International Journal of Phytopharmacy **Research Article**
Vol. 4 (2), pp.49-56, Mar-Apr 2014
ISSN: 2277-2928 (Online)
Journal DOI:10.7439/ijpp

www.ssjournals.com

Antinephrotoxic efficacy of *Operculina turpethum* and its isolated Stigma-5,22 dien-3-O-b-D-glucopyranoside against N-Nitrosodimethylamine induced renal carcinogenesis in male mice

Veena Sharma* and Manu Singh

Department of Bioscience and Biotechnology, Banasthali University, Banasthali 304022, Rajasthan

***Correspondence Info:**
Veena Sharma
Department of Bioscience and Biotechnology,
Banasthali University, Banasthali 304022, Rajasthan
E-Mail: drvshs@gmail.com

Abstract
Objectives: Human exposure to nitrosamines can result from the formation of N-nitroso compounds either in food during storage or preparation or *in vivo*, usually in the stomach. N-Nitrosodimethylamine (NDMA) is one of the main N-nitroso compounds which is commonly found in drinking water and is a potent carcinogen. The therapeutic effect of ethanolic root extract of *Operculina turpethum* was studied for its possible anti-cancerous potential induced by N- Nitrosodimethylamine in male albino mice as the *in vivo* model for the study.
Methods: Renal malondialdehyde (MDA), Superoxide dismutase (SOD), catalase (CAT), glutathione content (GSH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), protein, cholesterol, urea and creatinine content were measured as oxidant/antioxidant markers. Electron Microscopy investigations of the renal tissue were also performed. One-way ANOVA test was used for comparisons of parameters in study groups.
Results: Renal antioxidant defense systems, such as superoxide dismutase, catalase, glutathione peroxidase activities and reduced glutathione level, depleted by NDMA were restored to normal by the treatment. Oral administration of *Operculina turpethum* ethanolic extract, both crude and isolated groups recovered the enzyme activities and caused significant increase in serum protein, the treatment significantly reduced the elevated serum creatinine and urea levels ($p>0.05$), ($p<0.01$), ($p<0.001$). The ultrastructural electron microscopical analysis showed a decrease in cellular degradation comparing to the intoxicated mice.
Conclusion: These findings prove the potential of *Operculina turpethum* as an antioxidant therapy to counteract mitochondrial and post-mitochondrial oxidative stress generated in kidney upon NDMA treatment thereby acting against renal toxicity by a carcinogen.
Keywords: Antioxidant, Glycoside, Kidney, Isolated, Oxidative stress

1. Introduction

Plants are known to provide a source of inspiration for novel drug compounds and this is sequel to the fact that medicines derived from plants have made large contributions to human health and well being. Antioxidants from natural products used in traditional medicine may reduce the risk of toxicity and maintain the therapeutic effectiveness when the drug is used clinically[1-2]. WHO therefore approved the use of herbal products for national policies and drug regulatory measures in order to strengthen research and evaluation of the safety and efficacy of these products[3].

Researchers and the pharmaceutical industry are continually searching for new medications with novel or improved actions. Botanicals provide an extensive natural source of new drugs, with various chemical compounds that have been discovered from plant sources, leading to subsequent formulations of potential new drugs. Medicinal plants have bioactive compounds acting to block or reverse carcinogenesis at early stages[4]. Phytochemicals are naturally occurring substances found in plants. There has been considerable public and scientific interest in the use of phytochemicals to combat human diseases. Moreover, they are considered to be an inexpensive, effective and easily applicable approach to control cancer[5]. Cancer is a global health problem with high morbidity and mortality and poses both economic and psychological challenges. Thousands of herbal and traditional compounds are being screened worldwide to validate their use as anti-cancerous drugs[6].

A chemopreventive agent could be effective at any of the classically defined stages of carcinogenesis: initiation, promotion, and progression[7-8]. Studies of natural products provide opportunities to reveal interesting biology and generate leads pertaining to specific cellular targets, activities and therapeutic manipulations. A large number of plants possessing anti-cancerous toxicity properties have been documented[9-12]. Kidney is highly susceptible to toxicants and xenobiotics as a high volume of blood flows through it and it filters large amounts of toxins which can concentrate in the kidney tubules. It can result in systemic toxicity causing; decreased ability to excrete body wastes, inability to maintain body fluid and electrolyte balance and decreased synthesis of essential hormones[13].

Operculina turpethum Linn is convolvulaceous plant which is found throughout India, China, Ceylon, Australia. The plant *Operculina turpethum* contains various constituents such as carotenoids, terpenoids, flavonoids, polyphenols, alkaloids, tannins, glycosides, saponins, enzymes, and minerals etc. These phytochemicals possess antioxidant activities, which can be used in the treatment of multiple ailments[14]. They are useful in colic constipation, dropsy, vitiated conditions of vata, paralysis, myalgia, hyperlipidemia, arthralgia, pectoralgia, bronchitis, obesity,

helminthiasis, inflammations, intermittent fever, leucoderma, ulcers, erysepelas, haemorrhoids, tumors, jaundice, employed in drug formulations, dropsical effusions and rheumatism. Steroidal glycoside isolated from the roots of *Operculina turpethum* belongs to the class of phytosterols, which are shown to possess cholesterol lowering, immune-modulating as well as anticancer property[15-16]. Glycoside is a molecule in which sugar is bound to another functional group via a glycosidic bond. The sugar group is then known as the glycone and the non-sugar group as the aglycone or genin part of the glycoside.

Almost all the degenerative diseases involve Reactive oxygen species (ROS) as causative factors, including kidney failures and they are also involved in the promotion phase of carcinogenesis. Toxic effects of N-nitrosodimethylamine (NDMA), a potent carcinogenic and mutagenic substance, were also proposed to be due to reactive oxygen species formed by its metabolic activation [17-18]. The formation of reactive oxygen species (ROS) is apparent during the metabolic biotransformation of NDMA resulting in oxidative stress. Oxidative stress leads to carcinogenesis by several mechanisms including DNA, lipid and protein damage, change in intracellular signaling pathways and even changes in gene expression. Lipid peroxidation (LPO) may also result in several changes, including structural and functional membrane modifications, protein oxidation and generation of oxidation products such as acrolein, crotonaldehyde, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), which are considered strong carcinogens[19-20]. Hence, a major portion of the current pharmacological research is devoted to anticancer drug design customized to fit new molecular targets.

