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Rhinacanthus Nasutus Leaf Methanolic Extract Improves Antioxidant Levels In Rat Liver Under The Stress of Acrylamide

JAYALAKSHMI KUGARTHI	Department of Biochemistry, Sri Venkateswara University,Tirupati – 517 502, A.P., INDIA
MALLEPOGU VEN-KATASWAMY	Department of Biochemistry, Sri Venkateswara University,Tirupati – 517 502, A.P., INDIA
SUMAN BUKKE	Department of Biochemistry, Sri Venkateswara University,Tirupati – 517 502, A.P., INDIA.
SAJEDA DUDEKULA	Department of Biochemistry, Sri Venkateswara University,Tirupati – 517 502, A.P., INDIA.
Prof. Dr. THYAGA RAJU KEDAM	Department of Biochemistry, Sri Venkateswara University,Tirupati – 517 502, A.P., INDIA.

ABSTRACT

Rhinacanthus nasutus (RN), an herb available in our premises was analyzed for active molecules and used the extract of leaf for the induction of vitamin-C and glutathione (GSH) contents in the liver of rats under the stress of a neurotoxicant and prooxidant, acrylamide (AC). The rats on treatment with acrylamide showed decrease in GSH content from 4.55±0.146 (control) to 2.79±0.035µg/gm tissue of rat liver, the extract of *Rhinacanthus* (RN) has showed elevation of these levels from 4.55±0.146 to 5.34±0.068µg/gm of liver tissue and on combination of acrylamide and Rhinacanthus observed to replenish the GSH content on treatment of 300mg of RN. Similar to above the vitamin-C content was also found upon the treatments of AC, RN and AC+RN combination. These results were found to be statistically significant, however the mechanism of induction of GSH and Vitamin –C contents in liver of rat on treatment with RN extract require further investigation.

KEYWORDS Acrylamide,glutathione,vitamin-c,antioxidants,Rhinacanthus

INTRODUCTION

In aerobic organisms, oxygen is essential for efficient energy production but paradoxically, produces chronic toxic stress in cells. Thus, protective mechanisms must exist for the removal of toxic oxygen byproducts. Several protective systems have evolved in living organisms to enable adaptation to oxidative environments. These antioxidant defense systems are critical for survival in both prokaryotic and eukaryotic organisms. Several environmental insults as well as the aging process, are associated with oxidative stress (OS) due to elevation of Reactive oxyzen species (ROS) or insufficient ROS detoxification (Sies et al ., 1999). According to Janero et al., 2004 redox regulation by nitrosylation must be tissue-specific, since nitrosylation is determined by NO production and metabolic rate (Feelisch et al., 2002). Other studies indicate that differences among species also exist. (Bryan et al., 2004). In mammals, a relationship exists among OS, GSH/GSSG status, formation of nitrosylation products, Vitamin-C content and life span. Environmental toxicant-induced generation of ROS and RNS might modulate the structure and the function of signal transduction proteins and consequently, transcription, allowing cells to respond to environmental stressors through changes in gene expression.

Antioxidants

The amount of antioxidants present under normal physiological conditions is just adequate to quench the free radicals that are generated at a normal physiological rate. Any further increment in the concentration of free radicals (due to environmental or natural causes) can cause an imbalance between the free radicals and antioxidants, leading to oxidative stress (Blokhina et al.,2003).Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms (Cadenas, 1997). Defence mechanisms against free radical-induced oxidative stress involve: (i) preventative mechanisms, (ii) repair mechanisms, (iii) physical defences and (iv) antioxidant defences. Enzymatic antioxidants defences include within the body are Superoxide Dismutase (SOD), Glutathione Peroxidase (GPX), Xanthine Oxidase (XOD), Catalase (CAT) etc. (Cadenas, 1997). Dietary micronutrients also contribute to the antioxidant defense system. These include β-carotene, vitamin C, and vitamin E. Water-soluble molecule, such as vitamin C, is potent radical scavenging agent in the aqueous phase of the cytoplasm, whereas lipid soluble forms, such as vitamin E and β-carotene, act as antioxidants within lipid environments. The minerals such as selenium, copper, zinc and manganese are important for oxidation regulation, since they act as cofactors for antioxidant enzymes. In addition to internal and external defence sources the internally available micro molecules potentially participate are GSH and Vitamin-C.

