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Pharmacokinetics and Toxicities of Cisplatin Among Bangladeshi Cancer Patients and Measures to Reduce the Toxicity

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Pharmacokinetics and Toxicities of Cisplatin Among Bangladeshi Cancer Patients and Measures to Reduce the Toxicity

THESIS SUBMITTED FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY
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BANGLADESH

BY

A. K. M. SHAHIDUR RAHMAN
Roll No: P-23
Session: 2010-11

June, 2016 **Institute of Biological Sciences (IBSc)**
University of Rajshahi
Rajshahi, Bangladesh

Dedicated to the loving memories

of

my parents late Borhan Uddin & Sufia Begum

my wife Dr. Nishat Parvin

my son Nahan Shahab (Shihan)

&

my cutest daughter Lubaba Tabassum (Parisha)

Declaration

I hereby declare that this thesis entitled **"Pharmacokinetics and Toxicities of Cisplatin Among Bangladeshi Cancer Patients and Measures to Reduce the Toxicity''** is based on work carried out by me. I further declared that no part of this has been presented previously for higher degree.

Dr. A. K. M. Shahidur Rahman

Certificate

"Pharmacokinetics and Toxicities of Cisplatin Among Bangladeshi Cancer Patients and Measures to Reduce the Toxicity'' submitted by Dr. A. K. M. Shahidur Rahman for the award of Ph.D degree in Pharmacology, is an independent research work done at a) Indoor patient Department, Department of Oncology, Khwaja Yunus Ali Medical College Hospital, Enayetpur, Sirajgonj. b) Centre for bioequivalence study, Khwaja Yunus Ali Medical College Hospital, Enayetpur, Sirajgonj and c) Pharmacology Research Lab., Department of Pharmacology, BSMMU, Shahbag, Dhaka, under Institute of Biological Sciences (IBSc), University of Rajshahi and this dissertation has not been used as the basis for the award of any degree or fellowship.

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A. K. M. Shahidur Rahman

ABSTRACT

Background: Cisplatin, an old chemotherapeutic agent used to treat various types of cancers, including sarcomas, some carcinomas e.g. small cell lung cancer and ovarian cancer, lymphomas and germ cell tumors etc. Most important toxicity of cisplatin is nephrotoxicity produced by cisplatin itself and its few metabolites, which are some time fatal, various attempt were taken to risk reduction previously, use of the medicinal plant materials was the new concept, such as extract of *D.alata* and *M. olifera* which was proved promising activity in this study. **Methods:** 250 blood samples corresponding 250 urine samples of 50 adult cancer patients admitted in the IPD of KYAMC Cancer Centre, Khwaja Yunus Ali Medical College, Enayetur, Sirajgonj were collected at 0 (predose), 3, 6, 12 and 24 hour and placed in HPLC for identifying the RT and Area of Cisplatin and its possible metabolites and their concentrations in plasma and urine at different time interval, where nickel chloride used as internal standard. For isolation and purification of cisplatin and its possible metabolites 24 hours urine of 5 patients were collected and were placed in long chromatographic procedure such as column chromatography, glass chromatography, thin layer chromatography gave rise to 5 single compounds which was detected under UV spectrometry. Cytotoxicy of 5 compounds were done by using brine shrimp lethality bioassay. *D. alata* and *M. olifera* extracts were applied the cisplatin and its metabolites induced nephrotoxicity in mice and histo-pathological slides were prepared from the liver and kidney of mice to see the pathological. A series of pathological & biochemical investigations of blood and urine, various images technique of 50 patients were done. The data were statistically analyzed by ANOVA (one way of analysis of varience) and post-hoc Dunnett's tests the Statistical Package for Social Sciences (SPSS 16.0, USA) program.

Result & Discussion: A potent anti-cancer drug, cisplatin, used in various types of malignancies, but its use is limited due to it produce nephrotoxicity. 50 male patients treated with cisplatin in IPD of Khwaja Yunus Ali Medical College Cancer Centre (KYAMC Cancer Centre) on Sirajgonj showed most vulnerable age group 53-60 years 56% (n=28), moderate to severe anemic 2% (n=1), moderate to severe jaundice 4% (n=2), about 8% (n=4) were suffered from edema, Moderate increased of serum creatinine (µg/dl), blood urea (µg/dl) and albuminurea levels were found as 28% (n=14), 20% (n=10) and 32% (n=16) of the total patients respectively, strongly suggesting that cisplatin or its metabolites produced nephrotoxicities. From the evidence of HPLC data sheet, four suspected metabolites (CM2, CM3, CM4 and CM5) were identified with their specific RT (retention time) and Area, where mean plasma concentration of one suspected metabolite CM2 was gradually increased highest level to 234.64 µg/dl (±6.30) after 12 hours (*p>0.01*) indicating the finding not significant and its corresponding urine concentration highest after 24 hour were 269.43 µg/dl (±6.98) indicating the findings significant (*p<0.001*), for another metabolite CM5 was found after 3 hour to minimum to 8.02 µg/dl (±8.75) which was sharply increased to 53. 86 (±2.73) after 12 hours then gradually decreased to 39.06 µg/dl (±5.44) after 24 hours (*p>0.01*). Among 50 patients about 28% (n=14), 16% (n=8) & 20% (n=10) of the patient suffered increased serum creatinine level, increased albuminuria and increased blood urea level respectively. Primary screening for cytotoxicity of these five compounds (C, CM2, CM3, CM4 and CM5) along with cisplatin (as standard solution) were done by using brine shrimp lethality bioassay (figure 20-25 & table 4-9). Among them, comparative to cisplatin, its metabolites are more toxic, specially CM2 (Meta2, LC_{50}=1.2µgm/ml), CM3 (Meta 3, LC_{50}=1.08µgm/ml) and CM4 (Meta 4, LC_{50}=1.182µgm/ml)

which was correlate to the previous study indicating the findings significant ($p > 0.01$). A promising results showed that the use of some medicinal plant extracts (*D. alata and Morienga olifera*) gave rise to moderate restoration of normal physiology of kidney and liver of mice. The effects of the methanolic extracts of *D. alata and Morienga olifera* on biochemical parameter in mice also observed and found significant decrease in blood urea and creatinine levels and increase activity of GSH in all experimental mice groups which is proven antioxidant and nephroprotective.

Conclusion: Metabolites of Cisplatin induced nephrotoxicity was more prominent than cisplatin in our study. After adequate hydration of the admitted cancer patients in Khwaja Yunus Ali Medical College they were suffer nephrotoxicities. Extracts of *D. alata and Morienga olifera* had some effects on biochemical parameters; its effect on renal histology in injured mice kidney was very promising and significant.

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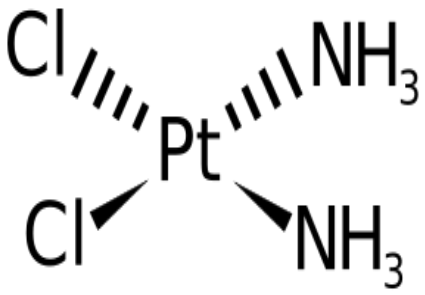
Chapter 1

Introduction

1. 1 Background

The compound cisplatin, a potent anti-cancer drug designed as cis-diamminedichloroplatinum (II) (CDDP), was first described by Michele Peyrone in 1845, and known for a long time as Peyrone's salt[1]. The structure was deduced by Alfred Werner in 1893[2]. In 1965, Barnett Rosenberg, van Camp *et al.* of Michigan State University discovered that electrolysis of platinum electrodes generated a soluble platinum complex which inhibited binary fission in *Escherichia coli* bacteria. Confirmation of this discovery, and extension of testing to other tumour cell lines launched the medicinal applications of cisplatin. Cisplatin was approved for use in testicular and ovarian cancers by the U.S. Food and Drug Administration on 19 December 1978[2-4] and in the UK (and in several other European countries) in 1979[5].

Figure 1 : Structure of cispatin.



Cisplatin, is a platinum-based chemotherapeutic agent used to treat various types of cancers, including sarcomas, some carcinomas e.g. small cell lung cancer and ovarian cancer, lymphomas and germ cell tumors etc. It is used in combinations with bleomycin and vinblastine in testicular cancer[6].

1.2 Route of administration of Cisplatin

Cisplatin is only available in the injectable form. The parenteral routes, intravenous, intra-arterial and intraperitoneal, have all been used in cisplatin therapy. Cisplatin is not effective when administered orally or other route such inhalation, dermal, eye as well[7].

Dose of cisplatin scheduled by different ways, 20 mg/m^2/day for 5 days, 20-30 mg/week for 3-4 weeks or 100mg/m^2 given once every 4 weeks[7]. One study suggested that pharmacokinetic and demographic data were collected from 32 adult patients (20 males/12 females, age range 47-76 years) receiving 30-min infusions or an oral formulation of cisplatin, 10-30 mg/m^2, for various malignancies resulting the oral bioavailability (F) population estimates were, respectively, 0.39 and 0.30 with associated inter-subject variabilities (ISV) of 24% and 26%. Peak concentrations following oral dosing occurred at 1.0 hour and 1.6 hour for unbound and total platinum, respectively. Clearance (CL) and central distribution volume (V1) of unbound platinum were significantly related to body surface area (BSA). The CL and V1 mean estimates were, respectively, 37 l/hour and 23 l with an associated ISV of 15% [8]. Some scientists reveals that platmum levels have been determined in 145 samples from 24 tissues and 7 types of tumours from patients, treated with cisplatin. Sampled were 27 males (average age: 36 years, range: 22–63) and 19 females (average age: 52 years, range: 17–69), with an average total intravenous dose of 820 mg and 545 mg cisplatin, respectively. Samples were obtained via biopsy and autopsy, mostly 4–15 weeks after the last cisplatin administration. The long-term clearance of platinum from the tissues analyzed could be described by a first-order process with a half-life in the range of 175–300 days.

The platinum fraction involved in the long term total body clearance is estimated to be 22–38% of the total dose administered. However, the platinum clearance from a mature teratoma appeared to be much slower as measurable levels were detected 7.6 years after administration of the drug. The highest long-term platinum accumulations were found in liver, uterus, testes, ovary, and thyroid, and the lowest in brain and blood.

1.2 Distribution of Cisplatin

Another study revealed that the plasma disposition kinetics and tissue distribution of platinum was evaluated following intravenous bolus administration to CD1 immune-competent mice of cisplatin, cisplatin conjugated to anti-CEA monoclonal antibody A5B7 via a carboxymethyl dextran (CMdextran) carrier molecule, and cisplatin coupled to the CMdextran in the absence of antibody. In addition, the in vivo characteristics of 125I-labeled A5B7 were compared with and without conjugation to CMdextran.

Conjugation of cisplatin [clearance (CL = 0.62 ml/min/g, volume of distribution at steady-state (Vdss) = 16 ml/g] to CMdextran restricted its tissue distribution (Vdss = 0.43 ml/g) and reduced its systemic clearance (CL = 0.055 mL/min/g). Subsequent conjugation of the complex to A5B7 further reduced both its distribution (Vdss = 0.20 mL/g) and clearance (CL = 0.016 ml/min/g). Clearance of A5B7 (CL = 0.002 ml/min/g) was increased by conjugation to CMdextran (CL = 0.014 ml/min/g); tissue distribution was unchanged. A5B7-Cmdextran-cisplatin was relatively stable in plasma and other tissues, except the liver.

The extent of distribution of platinum into tissues (lung, liver, muscle, kidney) was markedly influenced by conjugation, with the influence being greatest for unmodified cisplatin and least for the A5B7-Cmdextran conjugate. However, the time courses of tissue distribution, expressed in mean residence time scales, were similar, implying a common mechanism controlling tissue uptake[9].

Another study showed that the pharmacokinetics of platinum and cisplatin (CDDP)-DNA adducts were studied in nude mice after single-dose CDDP treatments. Whole blood, serum, kidney, lever, testis, brain, and tumor were collected at different intervals after injection of CDP at different dose levels. Platinum was measured with flameless atomic absorption spectrometry (FAAS) or adsorptive voltammetry (AdV) and CDDP-DNA adducts with quantitative immunohistochemistry. The drug was immediately absorbed into the blood circulation (peak serum platinum levels were reached within 5 min) after i.p. CDDP administration, and distribution into most tissues also occurred rapidly (tissue platinum levels peaked at 15 min). With a sampling period of 7 days there was a biphasic elimination of Pt from blood, serum, and tissues. In the brain the pharmacokinetics differed with a gradual accumulation of platinum occurring during the 1st week. Formation of CDDP-DNA adducts in tissues was a slower process, with maximal levels being achieved at between 30 min and 4 hour after drug administration, followed by a steady state lasting for at least 24 hour. Each tissue type had its specific immunohistochemical staining pattern of adducts. With escalating CDDP doses there was a linear, or almost linear, increase in platinum concentrations and CDDP-DNA adduct levels in all sample types examined.

These results suggest that a fair estimation of the amount of drug in tumor and normal tissues can be made from analysis of serum platinum at a fixed time point after a single dose of CDDP[10].

Figure 2: Time concentration curve after single intravenous injection in rabbits

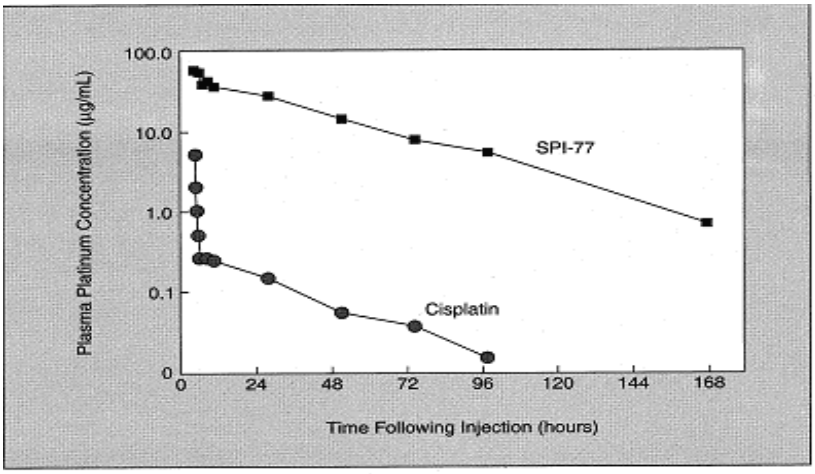
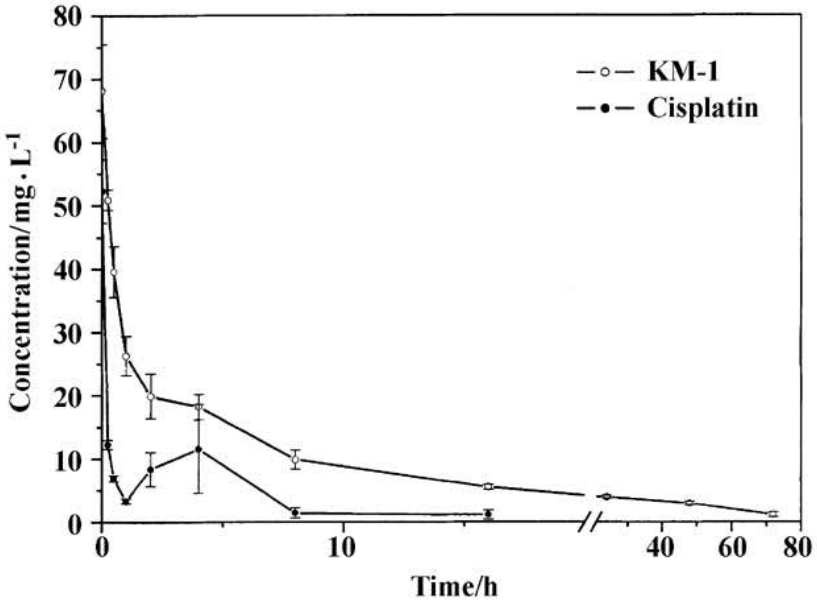


Figure 2: Plasma clearance of cisplatin or SPI-77 after single intravenous injection in rabbits.

Figure 3: Time concentration curve in plasma.



A group of scientists suggested in a research that tumour source or type is a much more important correlate of human tumour cisplatin concentrations than is intracranial vs extracranial location. Serum calcium, chloride and bilirubin levels may affect tumour cisplatin uptake or retention. CT scan characteristics may help predict cisplatin concentrations in intracranial tumours[11].

A radiochemical neutron activation analysis were performed by a group of scientists mentioned us that platinum were found to depend not only on the total amount of cisplatin ingested, but also on the time between the last dose and death. Highest concentrations were found in the liver, kidney and testis, and a significant drop could be seen during the first two months after the treatment was stopped; concentrations in nerve tissue were much lower than in the organs and showed clearly the effect of the blood-brain barrier[12].

Another study revealed that reactions of cisplatin with both recombinant human albumin and human albumin isolated from blood serum were studied. Although rHA and HAS are similar in amino acid composition, secondary structure and globular packing, they differ in two respects, namely in free thiol content (approximately 0.9 mol SH mol^{-1} rHA and 0.4–0.5 mol SH mol^{-1} HAS) and in the greater structural heterogeneity of HAS compared with rHA . Cisplatin-albumin interactions were studied mainly at pH 6.4, where albumin exists predominantly as one structural isomer, namely the N-form. Several experiments were also carried out at pH 7.4 and 5.0. In most experiments, KCl was added to suppress cisplatin hydrolysis (as would be the case under extracellular conditions)[11-14].