NDMA induced nephrotoxicity is a model of acute renal cancer. Nitrosodimethylamine (NDMA) belongs to a group of extremely toxic and mostly carcinogenic substances, the N-nitrosamines. NDMA has been detected in ambient air, water and soil, low levels of NDMA are commonly found in the air of car interiors, food, malt beverages, toiletry and cosmetic products, rubber baby bottle nipples and pacifiers, tobacco products and tobacco smoke, pesticides used in agriculture, hospitals, and homes, and sewage sludge. Several lines of evidence indicate that free radicals are involved in the nephrotoxicity caused by NDMA, and the damage is suggested to be the consequence of decreased renal antioxidant enzyme activity with enhanced lipid peroxidation. NDMA gets accumulated in the tubular epithelial cells of proximal kidney tubule, causing nephrotoxicity characterized by morphological destruction of intracellular organelles, cellular necrosis, loss of microvilli, alterations in the number and size of the lysosomes and mitochondrial vacuolization, followed by functional alterations including inhibition of protein synthesis, GSH depletion, lipid peroxidation and mitochondrial damage. Several distinct mechanisms have been proposed for NDMA cytotoxicity in renal tubule cells, including direct DNA damage, activation of caspase, mitochondrial dysfunction, formation of reactive oxygen species, effects on the endoplasmic reticulum and activation of TNF-α mediated apoptotic pathways[21].

The mechanism by which N-Nitrosodimethylamine produces cancer is well understood to involve biotransformation by microsomal enzymes, generating the methyldiazonium ion. In fact, various reports have enumerated the structural alterations in organelles, observed in different kinds of cancer [22]. It extends the study to the structural level, so the histological examination of the kidney tissues of mice exposed chronically to NDMA and the ultrastructural examination with electron microscopy is required. Moreover, no published information about *Operculina turpethum* and Stigma-5,22dien-3-O-b-D-glucopyranoside against renal carcinogenesis by NDMA is available. Therefore we examined its renoprotective effects in mice model.

2. Experimental Methodologies

2.1 Chemicals

TBA, TCA, HCl, pyrogallol, H_2O_2, triton-x, BSA, copper sulphate, ascorbic acid, thiourea etc. All chemicals used in the study were of analytical reagent grade and were purchased from reliable firms (SRL (India), MERCK, RANBAXY, HIMEDIA, TRANSASIA). NDMA was purchased from SIGMA.

2.2 Animal care and Monitoring

Healthy male Swiss albino mice (*Mus musculus*) (4-6 months old, weighing 20-30 g) were procured from C.C.S. Haryana Agricultural University (Hisar, India). They were housed under standard laboratory conditions of light (12:12 h L: D cycle), temperature (23 ± 2°C) and relative humidity (55 ± 5%).Animals had free access to standard food pellet diet (Hindustan Lever Limited: metal contents in parts per million dry weight: Cu 10.0, Zn 45.0, Mn 55.0, Co 5.0, Fe 75.0) and drinking water *ad libitum* throughout the study. The animal experiments were carried out according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The Institutional Animal Ethics Committee approved experimental design performed in this study for the use of Swiss Albino mice as an animal model for the study.

2.3 Plant Material

Operculina turpethum was collected from Pharmacological garden of CCSHAU Hisar, Haryana, India in the month of November 2012.The plant was identified with the help of available literature and authenticated by Botanist of Krishi Vigyan Kendra Rohtak, Haryana, India.

2.4 Preparation of Ethanolic extract

The freshly collected *Operculina turpethum* roots were dried in shade and coarse powder was extracted. Dried powdered material was placed in the Soxhlet thimble with ethanol in 500 ml flat bottom flask. It was further refluxed for 18 h at 80^0C for two days. Collected solvent was cooled and poured in a glass plate. The filtrate was dried in hot air oven below 50^0C for 48 h and kept in dissector for 2 days. The yield of the extract was 12.5% w/w of powdered plant material for further exploration. Collected dried extract was stored at 5^0C in air tight containers.

2.5 Isolation and Characterisation of Stigma-5,22 dien-3-O-b-D-glucopyranoside (Isolated Glycoside; IG)

Isolation of IG was achieved by Thin layer chromatography (TLC), Column Chromatography and High performance liquid chromatography (HPLC) whereas the characterization was achieved by Fourier transform infrared spectroscopy (FTIR), Nuclear magnetic resonance (NMR) and Liquid chromatography mass spectroscopy (LCMS). The nomenclature of the IG was achieved and it was then assessed for its anti-carcinogenic properties.

2.6 Acute Oral Toxicity Studies (LD50)

The acute toxicity of the plant extract was evaluated in mice (six per group) by preparing five different doses (100, 500, 1000, 1500 and 2000 mg/kg) and administered orally using gavages. Animals were kept without food for 18 h prior to dosing and were monitored continuously for 3 days after dosing for any sign of toxicity. The LD_{50} value of the extract was calculated arithmetically using the method described by Hamilton[23].

LD_{50} = Lethal dose - Σ (a x b)/N

Where a is the dose difference, b is the mean mortality and N is the number of animals in each group.

2.7 Treatment Regime

Adult Swiss albino male mice divided into ten groups of 6 mice in each group and were treated by oral gavage. Treatment consisted of simultaneous dosing of NDMA (N-Nitrosodimethylamine, 10mg/kg; intraperitoneal) followed by OTE (*Operculina turpethum* extract; oral). The animals were then euthanized 21 days after NDMA administration. NDMA was given on three consecutive days of each week for three successive weeks along with the plant extract.

The groups were as follows-

Group 1 - Control
Group 2 - NDMA treated (10 mg/kg body weight)
Group 3 - NDMA + OTE (300mg/kg body weight)
Group 4 - NDMA+ OTE (400mg/kg body weight)
Group 5 - OTE (300 mg/kg body weight)
Group 6 - OTE(400mg/kg body weight)
Group 7 - NDMA + Standard antioxidant(BHA1%)
Group 8 - BHA (1%)
Group 9 - IG (Isolated Glycoside; 50 mg/kg body weight)
Group 10 - NDMA + IG (Isolated Glycoside; 50 mg/kg body weight)

The doses of the plant extract, NDMA and standard were decided on the basis of previously published reports [24].

2.8 Biochemical assays

After 21 days of duration the mice were fasted overnight, then sacrificed and blood was collected by cardiac puncture. Kidneys were dissected out, washed immediately with ice-cold saline to remove blood, and the wet weight noted and then stored at -80°C for various biochemical assays, and histological studies.

2.8.1 Preparation of kidney homogenate

Kidney were minced and homogenized (10% w/v) in ice-cold 0.1 M sodium phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 rpm for 15-20 min at 48°C twice to get the enzyme fraction. The supernatant was used for biochemical assays[25].