Glutathione

The most abundant cellular antioxidant is the tripeptide, GSH(ɤ-glutamyl-l-cysteinyl glycine). GSH is synthesized in two steps. First, ɤ-glutamyl cysteine synthetase (ɤ-GCS) forms a di-peptide bond between glutamic acid and cysteine, and then GSH synthetase adds glycine and produces GSH. The formed GSH prevents the oxidation of protein thiol groups, either directly by reacting with reactive species or indirectly involving them. The main protective roles of glutathione against oxidative stress include (i) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathione-S-transferase and others; (ii) GSH participates in amino acid transport through the plasma membrane; (iii) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase (iv) glutathione is able to regenerate the most important

antioxidants, Vitamins C and E, back to their active forms; glutathione can reduce the tocopherol radical of Vitamin E directly, or indirectly, via reduction of semidehydro ascorbate to ascorbate through glutathione reductase. (Masella et al., 2005). This is a primary nucleophile used for scavenging free radicals. The glutathione plays a critical role in protecting cells from oxidative stress and xenobiotics. GSH and GSSG are important components in the cellular red-ox system. The GSH/GSSG ratio is the major red-ox buffer in the cell. GSH is found ubiquitously in eukaryotic cells at a concentration between 1 and 10mM. The relationship among intracellular GSH, GSSG, and GSH/GSSG or [GSH] 2/ [GSSG] ratio is more complicated. They are governed by the rate of synthesis of GSH, the prevailing oxidative state, the activities of GPX and GR, the degree of export of GSH and GSSG from the cell, and compartmentalization of these molecules. (Marí et al., 2009).

Vitamin -C

The U.S. studies of health professionals, at first, did not find any association between vitamin-C supplementation and coronary risk. However, several studies have shown effects on increased risk of heart attacks and death rate due to deficiency of vitamin- C in blood of human beings. Studies conducted on European populations also indicated that coronary heart disease mortality is higher in those with low vitamin -C blood. An analysis of a 10-year follow-up study of a representative sample of the U.S. population found that men with the highest vitamin- C consumption (at least 50 mg/day from diet plus regular use of supplements) had a 42% lower rate of death from cardiovascular diseases and women had a 25% lower rate. Further research is needed to resolve the discrepancy in epidemiologic findings on vitamin- C intake and heart disease risk.

Natural plant extracts which can correct redox balance

Enzymatic antioxidants like SOD,CAT,and GPX are produced endogenously in the cells where as non enzymatic antioxidants like carotenoids,flavonoids,vitamins,minerals,etc. are constituents of many fruits,vegetables,nuts,grains, and other plant parts.(Flora,2009).The use of natural substances particularly those derived from plants, to control diseases is a centuries old practice that has led to the discovery of more than half of all modern pharmaceuticals. A growing worldwide interest in the use of phytopharmaceutical as complimentary or alternative medicine. Rhinacinthus nasutus plant is rich in biological compounds with antioxidant properties contributed to the protection of cells and tissues against deleterious effects of reactive oxygen species.