One study said that a model of human advanced ovarian cancer was made by intra-abdominal inoculation of a cloned ovarian adenocarcinoma cell line of the Sprague-Dawley rat (ROT68/C1) into isologous newborn rats. Intra-abdominal tumors and tumors metastatic to the lung developed in 100% of the animals within 3 weeks after inoculation. With use of this model the intraperitoneal and intravenous routes of cisplatin (cis-diamminedichloroplatinum) administration were compared with regard to both the pharmacokinetics and the antitumor activity. After 2 hours of administration the serum cisplatin values were greater following use of the intraperitoneal route than with use of the intravenous route. Cisplatin values in the intra-abdominal tumor tissues were greater after the intraperitoneal route of administration than the intravenous route, and the growth was suppressed more prominently after intraperitoneal administration. No difference in drug values in the kidney tissues was found between the two administration routes. Thus the intraperitoneal route of cisplatin administration seems to be much more effective against advanced ovarian cancer confined to intra-abdominal cavity than does the intravenous route[15].

1.4 Cispatin toxicities

CDDP undergoes ligand-exchange reactions, which are virtually irreversible[16,17]. In biological fluids, CDDP is transformed immediately into aquated CDDP as a result of the release of chloride ion and equilibrium between CDDP and its aquated form is maintained. Aquated and unchanged CDDP also react readily with nucleophiles[17-19].

CDDP is biotransformed through binding to low molecular mass substances (such as glutathione, methionine and cysteine) and high molecular mass substances (such as albumin and nucleotides) and the resulting metabolites are called 'mobile' and 'fixed' metabolites, respectively[19]. The pharmacokinetic characteristics of these platinum species are different because their structures are different[20,21]. The CDDP in plasma is instantly eliminated and mobile and fixed metabolites are gradually increased. The mobile metabolites in plasma are eliminated more slowly than that of CDDP and fixed metabolites are little decreased after CDDP and mobile metabolites have been eliminated[20,21].

Recent studies in mice and rats have shown that the nephrotoxicity of cisplatin can be blocked by acivicin or (aminooxy) acetic acid, the same enzyme inhibitors that block the metabolic activation of a series of nephrotoxic halogenated alkenes. In this study, it was hypothesized that cisplatin is activated in the kidney to a toxic metabolite through the same pathway that has been shown to activate the halogenated alkenes. This activation begins with the formation of a glutathione-conjugate that is metabolized to a cysteinyl-glycine-conjugate, to a cysteine-conjugate, and finally to a reactive thiol. In this study, a protocol was developed in which confluent monolayers of LLC-PK1 cells were exposed to clinically relevant concentrations of cisplatin or cisplatin-conjugate for 3 h. Cell viability was assayed at 72 h. The role of gamma-glutamyl transpeptidase (GGT) and cysteine-S-conjugate beta-lyase in the metabolism of each of the cisplatin-conjugates was investigated. Pre-incubation of cisplatin with glutathione, cysteinyl-glycine, or N-acetyl-cysteine to allow for the spontaneous formation of cisplatin-conjugates increased the toxicity of cisplatin toward LLC-PK1 cells.

Inhibition of GGT activity showed that GGT was necessary only for the toxicity of the cisplatin-glutathione-conjugate. Inhibition of cysteine-S-conjugate beta-lyase reduced the toxicity of each of the cisplatin-conjugates. These data demonstrate that metabolism of cisplatin in proximal tubule cells is required for its nephrotoxicity. The elucidation of this pathway provides new targets for the inhibition of cisplatin nephrotoxicity[22].

1.5 Mechanism of action of Cisplatin

The platinum complexes react in vivo, binding to and causing crosslinking of DNA which ultimately triggers apoptosis (programmed cell death). Cisplatin crosslinks DNA in several different ways, interfering with cell division by mitosis. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible[1,2].

Figure 4: Mechanism of action of Cisplatin.

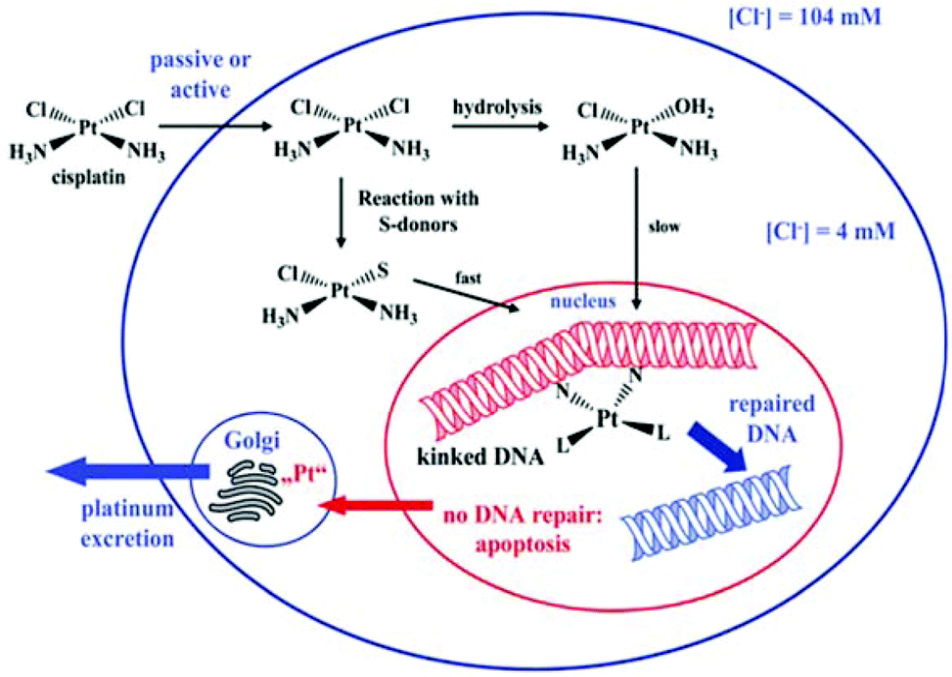


Figure 5 : Mechanism of action of cisplatin

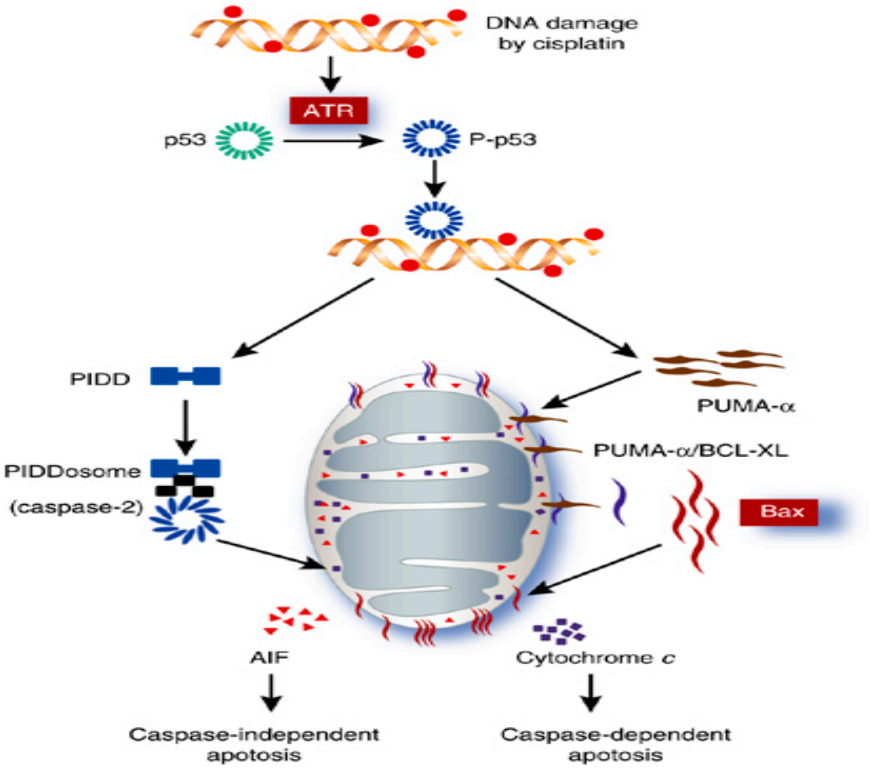
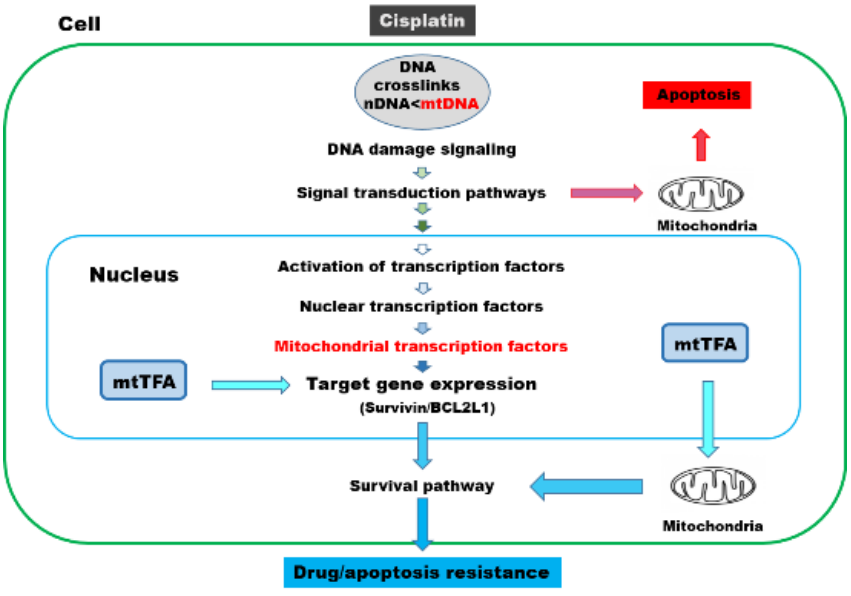


Figure 6: Mechanism of development of cisplatin resistance.



1.6 Mechanism of Cisplatin resistance

DNA damage-mediated apoptotic signals, however, can be attenuated, and the resistance that ensues is a major limitation of cisplatin-based chemotherapy. The mechanisms responsible for cisplatin resistance are several, and contribute to the multifactorial nature of the problem. Resistance mechanisms that limit the extent of DNA damage include reduced drug uptake, increased drug inactivation, and increased DNA adduct repair. Origins of these pharmacologic-based mechanisms, however, are at the molecular level. Mechanisms that inhibit propagation of the DNA damage signal to the apoptotic machinery include loss of damage recognition, overexpression of HER-2/neu, activation of the PI3-K/Akt (also known as PI3-K/PKB) pathway, loss of p53 function, overexpression of antiapoptotic bcl-2, and interference in caspase activation. The molecular signature defining the resistant phenotype varies between tumors, and the number of resistance mechanisms activated in response to selection pressures dictates the overall extent of cisplatin resistance[23].

Cisplatin causes acute and delayed toxicities such as in acute anorexia, nausea, vomiting and in delayed toxicities are nephrotixicity, peripheral sensory neuropathy, ototoxicity, nerve dysfunction and also causing dose limiting myelosuppression and hyperuricaemia, anaphylactic reactions. It also cause hypokalamia, hypomegnasimia, hypophospataemia, altered colored perception, abnormal liver function test and reversible focal encephalopathy that often causes cortical blindness[24-26] . Reduction of the systemic toxicity of cisplatin by intra-arterial hepatic route administration for liver malignancies[27];

1.7 Cisplatin Nephrotoxicity

Nephrotoxicity (kidney damage) is a major concern. The dose is reduced when the patient's creatinine clearance (a measure of renal function) is reduced. Adequate hydration and diuresis is used to prevent renal damage. The nephrotoxicity of platinum-class drugs seems to be related to reactive oxygen species and in animal models can be ameliorated by free radical scavenging agents (e.g., amifostine). Nephrotoxicity is a dose-limiting side effect [28].

Neurotoxicity (nerve damage) can be anticipated by performing nerve conduction studies before and after treatment. Common neurological side effects of cisplatin include visual perception and hearing disorder, which can occur soon after treatment begins[29].

While triggering apoptosis through interfering with DNA replication remains the primary mechanism of cisplatin, this has not been found to contribute to neurological side effects. Recent studies have shown that cisplatin noncompetitively inhibits an archetypal, membrane-bound mechanosensitive sodium-hydrogen ion transporter known as NHE-1[29]. It is primarily found on cells of the peripheral nervous system, which are aggregated in large numbers near the ocular and aural stimuli-receiving centers. This noncompetitive interaction has been linked to hydroelectrolytic imbalances and cytoskeleton alterations, both of which have been confirmed in vitro and in vivo. However, NHE-1 inhibition has been found to be both dose-dependent (half-inhibition = 30 μg/mL) and reversible [29].

1.8 Other toxicities of Cisplatin

Nausea and vomiting: cisplatin is one of the most emetogenic chemotherapy agents, but this symptom is managed with prophylactic antiemetics (ondansetron, granisetron, etc.) in combination with corticosteroids.

Aprepitant combined with ondansetron and dexamethasone has been shown to be better for highly emetogenic chemotherapy than just ondansetron and dexamethasone [29].

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Ototoxicity (hearing loss): there is at present no effective treatment to prevent this side effect, which may be severe. Audiometric analysis may be necessary to assess the severity of ototoxicity. Other drugs (such as the aminoglycoside antibiotic class) may also cause ototoxicity, and the administration of this class of antibiotics in patients receiving cisplatin is generally avoided. The ototoxicity of both the aminoglycosides and cisplatin may be related to their ability to bind to melanin in the stria vascularis of the inner ear or the generation of reactive oxygen species [29]. Electrolyte disturbance: Cisplatin can cause hypomagnesaemia, hypokalaemia and hypocalcaemia. The hypocalcaemia seems to occur in those with low serum magnesium secondary to cisplatin, so it is not primarily due to the cisplatin[29].

Myelotoxicity: This agent can also cause profound bone marrow suppression[30]. Hemolytic anemia can be developed after several courses of cisplatin. It is suggested that an antibody reacting with a cisplatin-red-cell membrane is responsible for hemolysis[31]. Following administration, one of the chloride ligands is slowly displaced by water (an aqua ligand), in a process termed aquation.

The aqua ligand in the resulting [PtCl(H2O)(NH3)2]+ is itself easily displaced, allowing the platinum atom to bind to bases. Of the bases on DNA, guanine is preferred. Subsequent to formation of [PtCl(guanine-DNA)(NH3)2]+, crosslinking can occur via displacement of the other chloride ligand, typically by another guanine[31]. Cisplatin crosslinks DNA in several different ways, interfering with cell division by mitosis. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible. In 2008, researchers were able to show that the apoptosis induced by cisplatin on human colon cancer cells depends on the mitochondrial serine-protease Omi/Htra2[32]. Since this was only demonstrated for colon carcinoma cells, it remains an open question if the Omi/Htra2 protein participates in the cisplatin-induced apoptosis in carcinomas from other tissues.

1.9 An approach to treat cisplatin resistance case

Cisplatin combination chemotherapy is the corner stone of treatment of many cancers. Initial platinum responsiveness is high but the majority of cancer patients will eventually relapse with cisplatin-resistant disease. Many mechanisms of cisplatin resistance have been proposed including changes in cellular uptake and efflux of the drug, increased detoxification of the drug, inhibition of apoptosis and increased DNA repair[33]. Oxaliplatin is active in highly cisplatin-resistant cancer cells in the laboratory; however, there is little evidence for its activity in the clinical treatment of patients with cisplatin-resistant cancer[33]. The drug paclitaxel may be useful in the treatment of cisplatin-resistant cancer; the mechanism for this activity is unknown[34].

1.10 Metabolism of cisplatin

In biological fluids, CDDP is transformed immediately into aquated CDDP as a result of the release of chloride ion and equilibrium between CDDP and its aquated form is maintained. Aquated and unchanged CDDP also react readily with nucleophiles[35-37].

CDDP is biotransformed through binding to low molecular mass substances (such as glutathione, methionine and cysteine) and high molecular mass substances (such as albumin and nucleotides) and the resulting metabolites are called 'mobile' and 'fixed' metabolites, respectively[37]. The pharmacokinetic characteristics of these platinum species are different because their structures are different[38.39]. The CDDP in plasma is instantly eliminated and mobile and fixed metabolites are gradually increased. The mobile metabolites in plasma are eliminated more slowly than that of CDDP and fixed metabolites are little decreased after CDDP and mobile metabolites have been eliminated[38,39].

Another study revealed that The metabolic fate of cisplatin has not been completely elucidated. There is little evidence to date that the drug undergoes enzymatic biotransformation. The cisplatin molecule has chloride ligands on it and it is believed that these are displaced by water thus forming positively charged platinum complexes that react with nucleophilic sites. Their rate and extent depends on the strength, concentration and accessibility of the nucleophiles. The chemical identities of the metabolites of cisplatin have been found but have yet to be identified. There is a strong possibility that cisplatin and its metabolites undergo enterohepatic circulation[40].

In an animal study, it was revealed that For rats dosed with cisplatin the rate of appearance in plasma of ultrafilterable metabolites containing platinum has been investigated using HPLC. At least seven species containing platinum in addition to cisplatin are present 15 min following injection i.p. of 15 mg kg-1. Unchanged cisplatin has been almost completely eliminated from the plasma within 3 hr of dosing; however, metabolite species are still present.

The same metabolite species form when cisplatin is incubated in vitro with plasma although in different proportions. After incubation for 24 hr at 37 degrees a mixture of metabolites is produced which contains less than 4% cisplatin. This mixture, when injected i.p. into rats, is nephrotoxic at doses of platinum at which cisplatin is not. The mixture of metabolites has considerably less antitumour activity than cisplatin when tested against the mouse L1210 leukemia assay[40].

Although no metabolite species has been unequivocally identified we present evidence which suggests that amongst the principle metabolite species are an hydrolysis product and methionine substitution products of cisplatin[40].

A mixture of cisplatin methionine substitution complexes showed neither antitumour nor nephrotoxic properties. However, an hydrolysis product was shown to be nephrotoxic at a dose of platinum at which cisplatin is not. The work reported here is the first direct experimental demonstration that cisplatin metabolites are more nephrotoxic but less effective antitumour agents than the parent compound[41].

