provide an insight into the understanding of the forces that determine the conformation of a protein and sometimes it may reveal additional features of a protein like existence of domains or the presence of stable folding intermediates [75]. Almost all the estimates of protein stability come from thermal denaturation and GdmCl or urea-induced denaturations and microcalorimetry.

- ***Differential Scanning Calorimetry (DSC)***

Differential scanning calorimetry is a direct, non-perturbing technique, developed in early 1960s to estimate protein stability. This technique measures the thermodynamic properties of a thermally induced transition and has been applied to variety of biological macromolecules such as lipids and proteins [76,77]. DSC endotherm gives direct estimate of constant-pressure heat capacity (ΔC_p), T_m (mid-point of thermal denaturation) and enthalpy change (ΔH_m) of a protein solution against a reference buffer. Thus DSC provides all the thermodynamic parameters of unfolding of a protein in a single experiment as shown in **Figure 3**.

DSC measures the excess heat capacity of a solution (C_p) of the molecule of interest as a function of temperature. The transition is recognized as a sharp endothermic peak centered at Tm and the maximum in C_p occurs directly at T_m. Integration of the C_p versus T curve yields the transition enthalpy (ΔH_m) and the shift in the baseline yields the ΔC_p (see **Figure 3**). DSC is the only method for the direct determination of ΔH_m. The value ΔH_m is calculated from the area under the transition [78]. DSC curve can be integrated incrementally to give a progress curve (C_p vs T), i.e. the proportion of the total heat absorbed as a function of temperature. Fitting of the progress curve will yield a van't Hoff enthalpy (ΔH_{vH}) which can be different from the calorimetric enthalpy, ΔH_m. The calorimetric enthalpy is the total enthalpy change including the contribution from all processes and determined independently of any model while the corresponding ΔH_{vH} assumes a simple two-state transition [79]. Comparison of ?Hm with ΔH_{vH} therefore allows one to assess whether the transition occurs as a two state or any intermediate is involved [80]. If $\Delta H_{vH} = \Delta Hm$ then the denaturation is considered to be well approximated by a two-state process. If $\Delta H_{vH} < \Delta H_m$ then most likely there is an unfolding intermediate (i.e. not a two-state process). If $\Delta H_{vH} > \Delta H_m$ then there is intermolecular association; the molecule may be a dimer or multimer or may be due to aggregation [78]. In DSC, ΔC_p is obtained by the difference between pre-transitional and post-transitional baselines of endotherm [81], though a good approach is to take several DSC scans in which Tm is perturbed as a result of change in pH. The slope plot of ΔH_m against corresponding T_m gives ΔC_p [54 82].

A concern regarding DSC studies is the concentration of the protein needed. Considerably, higher concentration of protein is needed, more than 1mg ml^{-1} and volume inbetween. 5 ml to 1 ml. This high concentration of protein may lead to difficulties arising from aggregation or intermolecular association of the denatured state, or possibly self-association of the native state. Accurate DSC studies thus require an assessment of the concentration dependence of the thermodynamics. Thus accurate determination of protein concentration is needed. However, sensitive commercial instruments are now available with higher sensitivity and quality data can be obtained from samples at $1/10^{th}$ the concentration previously required.

- *Equilibrium Method*

Protein stability is mainly discussed in terms of thermodynamic stability of a protein that unfolds and refolds rapidly reversibly with a two-state mechanism: N (native state)↔D (denatured state); the equilibrium between N and D is given by equilibrium constant KD, as, KD=[D]/[N]. The

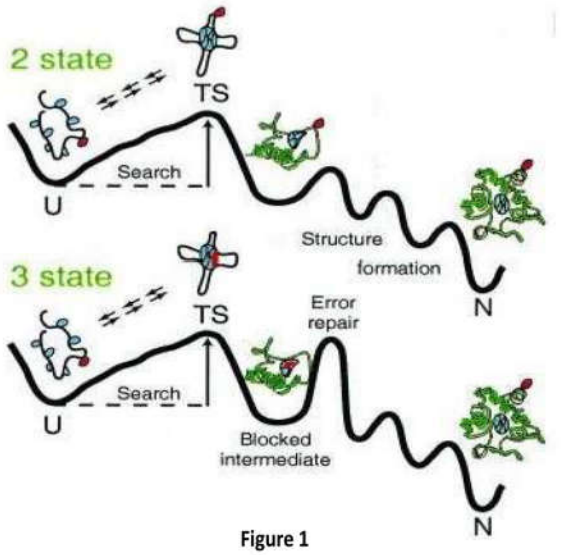

Fig. 1: A schematic energy profile representation of a two-state and three-state folder having hidden intermediates as described by the "Predetermined Pathway – Optional Error" method [20]. U represents the unfolded state, TS is the transition state and N is the native state. "Copyright (2001) National Academy of Sciences, U.S.A."

