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# Alteration in Levels of Minerals in DEN induced Hepatocellular carcinoma in Wistar Albino Rats

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**ABSTRACT**

The present attempt has been made to evaluate, and examine the levels of minerals in serum and liver in DEN induced hepatocellular carcinoma in wistar albino rats for possible chemopreventive effect. In hepatocellular carcinogenesis complications such as hepatic fibrosis and cirrhosis may lead to several abnormalities in mineral metabolism, hence attempt is made to evaluate on the level of minerals. Hepatic cancer was induced by single dose of intraperitoneal injections of DEN (200mg/kg body weight) followed by phenobarbital of 0.05% mixed with drinking water for 20 weeks. Concentration of calcium, magnesium, sodium and potassium were assessed in the serum and liver at the end of experimental period. Negative correlations were observed between liver function tests and serum mineral levels, except with albumin. Calcium, magnesium, potassium and sodium concentrations in the serum were decreased after the induction of hepatic cancer. The liver calcium content was increased after DEN treatment. No change occurred in liver sodium content. However, magnesium and potassium content was significantly reduced in the hepatic tissue. The results suggest that in DEN-induced hepato cellular carcinoma alteration of essential elements is noted. The low levels of albumin and the related ascites may be one of the major causes of the imbalance of mineral metabolism in hepatocellular carcinoma.

**Abbreviations:** N-Nitrosodiethylamine (DEN), Hepatocellular carcinoma (HCC), Captain Srinivasa Murti Drug Research Institute for Ayurveda (CCRAS), Tamilnadu Veterinary and animal Science University (TANUVAS), Institutional Animal Ethics Committee (IAEC).

## INTRODUCTION

Several disorders associated with mineral metabolism, have been described in hepatic diseases, but their cause, significance and relationship to clinical complications have yet to be identified. Many elements play important roles in the living body as components of metallo-proteins and metallo-enzymes as well as enzyme cofactors (McDowell, 2003). Since the metabolism of these compounds takes place mainly in the liver, studies of alterations of minerals and trace elements during liver disorders have been of considerable importance in recent years. However, the factors associated with liver diseases and mineral metabolism is still unclear. Sodium has a major role in the development of ascites in patients with liver cirrhosis. Impaired water and sodium excretion has been implicated in the patho-genesis of ascites formation (Rosner *et al.,* 2006; Sandhu *et al.,* 2005; Arroyo *et al.,* 1987). Potassium is the principal intracellular cation and its metabolism may be altered during liver fibrosis. The frequent finding of hypokalemia in liver diseases (Weiner and Wingo, 1997) is often attributed to total body potassium deficiency. Disturbances in calcium metabolism have been reported in hepatic fibrosis and related diseases (Castro *et al.,* 1994). Magnesium is involved in carbohydrate metabolism and also required for the synthesis of all proteins and nucleic acids. It was reported that DEN induced liver injury in rats is a suitable and reproducible animal model for studying various events associated with development of hepatic fibrosis and cirrhosis in human beings (George *et al.,* 2004; George *et al.,* 1996). Even though considerable data are available with regard to the role of minerals in liver diseases, the correlation between alteration of minerals and development of HCC is not clear. Therefore, concentrations of biochemically and physiologically important minerals such as sodium, potassium, calcium and magnesium were studied in serum and liver tissues during the pathogenesis of DEN-induced hepatic cancer in adult wistar albino rats and the data correlated with liver functions.

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*R.tuberosa* belongs to the family of *Acanthaceae*. In folk medicine; it has been used as diuretic, antidiabetic, antipyretic, analgesic, anti-hypersensitive, thirst-quenching, and antidotal agent (Chen, 2006; Satyajit, 2009), It has potential antioxidant properties to scavenge free radical and prevent oxidative damage (Arirudran *et al.,* 2014a), and it act as alternative chemotherapeutic agent for HCC (Arirudran *et al.,* 2014b). Present study was carried out to evaluate and examine the level of minerals after administration of ethyl acetate and ethanolic extracts of *R.tuberosa* in DEN-induced hepatic cancer in rat using doxorubicin as control drug.

## MATERIALS AND METHODS

### Chemical reagents

N-Nitrosodiethylamine (DEN), Doxorubicin Hydrochloride and Phenobarbital were purchased from Sigma Chemical Company, USA. All other chemicals including solvents were of high purity and of analytical grade purchased from Glaxo Laboratories, Mumbai and Sisco Research Laboratories Pvt, Ltd, Mumbai, India.