In another study performed *in vivo* showing reaction products were characterized using HPLC with inductively coupled plasma – mass spectrometry (HPLC-ICP-MS), (1)H and (13)C NMR and fast atom bombardment – mass spectrometry (FAB-MS). Three Pt-creatinine complexes, cis-[Pt(NH(3))(2)Cl(Creat)](+), cis-[Pt(NH(3))(2)(H(2)O)(Creat)](2+) and cis-[Pt(NH(3))(2)(Creat)(2)](2+), were synthesized and the platinum was shown to coordinate to the ring nitrogen, N(3)[42]. Human urine samples from patients on cisplatin chemotherapy were shown to contain cisplatin, its hydrolysis product and biotransformation products containing Pt-creatinine, Pt-urea and Pt-uric acid complexes[42].

It is suggested that cisplatin requires metabolic activation to become nephrotoxic. The activation is proposed to be via the metabolism of a glutathione-platinum conjugate to a cysteinyl-glycine-platinum conjugate, which is further processed to a cysteine conjugate[43]. HPLC analysis of the cisplatin-GSH, cisplatin-cysteinyl-glycine, and cisplatin-NAC preincubation solutions revealed two new platinum-containing peaks in each of the solutions. MS-MS analysis of the peaks revealed a diplatinum- and a monoplatinum conjugate in each of the solutions[43].

Daley-Yates and McBrien showed the first evidence that indicated biotransformation products of cisplatin were the nephrotoxic compounds[44]. These investigators reported that seven platinum containing species were present in plasma that could be separated via HPLC[2] following a single dose of cisplatin. The mixture of platinum-containing species was injected into rats and was more nephrotoxic than cisplatin. However, the anti-cancer activity of the mixture of platinum-containing species was less effective than cisplatin in a mouse leukemia model.

The mechanism of cisplatin-induced nephrotoxicity was not identified until studies in our laboratory demonstrated that cisplatin-induced renal toxicity is due to the metabolism of cisplatin to a nephrotoxin via γ-glutamyl transpeptidase (GGT) and a cysteine *S*-conjugate β-lyase[45, 48]. According to above the study, MS analysis revealed that the structures of the GSH-monoplatinum conjugate, GSH-2, the Cys-Gly-monoplatinum conjugate, Cys-Gly-1, and the NAC-monoplatinum conjugate, NAC-1, are similar. In each of the monoplatinum conjugates, the sulfur moiety of the GSH, Cys-Gly, or NAC is bound to the platinum with the loss of a chloride from the platinum. These compounds are substrates for the enzymes that we have found to be essential for the nephrotoxicity of cisplatin[46-48].

From the same study, the scientists was proposed that GSH-2 is the GSH-platinum conjugate that is metabolized to a nephrotoxin. The monoplatinum-NAC conjugate, NAC-1 would be deacetylated to a platinum-cysteine conjugate by the cell. Following deacetylation, this conjugate would be a substrate for PLP-dependent cysteine *S*-conjugate β-lyase[49].

Some study reported that, the diplatinum-GSH, GSH-1, and diplatinum-Cys-Gly, Cys-Gly-2, conjugates are both composed of the corresponding monoplatinum adduct with a second cisplatin bound to the nitrogen of the free amine of the amino acid with the loss of a chloride from the platinum. GSH-1 is not a substrate for GGT due to the lack of a free γ-glutamyl group[50]. Diplatinum-NAC, NAC-2, consists of the monoplatinum-NAC with the second cisplatin also bound to the sulfur with the loss of a chloride from the platinum.

In NAC the amine group of the amino acid is acetylated, therefore, there is no free nitrogen to bind the platinum. The NAC-2 conjugate is unlikely to be a substrate for the PLP-dependent cysteine *S*-conjugate β-lyase due to the two platinums bound to the sulfur, which would not favor the β-elimination reaction necessary to form the toxic thiol[51].

Formation of platinum-GSH conjugates and platinum-cysteine conjugates has been demonstrated in several laboratories[52,53]. Ishikawa and Ali-Osman reported a monoplatinum-diglutathione complex that was not toxic in tumor cells[52].

The increased concentration of GSH would favor the formation of the inactive monoplatinum-diglutathione complex identified by Ishikawa and Ali-Osman. Bose and coworkers reported a kinetic analysis of the reaction of cisplatin with cysteine[54]. Cysteine conjugates of cisplatin have been identified in the kidney[55].

Peripheral neurotoxicity is the most important dose-limiting problem associated with cisplatin[56]. A number of pathophysiological mechanisms have been proposed to explain this phenomenon, with some data suggesting that cisplatin kills malignant cells and peripheral neurons by means of a similar mechanism of apoptosis[57]. Peripheral neurotoxicity develops in approximately 50% of patients receiving cisplatin[58], but the onset of toxicity is delayed until a cumulative dose higher than 300 mg/m^2 has been given[59,60]. Signs and symptoms of peripheral neurotoxicity involve the upper and lower extremities and include loss of vibration sense, loss of position sense, tingling paraesthesia, weakness, tremor, and loss of taste[61-63]. Seizures and leukoencephalopathy have also been described[64,65].

After discontinuation of treatment, the neurological dysfunction may gradually improve, but it may persist for a period of time, or it can be permanent[58, 66].

Cisplatin is ototoxic. Tinnitus and hearing loss have been observed in up to 31% of patients treated with initial intravenous cisplatin dose of 50 mg/m^2 [67,68]. Transient hearing loss and mild audiometric abnormalities were observed in 30% of patients receiving 150 mg/m^2 of cisplatin[69, 70]. The mechanisms of cisplatin-induced damage to the outer hairy cells of the cochlea probably include the formation of reactive oxygen radicals and depletion of glutathione[71]. Other risk factors include simultaneous use of other potentially ototoxic agents (e.g., aminoglycosides), previous cranial irradiation, preexisting renal dysfunction, or inner ear damage[68, 70, 72, 73.]

1.11 Prevention and Treatment of Platinum-Induced Neurotoxicity

Many studies have examined the efficacy of a number of potential neuroprotective agents administered together with platinum analogues. The use of these agents generally aims to reduce the incidence and severity of the neurotoxicity without impairing the antitumor efficacy of the platinum drugs.

One study revealed that three thiol compounds have been studied as neuroprotective agents in patients receiving cisplatin: amifostine, glutathione, and the melanocortin Org 2766. Among these agents, glutathione seems to have some neuroprotective effects in platinum-induced neurotoxicity. Published data are conflicting as some of the studies have shown that glutathione may provide neuroprotection in patients treated with cisplatin without altering its antineoplastic effect[74-76], while others found no reduction in toxicity[77,78].

Cascinu et al. were the first to study the potentially protective effect of glutathione on oxaliplatin neurotoxicity in a randomized, placebo-controlled clinical trial[79].

1.11.1 Vitamin E, a protective approach against cisplatin induced neurotoxicity

One research study has recently been evaluated by Pace et al. in a randomized, placebo-controlled trial the neuroprotective role of vitamin E against cisplatin neurotoxicity [80]. This was a phase III study in which 108 patients, treated with cisplatin, were randomized to receive vitamin E (alpha-tocopherol 400 mg/day) or placebo. Class II evidence that vitamin E supplementation significantly reduces the relative risk of developing signs or symptoms of neurotoxicity (relative risk = 0.14) (95% confidence interval = 0.02–1.00, $P < 0.05$) was provided[80]. Another research study showed that Ca/Mg infusions have been used to decrease the incidence of oxaliplatin-induced neuropathy without any influence on antitumor activity[81-84].

1.11.2 Prevention of cisplatin induced nephrotoxicity, several approach

Many strategies have attempted to prevent or reduce its nephrotoxicity[85, 86]. Early experience suggested that the administration of cisplatin by prolonged continuous infusion and saline hyperhydration, with or without frusemide or mannitol osmotic diuresis, reduces nephrotoxicity.[85] Although contemporary protocols invariably employ hyperhydration and many use low-dose rates of cisplatin, it is clear that nephroprotection is not complete[87]. Hydration reduces the risk of nephrotoxicity with high-dose carboplatin[88] but appears unnecessary with conventional doses of 400–600 mg/m^2.

The consequences of tubular toxicity may be ameliorated by prophylactic intravenous magnesium supplements in hydration fluid to reduce the frequency and severity of hypomagnesemia[89]. Many pharmacological agents that may ameliorate cisplatin nephrotoxicity have been investigated[86], but none have found widespread acceptance yet owing to uncertainty about the mechanism of toxicity, and the lack of clear evidence to demonstrate improvements in the therapeutic index of cisplatin.

Amongst many others, the drugs studied have included a variety of sulfur-containing compounds, such as sodium thiosulfate, WR-2721 (amifostine), DDTC (sodium diethyldithiocarbamate), mesna, biotin, cephalexin and sulfathiazole, all of which probably react with nephrotoxic cisplatin metabolites to form less toxic products[86,90].

The dephosphorylation of amifostine to the active free thiol metabolite (WR-1065) by membrane-bound alkaline phosphatase is reduced in tumor cells compared with normal cells, therefore allowing healthy tissues (e.g., kidneys and bone marrow) a degree of selective protection against cisplatin toxicity[91].

Based on data from adult studies, guidelines from the American Society of Clinical Oncology suggest that its use may be considered for protection against cisplatin nephrotoxicity[92]. Although there is some experience of the use of amifostine to prevent cisplatin ototoxicity in children[91], only anecdotal case reports exist of its use to prevent cisplatin nephrotoxicity[93]. A randomised controlled trial of amifostine found no evidence of reduction of cisplatin ototoxicity or tubular nephrotoxicity in the amifostine group but did not report any measures of glomerular function[94].

Furthermore, the value of amifostine may be limited by side effects, including nausea, vomiting, flushing and infusion-related hypotension[92]. Animal studies have also suggested that cisplatin nephrotoxicity may be reduced by a variety of antioxidants including capsaicin, glutamine, melatonin, *N*-acetylcysteine and selenium[95]. Liposomal and microsphere preparations have been designed to improve the therapeutic index of platinum compounds.

Although a Phase I clinical study of liposomal cisplatin in children demonstrated reduced renal toxicity, cisplatin appeared to be retained within the liposomes, thereby compromising efficacy[96]. Additional protective strategies proposed have included a blockade of possible mediators of renal vasoconstriction, including aminophylline (to inhibit adenosine) and BN-52063 (to antagonize platelet-activating factor),[97,98] and inhibition of cisplatin metabolism by procainamide[99]. Other strategies to selectively reduce nephrotoxicity by inhibition of tubular cisplatin transport, for example with probenecid (organic anion) or cimetidine (organic cation transport), have been investigated, but the uncertainty concerning which renal tubular pathways are responsible for cisplatin transport has limited the value of this approach[100,101].

Another approach has implemented previously by a drug named methimazole, an antithyroid drug containing a free SH group, has a nephroprotective potential against chemically-induced nephrotoxicity. We tried to explore the nephrotoxic effect of the experimentally therapeutic dose of cisplatin (7 mg kg(-1), i.p.), particularly on the nuclear level of kidney cells in male albino rats, as well as the possible protective effect of methimazole. Furthermore, the drug interaction regarding the oncolytic effect of cisplatin was examined in Ehrlich ascites carcinoma (EAC)-bearing mice.

A single dose of cisplatin caused kidney damage, 6 days after injection, manifested by 219% increase in serum creatinine, 384% increase in blood urea nitrogen and 170% increase in kidney content of lipid peroxides. Kidney DNA showed clear fragmentations detected by gel electrophoresis. However, kidney reduced glutathione was unchanged at that time period. Histological examination of kidney confirmed the toxic effect of cisplatin. Methimazole (40 mg kg(-1), i.p., 30 min before cisplatin injection) significantly protected the kidney from the nephrotoxic effect of cisplatin as judged from the biochemical parameters investigated as well as the histopathological examination. On the other hand, the survival data in EAC-bearing mice treated with both drugs indicated the persistence of an effective cytotoxic action. This study points to a promising use of this combination and necessitates further experimental and clinical studies[102].

1.11.3 Protective effects of plants materials in drug-induced nephrotoxicites

Several medicinal plants extract were used to prevent cisplatin induced nephrotoxicity. One study suggested that protective effect of terpenes isolated from the fruiting bodies of *Ganoderma lucidum* (Fr) P.Karst found promising reduction of nephrotoxicity caused by the cisplatin, in mice. Intraperitoneal administration of cisplatin (16 mg/kg body wt) resulted in significant nephrotoxicity in mice. The results suggest the potential therapeutic use of *Ganoderma* terpenes to prevent nephrotoxicity caused during chemotherapy using cisplatin[103].

Several other studies regarding plant extract showed nephro-protection. One study revealed that the extracts of medicinal plants Hemidesmus indicus L. (Apocynaceae) and Acorus calamus L. (Araceae) protected the renal tissue effectively from cisplatin-induced toxicity.

Cisplatin treatment increased serum urea level to 41.3 +/- 2.86 mg/dL and administration of the extracts of H. indicus and A. calamus brought down the level to 34.54 +/- 0.37 and 30.12 +/- 0.95 mg/dL, respectively. The histopathological observations indicated that treatment with the H. indicus and A. calamus extracts restored the cisplatin-induced structural alterations in the renal tissue[104].

Very much known plant Azadirachta indica A. Juss. (neem, family: Meliaceae have been using as medicinal plant in various disease since prehistoricperiod. One study suggested that after treatment with MNLE (methanolic neem leaves extract), the histological damage and apoptosis induction caused by cisplatin were improved[105].

Conversion of cisplatin to nephrotoxic molecules in the proximal tubule cells is required for cell injury[105]. Cisplatin is conjugated to glutathione and then metabolized through a γ-glutamyl transpeptidase and a cysteine S-conjugate β-lyase-dependent pathways to reactive thiol, a potent nephrotoxin[105-107]. Inhibition of these 2 enzymes has no effect on the uptake of cisplatin into the kidneys but reduces nephrotoxicity[105-107]. Cisplatin can form monohydrated complexes by hydrolytic reactions. The monohydrated complex is more toxic to the renal cells than cisplatin but it is not kidney specific[108].

The normal low intracellular chloride concentrations promote its formation[108]. Using hypertonic saline to reconstitute cisplatin can decrease the amount of monohydrated complex formed. This approach attenuates nephrotoxicity but may also compromise its anti-tumor activity[109].

In the previousworks several attempts was taken to reduce cisplatin induced nephrotoxicities. One study suggested that, *Taxilli Ramulus* could protect against cisplatin induced acute renal failure in mice. *Taxilli Ramulus* treated group had significantly reduced levels o pro-inflammatory cytokines when compared to the control group[110-112].

Another study reveals that *Scutellaria Barbara* (*S. barbara*), a perennial herb, is used to treat types of cancer, inflammation and urinary disease throughout Asia can also prevent the nephrotoxicity by reducing the levels of pro-inflammatory cytokines[113].

Scientists of Amala Cancer Research Centre, Thrissur 680 553, India have noticed us about the methanolic extract of Ganoderma lucidum possessed significant in vitro antioxidant activity. This prompted us to investigate the preventive effect of the methanolic extract of G. lucidum to ameliorate cisplatin-induced nephrotoxicity in mice. Nephrotoxicity was assessed by determining the serum creatinine and urea levels and renal antioxidant status in mice after cisplatin administration (16 mg/kg body wt, i.p). Methanolic extract of G. lucidum (250 and 500 mg/kg body wt) was administered orally 1 h before cisplatin injection. The extract significantly reduced the elevated serum creatinine and urea levels. Renal antioxidant defence systems, such as superoxide dismutase, catalase, glutathione peroxidase activities and reduced glutathione level, depleted by cisplatin therapy were restored to normal by treatment with the extract[114-124].

Another Scientists group revealed that Aged garlic extract protects against oxidative stress and renal changes in cisplatin-treated adult male rats. This study showed most of CP-induced histomorphological, ultrastructural and biochemical changes were improved in animals pretreated with AGE (Aged Garlic Extract)[125-134].

Some authors suggested that the abnormalities that were produced by the cisplatin induced hepatotoxicities with a significant increase in activity of ALT, AST, γGT, ALP, and TB levels in mice was significantly restored these levels to normal values ($p< 0.05$) while treatment with MNLE (Methanolic Neem Leaves Extract)[135].

Many other herbal medicines such as Dioscorea species may reduce cisplatin induced nephrotoxicities has been reported (38). One study revealed that M. oleifera seed powder at the dose rate of 600 mg/kg is as efficacious as silymarin in exerting nephroprotective and antioxidant effects[154]. We reviewed clinical and experimental literature on cisplatin nephrotoxicity to identify new information on the mechanism of injury and further study will be emphasis the potential approaches on some medicina plants *Dioscorea alata* and *Moringa olifera* [154-158].

1.12 Hypothesis

In this study our hypothesis is whether the nephrotoxic effect of cisplatin is due to the active ingredients or its metabolites among the cancer patients of Bangladeshi population.

We reviewed clinical and experimental literature on cisplatin nephrotoxicity to identify new information on the mechanism of injury and further study will be emphasis the potential approaches on some medicinal plants *Dioscorea alata* and *Moringa olifera* to prevention of cisplatin induced nephrotoxicities on mice.

1.13 Justification of the study

The success in cisplatin based chemotherapy, however, strongly depends on how careful the drug's dosages are monitored in order to reduce severe side-effects and overcome cellular resistance. So the main aim of our study is to determine the pharmacokinetics of cisplatin and its active metabolites and to see correlation with these drugs induced nephrotoxicities and also to see the absorption, distribution of this drug and its metabolites that will be determined in tissues and to mark any organ toxicities in mice that will be needed to find out how to improve the therapeutic effects and lower the toxic ones.