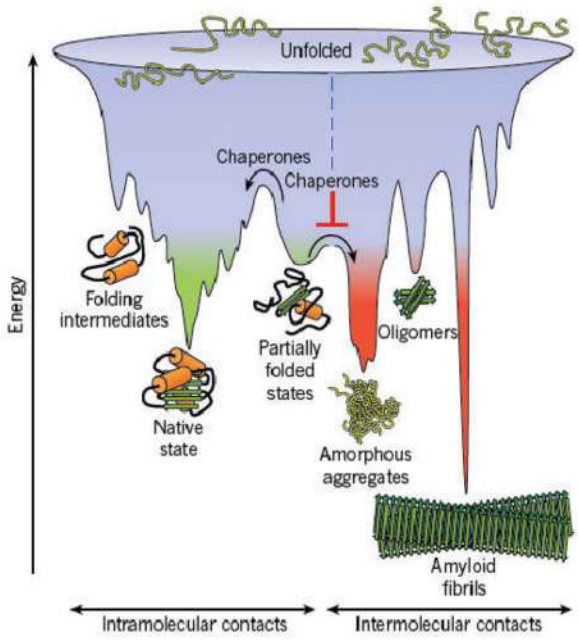

Fig. 2: Rugged "folding funnel" showing protein folding and aggregation. Reprinted by permission from Macmillan Publishers Ltd: [Nature] [23], copyright (2011).

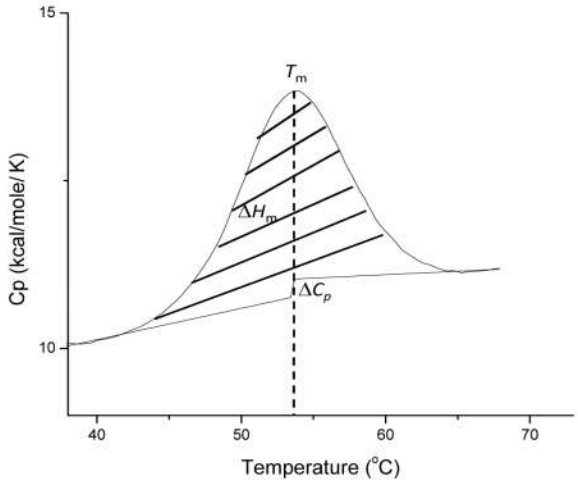

Fig. 3: DSC endotherm of yeast iso-1-cytochrome *c* at pH 6.0 in native buffer

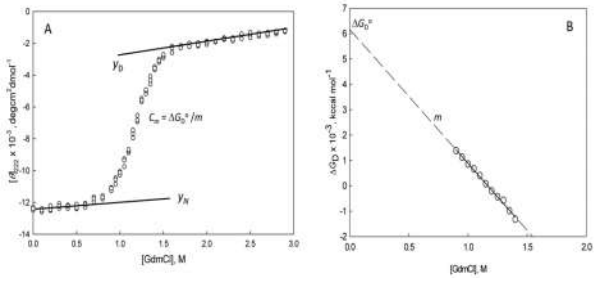

Fig. 4: A. GdmCl-induced denaturation curve of yeast iso-1-cytochrome *c* at pH 6.0 obtained by monitoring $[\varphi]_{222}$ Vs [GdmCl]. **B.** Plot of DG_D as a function of [GdmCl] and extended using least-squares analysis to zero concentration of denaturant to calculate DG_D^o. The slope of the line gives '*m*'.