### Plant materials and extract preparation

Fresh plant materials of *R.tuberosa* were collected from Tiruvallur district of Tamilnadu. The plant materials were identified and authenticated by botanist of this institute using the Flora of Presidency of Madras and voucher specimen (No: 00628) was deposited in the museum of CCRAS, Arumbakkam, Chennai. The shade dried and coarsely powdered plant material (100g) was successively extracted with ethyl acetate and ethanol successively using Soxhlet apparatus, filtered and concentrated to dryness (Arirudran *et al.*, 2014a) One gram of successive ethyl acetate and ethanolic extract from whole plant of *R.tuberosa* was weighed in dry weighing bottle. The working concentration of the each extract was diluted with Tween-80 to make a concentration of 100mg/ml and then the diluted solution was used for further chemopreventive study.

### Experimental animals

The procedure for animal experiments were reviewed and approved by IAEC of CCRIS (Approval No: 109/PHARMA/SCRI, 2011). Wistar albino male and female rats weighing 160-180g were purchased, from TANUVAS, Madhavaram, Milk colony, Chennai, Tamilnadu, India, for this study. The animals were maintained under standard conditions of humidity, temperature (25 ± 2ºC) and light (12hr light and 12hr dark).

The animals were acclimatized and maintained over husk bedding in polypropylene cages in central animal house facility of the institution for one week before use. The animals were fed with commercial pelleted diet (Hindustan lever Ltd, Bangalore, India with composition of 5% fat, 21% protein, 55% nitrogen free extract and 4% fibre (w/w) with adequate mineral, vitamin levels and free access to water throughout the experimental period. Experimental animals were handled according to the University and Institutional legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

### Experimental design

The experimental animals were divided into five groups with six animals in each group as shown in the table 1.

**Table 1:** The experimental animals were divided into five groups with six animals in each group.

| Treatment | No. of rats | Study period |
|---|---|---|
| **Group I** - Normal control animals | (3 Male and 3 Female) | 20 weeks |
| **Group II** - Control animals with single intra-peritoneal injection of DEN 200mg/kg body weight followed by phenobarbital of 0.05% mixed with drinking water for 20 weeks. | (3 Male and 3 Female) | |
| **Group III** - Animals with single intra-peritoneal injection of DEN 200mg/kg body weight, followed by phenobarbital of 0.05% mixed with drinking water for 16 weeks and ethyl acetate extract of *R.tuberosa* (400mg/kg body weight) for 4 weeks. | (3 Male and 3 Female) | |
| **Group IV** - Animals with single intra-peritoneal injection of DEN 200mg/kg body weight, followed by phenobarbital of 0.05% mixed with drinking water for 16 weeks and ethanolic extract of *R.tuberosa* (400mg) for 4 weeks. | (3 Male and 3 Female) | |
| **Group V** - Animals with single intra-peritoneal injection of DEN 200mg/kg body weight, followed by phenobarbital of 0.05% mixed with drinking water for 16 weeks and standard doxorubicin drug 5mg/kg body weight of one dose per week for 4 successive weeks. | (3 Male and 3 Female) | |
| **Total number of rats** | **30** | |

### Tumour induction and drug treatment

The experimental rats were fasted overnight and induced by a single intraperitonial injection of DEN at a dose of 200mg/kg body weight in saline to induce liver cancer. The control rats were similarly injected with saline. Neither death nor any other aggressive effect was not observed. Two weeks after the administration of DEN, phenobarbital at a concentration of 0.05% was incorporated into drinking water for about 14 successive weeks to promote liver cancer.

The changes in body weight in all groups of rats were recorded at regular intervals (every week). After the 16$^{th}$ week the animals with liver cancer was confirmed by α-fetoprotein which was measured quantitatively by solid phase enzyme linked immunosorbent assay, and the activity of γ-glutamyltransferase in serum. The plant extract of 400mg/kg /body weight of ethyl acetate extract from *R.tuberosa* whole plant was given for group III animals and 400mg/kg /body weight of ethanolic extract from *R.tuberosa* whole plant was given for group IV animals orally for about 4 weeks and the group V animals were treated with standard

doxorubicin 5mg/kg body weight of one dose per week for 4 successive weeks. After the end of the experimental period the animals were fasted overnight and sacrificed by cervical dislocation and the serum sample was collected.