Rhinacanthus nasutus Plant

Rhinacanthus nasutus is a small shrub, its trunk is edge shaped. The short twigs and young leaves are covered with hair. The blooms in bunch at the lane of twigs. Herbal tea preparation using Rhinacathus nasutus lowers blood pressure and diabetes. Leaves and roots of the plant have antifungal activity

various parts of Rhinacanthus nasutus plants have also been used for the treatment in many other diseases such as eczema, pulmonary tuberculosis, herpes, hepatitis, diabetes, hypertension, and various skin diseases, and the active components of this plant have been widely investigated The candidate plant chosen for the study is Rhinacanthus nasutus and the common name for Rhinacanthus nasutus is nagamalli belongs to acanthaceae family. According to Nirmala devi and Vasuki 2009., and studies conducted in our laboratory has revealed that the leaf part of Rhinacanthus nasutus was found to be rich in both enzymic and nonenzymic antioxidants. Thus the present study was conducted on the strong free radical scavenging activity and antioxidant potential of Rhinacanthus nasutus leaves to validate the use of these leaves in medicinal preparations for treatments of disorders and disease caused by oxidative stress.

MATERIALS AND METHODS

Chemicals

Acrylamide (99.9%), a monomer form, Glutathione reduced (GSH) and Vitamin-C were purchased from Bio-Rad laboratories (Richmond, USA) and other chemicals purchased indigenously were of pure and used for the analysis of various samples of our research. All other chemicals procured from the local companies were of high quality.

Experimental animals

The male wistar rats weighed about 150-200gms with an age of three months old were purchased

from Sri Venkateswara Enterprises (Animal Agency), Bangalore, India. These rats were acclimatized for seven days after arrival from the supplier (control and treatment groups consisted of six animals each). Temperature was maintained at 25°C with relative humidity of 40-50% on 13L:11D hrs (5.00am – 5.00pm) cycle. Animals were housed individually in polycarbonate cages and provided food (Purina Certified Rodent Chow 5002 and tap water ad libitum).

Collection of plants and preparation of extracts:

The Rhinacanthus nasutus (L) were identified and authenticated by plant Taxonomist, Department of Botany, Sri Venkateswara University,Tirupati,Andhra Pradesh and voucher specimennoSVUBH/579. The fresh leaves of R.nasutus were collected from Sesaschalam hills (Tirumala Hills and Tirupati) Chittoor district of Andhra Pradesh. Fresh leaves of Rhinacanthus nasutus (L) were shade dried and milled to Fine powder using pestle and mortar. The powdered plant material was macerated with hexane,Ethyl acetate, methanol and water separately. The extract was then filtered with filter paper (Whatman No. 1) under reduced pressure using Rota evaporator at 40°C. The concentrate was to obtained into a dark molten mass then layered on aluminum foil and freeze dried for further use.

Experimental Design:

The rats were divided into 12 groups in addition to control and each group consisted of six rats, treatment was given for six weeks as follows. The Control rats received water equivalent to the volume of treatment. The groups I, II and III have received 0.1mg, 0.2mg and 0.3mg, respectively, of acrylamide per Kg bw of rat, groups IV, V and VI have received 100mg, 200mg and 300mg of Rhinacanthus nusutus leaf powder methanolic extract, respectively, as Kg body weight of animal, groups VII, VIII and IX have received combination of Acrylamide and Rhinacanthus nusutus as 0.1mg + 100mg, 0.2mg+200mg and 0.3mg+300mg respectively per kg body weight of animal and group X, XI and XII have received 0.3mg of acrylamide mixed with 100mg of RN, 200mg of RN and 300mg of RN, respectively, for kg body weight of animals. After the treatment the animals were decapitated and collected the liver and stored until use at -20°C in defreezer.

Processing of tissue for antioxidant assay

The frozen normal and treated rat livers were thawed slowly, minced with scissors and homogenized in 50mM Tris-HCl buffer, pH 8.0, containing 0.25M sucrose and 1mM PMSF using a glass homogenizer. Homogenization was done by using the Potter Elvijhem homogenizer and care was taken to minimize the froth formation. The homogenate was passed through two layers of cheese cloth to remove fat and the resulting supernatant was centrifuged at 35, 000 x g on high speed refrigerated centrifuge (Remi) for 30min. The resulted supernatant was used as the enzyme source for the determination of GSH and vitamin-C. All the purification procedures were conducted at 4°C unless otherwise stated.