2.8.2 LPO (Lipid peroxidation)

LPO was estimated colorimetrically by measuring malondialdehyde (MDA) formation as described by Nwanjo and Ojiako[26]. In brief, 0.1 ml of homogenate was treated with 2 ml of a 1:1:1 ratio of TBA-TCA-HCl (TBA 0.37%, TCA 15%, HCl 0.25 N) and placed in water bath at 65°C for 15 min, cooled, and centrifuged at 5,000 rpm for 10 min at room temperature. The optical density of the clear supernatant was measured at 535 nm against a reference blank. The MDA formed was calculated by using the molar extinction coefficient of thiobarbituric acid reactants (TBARS; 1.56×10^5 l/mole cm^{-1}). The product of LPO was expressed as nmol of MDA formed per g of tissue.

2.8.3 Superoxide dismutase (SOD)

Renal SOD activity was assayed according to the method of Marklund and Marklund [27]. For the control, 0.1 ml of 20 mM pyrogallol solution was added to 2.9 ml of Tris buffer and mixed, and reading was taken at 420 nm after 1.5 and 3.5 mins. The absorbance difference for 2 min was recorded and the concentration of pyrogallol was adjusted in such a way that the rate in change of absorbance per 2 min was approximately 0.020-0.023 optical density units. Liver extract (200 µl) was treated with 10 µl of 25% triton X-100 and kept at 48°C for 30 min. To 2.8 ml of Tris buffer, 0.1 ml of treated sample was added and mixed, and the reaction was started by adding 0.1 ml of adjusted pyrogallol solution (as for control). Reading was taken at 420 nm after 1.5 and 3.5 mins and the difference in absorbance was recorded. The enzyme activity was expressed as U/ml of kidney extract and 1 U of enzyme is defined as the enzyme activity that inhibits auto-oxidation of pyrogallol by 50%.

2.8.4 Catalase (CAT)

Catalase (CAT) activity was estimated following the method of Aebi[28]. Kidney extract (100 ml) was treated with ethanol (10 ml) and placed on an ice bath for 30 min. To this, 10 ml of 25% triton X-100 was added and again kept for 30 min on ice. To 200 µl phosphate buffer (0.1 M), 50 µl of treated kidney extract and 250 µl of 0.066 M H_2O_2 (prepared in 0.1 M phosphate buffer, pH 7.0) were added in a cuvette. The decrease in optical density was measured at 240 nm for 60s. The molar extinction coefficient of 0.036/µmole/ml was used to determine CAT activity. One unit of activity is equal to the nanomoles of H_2O_2 degraded/min/mg tissue.

2.8.5 Aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed by the method of Reitman and Frankel[29]. In brief, 0.2 ml of kidney fraction and 0.5 ml of substrate solution (for AST: aspartate and 2-ketoglutarate; for ALT: alanine and 2-ketoglutarate) were incubated at 37°C for 60 min for AST and 30 min for ALT. After incubation, 0.5 ml of DNPH solution was added to arrest the reaction, which was kept for 20 min at room temperature. To this, 1 ml of 0.4 N NaOH was added and absorbance was read at 510 nm. Activities were expressed as m M/ml.

2.8.6 Alkaline phosphatase (ALP)

Activities of alkaline phosphatase (ALP) were determined according to the protocol described in a laboratory practical manual [30]. Substrate solution (3 ml) was incubated at 37°C for 15 min and then 0.5 ml kidney homogenate was added. It was mixed well and immediately 0.05 ml of the mixture was removed and mixed with 9.5 ml of 0.085 N NaOH. This corresponded to zero time assay (blank). The remaining solution (substrate-enzyme) was incubated for 15 min at 37°C and then 0.5 ml was drawn and mixed with 9.5 ml of 0.085 N NaOH. Absorbance was measured at 405 nm against the reference blank. Specific activities were expressed as µmoles of p-nitrophenol formed/ min/ g tissue.

2.8.7 Total Protein

Protein content was determined by the method of Lowry[31] and bovine serum albumin as a standard.

2.8.8 Total Cholesterol

Cholesterol was determined by the method of Zak[32] with cholesterol as a standard.

2.8.9 Urea

The Urea content was determined by GLDH Urease method[33].

2.8.10 Creatinine

Creatinine level was determined by Jaffe's method[34].

2.8.11 Glutathione-S-Transferase (GST)

The GST activity was measured as described by Habig's method [35]. Briefly, 0.85 mL of phosphate buffer (pH 7.4), 50 µL homogenate and 50 µL of 1 mM CDNB were mixed in a cuvette, to which 50 µL of 10 mM GSH was added to initiate the reaction. The rate of formation of GSH-CDNB complex was monitored for 5 min at 340 nm. The results were expressed as nmol of CDNB conjugate formed/min/mg protein.

2.8.12 Reduced Glutathione (GSH)

Renal reduced glutathione (GSH) level was determined by the method of Ellman modified by Jollow [36]. Sulphosalicylic acid (0.5 ml 10%) was added to mixture of 0.4 ml homogenate and 0.6 ml of distilled water as protein precipitant. Supernatant (0.5 ml) was mixed with the reaction mixture of 4.5 ml of 0.5M Tris-buffer and 0.5 ml of 10mM DTNB and the absorbance was measured immediately at 412 nm .The GSH contents were calculated using GSH as standard and expressed as m M /g tissue.

2.9 Scanning Electron Microscopy (SEM)

For SEM, kidney samples (2–3 mm) were quickly isolated from the sacrificed animals and kept in 5% glutaraldehyde made in 0.1 M phosphate buffer pH 7.4, for 4–5 h. The samples were then washed in 0.1 M phosphate buffer and the dehydration steps followed.

2.10 Statistical Analysis

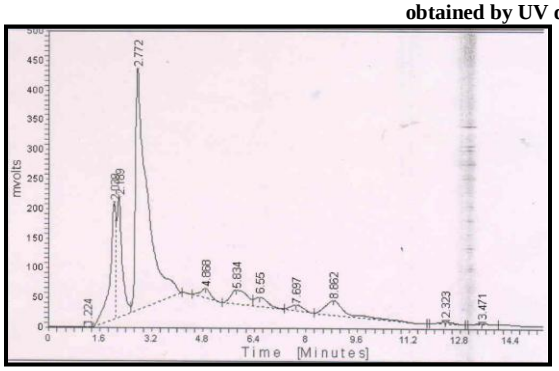
Data are expressed as the mean±SEM. The data was analyzed by analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (S.P.S.S. 11). The results were evaluated at a significance level of $p<0.05$ and 0.001 which were considered significant and highly significant, respectively.