**Collection of blood sample for plasma biochemistry**

In the other tube 11% Tri sodium citrate was used for the separation of plasma. Blood was allowed to coagulate before being centrifuged and the serum was separated. Estimation of total protein was carried out by Biuret method of (John Savory *et al.*, 1976), Estimation of albumin by Bromocresol green Dye binding method (Varley, 1966), Estimation of Minerals: Calcium, Magnesium, Sodium and Potassium were analysed in serum using Auto analyzer COBAS C III.

**Preparation of tissue homogenate**

The liver tissue was excised, washed in ice-cold saline; 1g of tissue was weighed and homogenized in 0.1M cold Tris-HCl buffer of pH 7.4 in a potter-Elvehjam homogenizer fitted with a Teflon plunger at 600 rpm for 30min. The liver homogenate was prepared in cold 50mM potassium phosphate buffer of pH 7.4. The unbroken cells and debris were removed by centrifugation at 10,000 rpm for 15min at 4°C using a REMI cooling centrifuge and the supernatant was used for the biochemical assays.

**Statistical analysis**

Hypothesis testing methods included one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison method was used to compare the means of different groups by using SPSS version 16.0 software (Chicago, USA), comparisons were made between DEN induced cancer group animals with other group animals. $P<0.05$ was considered significant between study groups. All the results were expressed as mean ± S.D for six animals in each group.

## RESULTS AND DISCUSSION

The table 2 shows the change in total protein, albumin, globulin which are significantly decreased ($p < 0.01$) and A/G ratio is significantly increased ($p < 0.001$) in DEN induced HCC group II animals when compared with group I control animals. This significant decrease in total protein, albumin and globulin may be due to hepatoxicity which leads to hepatocellular damage which in turn cause defective protein biosynthesis in liver. On treatment with ethyl acetate extract of *R.tuberosa* the level of globulin is significantly increased ($p < 0.05$) and the level of total protein, albumin and A/G ratios are significantly increased ($p < 0.001$) in group III animals. Serum proteins have many functions, including the transport of substances, immune defense, blood clotting, and inflammation defense. Serum protein levels are useful for evaluating nutritional status, infection, and various other disorders. Within the human body, albumin is an important component of life (Aiad *et al.*, 2004; Goyal and Soni, 2011, Honarmand *et al.*, 2011). Albumin is synthesized in liver. In the human body albumin transports essential fatty acids from adipose tissue, to muscle tissue. Consequently, decreased albumin levels may be associated with liver diseases (Honarmand *et al.*, 2011). Albumin/Globulin (A/G) ratio indicates the calculated ratio of levels of these two serum proteins. A low A/G is found in certain liver diseases, kidney disease, myeloma, and inflammation, as well as other disorders (AL-Shinnawy, 2009). The total protein levels including albumin and globulin levels that have been reported to decrease in hepatotoxic conditions due to defective protein biosynthesis in liver (Clawson, 1989).

**Table 2:** Effect of extract of *R.tuberosa* on Total protein, Albumin, Globulin and A/G ratio in serum of control and experimental animals.

| Parameters | Group I | Group II | Group III | Group IV | Group V |
|---|---|---|---|---|---|
| Totalprotein | 6.42 ± 0.2 | $4.4 \pm 0.1^{b}$ | $4.8 \pm 0.2^{c}$ | $6.1 \pm 0.2^{c}$ | $6.2 \pm 0.1^{c}$ |
| Albumin | 4.7 ± 0.1 | $3.4 \pm 0.2^{b}$ | $3.8 \pm 0.1^{c}$ | $4.5 \pm 0.1^{c}$ | $4.6 \pm 0.2^{c}$ |
| Globulin | 1.7 ± 0.07 | $1.0 \pm 0.09^{b}$ | $1.0 \pm 0.08^{a}$ | $1.6 \pm 0.06^{c}$ | $1.6 \pm 0.04^{c}$ |
| A/G ratio | 2.7 ± 0.09 | $3.4 \pm 0.1^{c}$ | $3.8 \pm 0.25^{c}$ | $2.8 \pm 0.09^{c}$ | $2.87 \pm 0.1^{c}$ |

Each value is expressed as mean ± S.D, for six rats in each group. The total protein, albumin, globulin are expressed in gm% and A/G ratio. Group I- control animals, Group II- cancer bearing animals, Group III- ethyl acetate extract post treated, Group IV- ethanolic extract post treated and Group V- standard Doxorubicin treated animals. Statistical significance (a = $p < 0.05$, b = $< 0.01$, c = $p < 0.001$) and non significant (NS), when Group II compared with Group I, when Group III, IV and V compared with Group II.