It is suggestive that the population approach has been widely used in clinical pharmacokinetics as an effective technique, because it can be used to obtain population mean values, including regression relationships with specific patho-physiological characteristics, together with estimates of inter individual and residual variability.

1.14 Aim & objectives

General

To determine the active or inactive metabolites in blood and urine sample after administration of cisplatin and their correlation with organ toxicities specially nephrotoxicities among Bangladeshi cancer patients.

Specific

1. To find out the extent of metabolism of cisplatin in blood and urine sample by using HPLC method in male cancer patients aged between 18-60 years, attending Oncology In Patient Department, Khwaja Yunus Ali Medical College Hospital, Sirajgonj.
2. Isolation and purification of cisplatin and its metabolites by using column chromatography, PTLC and glass chromatography.
3. Determination and comparison cytotoxicity of isolated compounds by using brine shrimp lethality bioassay.
4. To detect & Compare the accumulation of cisplatin & its metabolites in different organ in mice.
5. To find out the histopathological changes in vital organs in mice treated with cisplatin and its metabolites along with some medicinal plants Dioscorea alata and Dioscorea bulbifera of Dioscorea species, antioxidants, antioxidant rich foods, minerals to prevent of cisplatin induced nephrotoxicities on mice.

6. To find out the correlation between the development of organ toxicities such as nephrotoxicities and active ingradients or its metabolites.
7. To determined the structure of active or inactive metabolites of cisplatin among Bangladeshi cancer patients by using AAS(Atomic absorption spectrometry), HPLC- mass spectrometry.

Figure 7 : Cisplatin compound by HPLC-ICP-SFMS.

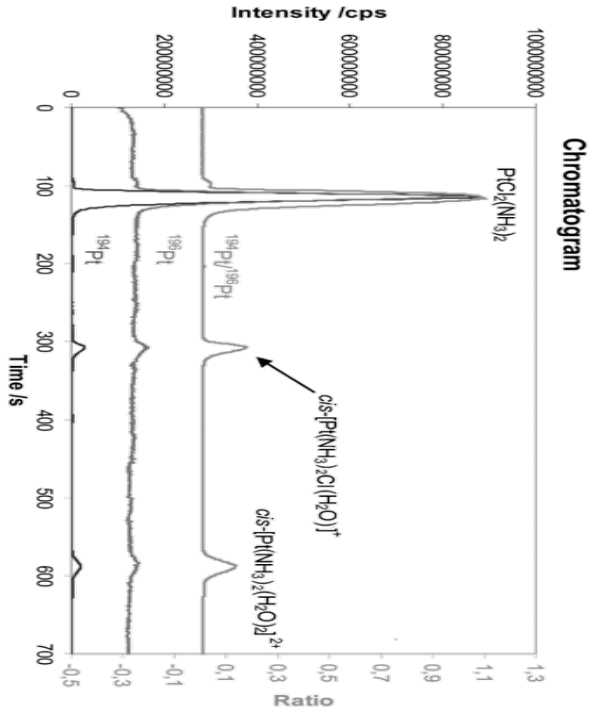


Figure 8 : An ion attachment mass spectrum of a cisplatin sample heated to ~270 °C.

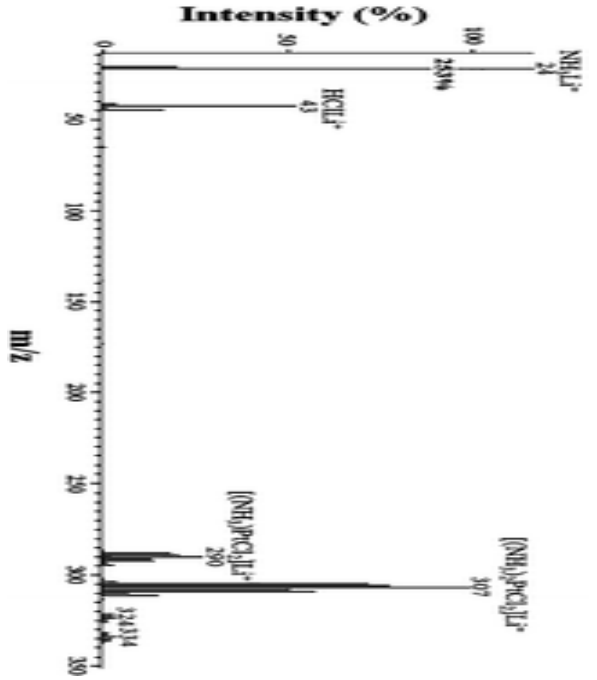
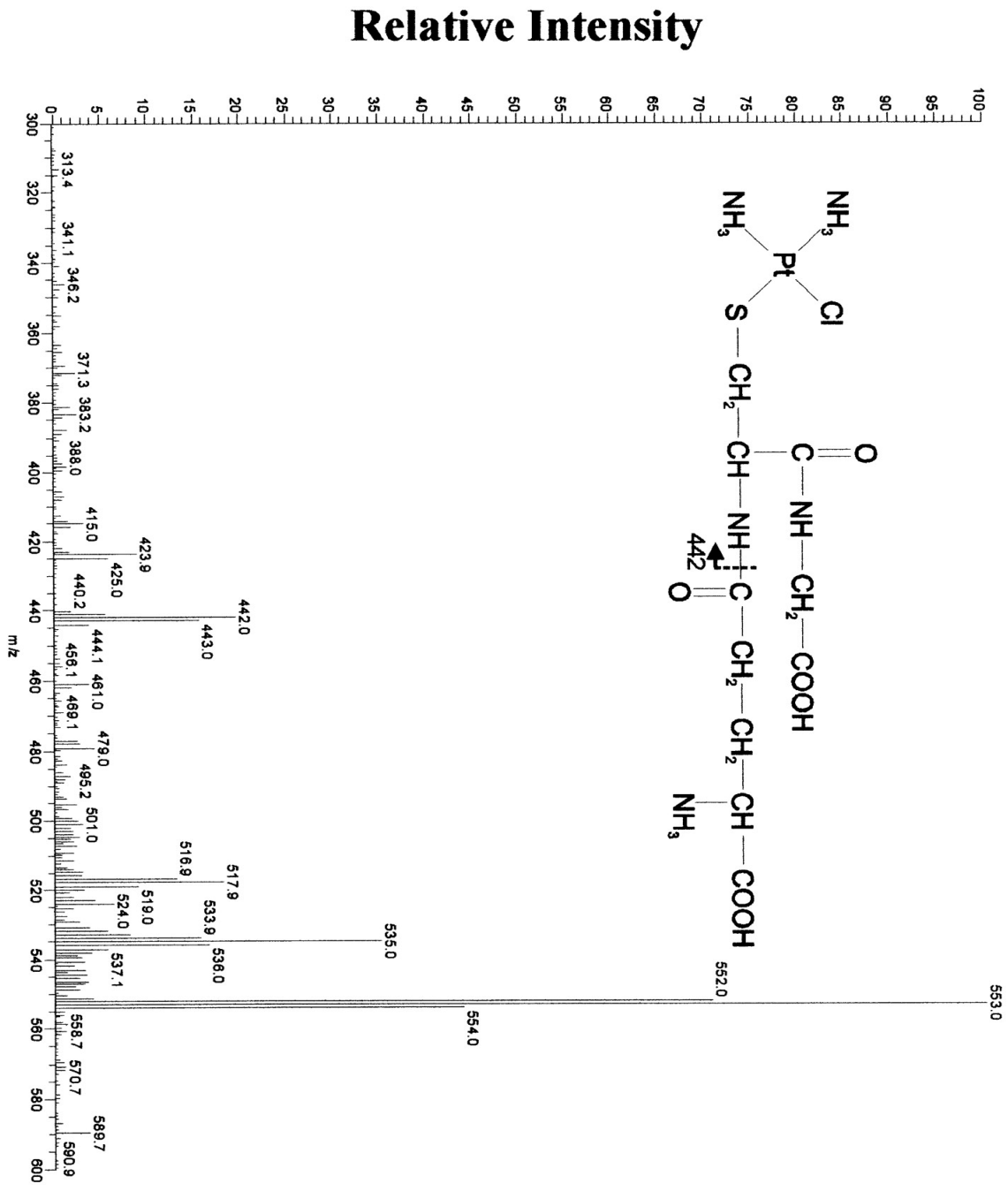


Figure 9 : High Pressure Liquid Chromatography and Mass Spectrometry Characterization of the nephrotoxic biotransformation products of Cispltin.



Chapter 2

Materials and methods

2.1 Place of study

a) Indoor patient Department, Department of Oncology, Khwaja Yunus Ali Medical College Hospital, Enayetpur, Sirajgonj.

b) Centre for bioequivalence study, Khwaja Yunus Ali Medical College Hospital, Enayetpur, Sirajgonj

c) Pharmacology Research Lab., Department of Pharmacology, BSMMU, Shahbag, Dhaka.

2.2 Duration of study

From January 2012 to December 2014

2.3 Study sample

Total 50 adult male (age 18-60 years) hospital admitted cancer patients receiving cisplatin were selected for this study. Smoker, alcoholic, female and CVD (cerebrovascular disease) patients will be excluded from this study.

2.4 Ethical clearance

Protocol

The study was approved by the Ethics Committee of the Khwaja Yunus Ali Medical College, Enayetpur Sharif, Sirajgonj and written informed consent of all patients were collected.

2.5 Questionnaire

Questionnaire- A questionnaire was develop for this study with patient written consent along with all biochemical, pathological, histopathological, radiolgical and imaging records were collected (Annexure I).

2.6 Inclusion criteria

1. Cancer patients receiving cisplatin
2. Age – 18 to 60 years of age male patients
3. Hospital admitted patients
4. Non-smoker and non-alcoholic patients for at least previous 6 months

2.7 Exclusion criteria

1. Children (below 18 years), old age (above 60 years) and female patients.
2. Smokers for at least previous 6 months.
3. Chronic alcoholism.
4. Cerebrovascular disease (CVD) patients

2.8 Instuments

1. HPLC (High performance liquid chromatography)
2. Electronic centrifuge machine
3. Vortex meter
4. PH meter
5. Electronic ultra-filtration
6. Nitrogen cylinder
7. -4°C freezing system
8. Micropipette
10. PTLC

2.9 Chemicals

1. Cisplatin (>98.5%purity for HPLC) and Nickel chloride will be purchased from Sigma Aldrich (USA).
2. Methanol and acetonitrile (Merck, Germany), and ethyl acetate were of HPLC grade and will be obtained from Merck Laboratory Supplies (Germany).

3. Distilled and deionized water will be obtained by passage through ELGA® (a trade name of VivendiWater Systems Ltd.,Wycombe, Bucks, UK).
4. Stock solutions will be prepared by dissolving the compounds in water. The standard solutions will be prepared every day.
5. Sodium chloride (NaCl)
6. Dimethyl sulfoxaide (DMSO)
7. Additional solvent: Pet ether, Dichloromethane, Chloroform etc.
8. Silica gel (column grade)
9. Silica gel for glass chromatography

2.10 Statistical analysis

All the values in the test are expressed as mean ± standard deviation (SD). The data were statistically analyzed by ANOVA (one way of analysis of varience) and post-hoc Dunnett's tests the Statistical Package for Social Sciences (SPSS 16.0, USA) program. Dissimilarity between the means of the various groups were measured significant at *P < 0.05, **P < 0.01 and ***P < 0.001.

2.11 Chromatographic conditions[136-139]

The HPLC-UV diodearray system consisted of Agilent model 1200 series degasser, solvent delivery pump, autosampler, column oven, photo diode array detector. Chromatographic data were collected and analyzed using Chemstatin software.

A reverse-phase high performance liquid chromatography (HPLC) was used for the determination of cisplatin and its metabolites in plasma and corresponding urine of 40 patients at different time interval (0, 3, 6, 12 and 24 hours).

The chromatographic analysis were performed on an Agilent 5µm C18 column (150 x 4.6 mm). The mobile phase used for analysis consisted of 20% acetonitril (HPLC grade, E. Mark, Germany) and 80% distilled water and the flow rate was 0.5 ml/min. Separation was achieved at 40°C. The wavelength was set at 213 nm (bandwidth 1 nm). Injection of sample (20µl) was done using an autosampler. The peak with retention time and areas were defined using software. This is for the quantitative analysis of the drug in ultrafiltrate plasma and urine in the presence of nickel chloride as internal standard. Here nickel chloride will be used as internal standard.

2.12 Solutions of external and internal standards

In HPLC, narrow columns with internal diameters 2-80 mm was used. These columns are packed with particles having an average diameter of less than 50 microns (50 x 10-6m).

Reversed phase chromatography, which is the most common form of HPLC, is a type of partition chromatography was used in this research work. Cisplatin and its metabolites separation were carried out on a reversed-phase column using acetonitrile-water as the mobile phase.

The flow rate was maintained constant at 0.5 ml/min and analysis was performed at 40 degrees C. Detection will be carried out by absorbance at 213 nm. The more polar components of a mixture elute first, since these partition relatively unfavorably on the highly non-polar packing. Increasing the polarity of the solvent increases the retention time of a particular component. This is a simple, rapid and sensitive high performance liquid chromatographic (Solvent: Acetonitrile-water as the mobile phase) assay for cisplatin in human plasma ultrafiltrate and urine among Bangladeshi population.

This is for the quantitative analysis of the drug in ultrafiltrate plasma and urine in the presence of nickel chloride as internal standard. Here nickel chloride will be used as internal standard.

50 (Fifty) patients was taken for experiment and these patients will be received cisplatin (60–100 mg/m2) as single drug with intravenous constant infusion for 90 min.

Blood and urine samples was taken at about five points per patient (at 0, after 3, 6, 12 and 24 hours)

2.13 Sample collection and preparation of Blood

A total 250 blood samples of 50 patients was taken for this experiment. These patients was received cisplatin (60–100 mg/m2) with intravenous constant infusion for 60-90 min, as part of protocols for the treatment of various cancer diseases at Khwaja Yunus Ali Medical College, Enayetpur Sharif, Sirajgonj, Bangladesh.

Blood samples was taken at about five points per patient. Blood samples will be collected at various time points (0, after 1hour, 2 hours, 3 hours, 6 hours, 12 hours, 24 hours and 48 hours) after the end of each infusion.

5 ml x 5= 25 ml blood sample was collected from each patient at different time interval (at 0,3,6,12 & 24 hours). After complete mixing of samples with internal standard, 5 ml of ethyl acetate and vortex mixed for 2 minutes, then centrifuged at 3000 rpm for 15 minutes. 20 µL aliquots of the supernatant will be directly injected into the chromatography column. Each sample will be analyzed in duplicate. All samples or standard solutions will be stored at –5ºC until analyzed.

2.14 Sample collection and preparation of urine

Patient urine

The urine of a cancer patient will be collected after receiving intravenous chemotherapy with cisplatin (60-100mg/m2) drug (0, after 3 hours, 6 hours, 12 hours, 24 hours) respectively. The urine will be frozen instantly after collection. For measurement, the urine was diluted in ultrapure water (factor 20).

2.15 Determining the amount of cisplatin and their possible metabolites in plasma and urine

Determine the relative peak areas and correct for the molar detection responses of the individual components. Using the known concentration of the internal standard, calculate the concentration of those components present in the plasma and urine. The structures of the compounds will be elucidated by mass spectrometry.

2.16 Determining the changes in the biochemical parameter in patients treated with cisplatin

The retrospective study of the biochemical parameters from the of 50 cancer patients those were admitted in IPD of KYAMC Cancer Center, KYAMC&H obtained from hospital records. The biochemical parameters were as follows: Total WBC count: Neutrophil, Lymphocyte, eosinophil, monocyte, basophil; Total RBC count; Haemoglobin (gm%); ESR; Serum creatinine; Serum bilirubin; Alkaline phosphatase; SGPT; Blood urea; Random blood sugar, Fasting blood sugar; Urine routine examination.

2.17 Imaging & other investigations

Retrospective Ultrasonography; CT Scan; MRI; ECG; Echocardiography; Lipid profile; Histopathology reports of admitted 50 patients were studied.

2.18 Separation of cisplatin and its possible metabolites from urine Samples of 5 patients and their primary screening for cytotoxicity

Total 20 urine samples of 5 cisplatin treated patients at 3 hr, 6 hr, 12 hr and 24 hr respectively

Each sample consist of 150 ml urine. Evaporation of water by rotary evaporator to dryness of the samples.

Table 1: Weight of the 20 dry urine samples

Name of the patient	Sample	Weight in gram
1. Md. Nazar Ali	1	7.8
	2	6.9
	3	6
	4	5.8
2. Md. Rabiul Sarker	5	9
	6	6.1
	7	6.4
	8	7.6
3. Md. Jahangir Alam	9	7.7
	10	6.1
	11	6.3
	12	7.6
4. Md. Rabiul Islam	13	7.7
	14	6.1
	15	6.3
	16	7.6
5. Md. Chandu Sheikh	17	6.7
	18	6.4
	19	7
	20	5.1

2.19 Column chromatography preparation

Each sample dissolved in 10 ml methanol and mixed with column grade silica gel and dry in air.

The glass column, specially burette (Diameter=5mm, Length=50 cm) was packed with silica gel (Kieselgel 60, mesh 70- 230). When the desired height of adsorbent bed was obtained , a few hundred milliliter of di-chloromethane was run through the column for proper packing of the column. The sample was prepared by adsorbing 2 g of dried urine sample dissolved in 1 ml methanol and applied onto silica gel, allowed to dry and subsequently applied on the top of the adsorbent layer.

The column was then eluted with di-chloromethane, mixtures of di-chloromethane and chloroform , chloroform-ethyl acetate and ethyl acetate with methanol then methanol with increasing polarity.

Primarily 412 samples of 5 ml each were collected from 25 urine samples. After TLC analysis, the fractions with similar TLC pattern, were recombined and finally seventy three (73) samples were obtained.