3. Results

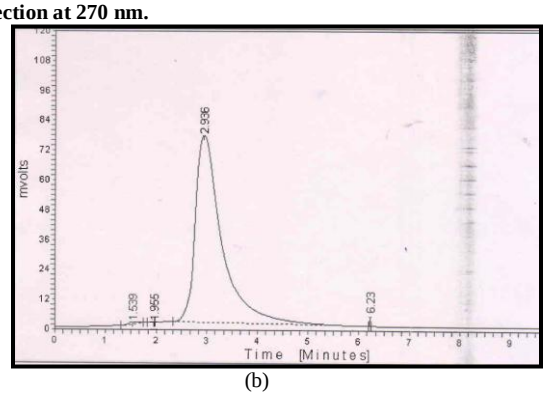
3.1 Isolation and Characterisation of Stigma-5,22 dien-3-O-b-D-glucopyranoside from the roots of *Operculina turpethum*

The isolated compound was found to be a steroidal glycoside. The HPLC profile of the crude extract and the isolated compound is shown in Figure1.

Figure 1: HPLC chromatogram of the crude ethanolic root extract (a) and isolated Stigma-5, 22dien-3-O-b-D-glucopyranoside (b) obtained by UV detection at 270 nm.



(a)



(b)

3.2 Acute Toxicity Study

The result of the toxicity test of *Operculina turpethum* extract for 3 days did not show any clinical adverse effect of substance-related toxicity on the animals, such as restlessness, hematuria, diarrhea and muscle-coordinated movement. Similarly, there was no mortality or morbidity observed at any tested doses except at the 2000 mg/kg dose. The LD_{50} value of the
Extract was found to be 1917.66 mg/kg.

3.3 Effect of *Operculina turpethum* on antioxidant enzymes

Treatment with different doses of the extract was well tolerated by all the animals, as there were no toxic effects observed by direct visual observation of the animals throughout the experiment. There was no death and apparent behavioural changes recorded during the course of the experiment in all treatment groups as compared to the control group. Administration of intraperitoneal NDMA to mice caused damage as indicated by a significant decrease in enzymes AST, ALT, ALP activity compared to control mice. The treatment of animals with the plant extract at the dose of 400mg/kg b.wt. and its IG at the dose of 50 mg/kg b. wt. significantly ($p<0.01$, $p<0.001$, $p<0.05$) recovered the normal range of enzymes. The decrease in the enzyme activities after administration of NDMA may be attributed to loss of membrane component (including alkaline phosphatase) into the extracellular fluids[37]. This reduction in alkaline phosphatase activities may lead to less availability of the phosphate group for the phosphorylation of ethanolamine and choline needed for the synthesis of two major membrane phospholipids, phosphatidylethanolamine and phosphatidylcholine, respectively with the attendant consequence of affecting membrane fluidity and decreasing the permeability of the epithelial cells. Biochemical parameters in the control and various experimental groups are depicted in Table I.

Table I Renoprotective effect of OTE and IG with different treatments in various experimental groups.

Groups	Dose(mg/kg)	AST (m M/ml)	ALT (m M/ml)	ALP (µmoles/min/g)
Control (1)	-	63.3±0.821#*	49.3±0.800#*	79.3±0.995#*
NDMA (2)	10	45.6 ±0.718# *	37.3±0.909# *	62.6±0.935# *
NDMA+OTE (3)	10+ 300	56.4±0.690# **	39.3±0.448#*	69.4 ±0.870#*
NDMA+OTE (4)	10+ 400	60.3±0.922#*	41.0±0.272#*	75.4±0.851#*
OTE (5)	300	55.8±0.491# **	43.2±0.745# **	72.7±0.524#**
OTE (6)	400	55.4±0.794# *	43.1±0.940# *	72.5±0.806#*
NDMA+BHA (7)	10+1%	54.7±0.605# *	44.1±0.498#*	69.2±0.927#*
BHA (8)	1%	57.4±0.695# *	46.5±0.544#*	76.7±0.691#*
IG (9)	50	55.2±0.879# *	47.5±0.580#*	72.7±0.392#*
NDMA+IG (10)	10+ 50	62.9±0.238# **	49.5±0.604#**	77.3±0.429#^

Values are expressed as mean ± S.E.M for six mice in each group. #p<0.001 vs. control group ; * p<0.01; **p<0.05; ^p<0.001 vs. treated (NDMA) group. NDMA (N-Nitrosodimethylamine, OTE (*Operculina turpethum* extract), BHA (Butylated Hydroxy Toluene), IG(Isolated Glycoside), AST (Aspartate aminotransferase),ALT(Alanine aminotransferase), ALP(Alkaline phosphatase).

3.4 Effect of *Operculina turpethum* on lipid peroxidation:

The level of TBARS which is an index of lipid peroxidation, a degradative process of membranous lipids, in tissues of NDMA treated mice was significantly ($p<0.01$) elevated (98.7±0.233) when compared to control animals (65.8±0.469). The remarkable increase of lipid peroxides in kidney tissue during NDMA administration indicates the formation of reactive oxygen species (ROS), which plays a major role in cell injury and pathogenesis of renal fibrosis. Treatment with OTE (300 and 400 mg/kg) and its IG (50mg/kg) significantly ($p<0.01$, $p<0.001$ and $p<0.05$ respectively) attenuated the increased LPO level in NDMA treated mice when compared with NDMA alone treated animals. Lipid peroxidation level was restored towards its normal value by treatment with the *Operculina turpethum* extract on NDMA induced toxicity. (Table II)

3.5 Effect of *Operculina turpethum* on Biochemical parameters

On renal SOD level

Effect of NDMA alone and co-treatment with *Operculina turpethum* on SOD activity has been shown in Table II. SOD activity in NDMA treated kidney tissue (2.4±0.698) was reduced markedly than the control group (7.7±0.641), co-treatment with *Operculina turpethum* at a dose of 400 mg/kg b.wt. and its IG to the NDMA treated mice significantly ($p<0.01$), ($p<0.05$), ($p<0.001$) recovered that SOD depletion.

On renal CAT level

CAT activity in the NDMA treated group inferred marked reduction compared to normal group (2.2±0.691 in NDMA treated group vs. 7.8±0.895 in control group). Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues. CAT decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals. Therefore the reduction in the activity of these enzymes may result in a number of deleterious effects. As shown in Table II, simultaneous treatment with *Operculina turpethum* at a dose of 400 mg/kg b. wt. and its IG at 50 mg/b. wt. to the NDMA treated mice significantly ($p<0.05$), ($p<0.01$), ($p<0.001$) restored the CAT activity.