**Table 3:** Effect of extract of *R.tuberosa* on Calcium, Magnesium, Sodium and Potassium in serum of control and experimental animals.

| Parameters | Group I | Group II | Group III | Group IV | Group V |
|---|---|---|---|---|---|
| Calcium | 109 ± 3 | $101 \pm 5^{NS}$ | $104 \pm 1^{NS}$ | $107 \pm 2^{NS}$ | $106 \pm 7^{NS}$ |
| Magnesium | 21.7 ± 1 | $15 \pm 0.7^{c}$ | $18 \pm 0.8^{c}$ | $20 \pm 0.4^{c}$ | $20.5 \pm 0.7^{c}$ |
| Sodium | 353 ± 14 | $312 \pm 10^{c}$ | $325 \pm 13^{NS}$ | $340 \pm 10^{c}$ | $350 \pm 20^{c}$ |
| Potassium | 28.1 ± 1.2 | $24 \pm 1.5^{c}$ | $25.2 \pm 1.8^{NS}$ | $27 \pm 1.3^{b}$ | $27.5 \pm 1.5^{c}$ |

Each value is expressed as mean ± S.D, for six rats in each group. The calcium, magnesium expressed as µg/ml and sodium, potassium expressed as µg/100ml. Group I- control animals, Group II- cancer bearing animals, Group III- ethyl acetate extract post treated, Group IV- ethanolic extract post treated and Group V- standard Doxorubicin treated animals. Statistical significance (a = $p < 0.05$, b = $< 0.01$, c = $p < 0.001$) and non significant (NS), when Group II compared with Group I, when Group III, IV and V compared with Group II.

The DEN intoxication causes disruption and disassociation of polyribosomes on endoplasmic reticulum and thereby reduces the biosynthesis of protein. On treatment of ethanolic extract of *R.tuberosa* the level of total protein, albumin and globulin are significantly increased ($p < 0.001$), but the A/G ratios are significantly decreased ($p < 0.001$) in group III & IV animals. Ethanolic extract of *R.tuberosa* was found to be highly significant than ethyl acetate extract. After administration of ethanolic extract of *R.tuberosa* the level of total protein, albumin synthesis regained to normal level.

Because of normal synthesis of total protein, albumin, globulin, and A/G ratio is also returned to normal as in group I animals. This indicates that ethanolic extract of *R.tuberosa* may have chemopreventive property similar to that of standard anticancer drug doxorubicin. The table 3 shows that the level of magnesium, sodium and potassium in serum is significantly decreased ($p < 0.001$) but no alteration in calcium level in group II animals when compared with group I control animals. The decreased albumin synthesis by the fibrotic liver contributes towards the reduction of serum calcium and magnesium

concentrations. Similarly, ascites plays an important role in the depletion of serum electrolytes in liver cirrhosis (Papadakis, 1988). Significantly decreased serum calcium levels in patients with liver cirrhosis have been reported by sullivan *et al.,* 1979. The most probable cause for depleted serum sodium in DEN-induced hepatic fibrosis is due to retention of excess water. The marked decrease of serum albumin level (table 2) observed in the present investigation may be partly responsible for the reduced serum calcium levels. It was reported that the principal pathogenesis of hepatic osteodystrophy is due to the intestinal calcium malabsorption due to lower serum albumin concentrations (Nakano *et al.,* 1996). A significant decrease in either plasma or serum magnesium level has been reported in patients with liver cirrhosis (Sullivan *et al.,* 1979; Suzuki *et al.,* 1996; Rocchi *et al.,* 1994). Magnesium is one of the most important micronutrients which play a vital role in the immune system, in both innate and acquired immune response (Tam *et al.,* 2003). The level of magnesium is significantly increased ($p < 0.001$) with ethyl acetate extract of *R.tuberosa* but the level of calcium, sodium and potassium are not significant in group III animals. The level of potassium is significantly increased ($p < 0.01$) with ethanolic extract *R.tuberosa* in group IV animals. After administration of ethanolic extract of *R.tuberosa* the level of magnesium, sodium and potassium are reversed back to normal as that of standard anticancer drug doxorubicin. The present investigation suggests that alterations of essential elements play an important role in the aggravation of DEN-induced hepatic fibrosis in rats is secondary to the disease process. The table 4 shows the level of magnesium, sodium and potassium in liver is significantly decreased ($p < 0.001$) but the level of calcium is significantly increased ($p < 0.001$) in DEN induced HCC of group II animals when compared with group I normal animals. Potassium is the principal cation of intracellular fluid and plays an important role in the maintenance of acid-base balance. Several disorders of potassium metabolism have been described in association with liver diseases (Pitts and Van-Thiel, 1986; Perez *et al.,* 1983). Hypocalcaemia is a common phenomenon in patients with hepatic cirrhosis (Weiner and Wingo, 1997; Podolsky *et al.,* 1973).