Assay of non-enzymatic antioxidants

Reduced Glutathione (GSH)

Reduced Glutathione content was determined according to the method of Ellman and Boyne (DTNB procedure., 1972). 0.5mL of homogenate was pipetted out and precipitated with 2.0mL of 5% TCA. 1.0mL of the supernatant was taken af-

ter centrifugation and added to it 0.5mL of Elman's reagent followed by 3.0mL of phosphate buffer. The yellow colour developed was read at 412 nm. A series of standards were made in a similar manner along with a blank containing 3.5mL of buffer.

Ascorbic acid (Vit-C)

Ascorbic acid levels were determined according to the method of Omaye et al., 1979. 0.5mL of tissue homogenate was mixed thoroughly with 1.5mL of 6%TCA and centrifuged for 20minutes at 3500 x g. To 0.5 mL of the supernatant, 0.5mL of DNPH reagent was added and mixed well. The tubes after 3h of incubation at room temperature placed in ice-cold water and added 2.5mL of 85% sulphuric acid and further allowed to stand for 30 minutes. A set of standards containing 10- 50mg of ascorbic acid were taken and processed similarly along with a blank, containing 0.5mL of 4% TCA. The color developed was read at 530 nm.

Statistical analysis

Results were expressed as the means ± standard deviation (SD). Differences between groups were evaluated by using one-way ANOVA, followed by Dunett's t-test. All statistical analyses were performed using the statistical software SPSS11.0 (SPSS Ltd., Surrey, UK). The p value of less than 0.005 was considered as statistically significant.

RESULTS

Effect of acrylamide on non enzymatic antioxidants

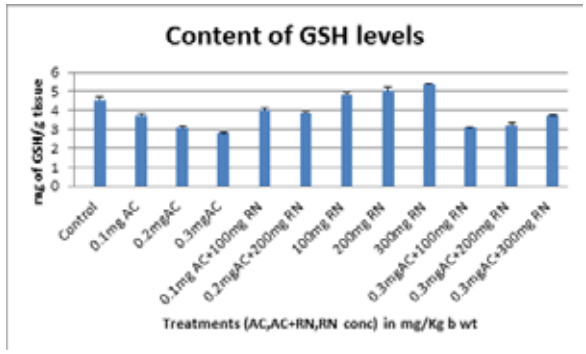
Glutathione (GSH)

The Reduced glutathione (GSH) levels in livers of control and AC-treated, 0.1mg - 0.3mg, and Rhinacanthus methanolic extract treated groups of 100mg-300mg, and also the groups of treated with combination of both acrylamide and rhinacanthus methanolic extract results were presented in Fig 1 are as follows. Glutathione (GSH) levels in control as well as 0.1mg, 0.2mg, and 0.3mg AC- alone treated groups (II to IV) were 4.55, 3.74, 3.05, 2.79, respectively, and there was a 1.216, 1.492 and 1.630 fold decrease in reduced glutathione was found with increased concentration of AC in treated than in the control. Groups which were given combination of both AC and RN treatments had reduced glutathione levels as 4.01, 3.86, 3.05, 3.23, 3.72 of V, VI, X, XI, XII groups respectively. There is 1.072, 1.265, 1.093, 1.157 and1.33 folds increase when compared to AC alone treated groups (AC treated controls) II, III, IV groups, respectively. The GSH values of X, XI, XII significantly elevated with increasing concentration of RN when compared to 0.3 AC treated control group(IV). Groups which were treated with only rhinacanthus leaf methanolic extract were having GSH concentrations as 4.80, 5.02, 5.34 in VII, VIII, IX, groups respectively. There is 1.0549, 1.1032, 1.1736 folds increase in GSH levels with increasing concentration of plant extract when compared to control these were statistically significant as shown in Table 1.