Table II: Effect of ethanolic extract of *Operculina turpethum* and IG on the levels of LPO, SOD, and CAT against NDMA induced renal damage.

roups	Dose (mg/kg)	LPO (nmoles/g)	SOD (U/ml)	CAT (nanomoles/min/mg)	Urea (mg/dl)	Creatinine (mg/dl)
Control (1)	-	65.8±0.469# *	7.7±0.641#*	7.8±0.895#*	32.3±0.859# *	0.7±0.983#*
NDMA (2)	10	98.7±0.233# *	2.4±0.698#*	2.2±0.691#*	49.0±0.367#	2.6±0.651#
NDMA+OTE (3)	10+ 300	83.4±0.706# *	3.5±0.767#*	4.4±0.605#^{NS}	45.5±0.859#*	1.9±0.993#*
NDMA+OTE (4)	10+ 400	75.2±0.181# *	5.4±0.671#*	4.7±0.621#*	41.4±0.810#^	1.5±0.975#^
OTE (5)	300	64.3±0.771# **	7.6±0.779#**	7.7±0.992#*	32.2±834#^	0.7±0.993#^
OTE (6)	400	63.7±0.291#*	7.8±0.889#*	7.9±0.952#*	32.1±0.990#^	0.6±0.959#^
NDMA+BHA (7)	10+1%	69.3±0.180# *	7.3±0.847#*	6.7±0.558#*	39.4±0.928#^	1.1±0.974#^
BHA (8)	1%	62.9±0.414#*	7.4±0.999#*	8.0±0.718#*	39.0±0.385#^	0.5±0.766#^
IG (9)	50	66.9±1.09#*	7.2±0.702#*	7.4±0.683#*	32.5±0.823#^	0.7±0.978#^
NDMA+IG (10)	10+ 50	71.3±0.692# *	7.6±0.735#*	6.6±0.724#*	38.4±0.931#^	0.9±0.992#^

Values are expressed as mean ± S.E.M for six mice in each group. #p<0.001 vs. control group; * p<0.01; ** p<0.05; ^p<0.001; NS: Non significant vs. treated (NDMA) group. NDMA (N-Nitrosodimethylamine, OTE (*Operculina turpethum* extract), BHA (Butylated Hydroxy Toluene), IG (Isolated Glycoside), LPO (Lipid peroxidation),SOD(Superoxide dismutase), CAT (Catalase).

3.6 On Kidney markers (urea, creatinine), total protein and total cholesterol level

Serum clinical chemistry showed some consistent changes. NDMA enhanced the levels of urea and creatinine in mice (49.0±0.367, 2.6±0.651). These abnormal elevations in serum urea levels returned to normal after the onset of treatment. Cholesterol level was enhanced in the NDMA treated animals, whereas it also depleted the protein level (59.2±0.222) which was significantly (p<0.05) recovered as (65.4±0.637) with the simultaneous dosing of the extract at 400 mg/kg body weight to the NDMA treated mice whereas the dosing with the isolated glycoside showed the (p<0.001) marked therapeutic values as shown in Table III.Enhanced protein catabolism and accelerated amino acid deamination for gluconeogenesis is possibly an acceptable postulate to interpret the elevated levels of urea. Creatinine is the last variable of non-protein nitrogenous blood constituents. It appears in the serum in amounts proportional to the body's muscle mass and is more readily exerted by the kidneys than urea and uric acid. Elevated creatinine concentration is associated with abnormal renal function, especially as it relates to glomerular function.

3.7 Effect of *Operculina turpethum* on GST and GSH level

GSH level as measured from the kidney tissue of all the experimental groups has been shown in Table III. NDMA administration caused massive reduction in kidney GSH and GST levels (1.5±0.669 vs. 3.3±0.924 in normal mice) and (92.4±0.830 vs. 124.5±0.859 in normal animals). Administration with the ethanolic extract at a dose of 400 mg/kg b.wt. and IG (50 mg/kg b.wt.) simultaneously with NDMA significantly (p<0.05),(p<0.01) elevated that reduction in animals.

Table III: Therapeutic effect of OTE and IG on various biochemical parameters against NDMA induced renal damage

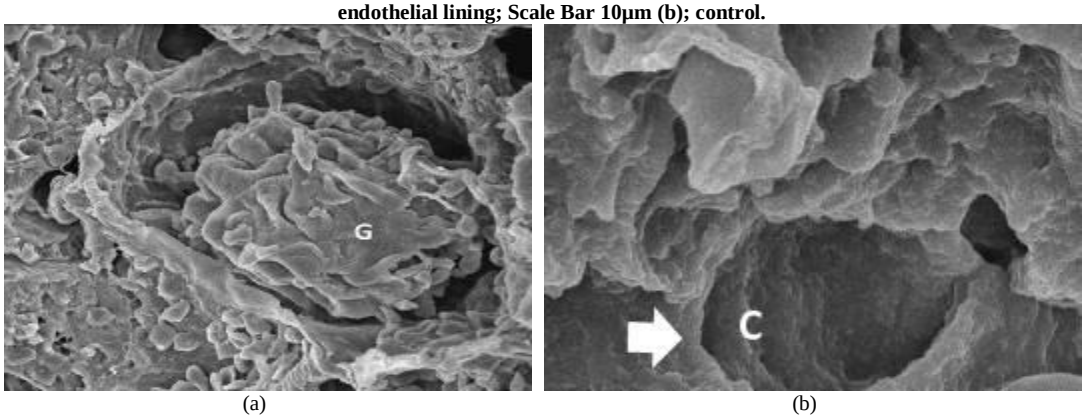
Groups	Dose(mg/kg)	GST (nmol/min/mg)	GSH (m M/g)	TP (mg/g)	TC (mg/g)
Control (1)	-	124.5±0.859# *	3.3±0.924#*	74.4±0.605#*	24.3±0.948#*
NDMA (2)	10	92.4±0.830#*	1.5±0.669# *	59.2±0.222#*	35.8±0.782#*
NDMA+OTE (3)	10+ 300	119.7±0.859# *	2.9±0.994#**	62.4±0.951#**	34.7±0.767#**
NDMA+OTE (4)	10+ 400	119.0±0.101# **	2.4±0.830# *	65.4±0.637# **	30.6±0.981#**
OTE (5)	300	124.1±0.434# *	3.2±0.840#*	74.5±0.648# **	24.5±0.610#**
OTE (6)	400	124.5±0.926# *	3.1±0.620#*	75.7±0.647# *	23.6±0.543#*
NDMA+BHA (7)	10+1%	120.3±0.887# *	2.9±0.931#*	71.6±0.604#*	28.5±0.862#*
BHA (8)	1%	124.7±0.470#*	3.7±0.988# *	76.2±0.933# *	22.7±0.775# *
IG (9)	50	124.2±0.300#*	3.2±0.798#*	74.4±0.657# **	24.4±0.641# *
NDMA+IG (10)	10+ 50	121.1±0.296#*	2.9±0.910# *	72.1±0.780#*	27.6±0.774# *

Values are expressed as mean ± S.E.M for six mice in each group. # p<0.001 vs. control group; * p<0.01; ** p<0.05 vs. treated (NDMA) group. NDMA (N-Nitrosodimethylamine, OTE (*Operculina turpethum* extract), BHA (Butylated Hydroxy Toluene), IG(Isolated Glycoside), GST (Glutathione-S-Transferase),GSH(Reduced Glutathione), TP(Total Protein), TC(Total Cholesterol).