The decreased potassium level observed in the present investigation in both serum (table 3) and liver tissue (table 4) may be attributed to the pathophysiology of hepatic fibrosis and the underlying disease process (Thier, 1986; Vitale *et al.,* 1985). The level of magnesium is significantly increased ($p < 0.01$) and .psodium is significantly increased ($p < 0.05$) with ethyl acetate extract *R.tuberosa* but calcium and potassium levels are not significant in group III animals. The level of calcium is significantly increased ($p < 0.01$), magnesium, sodium are significantly increased ($p < 0.001$) with ethanolic extract of *R.tuberosa* but the potassium is not significant in group IV animals. Many factors interfere with mineral metabolism in fibrotic animals. Some of the factors are gastrointestinal disturbances, malabsorption and interactions between elements. It is important to note that the animal body weight and liver weight were significantly reduced after the administration of DEN. The remarkable decrease of both serum and liver ascorbic acid concentrations reported (George, 2003) in DEN-induced hepatic fibrosis in rats may also have a relationship with the alterations of minerals. After administration of ethanolic extract of *R.tuberosa* the level of calcium, magnesium and sodium are reversed back to normal level and thereby liver cirrhosis was reduced along with immune system being protected.

**Table 4:** Effect of extract of *R.tuberosa* on Calcium, Magnesium, Sodium and Potassium in liver of control and experimental animals.

| Parameters | Group I | Group II | Group III | Group IV | Group V |
|---|---|---|---|---|---|
| Calcium | 9.0 ± 0.3 | 11.2 ± 1.0$^{c}$ | 10.1 ± 0.7$^{NS}$ | 9.8 ± 0.3$^{b}$ | 9.5 ± 0.4$^{a}$ |
| Magnesium | 65.1 ± 1.5 | 50 ± 2.0$^{c}$ | 54 ± 2.3$^{b}$ | 60 ± 2$^{c}$ | 62 ± 3.1$^{c}$ |
| Sodium | 998 ± 21 | 762 ± 32$^{c}$ | 800 ± 19$^{a}$ | 870 ± 17$^{c}$ | 920 ± 40$^{c}$ |
| Potassium | 258 ± 15 | 231 ± 20$^{a}$ | 238 ± 11$^{NS}$ | 245 ± 15$^{NS}$ | 250 ± 9$^{NS}$ |

Each value is expressed as mean ± S.D, for six rats in each group. The calcium, magnesium are expressed as mg/gm weight of tissue and sodium, potassium are expressed as µgm/100gm weight of tissue. Group I- control animals, Group II- cancer bearing animals, Group III- ethyl acetate extract post treated, Group IV- ethanolic extract post treated and Group V- standard Doxorubicin treated animals. Statistical significance (a = $p < 0.05$, b = $< 0.01$, c = $p < 0.001$) and non significant (NS), when Group II compared with Group I, when Group III, IV and V compared with Group II.

## SUMMARY AND CONCLUSION

Many of infectious diseases are known to be treated with herbal remedies throughout the history of mankind: even today plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries. The discovery of medicinal plants in different parts of the world is important for agriculture and medical sector. The total protein, albumin, globulin and A/G ratio were significantly decreased in DEN induced HCC animals. After administration of ethanolic extract of *R.tuberosa* the level of total protein, albumin, globulin and A/G ratio levels were back to normal. The level of magnesium, sodium and potassium were significantly decreased but the level of calcium was significantly increased in DEN induced HCC animals.

These changes were reverted to near normal with treatment of ethanolic extract of *R.tuberosa* and thereby liver cirrhosis was reduced along with immune system being protected. In conclusion, results of the present study suggest that DEN-induced hepaticcarcinogesis play certain role in mineral disturbances. Low levels of albumin and related ascites may be one of the major causes of the imbalance of mineral metabolism in liver diseases. Over all the present study gives scientific evidence for a new novel innovative chemotherapeutic agent for management of human hepato carcinoma to balance the mineral metabolism in hepatocarcinoma. However further studies are needed to isolate the active compounds from the ethanolic extract of *R.tuberosa* to confirm these properties for safe, efficacious, cost effective and eco-friendly anticancer drug.

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