Total 73 samples were found from 412 fractions of 20 urine samples due to same PTLC character [from sample1: 3 fractions , sample 2: 4 fractions, sample 3: 3 fractions, sample 4: 3 fractions, sample 5: 4 fractions , sample 6: 5 fractions, sample 7: 4 fractions, sample 8: 4 fractions, sample 9: 3 fractions , sample 10: 3 fractions, sample 11: 4 fractions, sample 12: 4 fractions, sample13: 3 fractions , sample 14: 5 fractions, sample 15: 3 fractions, sample 16: 3 fractions, sample17: 4 fractions , sample 18: 4 fractions, sample 19: 3 fractions, sample 20: 4 fractions.

2.20 PTLC of 73 fractions

(Solvent system: chloroform and ethyl acetate in different polarity)

PTLC of 73 fractions was done. All the column fractions were screened by TLC under UV light and spraying vanillin-sulfuric acid reagent.

Mixing the same fraction due to same PTLC character to give rise to 8 fractions(23.9 mg, 27.1 mg, 22.6 mg, 21.3 mg, 19.8 mg, 20.4 mg, 18.7 mg and 17.5 mg) containing probable single compound with some impurities in each fractions. Cisplatin 100 µg/ml was used as Standard solution .

2.21 Glass chromatograpy

Final 8 fractions containing probable single compound with some impurities were subjected to glass chromatography.

8" x 6" glass plate was prepared for glass chromatography with silica gel

Solvent system: chloroform and ethyl acetate in different polarity

8 fractions were run in glass chromatography and identified the 5 single compound under UV light and scratch them and dissolved in methanol and collected in small beaker. 5 possible single compounds with some impurities, which were purified from the different sub-fractions employing washing techniques e.g. acetone.

PTLC of 5 single compounds were done and calculated the R*f* values and than dried. Finally weight of each compounds and stored in close brown bottle for biological test.

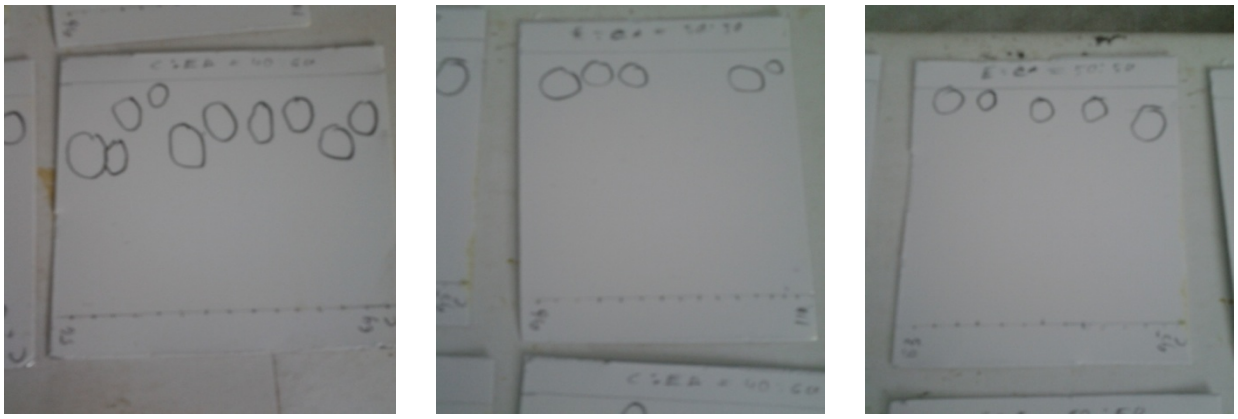
2.22 Weight of the compounds collected from urine samples of the patients

Compound 1 (C): 8.1 mg, Compound 2 (CM2): 7.4 mg, Compound 3 (CM3): 8.3 mg, Compound, 4 (CM4): 6.9 mg, Compound 5 (CM5): 8.6 mg

Table 2 : R*f* values of compounds

Compound	Mobile phase	R*f* value	Amount (mg)	Yield (%)	Compound ID
1	Chloroform: Ethyl acetate = 80:20	0.821	8.1	4.0	C
2	Chloroform: Ethyl acetate = 75:25	0.685	7.4	3.7	CM2
3	Chloroform: Ethyl acetate = 75:25	0.654	8.3	4.1	CM3
4	Chloroform: Ethyl acetate = 75:25	0.704	6.9	3.4	CM4
5	Chloroform: Ethyl acetate = 75:25	0.721	8.6	4.3	CM5

Photograph 1: A few photographs in PTLC of 73 samples showing the five compounds including cisplatin by using UV spectrophoyometry.



2.23 Phytochemical investigation

2.22.1 Collection and identification of plant materials

2 kg of tuber part of the *Dioscorea alata* and 2 kg of seed pods of *Moringa oleifera* were collected from the local market. Both plants were taxonomically identified by the National Herbarium , Mirpur, Dhaka, Bangladesh (vide memo no. 0354).

2.22.2 Drying, Grinding and weighting of plant material

The tuber parts of *Dioscorea alata* and seed pods of *Moringa oleifera* were cut into pieces and dried at room temperature. The dried cut pieces of both plants were grinded to powder form separately and 980 g *Dioscorea alata* and 760 g seed pods of *Moringa oleifera* powder were measured and preserved in air tight bottle for future phytochemical and biological studies.

2.22.3 Extraction of plant materials with MeOH

Dried powder of 980 g of tuber parts of *Dioscorea alata* and 760 g of seed pods of *Moringa oleifera* were dissolved in 2 liters and 1.5 liters in two separate jar respectively for 7 days. After 7 days two solutions were filtered to gave rise clear solutions and were placed to evaporate MeOH by using rotary evaporator at 40 to 50° C in the Pharmacology Research Lab., Bangabandhu Sheikh Mujib Medical University (BSMMU), Shahbag, Dhaka, Bangladseh.

2.22.4 Collection of powdered material from plant extracts

After evaporation gummy part of tuber parts of *Dioscorea alata* and seed pods of *Moringa oleifera* were collected from the wall of volume flask weighted to 56 g and 34 g respectively. Two extracts were preserved in two separate air tight bottle for future biological investigations.

Chapter 3

Biological investigation-

Cytotoxicity test

3 Cytotoxicity test of 5 compounds[140-143]

3.1 Brine shrimp lethality bioassay

Brine shrimp lethality bioassay is rapid general bioassay for the bioactive compounds of the natural and synthetic origin. Bioactive compounds are almost always toxic at high dose. Pharmacology is simply toxicology at a lower dose or toxicology is simply the pharmacology at a higher dose.

Brine shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer, antimicrobial, and pharmacological activities of natural products and it is a recent development in the bioassay for the bioactive compounds. By this method, natural products extracts, fractions as well as pure compounds can be tested for their biosphere-activity. Here, in vivo lethality in a simple zoologic organism (Brine shrimp nauplii) is used as a convenient monitor for screening and in the discovery of new bioactive natural products. This bioassay is indicative cytotoxicity and a wide range of pharmacological activity of the compounds.

Brine shrimp lethality bioassay stands superior to other cytotoxic testing procedures because it iis a rapid method utilizing only 24 hours, inexpensive and requires no special equipment. It utilizes a large a large number of organisms for statistical validation and a relatively a small amount of sample. Furthermore, unlike other methods, it does not require animal serum.

Brine shrimp eggs are hatched simulated sea water to get nauplii. Sample solution are prepared by dissolving the test materials in pre-calculated amount of DMSO. Ten nauplii are taken in a test tubes containing 5 ml of simulated sea water. The samples of different concentrations are added to the pre-marked test tubes with a micropipette. The assay is performed in three replicates. Survivors are counted after 24 hours and 48 hours. These data are processed in a simple program for probit analysis to estimate LC50 values.

3.2 Procedure

3.2.1 Preparation of sea water

38 gm salt (pure NaCl) was weighed, dissolved in 1 liter of distilled water and filtered off to get clear solution.

3.2.2 Hatching of brine shrimps

Artemia salina leach (Brine shrimp eggs) collected from pet shop was used as the test organism. Seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. One day (24 hour) was allowed to hatch the shrimps and to be matured as nauplii. Constant oxygen supply was provided throughout the hatching time. The hatched shrimps were attracted to the lamp through the perforated dam and with help of pasteur pipette 10 living shrimps were added to each of the test tubes containing 5 ml of sea water.

3.2.3 Preparation of the test solution

Measured amount (Table) of each sample was dissolved in specific volume of DMSO to obtained the desired concentration of the prepared solution as 1 mg/1 ml. Then a series of solutions of lower concentrations were prepared from this solution by serial dilution with DMSO. Thus the concentration of the solutions were obtained as 6.25 µg/ml, µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml and added to the pre-marked test tubes containing 5 ml of sea water and 10 shrimp nauplii. So, the final concentration of samples in the test tubes were 1.25 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml.

Log concentration of each sample were : 0.09691, 0.3979, 0.69897, 1, 1.30102, 1.60205

3.2.4 Preparation of the control group

Cisplatin served as the positive control. 1 mg of cisplatin was dissolved in DMSO to get an initial concentration of 1 mg/ ml from which serial dilutions were made using DMSO to get 6.25 µg/ml, µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml concentrations and added to the pre-marked test tubes containing 5 ml of sea water and 10 shrimp nauplii. So, the final concentration of samples in the test tubes were 1.25 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml.

The control group containing 10 living brine shrimp nauplii in 5 ml simulated sea water received the positive control solutions.

As for negative control, 1 ml of DMSO was added to each of three pre-marked glass vials containing 4 ml of simulated sea water and 10 shrimp nauplii to use for negative control. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as the nauplii died due to some reason other then the cytotoxicity of the compounds.

3.2.5 Counting of nauplii

After 24 and 48 hours, the vials were observed using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of the lethality of the brine shrimp nauplii was calculated for each concentration

Chapter 4

Biological Investigation-

Histopathological & Biochemical Investigation in Mice

4.1 Cisplatin & its metabolites induced Histo-Pathological changes in liver and kidney of mice

4.1. Introducton

The most common procedure used in histologic research is the preparation of tissue sections or slices that can be studied with the light microscope. Under the light microscope, tissues are examined visually in a beam of transmitted light. Because most tissues and organs are too thick to pass through them, they must be sliced to obtained thin, translucent sections that are attached to glass slides for microscopic examination[144].

4.1.2 Materials & methods

1. Specimens are Cisplatin & its metabolites induced liver and kidney of mice.
2. Biopsy material- Small pieces of tissues are cut from large specimens. The material is processed and stained for microscopic examination.

4.1.3 Collection of mice

Animal House, ICDDR'B, Mohakhhali, Dhaka. (vide memo no. 0461)

Total number of test animal (Mouse[145] :*Mus musculus*) : 50

Average wt of mouse: 40 g

Total number of groups =13

Group A- Cisplatin

Group B- Cisplatin + *D. alata*[146, 147]

Group C- Cisplatin + *Moringa oleifera* [148, 149]

Group D- Metabolite 1

Group E- Metabolite 1+ *D. alata*

Group F- Metabolite 1 + *Moringa oleifera*

Group G- Metabolite 2

Group H- Metabolite 2+ *D. alata*

Group I- Metabolite 2 + *Moringa oleifera*

Group J- Metabolite 3

Group K- Metabolite 3+ *D. alata*

Group L- Metabolite 3+ *M. olifera*

Group M- Control.

4.1.4 Animal handling/Feeding[150] - Properly maintained.

4.1.5 Animal Dose & Duration[151]

A. Injectable:

(a) Cisplatin : 16 mg / kg body wt, given every weeks for 2 weeks

(b) Metabolite 2 : 16 mg / kg body wt, given every weeks for 2 weeks

(c) Metabolite 4 : 16 mg / kg body wt, given every weeks for 2 weeks

B. Powdered extract: 250mg/kg body-wt

4.1.6 Drug dissolving solvent

a) Dimethyl sulfoxide (DMSO)

b) Propylene glycol (Merck Specialities Private Limited, Mumbai, India)

4.1.7 Steps in preparation of Slides[152]

I. Sectioning of Tissue- Paraffin sectioning: The steps in paraffin sectioning are-

a. Fixation of the Tissue: Formol-saline (Formalin 10 ml and normal saline 90 ml) is commonly used as a used as a fixative.

b. Dehydration of the tissue in ethyl alcohol and xylol

c. Preparation of Block-The tissue is casted in wax.

d. Section cutting- Sections are cut with microtome and taken on slide.

II. Staining of the sections.

Haematoxylin and Eosin (H & E) Stains

4.1.8 Characteristics of Haematoxylin and Eosin (H & E) Stains[153]

1. Nuclei stain blue
2. Cytoplasm stain pink or red
3. Collagen fibers stain pink
4. Muscle stain pink

4.1.9 Steps

1. Treat with xylol to remove wax
2. Treat with alcohol to remove xylol
3. Rinse in water
4. Stain in haematoxylin for 15-30 minutes
5. Rinse in water for 15-30 minutes
6. Differentiate in acid-alcohol till nuclei are only stained
7. Rinse in water for 20-30 minutes
8. Stain in 1% eosin for 5-15 minutes
9. Wash in running water till eosin is differentiated
10. Blot, dehydrate in alcohol, clear in xylol
11. Mount the section in Canada balsam on a slide

5 Biochemical investigations in Mice

5.1 Introduction

Several lines of evidence indicate that free radicals are involved in the nephrotoxicity caused by cisplatin, and the damage is suggested to be the consequence of decreased renal antioxidant enzyme activity with enhanced lipid peroxidation. However, administration of antioxidants has been shown to ameliorate cisplatin-induced nephrotoxicity in animals3.

Aqueous methanolic (50% MeOH) extracts of the tubers (peel and flesh) of nine cultivars of greater yam (Dioscorea alata) were determined to have relatively high antioxidant activities among which two cultivars (Ubing upo, purple; LA 096, white) had activities as high as those of α-tocopherol and butylhydroxyanisole (BHA). The aqueous methanol extract of the purple variety was fractionated on XAD-2 column chromatography serially with water, 25% MeOH, 50% MeOH, 75% MeOH, 100% MeOH and acetone. High activities were found in the 50% and 75% MeOH fractions; these were dried[165].

Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to human against infections and degenerative diseases. Current research is now directed towards natural antioxidants originated from plants due to safe therapeutics. Moringa oleifera is used in Indian traditional medicine for a wide range of various ailments. To understand the mechanism of pharmacological actions, antioxidant properties of the Moringa oleifera leaf extracts were tested in two stages of maturity using standard in vitro models.

The successive aqueous extract of Moringa oleifera exhibited strong scavenging effect on 2, 2-diphenyl-2-picryl hydrazyl (DPPH) free radical, superoxide, nitric oxide radical and inhibition of lipid per oxidation. The free radical scavenging effect of Moringa oleifera leaf extract was comparable with that of the reference antioxidants. The data obtained in the present study suggests that the extracts of Moringa oleifera both mature and tender leaves have potent antioxidant activity against free radicals, prevent oxidative damage to major biomolecules and afford significant protection against oxidative damage[166].

5.2 Steps

Reduced glutathione (GSH), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and diacetylmonoxime (DAM) were purchased from Bangladesh Medical Equipment, Hatkhola, Dhaka, Bangladesh. Cisplatin was purchased from Bangladesh Medical Equipment, Hatkhola, Dhaka, Bangladesh.. Other chemicals and reagents used were of analytical grade. Tuber part of *D. alata* and Seed pods of *Moringa oleifera* were collected from the local vegetable market of Sirajgonj district, Bangladseh.

The type specimen was deposited in the herbarium of Department of Botany, University of Dhaka, Dhaka, Bangladesh. The tuber parts and seed pods of *D. alata* and Seed pods of *Moringa oleifera* were cut into small pieces, dried at 45–50°C for 48 h, and powdered. Two hundred grams of the powdered material was extracted with petroleum ether using a Soxhlet apparatus. The defatted material was air-dried, then suspended in 70% methanol and boiled for 8 h.

The solvent was removed and the extraction repeated. The extracts were combined and filtered through Whatman No.1 filter paper. The solvent evaporated at low temperature under vacuum and the concentrated extract was finally lyophilized.

The methanolic extract tuber parts of *D. alata* and Seed pods of *Moringa oleifera* thus obtained (8.1 g and 6.7gm respectively) was employed in the experiments. The extract before administration was solubilized in distilled water. To investigate cisplatin-induced nephrotoxicity, animals were divided into seven groups of five animals each. Group 1 was kept as normal. Group 2 was given cisplatin (16 mg/kg body weight, i.p)[167], Groups 3 methanolic extract of the methanolic extract tuber parts of *D. alata*, Group 4 were given methanolic extract of seed pods of *Moringa oleifera* (250 and 500 mg/kg body wt; p.o), 1 h before the cisplatin injection (16 mg/kg body wt, i.p), , Group 5 were given cisplatin metabolite 3 (CM3), Group 6 methanolic extract tuber parts of *D. alata* Group 7 were given methanolic extract of seed pods of *Moringa oleifera* (same dose and duration), 1 h before the compound 3 (CM3 = cisplatin metabolite 3) injection (16 mg/kg body wt). The treatments in all groups were continued for 10 consecutive days. At the end of the 5th week, animals were sacrificed.

Mice in all groups were sacrificed 72 h after treatment. Blood was collected directly from the heart; serum was separated for creatinine and urea analyses. The kidneys were dissected and stored at –70°C until the analyses could be completed. The kidneys were homogenized in 50 mM phosphate buffer (pH 7) to give a 10% homogenate (w/v). The homogenate was centrifuged at 1000 rpm for 10 min in a cold centrifuge at 0C and the supernatant was used for enzyme assay and protein determination.