3.8 Scanning Electron Microscopy

Chemically induced nephrotoxicity is a well documented event, which involves many pathophysiological and histological processes. In the present study, NDMA (a potent carcinogen) was used to induce the toxicity. Scanning electron microscopy (SEM) has enhanced greatly our understanding of the complex morphology of the glomerulus of mice kidney. It revealed the three-dimensional appearance of normal podocytes and their interdigitating foot processes. In the control, the normal architecture of the glomerulus was observed with intact glomerulus with well preserved external morphology (Figure 2). Intact glomeruli appear to have clean surfaces with minimal extraneous deposits and the endothelial cells lining the capillary appears to be well in shape.

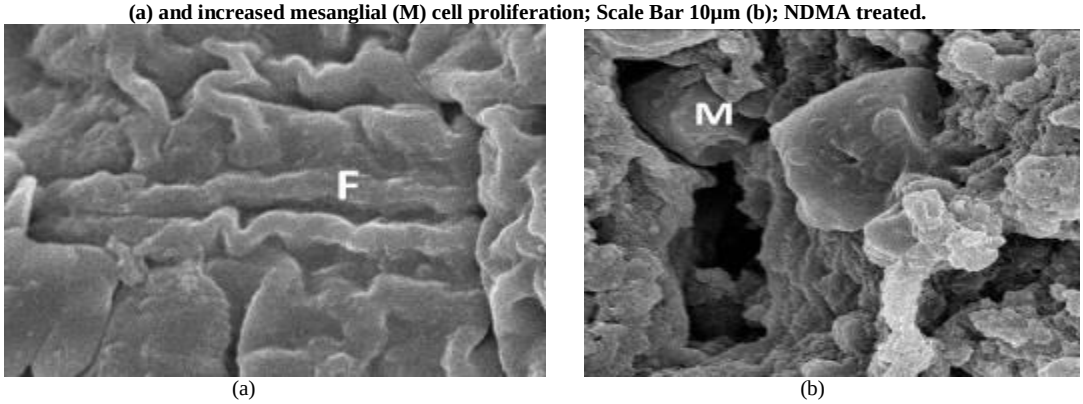
Figure 2: Scanning Electron Microscopy of the kidney showing intact Glomerulus (G) ; Scale Bar 40 µm (a) and capillary (C) with intact endothelial lining; Scale Bar 10µm (b); control.



(a) (b)

In the group treated with NDMA, the renal histoarchitecture of the mice resulted in severe necrotic changes with extensive degeneration of podocytes, increased vacuolated cytoplasm, inflammatory cell infiltration and fatty degenerative changes. It might be due to the formation of highly reactive radicals. The accumulated hydroperoxides can cause cytotoxicity, which is associated with peroxidation of membrane attributing to the degenerative structure. At a higher magnification the complex interdigitations of the podocyte swollen foot processes can be seen (Figure 3). The development of renal fibrosis was observed which may involve the progressive appearance of glomerulosclerosis, tubulointerstitial fibrosis and changes in renal vasculature. Glomeruli appeared remarkably congested and swollen, with leakage of red blood cells and shrunken capsular space. The tubule epithelial cells were collapsed, degenerated and broken down; the lumina were dilated and filled with protein casts and red blood cells, whereas increased mesanglial cell proliferation was also observed.

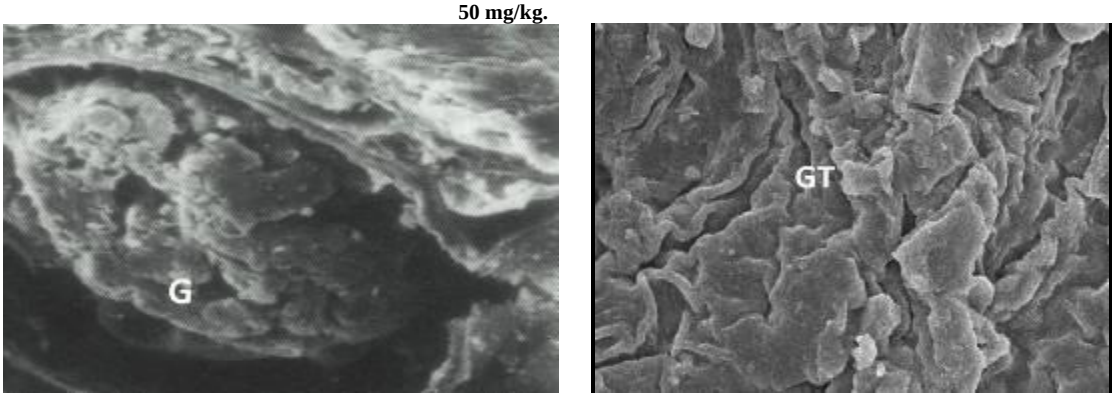
Figure 3: Scanning Electron Microscopy of the kidney showing swollen foot processes (F) of Glomerular podocyte slit ; Scale Bar 10 µm (a) and increased mesanglial (M) cell proliferation; Scale Bar 10µm (b); NDMA treated.



(a) (b)

SEM provided clear conceptualization of the three dimensional appearance and topographical distribution of cellular alterations. Reversal of these abnormalities to a near normal status reflects the causal association of therapeutic properties of the plant. In the treated animals, the filtration membrane was intact, podocyte foot processes were obvious and regularly arranged, capsular space was in focus, there was an intact glomerular basement membrane (Figure 4). The Basal Membrane of individual capillary loops of the glomerular tuft can be discriminated easily. They show a certain degree of intrinsic rigidity despite an absence of cellular components, although partial collapse of capillary loops is observed. The lack of appreciable glomerular basal membrane alteration is consistent with the uniform recovery with the treatment with the plant isolated compound. It can be stated that the histopathological changes coincide with our biochemical studies.

Figure 4: Scanning Electron Microscopy of the kidney showing recovering Glomerulus ;Scale Bar 10 µm; 400 mg/kg crude extract (a) and Glomerulus showing preservation of the glomerular tuft architecture and extraction of all cellular elements; Scale Bar 10µm (b); IG 50 mg/kg.