Ascorbic acid (Vit-C)

Vitamin-C levels in livers of control and AC-treated 0.1mg to 0.3mg and Rhinacanthus methanolic extract treated groups of 100mg to 300mg ,and also the groups of treated with combination of both acrylamide and Rhinacanthus methanolic extract results are presented in Fig-2 were as follows. Vitamin-C levels in control as well as 0.1mg, 0.2mg, and 0.3mg AC- alone treated groups were 8.17, 7.18, 6.18, 5.16, respectively and there was a 1.105, 1.322, and 1.583 fold decrease in Vitamin-C concentration with increase in AC concentration was found in treated than in the control. Groups which were given combination of both AC and RN treatments had Vitamin-C levels as 7.39, 7.63, 7.81, of V, VI, X, XI, XII groups, respectively. There is 1.029, 1.23, 1.038, 1.394 and 1.55 folds increase when compared to AC alone treated II, III, IV groups, respectively. The vitamin-C values of X, XI, XII significantly elevated with increasing concentration of RN when compared to 0.3 AC treated control group(IV). Groups which were treated with only Rhinacanthus methanolic (RN) extract were having vitamin-C concentrations as 8.39, 8.64, 8.89 in VII, VIII, IX, groups, respectively. There is 1.0269, 1.0575, 1.088 folds increase in vitamin-C levels with increasing concentration of plant extract when compared to control. These were statistically significant as shown in Table 2.

GSH Analysis



All groups are statistically significant at the level of p value less than 0.005 compared to control.

Note: The GSH content was expressed in mg of GSH present in gram tissue of liver.

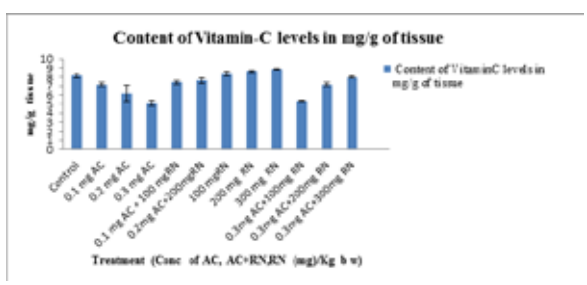
Figure-1: Effect of acrylamide on the GSH levels of rat liver and hepatoprotective role of Rhinacanthus methanolic extract by enhancing GSH levels The glutathione level is expressed as mg/g tissue

Table-1
ANOVA of GSH

GSH	Sum of Squares	df	Mean Square	F-value	p-value
Between Groups	18.67	9	2.07		
Within Groups	0.25	20	0.01	168.801	*0.000
Total	18.91	29			

***The p value of less than 0.0050 was considered as statistically significant.**

Vitamin-C



All groups are statistically significant at the level of p value less than 0.005 compared to control.

Figure-2: Effect of acrylamide on the ascorbic acid levels of rat liver and hepatoprotective role of rhinacanthus methanolic extract by enhancing ascorbic acid levels. The ascorbic acid was expressed as mg/g tissue.

Table-2
ANOVA of vitamin-C

Vitamin-C	Sum of Squares	df	Mean Square	F-value	p-value
Between Groups	36.93	9	4.10		
Within Groups	0.85	20	0.04	96.679	*0.000
Total	37.78	29			