Serum creatinine and urea were estimated by the method of Brod and Sirota[168] and Marshell *et al.*[169], respectively. The activity of glutathione peroxidase (GPX) by the method of Hafemann *et al.*[170], levels of reduced glutathione (GSH) by the method of Moron *et al.*[171] and malondialdehyde (MDA) by the method of Ohkawa *et al.*[172] using 1,1,3,3 tetramethoxypropane as standard. The protein content was estimatedby the method of Lowry *et al.*[173] using bovine serum albumin as standard.

Experimental data were expressed as mean ± SD. Student's '*t*' test was applied for expressing the significance, and *P* values less than 0.05 were considered as significant. Serum creatinine and urea levels were significantly elevated ($P < 0.001$) in the cisplatin-treated animals compared to the normal group. The increase of serum creatinine and urea levels was 7 and 5.7-fold, respectively. Treatment of animals with methanolic extract of *D.alata and M. olifera* significantly reduced the elevated levels of serum creatinine and urea. The extract treatment was able to lower the serum creatinine and urea to almost normal level (Table 1).

The renal GSH concentration decreased by over 40% ($P < 0.001$) in cisplatin-treated animals compared to the normal group. However, the administration of methanolic extract of *D. alata* and *M. olifera* at a concentration of 250 mg/kg body wt prior to cisplatin treatment increased the renal GSH concentration to the normal level (Table 2).

The present study was carried out using higher doses of cisplatin (16 mg/kg body wt, i.p). This corresponds to the dose of cisplatin normally used in clinical practice[167]. This dose is also found to produce nephrotoxicity and ototoxicity.

Several protective agents have been evaluated against cisplatininduced nephrotoxicity in experimental and clinical studies. They include diethyldithiocarbamates[174], glutathione3, glycine[175], methionine[176], procaine and procainamide[177]. However, none of these compounds has proved to be clinically efficacious as complete protection in patients.

The results of the present study show that reduced GSH level significantly decreased in the cisplatin-treated animals compared to the normal group. These observations support the hypothesis that the mechanism of nephrotoxicity in cisplatin-treated animals is related to depletion of antioxidant defence system. Treatment with *D. alata* and *M. olifera* extract (250 mg/kg body wt, p.o) prior to cisplatin administration prevents the depletion of renal antioxidants.

The tested doses of methanolic extract of with *D. alata* and *M. olifera* (250 mg/kg body wt, p.o) show no signs of toxicity in mice. Survival rate of animals treated with cisplatin and with *D. alata* and *M. olifera* extract (250 mg/kg body wt, p.o) supports the nephroprotective effect of this mushroom.

Free radicals are known to play an important role in cisplatin-induced nephrotoxicity. The free radicals and reactive oxygen species induce oxidative stress in kidneys[180,181]. Due to cisplatin administration, platinumsulphydryl group complexes formed are taken up by renal cells and stabilized by intracellular GSH for several hours. In case of intracellular GSH depletion, the complexes undergo rapid transformation to reactive metabolites[182]. Thus GSH depletion results in increased toxicity of cisplatin. GSH depletion also results in lipid peroxidation and this seems to be the prime factor that permits lipid peroxidation and impaired antioxidant enzyme activities.

These observations support the conclusion that the mechanism of nephrotoxicity in cisplatin-treated rats is related to depletion of antioxidant systems. Thus, nephroprotection by the mushroom extract might be directly related to its antioxidant activity.

Preliminary phytochemical analysis indicates that the major constituent of the extract is a polysaccharide. This conclusion is based on the positive reaction of the extract to anthrone test[183] and also to phenol-sulphuric acid reagent[184].

Because it has become naturalized throughout tropical South America, Africa, Australia, the US southeast, *D. alata* has many different common names from these regions. In English alone, aside from purple yam, other common names include greater yam, Guyana arrowroot, ten-months yam, water yam, white yam, winged yam, or simply yam**.** In other cultures and languages it is known variously as *ratalu* or *violet yam* in India and one study revealed that *M. olifera* seed powder and tuber part of *D. alata* at the dose rate of 600 mg/kg is as efficacious as silymarin in exerting nephroprotective and antioxidant effects[154].

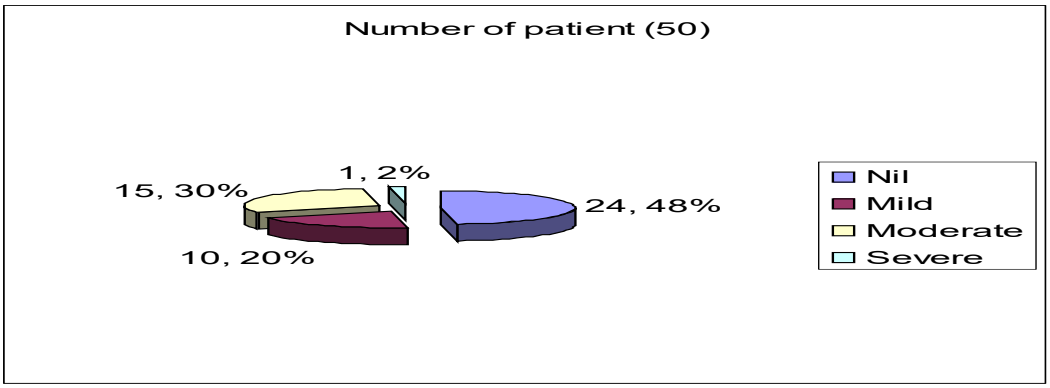
The specimen was identified with the help of the available literature and the identification was confirmed by the Bangladesh National Herbarium , Mirpur, Dhaka, Bangladesh (vide memo no. 0354). The experimental results reveal that the methanolic extract of *M. olifera* seed powder and tuber part of *D. alata* could help prevent nephrotoxicity manifested consequent to cisplatin chemotherapy. The effect is mainly due to the capacity of the extract to restore renal antioxidant defence system.

Our earlier investigations have shown that methanolic extract of *M. olifera* seed powder and tuber part of *D. alata* occurring in South India possessed significant antioxidant and antitumour activities.

Hence, a combined therapy with *M. olifera* seed powder and tuber part of *D. alata* extract and cisplatin would be more beneficial than cisplatin alone. The experimental findings thus suggest the potential therapeutic use of extracts *M. olifera* seed powder and tuber part of *D. alata* in cancer chemotherapy.

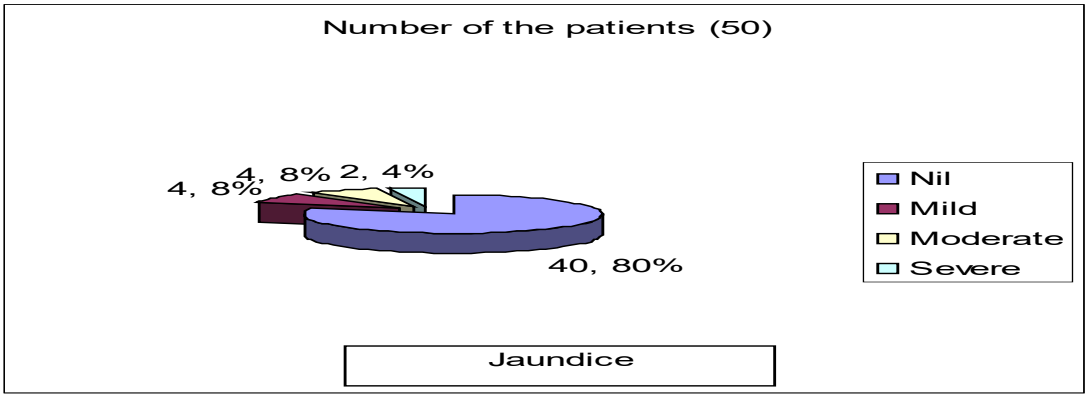
Chapter 5
Results

Figure 10 : Distribution of cancer patient according to the state of haemoglobin level.



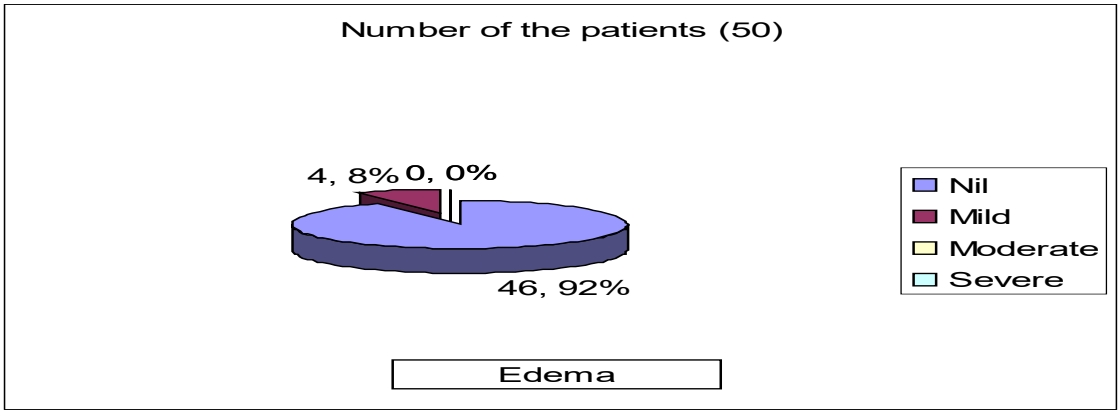
In figure 7 a retrospective study of 50 admitted cancer patients in KYAMC Cancer Center, Sirajgonj demonstrated that , a good number of patients (48%, n=24) were not suffered anaemia, only 2% (n=1) and 30% (n=15) of the patients were suffered from severe and moderate anaemia after cisplatin administration.

Figure 11 : Distribution of cancer patient according to jaundice condition



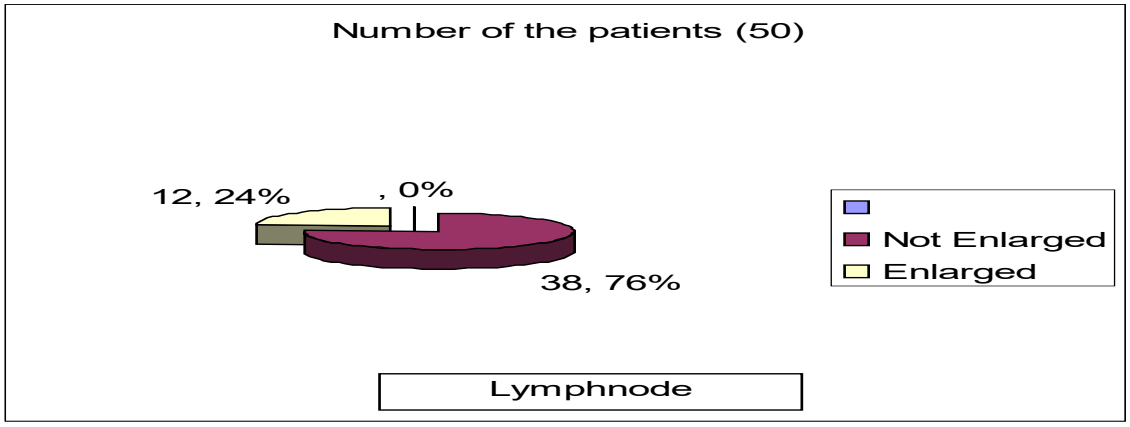
In figure 7 a retrospective study of 50 admitted cancer patients in KYAMC Cancer Center, Sirajgonj demonstrated that only 4% (n=2) and 8% (n=4) of the patients were suffered from severe and moderate jaundice which was not significant in relation with hepatic involvement of cisplatin toxicities.

Figure 12 : Distribution of cancer patient according to edematous condition



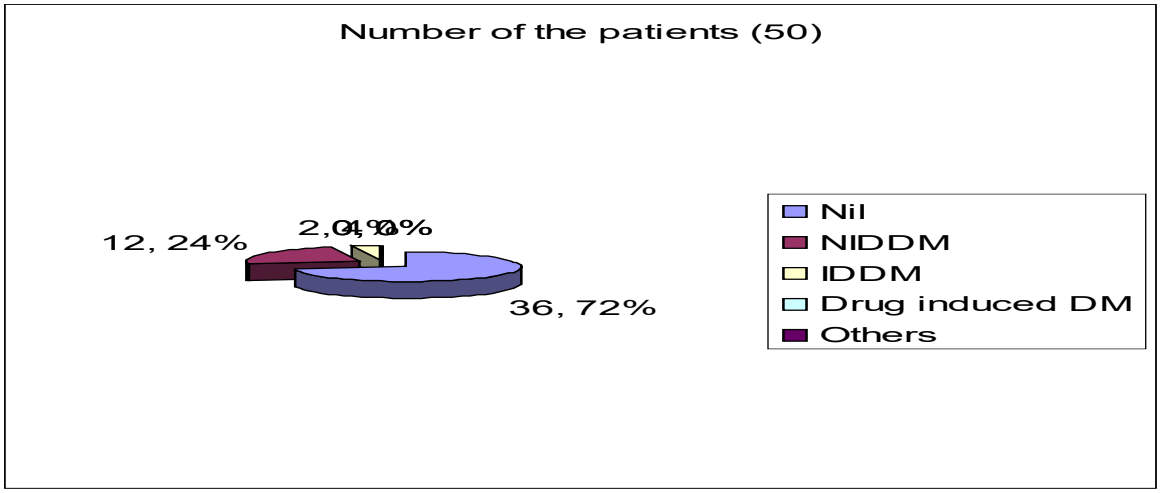
Only 8% (n=4) of the patients were suffered from mild edema that was reflected from a retrospective study of 50 admitted cancer patients in KYAMC Cancer Center, Sirajgonj, rest of the patients were non-edematous

Figure 13 : Distribution of cancer patient according to lymph node enlargement.



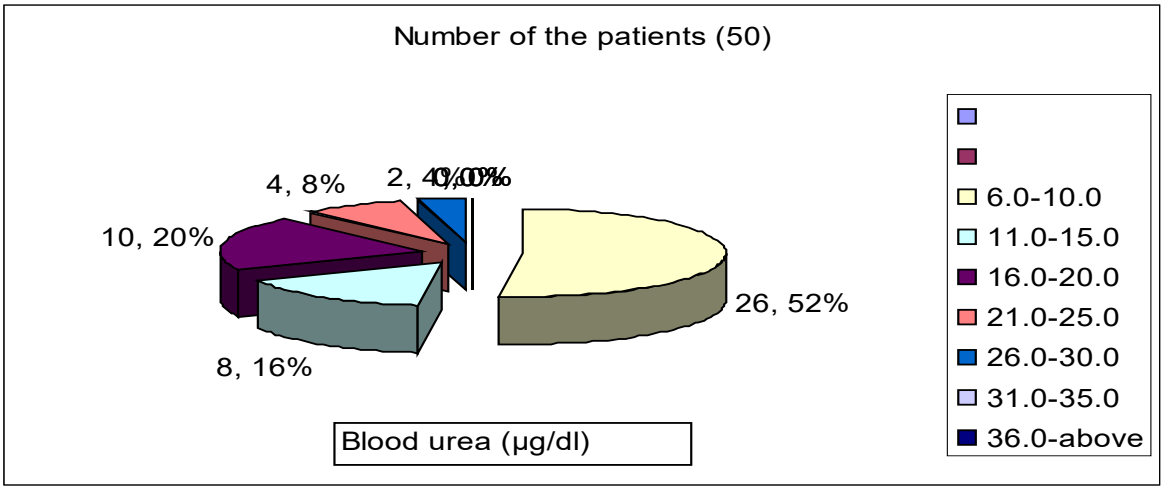
In figure 10 a retrospective study of 50 admitted cancer patients in KYAMC Cancer Center, Sirajgonj demonstrated that most of the cancer patients (76%, n=38) were freed from lymph node enlargement after cisplatin administration though 24% (n=12) remain unchanged.

Figure 14 : Distribution of cancer patient according to the state of glyceamic condition.



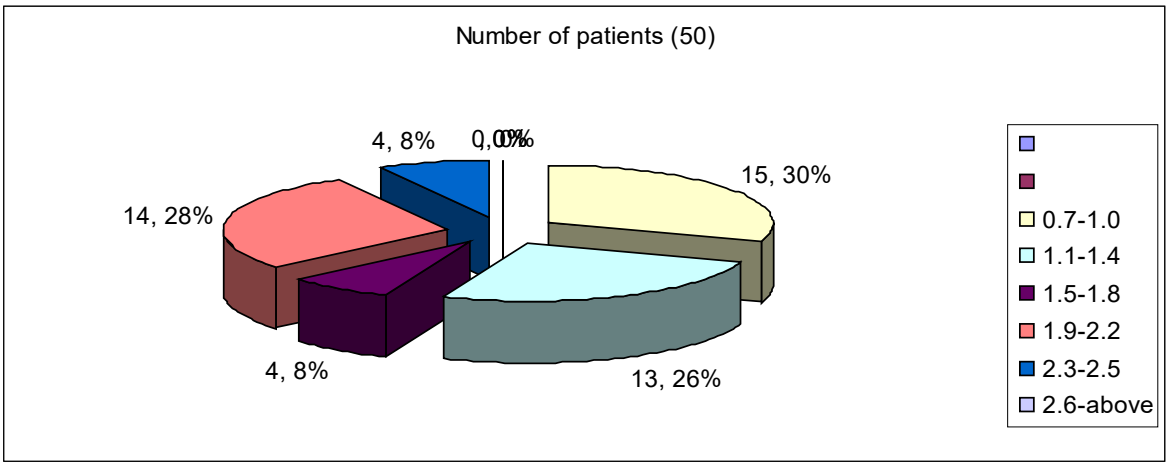
In figure 10 a retrospective study of 50 admitted cancer patients in KYAMC Cancer Center, Sirajgonj demonstrated that 4% (n=2) of the were suffered from IDDM, 24% (n=12) were NIDDM and 72% were free of DM.

Figure 15 : Distribution of cancer patient according to the state of uremia.