(a) (b)

4. Discussion

Renal cancer is one of the most common types of cancers worldwide, the principal processes involved in the progression of chronic kidney cancer is fibrosis. At a molecular level, fibrosis can be defined as an excessive accumulation of extracellular matrix such as collagen and fibronectins. The presence of kidney fibrosis seems mostly to be viewed as an endpoint or marker of tissue or organ failure and loss of function. Mitochondria generate ATP through oxidative phosphorylation machinery. Under normal conditions, damage by the toxic free radicals is physiologically counteracted by the intracellular antioxidant systems: antioxidant enzymes and endogenous free radical scavengers. However, when the rate of free radical generation exceeds the capacity of antioxidant defences, oxidative stress ensues with consequential severe damage to DNA, proteins and lipids.

The present results show therapeutic effects of *Operculina turpethum* and it isolated Steroidal glycoside against NDMA induced kidney toxicity through reduction of oxidative stress and improving kidney function. Steroids have very important physiological impact on biological system. The pharmacological value derives from the fact that the sterols have a similar structure to cholesterol and have the capacity to lower plasma cholesterol and LDL cholesterol along with the anticancer properties. Reactive oxygen species (ROS) and organic free radical intermediates formed from many carcinogens are suggested to be involved in the initiation and progression of carcinogenic transformation. Antioxidant and detoxicant enzymes play important protective roles in the kidney primarily because of its transport functions, the kidney has a very active oxidative metabolism. NDMA induced oxidative stress in kidney of mice as evidenced by the marked elevation in the MDA levels as compared to the normal untreated ones.

Lipid peroxidation generates a complex variety of products, many of which are reactive electrophiles some of which react with protein and DNA and as a result are toxic and mutagenic [38]. Malondialdehyde (MDA) is one of the products of lipid per oxidation that reacts with DNA to produce MDA-DNA adducts, which have been implicated in the induction of G→T transversions and A→G transitions [39]. The ability of MDA-DNA adducts to induce frame shift mutations in sequences for genetic instability is emerging as a possible direct link between oxidative stress and human cancers [40-41]. SOD protects the cells against superoxide and hydrogen peroxide mediated LPO. CAT is widely distributed in all tissues and catalyses the breakdown of hydrogen peroxide. The source of hydrogen peroxide is mainly SOD-mediated dismutation of superoxide radical, which is generated by various enzyme systems. Several reports have cited decreased activities of SOD and CAT in various carcinogenic conditions that may be due to the increased LPO.

Glutathione, a potent inhibitor of neoplastic process, plays an important role as an endogenous antioxidant system and is known to have key functions in the protective process. GSH acts directly as a free radical scavenger by donating a hydrogen atom and thereby neutralizing the hydroxyl radical. It also reduces peroxides and maintains protein thiols in the reduced state. Changes in the rate of cancer cell proliferation are accompanied by changes in their intracellular GSH levels and consequently these could be reflected in their antioxidant machineries. The reduction in activity of these enzymes may be caused by the increase in radical production during NDMA metabolism. GST catalyzes the conjugation of the thiol functional groups of GSH to electrophilic xenobiotics and results in escalating solubility. The xenobiotic–GSH conjugate is then either eliminated or converted to mercapturic acid. As the activity of GST increased in NDMA treated mice, it appears that the drug induces greater coupling of electrophilic intermediates with GSH. GSH has been endowed with an important function in maintaining the reducing milieu of cells, in addition to its conjugating ability owing to nucleophilic center and is involved in detoxification of xenobiotics that cause toxicity and carcinogenicity.

Urea and creatinine are waste products of protein metabolism that need to be excreted by the kidney. Therefore, marked increase in serum urea and creatinine are indications of functional damage to the kidneys and increased protein catabolism [42]. At the onset of treatment, the elevation in the levels of urea and creatinine of the intoxicated mice, may indicate renal function impairment due to NDMA toxicity [43]. Alkaline phosphatase is a "marker" enzyme for the plasma membrane and endoplasmic reticulum It is often employed to assess the integrity of plasma membrane and endoplasmic reticulum. The findings of biochemical observations are further supplemented by ultrastructural analysis with Scanning Electron Microscopy which implicates the degenerative changes induced by NDMA treatment is prevented by administration of the crude root extract and isolated glycoside.

5. Conclusion

In conclusion, the renoprotective effects of root extract are attributed for its suppression of lipid peroxidation, free radical scavenging activity, ability to induce GST and other phase II enzymes involved in carcinogen detoxification and maintenance of structural integrity of the tissues against NDMA as cancer inducing agent. The obtained results provide ample evidences for the potential of this plant in combating kidney toxicity by NDMA and ameliorating the kidney in mice, a mammalian model. Therefore, the ability of this drug in producing similar reno-protective effects in human, which has a similar genome, is a possibility and it has a potential for use as a supporting palliative medicine in cancer therapy. Hence, there is a great potential for the development of anticancer drugs from the essentially untapped reservoir of the plant kingdom.

References

1. Ali SA, El-Rigal N, Rizk MZ. Nutritional supplementation with Ailanthus altissimia and Ziziphus spina Christi to compensate for some metabolic disorders in Schistosoma mansoni infected mice. *Pak J Biol Sc*. 2006; 9: 1700-1706
2. El-Ansary AK, Ahmed SA, Aly SA. Antischistosomal and protective effects of Curcuma longa extract in Schistosoma mansoni infected mice. *Indian J Exp Biol*. 2007; 45: 791-801.
3. Saxena MJ. Relevance of herbs in improving health index of livestock animals. Proceeding of 38th Congress of the Nigerian Veterinary Medical Association held in Topo. Badagry, Lagos State, Nigeria, 2011; October 9-13.
4. Lippman SM, Benner SE, Hong WK. Cancer Chemoprevention. *J Clin Oncol.*1993; 12: 851-873.
5. Wattemberg LW. Chemoprevention of cancer. *Cancer Res.*1985; 45:1-8.
6. Pandey G. Anticancer herbal drugs of India with special reference to Ayurveda. New Delhi. Sri Satguru Publications. 2002;18–121.
7. Shureiqi I, Reddy P, Brenner DE. Chemoprevention: general perspective. *Crit Rev Oncol Hematol.* 2000; 33: 157–67.
8. Guilford JM, Pezzuto JM. Natural products as inhibitors of carcinogenesis. *Expert Opin Investig Drugs.* 2008; 17: 1341–52.
9. Kim JB, Koo HN, Joeng HJ. Introduction of apoptosis by Korean medicine Gagam-whanglyun-haedoktang through activation of capase-3 in human leukemia cell line, HL-60 cells. *J Pharmacol Sci.* 2005; 97: 138-45.
10. Aoki K, Yamakuni T, Yoshida M, Ohizumi Y. Ephedorae herba decreases lipopolysaccharide induced cyclooxygenase-2 protein expression and NF-kB-dependent transcription in C6 rat glioma cells. *J Pharmacol Sci.* 2005; 98:327-30.
11. Gupta M, Mazumder UK, Sambath Kumar R, Sivakumar T, Vamsi MLM. Antitumor activity and antioxidant status of Caesalpinia bonducella against Ehrlich ascites carcinoma in Swiss albino mice. *J Pharmacol Sci*. 2004; 94: 177-84.
12. Shimizu M, Azuma C, Taniguchi T, Murayama T. Expression of cytosolic phospholipase A2α in murine C12 cells, a variant of L929 cells, induces arachidonic acid release in response to phorbol myristate acetate and Ca2++ ionophores, but not to tumor necrosis factor-α. *J Pharmacol Sci.* 2004; 96: 324-32.