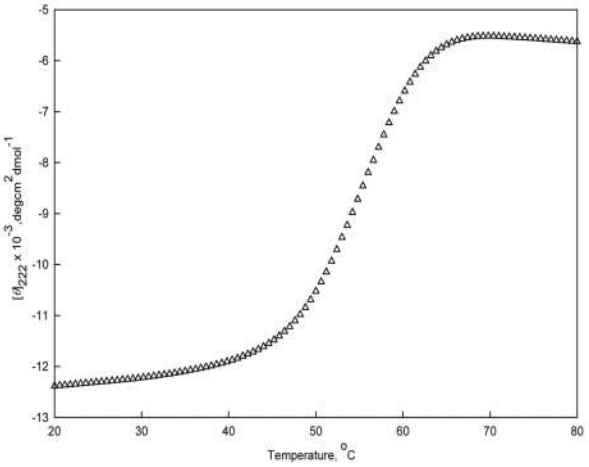

Fig. 5: Thermal denaturation curve of yeast iso-1-cytochrome *c* monitored by following change in $[\varphi]_{222}$ Vs temperature at pH 6.0.

difference in free energy between denatured and native state (stability) is give by $\Delta G_D^o = -RT \ln K_D$. More the ΔG_D^o, more stable is the protein to denaturation. Thus to estimate ΔG_D^o, we have to study the equilibrium between the N and the D state. The equilibrium can only be studied by perturbing this equilibrium using denaturants like GdmCl, urea or other solvents [83, 84] and heat. The method involves measuring of some optical property (at which N and D differ) as a function of denaturant concentration. This conformational transition is used to evaluate ΔG_D^o of a protein. Some of the methods used are;

Chemical Denaturant-Induced Denaturation

In these denaturation transition, a chemical denaturant is used to induce denaturation, mainly three procedures are used to evaluate ΔG_D^o from the analysis of conformational transitions; [85]. These are linear extrapolation method, denaturant binding model and transfer free energy model. Here, we will discuss the first method, i.e., linear extrapolation model since this is widely used model to evaluate ΔG_D^o of chemical-induced transition [39, 55, 83-88]. In this method, an observable spectral property is plotted as a function of denaturant concentration. The resulting plot, i.e., iso-thermal denaturation curve is sigmoidal (**Figure 4A**), signifying a denaturant concentration-induced cooperative transformation of N state to the D state. Assuming this represents only two states N and D and the denaturation process is reversible (to be checked), the equilibrium is put as;

N (Native Conformation)↔D (Denatured Conformation) (1)

For this process, one can calculate f_N (the fraction

of native protein molecules) and f_D (the fraction denatured protein molecules, where;

$$f_N + f_D = 1 \quad (2)$$

the value of any observed conformational property, y, is directly related to the fraction of the native and denatured protein

$$y = f_N y_N + f_D y_D \quad (3)$$

fD, the fraction of dentured protein can be calculated from

$$f_D = (y - y_N)/y_D - y_N) \quad (4)$$

where y is the observed optical property at the particular pH, temperature and denaturant concentration, while y_N and y_D are respectively the properties of the native and denatured states measured under the same experimental conditions in which *y* has been measured. The values of y_N and y_D for any point in the transition region are obtained by the intrapolation of the pre- and post-transition baselines, which is generally obtained by least-squares analysis [55] under the same experimental conditions in which y has been observed. Plots of f_D versus molar concentration of denaturant, at which they were calculated, gives the normalized transition curve. For each observed point within the transition region and equilibrium constant, KD can be calculated using the relation:

$$K_D = f_D/(1 - f_D) = (y - y_N) / (y_D - y) \quad (5)$$

The free energy change (ΔG_D) for folding unfolding reaction ($N \leftrightarrow D$) can be calculated using the relation;

$$\Delta GD = - RT \ln K_D \quad (6)$$

where R is the gas constant (1.987 calories/deg/mol) and T is the absolute temperature in Kelvin (K). The value of equilibrium constant, KD can be measured most accurately near the mid-point of denaturation curve, and the value of KD outside the range 0.1—1.0 ($0.1 \leq f_D \leq 0.9$) the error becomes substantial [30]. It has been observed that, the plot of -1.3 ≤ dGD (kcalmol-1) ≤ 1.3 versus [denaturant] is linear in the transition region. From this linear plot of ΔG_D values against the molar concentration of each denaturant, ΔGD^o can be estimated from the least-squares analysis according to the relation (**Figure 4B**) [86],

$$\Delta G_D = \Delta G_D^o - m\,[d] \quad (7)$$

where ΔG_D^o is the value of ΔG_D in the absence of denaturant and *m* is the slope of the line i.e., $(\partial \Delta G_D / \partial [d])_{T,P.}$ and [d] is molar denaturant concentration. The midpoint of transition curve, Cm is calculated from $C_m = \Delta G_D^o / m$.