***The p value of less than 0.0050 was considered as statistically significant.**

DISCUSSION

In rat liver under acrylamide stress depletes the level of vitamin C and glutathione significantly when compared to respective control groups. This reduction reflects exploitation of vitamin C and reduced glutathione against oxidative damages caused by the acrylamide. Acrylamide treatment causes oxidative stress by inducing the generation of reactive oxygen species (ROS) there by reducing the antioxidant defense systems of the cell by depleting non enzymatic antioxidants systems (Vitamin-C and GSH) and increasing susceptibility of cells to oxidative attack by altering the membrane integrity and fatty acid composition (Lee and Jacobs, 2005,Thyagaraju et al., 2013). Free radicals cause damage in biological systems. This in turn cause cellular damage that may lead to cancer, liver injury, heart diseases etc (Halliwell and Gutteridge, 1985). AC like other α,β-unsaturated electrophiles reacts with cellular nucleophiles possessing -SH,-NH or OH as the reactive group. Therefore, it reacts with GSH in a similar manner and formed GSH-S-conjugates, which is the initial step in the biotransformation of nucleophiles into mercapturic acid(Awad et al., 1998). Glutathione is primary nucleophile required to react with any electrophile present in the body or entered in the biological system from the environment in the absence of GSH the next nucleophile involved for reaction with electrophile is DNA. So to avoid damage to DNA the GSH, as major antioxidant molecule, present in the system scavenges free radicals or ROS and protects almost all organs from oxidative stress. A decline in glutathione content was observed when the rat liver which were treated with standard oxidant. GSH, a key antioxidant, is an important constituent of intracellular protective mechanisms against various noxious stimuli, including oxidative stress. GSH depletion in hepatocyte mitochondria, has been shown to be an important mechanism in the pathology of experimental liver injury.(Yuan et al ., 2007, and Park et al 2002). This is also observed with other reports that showed significant decrease in the level of glutathione (GSH) in liver of rats and chicks upon acrylamide administration (Srivastava et al., 1983,and Venkataswamy et al 2015).The exact mechanism of influence of acrylamide on GSH content has not been established till to date (Sugawara et al., 1991, Bechara, 2004). There may be two possible reasons for decline in GSH levels 1.Impairment of GSH biosynthesis. Since AC binds to cysteine residues of proteins-(Srivastava et al., 1986) and is a known to limit substance in GSH biosynthesis.2.Involvement of GSH in AC metabolism. Three distinct pathways have been proposed:(1) AC conjugation with GSH, a metabolic process representing the major route of detoxification of AC. (2) liberation of glycidamide from AC metabolism. Glycidamide is a potent generator of ROS production (via inhibition of the mitochondrial respiratory chain) as well as an inhibitor of the activities of several antioxidant enzymes; (3) ROS generated as by-products of AC metabolism via cytochrome P450 2E1 oxidation (Griffin et al., 1998; Summer et al.,1999, Eikelenboom et al., 2002). Also it has been reported that 59% of AC metabolites were excreted in the urine of rats through mercapturic acid metabolism. administered AC as AC-GSH conjugates. Depletion of GSH caused by AC-GSH metabolites has been reported in vivo (Martensson et al .,1990))) and in vitro (Park et al., 2002). (Reed and Fariss.,1984)., and also the World Health Organization (WHO-2012) reported that in rats biotransformation of acrylamide occurs through gluthathoine conjugation and through decarboxylation at least four urinary metabolites have been found in rat urine. A reduction in GSH level during acrylamide toxicity has been reported in many studies (Pallavi, SVU, 2016) GSH decrease along with increased MDA content suggest that acrylamide induced lipid peroxidation and decreased GSH concentrations. Plants have evolved protective enzymatic and non-enzymic mechanisms to scavenge free radicals as well as reactive oxygen species and minimize their harmful effects and also could revert the damage caused to the cells as indicated by the increased levels of antioxidant vitamins in all the treated groups. An increase in the vitamin C and GSH levels in rat liver revealed the ability of the leaf extracts to counteract the oxidant induced changes (Haseena bhanu et al 2011). According to Kadri Mohamed Sadek (2012) and Sohn et al., the Carica papaya extract have showed hepatoprotective effect by increasing antioxidant levels (GSH and Vitamin-C) in acrylamide treated rats.