13. Panda NC. Kidney In: Textbook of Biochemistry and Human Biology, 2nd ed. Prentise Hall, India.1999; 290-296.
14. Sharma V, Singh M. Alterations induced by N- Nitrosodimethylamine and ethanolic root extract of *Operculina turpethum* in serum lipid profile of male albino mice. *Asian J Pharm Clin Res.* 2012; 5(3): 69-73.
15. Moreau RA, Whitaker BD, Hicks KB. Phytosterols, phytostanols, and their conjugates in foods: Structural diversity, quantitative analysis, and health-promoting uses. *Prog. Lipid Res.* 2002; 41:457– 500.
16. Bouic PJ. The role of phytosterols and phytosterolins in immune modulation: A review of the past 10 years. *Curr. Opin. Clin. Nutr. Metab. Care.* 2001; 4: 471–475.
17. Cerutti PA. Prooxidant status and tumor promotion. *Science.*1985; 227: 375±381.
18. Guttenplan JB. Inhibition by L-ascorbate of bacterial mu- tagenesis induced by two N-nitroso compounds. *Nature*.1977; 268: 368-370.
19. Saprin AN, Piette LH. Spin trapping and its application in the study of lipid peroxidation and free radical production with liver microsomes. *Arch Biochem Biophys.* 1977; 180: 480-492.
20. Ahotupa M, Bussacchini-Griot V, Bereziat J-C, Camus A-M, Bartsch H. Rapid oxidative stress induced by N-nitro- soamines. *Biochem Biophys Res Commun.*1987; 146: 1047-1054.
21. Sharma V, Singh M. N-Nitrosodimethylamine as a hazardous chemical toxicant in drinking water. *International research journal of pharmacy*. 2012; 3(3).
22. Zhang XH, Takenaka I. Interstitial stroma and carcinogenesis: ultrastructural observations in rat bladder treated with N-butyl-N- (4-hydroxybutyl) nitrosamine. *Urol Res*. 1995; 24:177–181.
23. Hamilton MA, Rosemarie CR, Robert VT. Trimmed Spearman- Karber Method for estimating Median Lethal Concentrations in Toxicity Bioassays. *Environmental Science & Technology.* 1977; 11(7):714-718.
24. Ahmad R. *Operculina turpethum* attenuates N-nitrosodimethylamine induced toxic liver injury and clastogenicity in rats. *Chemico-Biological Interactions*. 2009; 181: 145-153.
25. Sharma A, Sharma V, Kansal L. Amelioration of lead-induced hepatotoxicity by Allium sativum extracts in Swiss albino mice. *Libyan J Med*. 2010; 5: 4621.
26. Nwanjo HU, Ojiako OA. Effect of vitamins E and C on exercise induced oxidative stress. *Global J Pure Appl Sci*. 2005; 12: 199-202.
27. Marklund S, Marklund G. Involvement of superoxide anion radical in the autooxidation of pyrogallol and convenient assay for SOD. *Eur J Biochem.* 1974; 47: 469-74.
28. Aebi HE . Catalase (Bergmeyer HU, Bergmeyer J, Grabl M, eds) 1993; 3: 273-86.
29. Reitman S, Frankel AS. A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. *Am J Clin Pathol.* 1957; 28: 53-6.
30. Sadashivam S, Manickam A. Biochemical methods.1996; 2:121-4.
31. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurements with the folin phenol reagent. *J Biol Chem*. 1951; 193: 265-75.
32. Zak B. Cholesterol methodologies: a review. *Clin Chem*. 1977; 23: 1201-14.
33. Ambigo S, Ramolingam K, Muthuraman S. Development of a rapid assay for serum, plasma,urea estimation with an overview of literature. *Bioscience Discovery*. 2011; 2(1).
34. Harry H, Abraham R. Estimation of Creatinine by the Jaffe Reaction A Comparison of Three Methods. *Clinical Chemistry*. 1968; 14(3).
35. Habig WH, Pabst MJ, Jakoby WB. Glutathione Stransferase: the first enzymatic step in mercapturic acid formation. *J Biol Chem*. 1974; 249: 7130-39.
36. Jollow DJ, Mitchell JR, Zamppaglione Z, Gillette JR. Bromobenzene induced liver necrosis; Protective role of glutathione and evidence for 3-4 bromobenzene oxide as the hepatotoxic metabolite. *Pharmacology.* 1974; 11:151-156.
37. Malbica JO, Hart LG. Effect of adenosine triphosphate (ATP) and some anti-inflammatory agents on purified lysosomal fraction having high acid phosphatase and labile glucouronidase activity. *Biochemical Pharmacology*. 1971; 20: 2017–2022.
38. Marnett LJ. Lipid peroxidation-DNA damage by malondialdehyde. *Mut. Res.* 1999; 424(1-2): 83-95.
39. Benamira M K, Johnson A, Chaudhary K, Bruner C. Tibbetts and L.J. Marnett. Induction of mutations by replication of malondialdehydemodified M13 DNA in *Escherichia coli*: Determination of the extent of DNA modification, genetic requirements for mutagenesis and types of mutations induced. *Carcinogenesis.* 1995; 16(1): 93-99.
40. Valko M, Izkovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. *Mol. Cell Biochem*. 2004; 266: 37-56.
41. Zienolddiny S, Ryberg D, Haugen A. Induction of microsatellite mutations by oxidative agents in human lung cancer cell lines. *Carcinogenesis*. 2000; 21: 1521-1526.
42. Stryer L. Biochemistry. 4th Edn., W.H. Freeman and Company, New York, USA. 1995; Chapter 24: 607-610.
43. Wright PJ, Plummer DT. The use of urinary enzyme measurement of direct renal damage caused by nephrotoxic compound. *Biochemical Pharmacology.*1974; 23:65–73.