Alternatively, the method which is commonly used nowadays, the entire equilibrium transition curve obtained by measuring any observable property *(y)* as a function of denaturant concentration *(d)* can be fitted to a two-state unfolding model with the help of Sigma Plot, v.10 or Origin softwares to analyze the values for ΔG_D^o, m_d and C_m using the relation [89]

$$y = y_N(d) + y_D(d) * \mathrm{Exp}[-(\Delta G_D^o + m_d[d])/RT]/(1 + \mathrm{Exp}[-(\Delta G_D^o + m_d[d])/RT]) \quad (8)$$

where *y* is the observed optical property at [d], the molar concentration of any denaturant, $y_N(d)$ and $y_D(d)$ are optical properties of the native and denatured protein molecules under the same experimental conditions in which *y* was measured, ΔG_D^o is the value of the Gibbs energy change in the absence of the denaturant, m_d is the slope $(\partial \Delta G_D / \partial [d])$, *R* is the universal gas constant, and *T* is the temperature in Kelvin. It should be noted that the analysis of each equilibrium transition curve was done assuming that unfolding is a two-state process and reversible and dependencies of $y_N(d)$ and $y_D(d)$ are linear *(i.e.,* $y_N(d) = a_N + b_N[d]$ and $y_D(d) = a_D + b_D[d]$, where a and b are [d]-independent parameters, and subscripts *N* and *D* represent these parameters for the native and denatured protein molecules, respectively. Equation (9), fits the native state $a_N + b_N[d]$ and denatured state $y_D(d) = a_D + b_D[d]$ baselines as well as the unfolding transition region simultaneously, assuming a linear dependence of free energy on denaturant concentration [d].

Heat-Induced Denaturation

Protein stability in terms of Gibbs free energy change (ΔG_D^o) is determined from the measurements of reversible heat-induced denaturation of proteins using calorimetric or equilibrium methods [90]. DSC is a direct method which gives the thermodynamic parameters associated with the endotherm. While in the equilibrium method, transition is followed by measuring a suitable structural property as a function of temperature and analysis of the resulting transition curve (sigmoidal) for the equilibrium constant (KD). Hence this method is also called equilibrium method.

Since, temperature is a thermodynamic property; temperature dependence of equilibrium provides an access to the enthalpy (ΔH), entropy (ΔS), and heat capacity ΔCp) components of the Gibbs free energy (ΔG_D). These parameters can be used to establish the relative stability of different proteins or mutants or to estimate the size of the cooperative units of different proteins [91]. When the protein unfolds, the buried

non-polar side chains come in contact with water. In order to accommodate these side chains, cages of water molecules surround them so that the extent of hydrogen bonding is increased. The heat capacity of unfolded protein is greater than that of folded protein because now these cages must be melted in order to raise the temperature of the protein solution. Thus the same effect that gives rise to hydrophobic interaction also gives rise to larger ΔC_p. Privalov studied the effect of melting temperature (T_m, the midpoint of transition) and enthalpy of denaturation of a number of proteins. It was observed that the plots of enthalpy versus the corresponding melting temperature were linear for all proteins studied. It is concluded that in these experiments ΔH_m, the value of ΔH at T_m is a direct function of temperature. ΔH_m = Constant + $\Delta C_p T_m$, where T_m is the temperature at which $\Delta G_D = 0$ and ΔH_m is the value of ΔH at T_m. The apparent dependence of ΔH_m on pH results from the fact that pH changes T_m; the temperature at which ΔH is evaluated [91]. The heat capacity is the temperature derivative of basic thermodynamic function, the enthalpy. Therefore denaturation heat capacity increment determines the temperature dependence of the enthalpy and hence of the entropy on temperature i.e., the parameter that determines the native state stability.

The two-state heat-induced denaturation curve is also sigmoidal (Figure 5), which is used to derive the thermodynamic parameters T_m (midpoint of denaturation), ΔH_m (van't Hoff enthalpy change at Tm) and ΔCp (constant pressure heat capacity change), associated with it. The entire data points obatained are fitted to the equation (10) or (11) using softwares like Origin or Sigmaplot to obtain the thermodynamic paramaters [89, 92].

$$y(T) = \frac{y_N(T) + y_D(T) Exp\left[\frac{-\Delta H_m^{van}}{R}\left(\frac{1}{T} \quad \frac{1}{T_m}\right)\right]}{1 + Exp\left[\frac{\Delta H_m^{van}}{R}\left(\frac{1}{T} \quad \frac{1}{T_m}\right)\right]} \quad (10)$$

$$y_{obs} = \frac{(y_n + m_n T) + (y_d + m_d T) \quad \exp\{([\quad C_p \quad ((T_m/T \quad 1) + \ln(T/T_m))] \quad [\quad H_m \quad (T_m/T \quad 1)])/R\}}{1 + \exp\{([\quad C_p \quad ((T_m/T \quad 1) + \ln(T/T_m))] \quad [\quad H_m \quad (T_m/T \quad 1)])/R\}} \quad (11)$$

Where, yobs is the experimentally observed optical property of the protein at temperature T (K), yn and yd are the optical properties of the native (N) and denatured (D) molecules at same temperature, and R is universal gas constant. It should be noted these equation assume that the heat-induced denaturation is a two-state process.