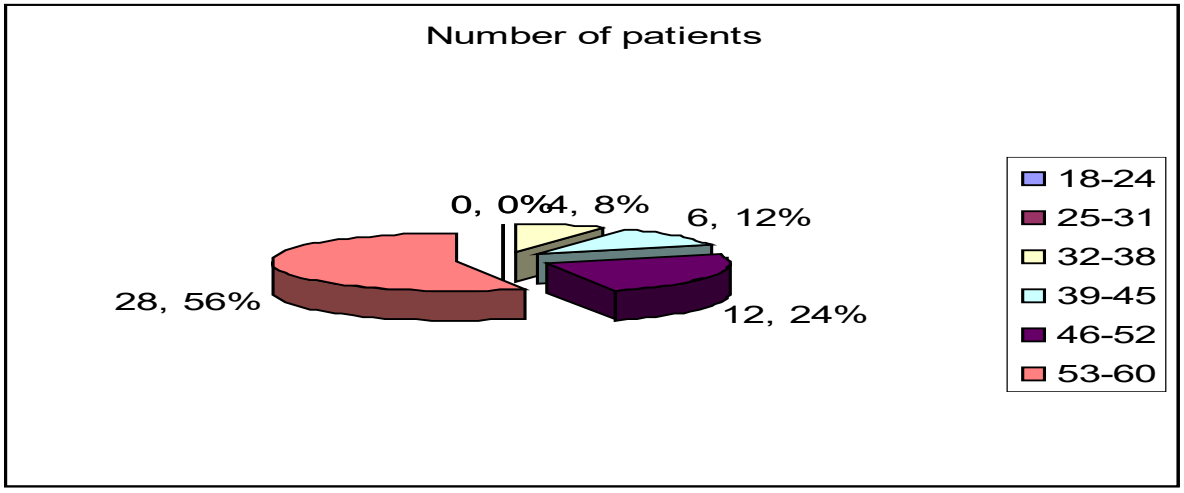
4% (n=2), 8% (n=4) and 20% (n=10) of total cancer patients were suffered from severe to moderate to mild hyperureamia respectively indicating strong involvement of renal system of cisplatin toxicity (figure 12)

Figure 16 : Distribution of cancer patient according to serum creatinine levels.



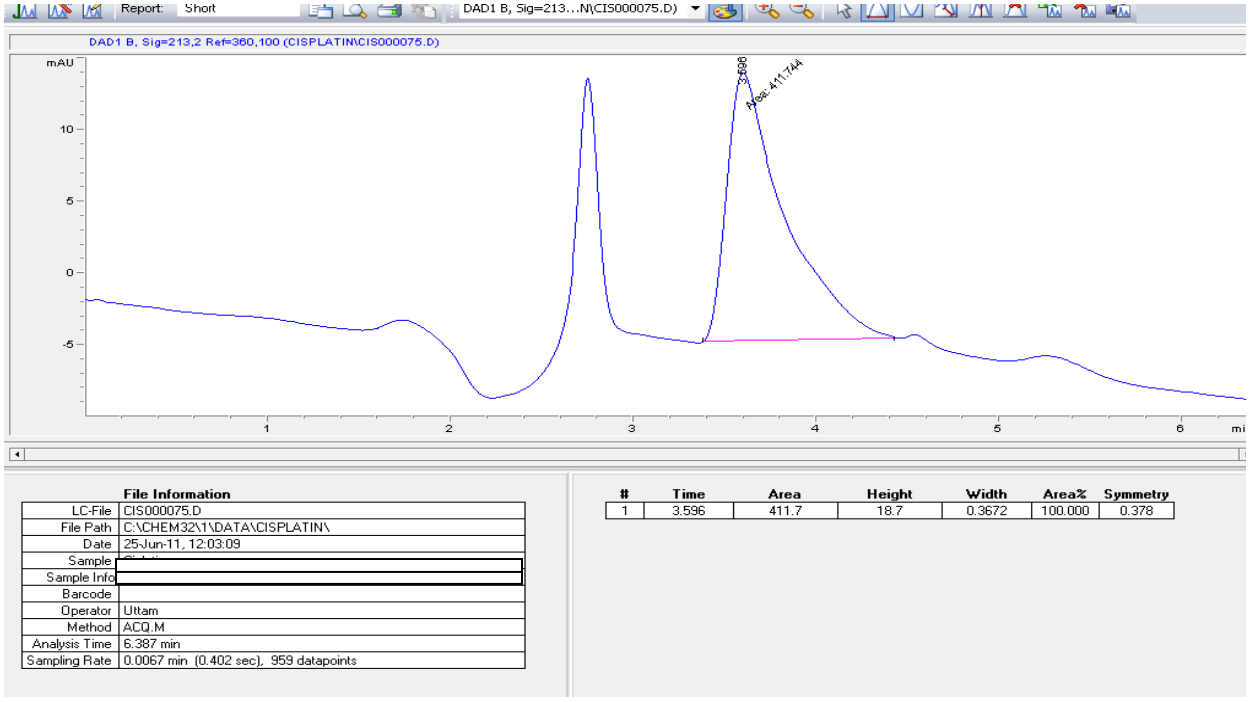
Serum creatinine level were increased (≥2.6 mg/dL) about 26% (n=13) of total cancer patients, where only 8% (n=4) of the patients suffered slight increase serum creatinine level (2.3-2.5 mg/dL) and the rest of patients were normal creatinine level.

Figure 17 : Distribution of cancer patients according to age group.



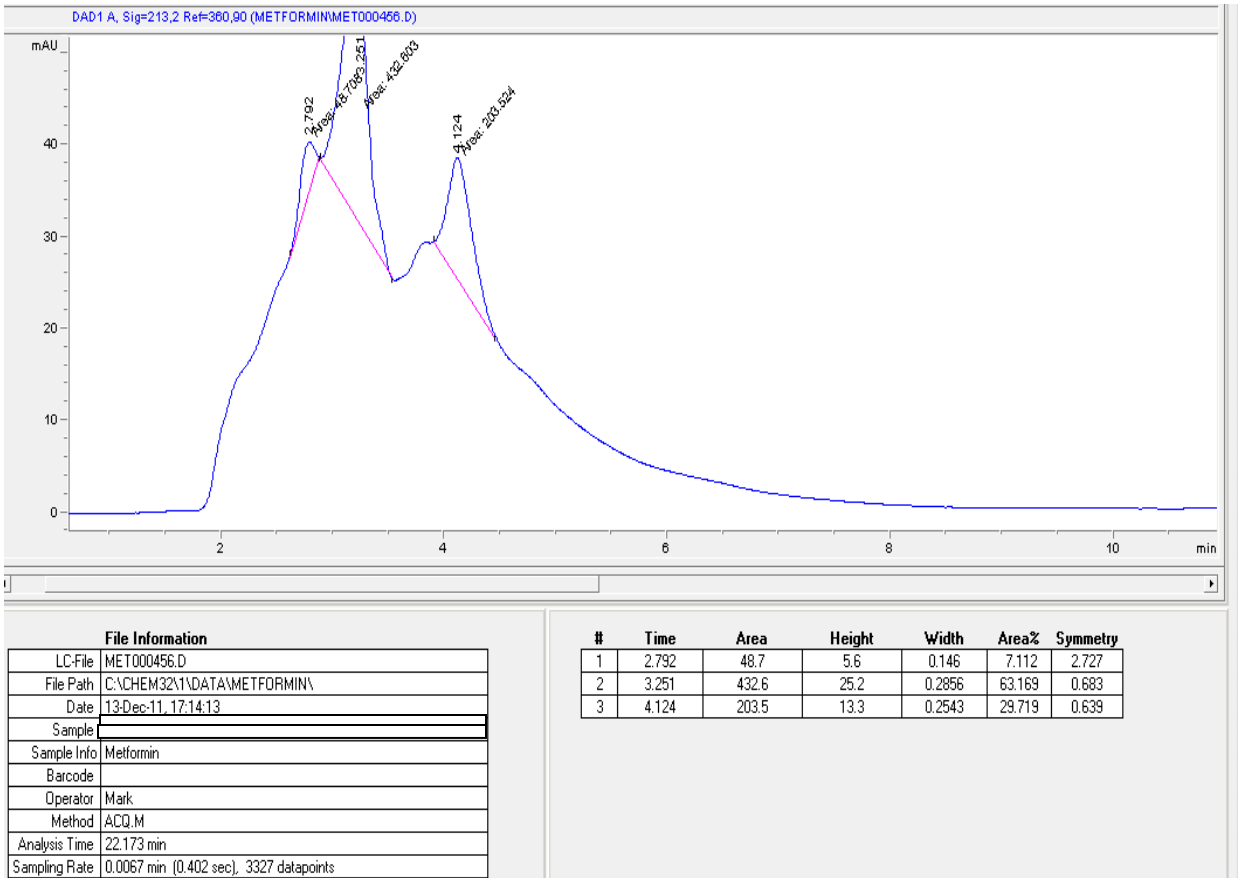
In figure 14, 56% (n=28) of cancer patients were in 53-60 years of age group, while second highest age group (46-52 years of age) suffering from cancer were 24% (n=12) on the other hand young adult cancer patients were found nil in this study.

Figure 18 : RT and Area of Cisplatin as standard solution and Nickel chloride as internal standard.



This is a simple, rapid and sensitive high performance liquid chromatographic (Solvent: Acetonitrile-water as the mobile phase) assay for cisplatin in human plasma ultrafiltrate and urine among Bangladeshi population. This is for the quantitative analysis of the drug in ultrafiltrate plasma and urine in the presence of nickel chloride as internal standard. Here nickel chloride will be used as internal standard. Here The flow rate was maintained constant at 0.5 ml/min and analysis was performed at 40 degrees C. Detection was carried out by absorbance at 213 nm. The mobile phase used for analysis consisted of 20% acetonitril (HPLC grade, E. Mark, Germany) and 80% distilled water. In figure 15, graphic presentation showed that the peak time (3.956 minutes) and area (411.7) of the Nickel chloride as internal standard and concentration were calculated as 2190.01 µg/ml.

Figure 19 : RT and are of cisplain and its metabolites in blood after 3 hour.

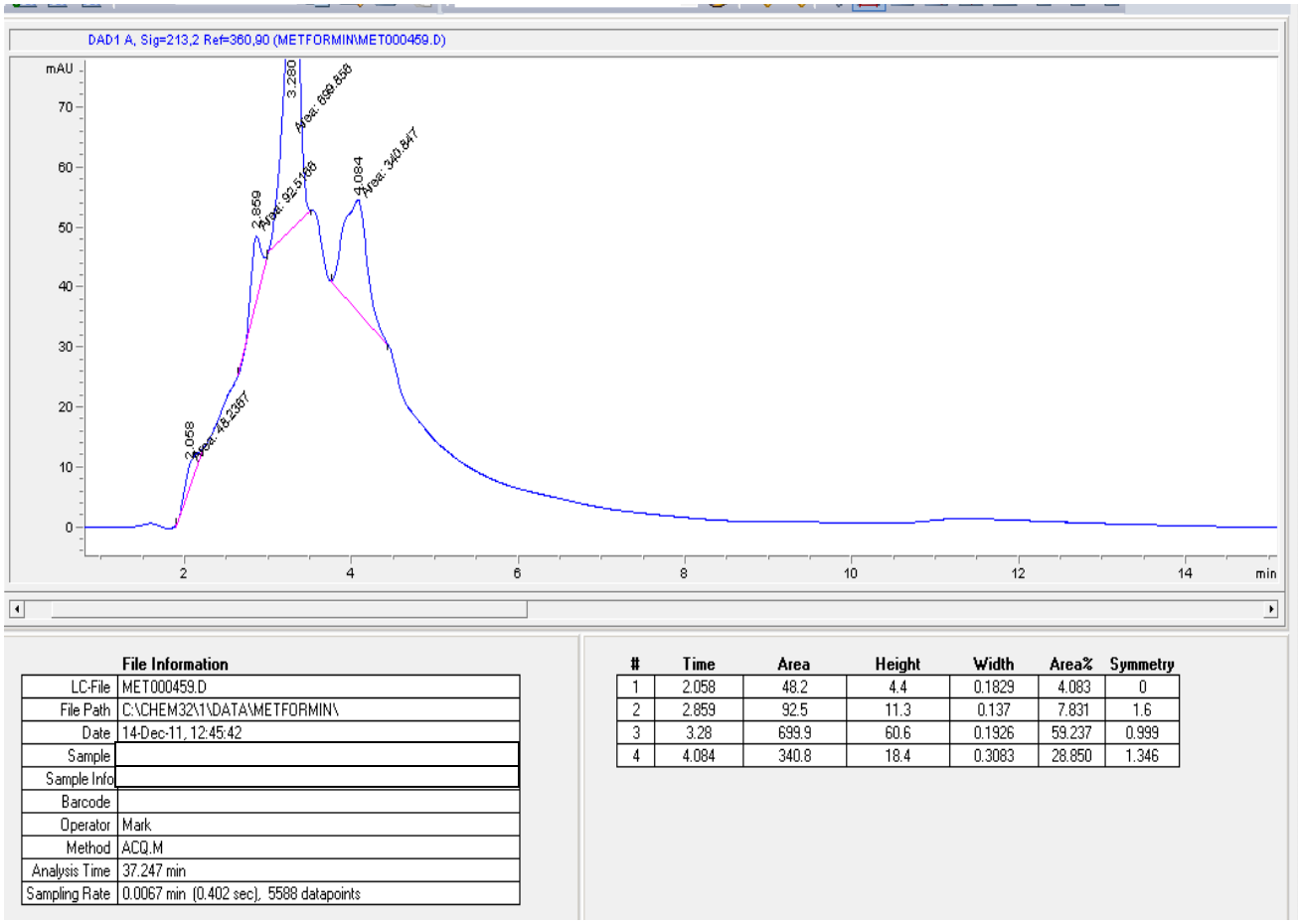


File Information	
LC-File	MET000456.D
File Path	C:\CHEM32\1\DATA\METFORMIN\
Date	13-Dec-11, 17:14:13
Sample	
Sample Info	Metformin
Barcode	
Operator	Mark
Method	ACQ.M
Analysis Time	22.173 min
Sampling Rate	0.0067 min (0.402 sec), 3327 datapoints

#	Time	Area	Height	Width	Area%	Symmetry
1	2.792	48.7	5.6	0.146	7.112	2.727
2	3.251	432.6	25.2	0.2856	63.169	0.683
3	4.124	203.5	13.3	0.2543	29.719	0.639

In figure 16, graphic presentation showed that the detection of cisplatin and its suspected metabolites by measuring the peak time and area by using HPLC after 3 hours of cisplatin administration in a cancer patient. Here peak time, area and concentration of cisplatin (C), metabolite 1 (CM2) and metabolite 2 (CM3) were 2.792 min., 48.7, 440.6 µg/ml, 3.25 min., 432.6, 135.46 µg/ml, 4.17 min., 203.5, 83.08 µg/ml respectively. Flow rate= 0.5 ml/min., Temp. = 40° C, solvent system=20% acetonitril: 80%MeOH, injected amount was 10 µl.

Figure 20 : RT and area of cisplain and its metabolites in blood after 6 hours

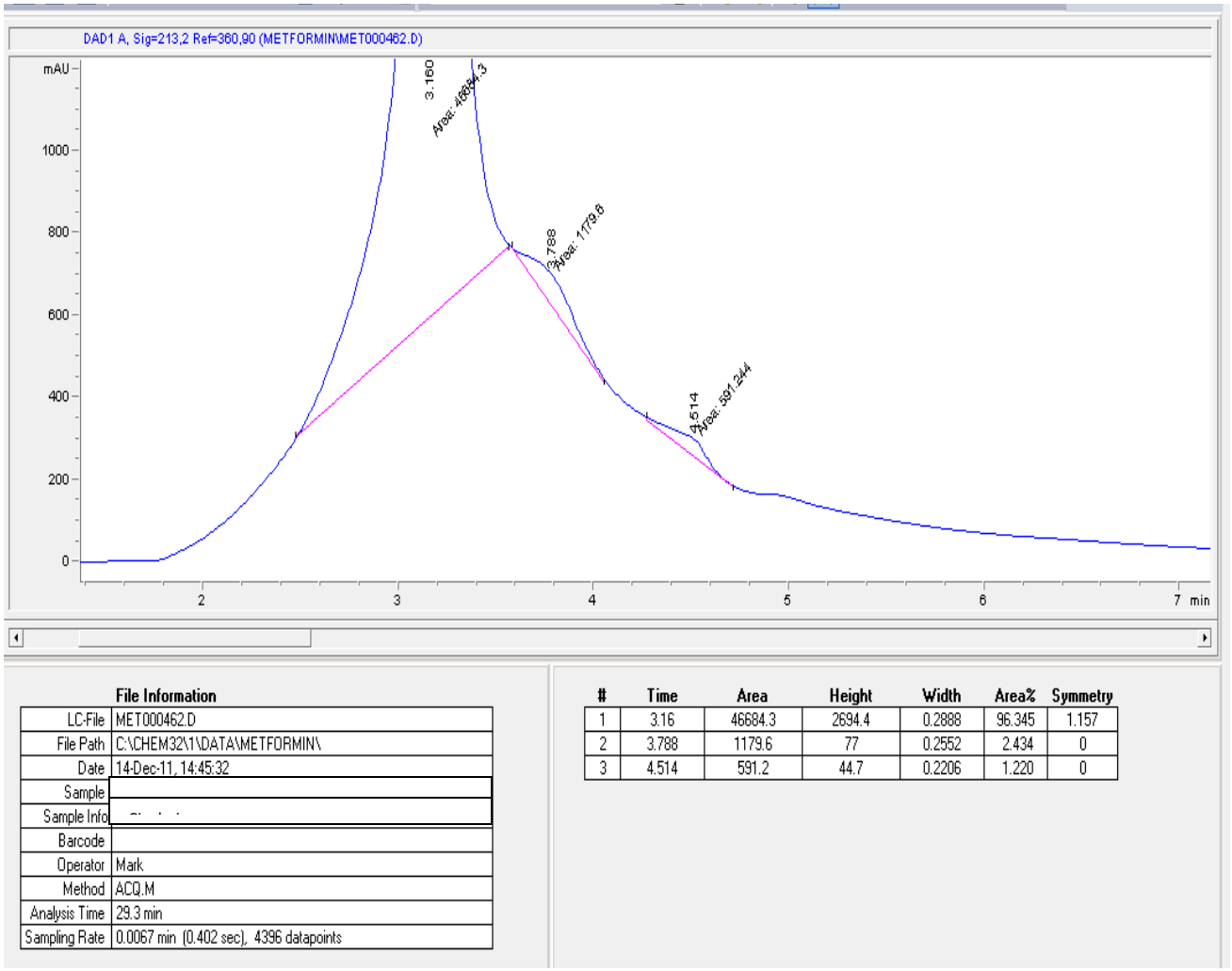


File Information	
LC-File	MET000459.D
File Path	C:\CHEM32\1\DATA\METFORMIN\
Date	14-Dec-11, 12:45:42
Sample	
Sample Info	
Barcode	
Operator	Mark
Method	ACQ.M
Analysis Time	37.247 min
Sampling Rate	0.0067 min (0.402 sec), 5588 datapoints

#	Time	Area	Height	Width	Area%	Symmetry
1	2.058	48.2	4.4	0.1829	4.083	0
2	2.859	92.5	11.3	0.137	7.831	1.6
3	3.28	699.9	60.6	0.1926	59.237	0.999
4	4.084	340.8	18.4	0.3083	28.850	1.346

In figure 17, graphic presentation showed that the detection of cisplatin and its suspected metabolites by measuring the peak time and area by using HPLC after 3 hours of cisplatin administration in a cancer patient. Here peak time, area and concentration of cisplatin ©, metabolite 1 (CM2) and metabolite 2 (CM3) were 2.659 min., 92.5, 339.10 µg/ml, 3.28 min., 699.9, 2324.91 µg/ml, 4.084 min., 340.8, 1210.06 µg/ml respectively. Flow rate= 0.5 ml/min., Temp. = 40° C, solvent system=20% acetonitril: 80%MeOH, injected amount was 10 µl.