In other studies also reported that there is increasing GSH levels in rat tissues following treatment with plant extracts. According to Shawkia et al., 2008 there is demonstrated that elevated GSH levels might be due to an induced denovo synthesis of GSH in hepatic cells in the presence of plant extract where gene expression of gluthamate cysteine ligase a key rate limiting enzyme stimulate GSH synthesis (Zheng et al., 2007).The control of oxidant levels is achieved by inducing the antioxidative defense mechanism. (Ali et al., 2006). In an another study it was demonstrated as the increase of hepatic GSH levels in the liver of RN + AFB1 treated rats indicate that the plant extracts have significant antioxidant effects, which is important for removing free radicals and reactive intermediates generated from the metabolism of environmental toxins. It has been reported that higher GSH levels help to lower AFB1 toxicity through conjugation with the toxin.(Dalvi and Sere, 1988).

The levels of glutathione and Vitamin C were depicted in Figure 1 and 2 ,of this study,showed a significant elevation in levels of Vitamin C and GSH are observed in RN treated groups. It is evident from the values that Rhinacanthus nasutus leaf extracts brought a significant increase in the levels of ascorbate and glutathione even in the presence of acrylamide. An increased vitamin-C and reduced glutathione levels in the groups of methanolic extract of Rhinacanthus nasutus leaves revealed its ability to counteract the oxidant –induced changes.(Nirmala Devi and Vasuki 2009). Nirmala Devi and Vasuki 2009 demonstrated that an increased Vitamin-C and reduced gluthathoine was observed in rat liver slices treated with acrylamide and Rhinacanthus leaf methanolic extract. Similar to it Rhinacanthus nasutus leaf extracts evoked a significant increase in the depleted Vitamin-C and reduced glutathione contents (Suman bukke et al., 2013). The above findings are supported by other similar studies.(Ip et al 1996., Sohn et al 2003.). The results of the present study indicate that RN whole plant extract inhibits oxygen radicals such as superoxide, hydroxyl and lipid peroxides. The concentration of RN (aerialparts) extract required for in vitro inhibition of these oxygen radicals was higher than that of a known antioxidant like curcumin (Halliwell et al.,1985) RN is reported to contain flavanoids, lignans, triterpenes etc (Sendi A, et al.,1996, and Kernan.,et al 1997), and these may be responsible for the observed antioxidant properties in the present study. Natural products like coumarins, flavonoids and lignans have the potential of reducing the deleterious effects of free radicals (Chen et al., 1976, . Hoefler et al 1980. Faure et al., 1991.). The free radical scavenging (antioxidant) property of RN extract may be responsible for the observed hepatoprotective effects of the present study. The RN extract is non - toxic up to 500mg/kg b w.(Kupradinun et al.,2009, Suja et al.,2004 and Visweswara Rao SVU, 2013) This is not surprising, as it is an ingredient of several tribal medicinal formulations used for liver diseases, and viral infections (Suja et al.,2004). This elucidation and study thus supports the present view still requires further studies for its bioactive principle, to evaluate its potential in the treatment of liver diseases.

CONCLUSION

On the basis of results obtained we conclude that acryl amide cause disturbances in the oxidative status and the effect was pronounced with the high doses, indicated enhanced liver damage risk during exposure to acryl amide. In rat liver GSH depletion leads to increased production of superoxide, hydroxyl radicals, and also H2O2. The intracellular GSH pool is important for limiting oxidative stress–induced hepatic injury and suppression of cell proliferation and cell differentiation. Because of reduction of glutathione and vitamin-c the free radical scavenger´s role suppressed in acrylamide treated groups from II to IV. But in groups treated with both acrylamide and rhinacanthus methanolic extract to VI and also in groups X, XI, XII, we can observe significant elevation in antioxidant levels than to only AC treated controls (II, III, IV).The groups which

are treated with only rhinacanthus methanolic extract were found to have elevated levels of reduced glutathione and Vitamin-C when compared to untreated control (I group). This can convey us that rhinacanthus could be a better choice for the preparation of medicine to treat many liver diseases

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