The thermodyanamic values thus obtained can be put in Gibbs–Helmholtz equation (12), to calculate the Gibbs free energy change (ΔG_D^o)

$$\Delta G_D(T) = \Delta H_m\left(\frac{T_m \quad T}{T_m}\right) \quad \Delta C_p\left[(T_m \quad T) + T ln\left(\frac{T}{T_m}\right)\right] \quad (12)$$

The dependencies of pre- and post-transition baselines in a thermally induced transition curve can be analyzed by three different methods; like linear model, mixed model and parabolic model. It has been shown that if analysis is carried out assuming that the temperature dependence of pre- and post-transition baselines is described by a parabolic function, there exists an excellent agreement between ΔH_m values of all proteins obtained from equilibrium and calorimetric methods [93,94].

There are several methods for the determination of ΔC_p from conformational transition curves. The earlier method involves the estimation of the values of ΔH_m^{van} as a function of temperature from vant's Hoff analysis of thermal denaturation curves measured at different pH values or chemical denaturation [95]. A second approach involves the measurement of ?Hmvan and Tm from thermal-transition curves obtained at different pH values and estimation of ΔC_p from plot of ΔH_m^{van} versus Tm [82]. Another approach developed by Swint and Robertson [96], ΔC_p is determined from the fit of the entire transition data (y,T) in equation that includes the temperature-dependence of ΔH_D (equation (3) in Swint and Robertson. Singh et al. [42] developed a non-calorimetric method for measurement of ΔC_p. This method involves the use of thermodynamic data obtained from isothermal GdmCl (or urea)-induced denaturation and heat-induced denaturation in the presence of the chemical denaturant concentration at which significant concentrations of both native and denatured states exist. The method involves the determination of ΔH_m (0) (enthalpy change in absence of denaturant), T_m (0) (mid-point of denaturation in the absence of the denaturant) and ΔG_D^o (Gibbs energy change in absence of denaturant). ΔH_m and Tm can be obtained from the measurement of effects of temperature on the equilibrium constant, KD. For this denaturation curves of the protein are measured in the presence of transition region concentration of the chemical denaturant by following changes in optical property as a function of temperature.

Conclusion

Predicting 3-D structure of a protein from its sequence is still a fascinating field of modern biology and biophysics and at the same how does a protein fold, the pathway involved and the thermodynamic aspects has remained and is still an area which has puzzled scientists over the decades. Still at present, there are unanswered questions to be answered and puzzles to be solved like, predicting the tendency of a protein to aggregate or to misfold, explaining the reason why cellular proteome doesn't precipitate because of so much crowding, preventing and intervening in misfolding diseases, function of intrinsically disordered proteins (IDP's) and so on. Thus, the future of proteins sciences is as persuasive as its past.

Conflict of Interest

The authors declare no conflict of interest.

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[1] Flink H, Tegelberg Å, Thörn M, Lagerlöf F. Effect of oral iron supplementation on unstimulated salivary flow rate: A randomized, double-blind, placebo-controlled trial. J Oral Pathol Med 2006; 35: 540-7.

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Article in supplement or special issue

[3] Fleischer W, Reimer K. Povidone iodine antisepsis. State of the art. Dermatology 1997; 195 Suppl 2: 3-9.

Corporate (collective) author

[4] American Academy of Periodontology. Sonic and ultrasonic scalers in periodontics. J Periodontol 2000; 71: 1792-801.

Unpublished article

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Personal author(s)

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Chapter in book

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No author given

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Reference from electronic media

[9] National Statistics Online—Trends in suicide by method in England and Wales, 1979-2001. www.statistics.gov.uk/downloads/theme_health/HSQ 20.pdf (accessed Jan 24, 2005): 7-18. Only verified references against the original documents should be cited. Authors are responsible for the accuracy and completeness of their references and for correct text citation. The number of reference should be kept limited to 20 in case of major communications and 10 for short communications.

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