Figure 21 : RT and area of cisplain and its metabolites in blood after 12 hour.

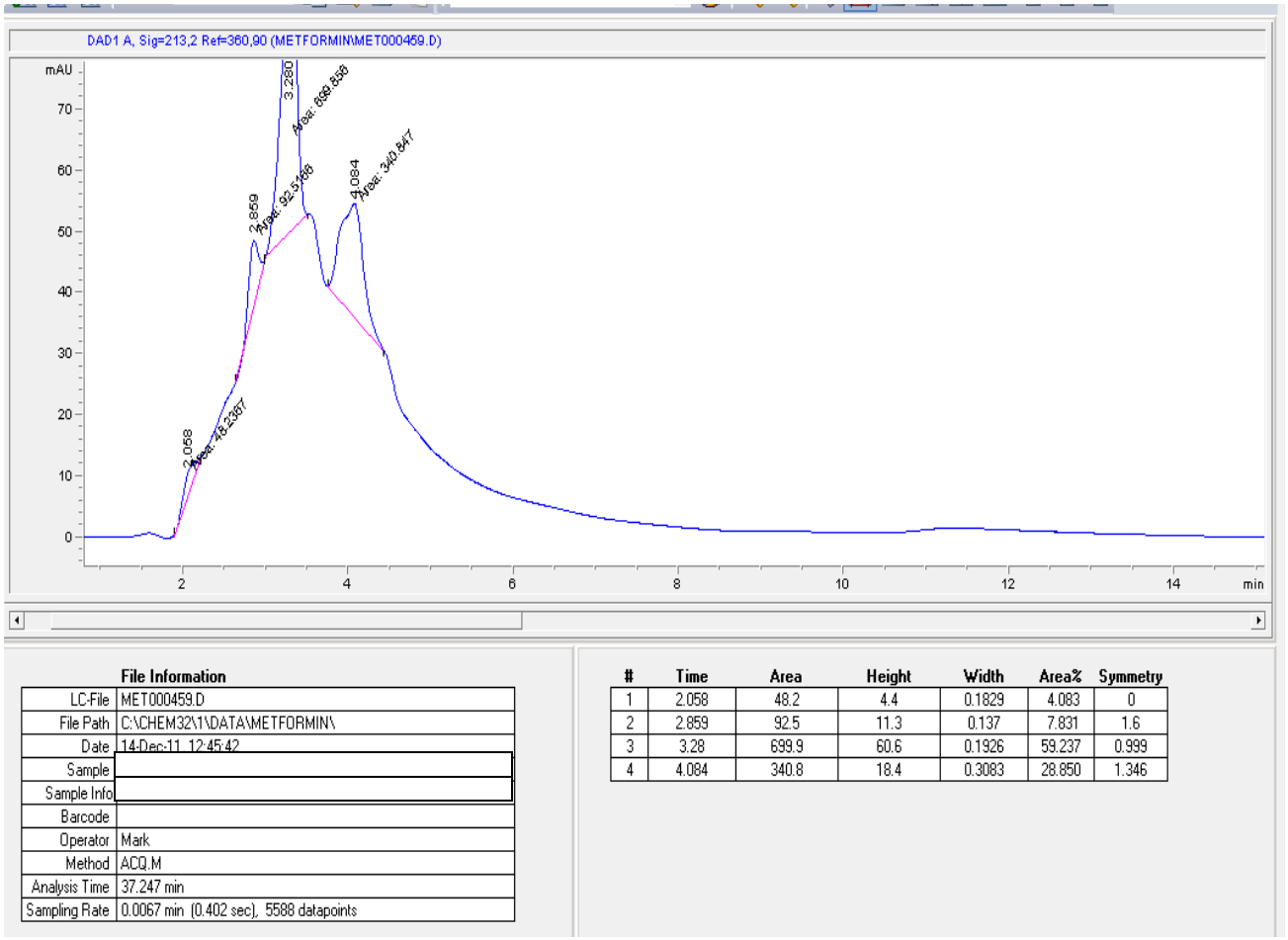


File Information	
LC-File	MET000462.D
File Path	C:\CHEM32\1\DATA\METFORMIN\
Date	14-Dec-11, 14:45:32
Sample	
Sample Info	
Barcode	
Operator	Mark
Method	ACQ.M
Analysis Time	29.3 min
Sampling Rate	0.0067 min (0.402 sec), 4396 datapoints

#	Time	Area	Height	Width	Area%	Symmetry
1	3.16	46684.3	2694.4	0.2888	96.345	1.157
2	3.788	1179.6	77	0.2552	2.434	0
3	4.514	591.2	44.7	0.2206	1.220	0

In figure 18, graphic presentation showed that the detection of cisplatin and its suspected metabolites in plasma by measuring the peak time and area by using HPLC after 3 hours of cisplatin administration in a cancer patient. Here peak time, area and concentration of metabolite 1 (CM2), metabolite 2 (CM3) and metabolite 3 (CM4) were 3.16 min., 46484.3, 723.40 µg/ml, 3.788 min., 1179.6, 165.19 µg/ml, 4.514 min., 591.2, 83.08 µg/ml respectively. Flow rate= 0.5 ml/min., Temp. = 40° C, solvent system=20% acetonitril: 80%MeOH, injected amount was 10 µl.

Figure 22 : RT and area of cisplain and its metabolites in blood after 24 hours.

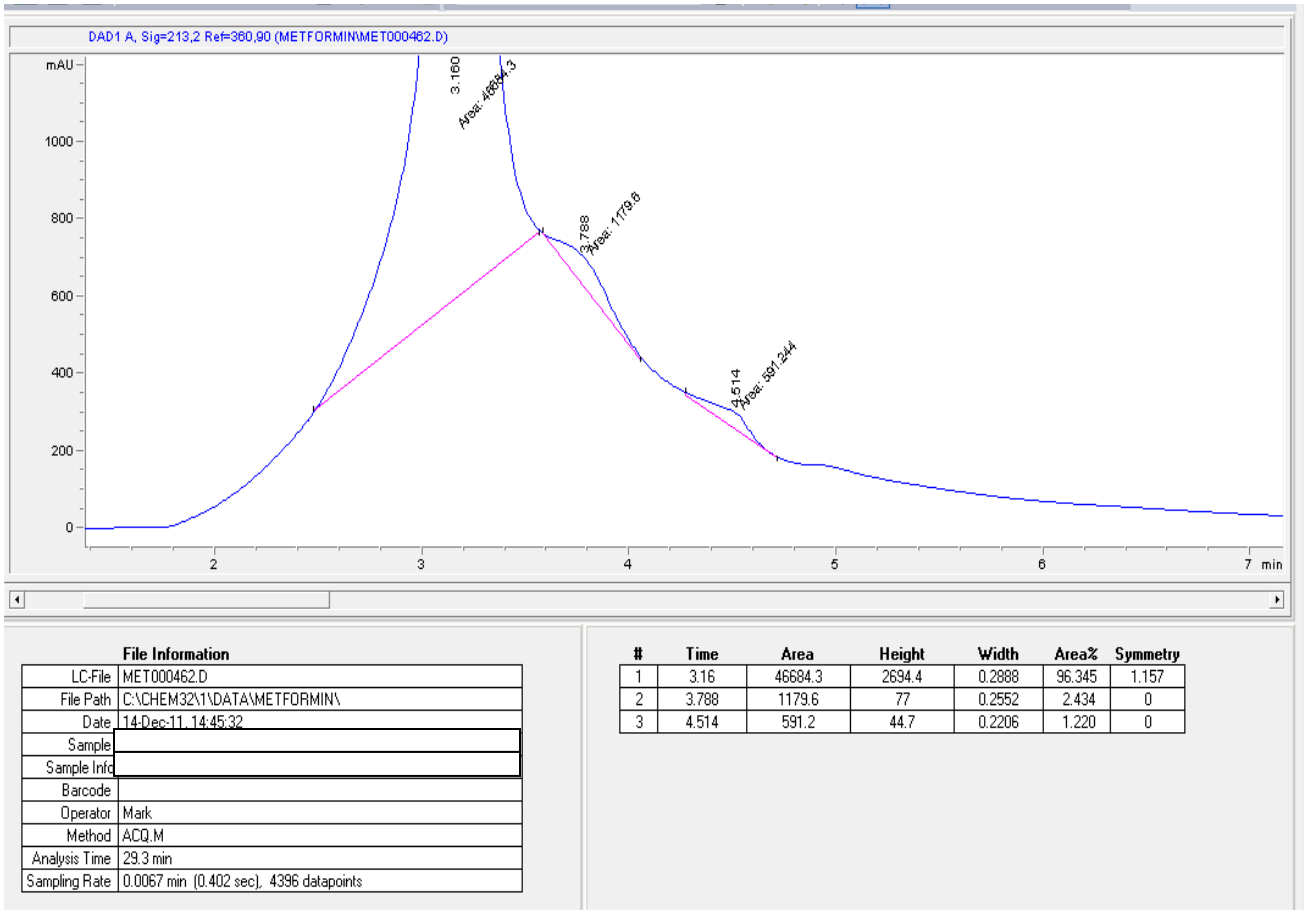


File Information	
LC-File	MET000459.D
File Path	C:\CHEM32\1\DATA\METFORMIN\
Date	14-Dec-11, 12:45:42
Sample	
Sample Info	
Barcode	
Operator	Mark
Method	ACQ.M
Analysis Time	37.247 min
Sampling Rate	0.0067 min (0.402 sec), 5588 datapoints

#	Time	Area	Height	Width	Area%	Symmetry
1	2.058	48.2	4.4	0.1829	4.083	0
2	2.859	92.5	11.3	0.137	7.831	1.6
3	3.28	699.9	60.6	0.1926	59.237	0.999
4	4.084	340.8	18.4	0.3083	28.850	1.346

In figure 19, graphic presentation showed that the detection of cisplatin and its suspected metabolites by measuring the peak time and area by using HPLC after 3 hours of cisplatin administration in a cancer patient. Here peak time, area and concentration of cisplatin (C), metabolite 1 (CM2) and metabolite 2 (CM3) were 2.859 min., 48.2, 44.16µg/ml, 3.28 min., 699.9, 2681.61 µg/ml, 4.084 min., 340.8, 80.22 µg/ml respectively. Flow rate= 0.5 ml/min., Temp. = 40° C, solvent system=20% acetonitril: 80%MeOH, injected amount was 10 µl.

Figure 23 : RT and area of cisplain and its metabolites in urine after 3 hours.

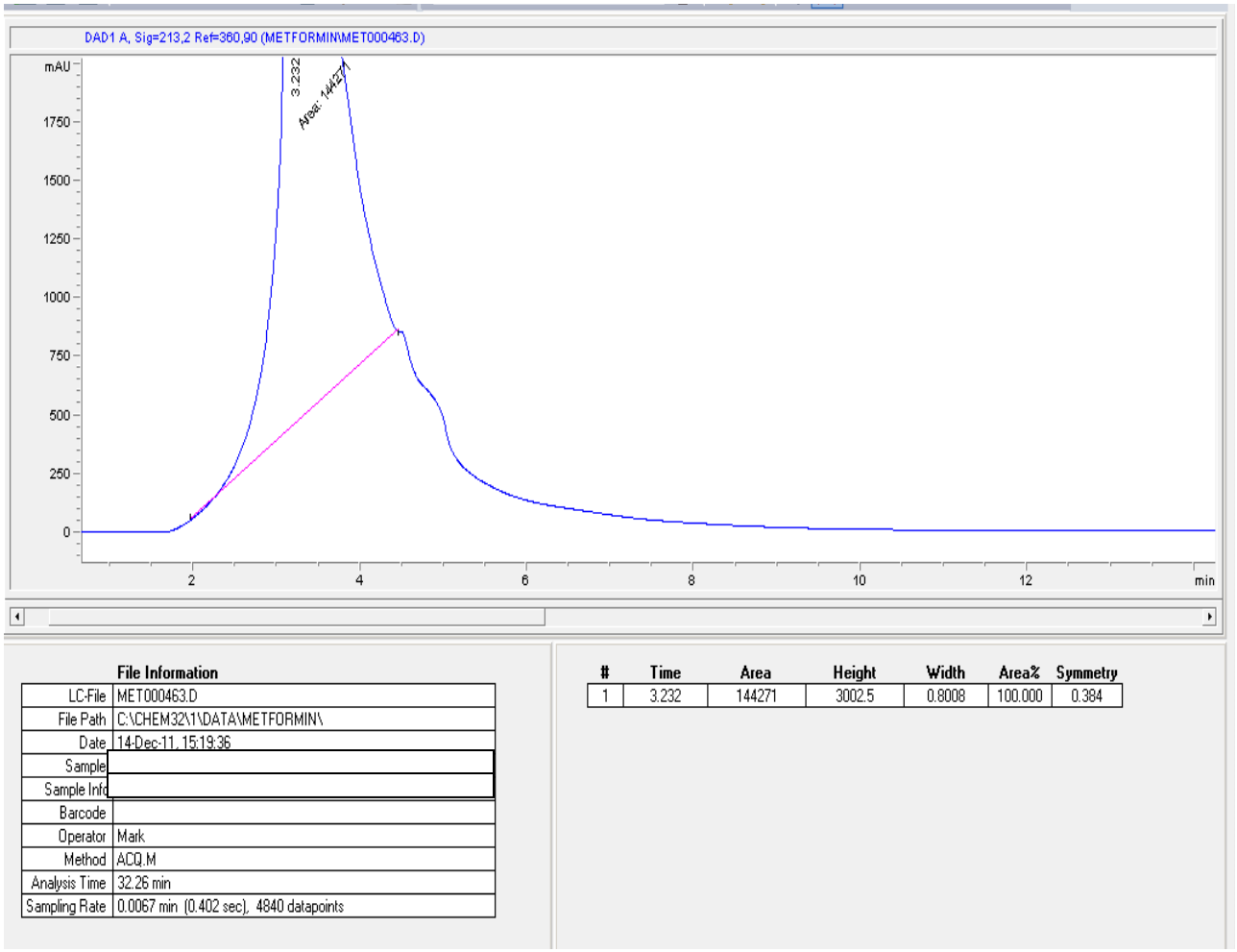


File Information	
LC-File	MET000462.D
File Path	C:\CHEM32\1\DATA\METFORMIN\
Date	14-Dec-11, 14:45:32
Sample	
Sample Info	
Barcode	
Operator	Mark
Method	ACQ.M
Analysis Time	29.3 min
Sampling Rate	0.0067 min (0.402 sec), 4396 datapoints

#	Time	Area	Height	Width	Area%	Symmetry
1	3.16	46684.3	2694.4	0.2888	96.345	1.157
2	3.788	1179.6	77	0.2552	2.434	0
3	4.514	591.2	44.7	0.2206	1.220	0

In figure 20, graphic presentation showed that the detection of cisplatin and its suspected metabolites by measuring the peak time and area by using HPLC after 3 hours of cisplatin administration in a cancer patient. Here peak time, area and concentration of cisplatin ©, metabolite 2 (CM3) and metabolite 3 (CM4) were 3.16 min., 46684.3, 723.4 µg/ml, 3.788 min., 1179.6, 167.34 µg/ml, 4.514 min., 591.2, 73.89 µg/ml respectively. Flow rate= 0.5 ml/min., Temp. = 40° C, solvent system=20% acetonitril: 80%MeOH, injected amount was 10 µl.

Figure 24 : RT and area of cisplain and its metabolites in urine after 6 hours.

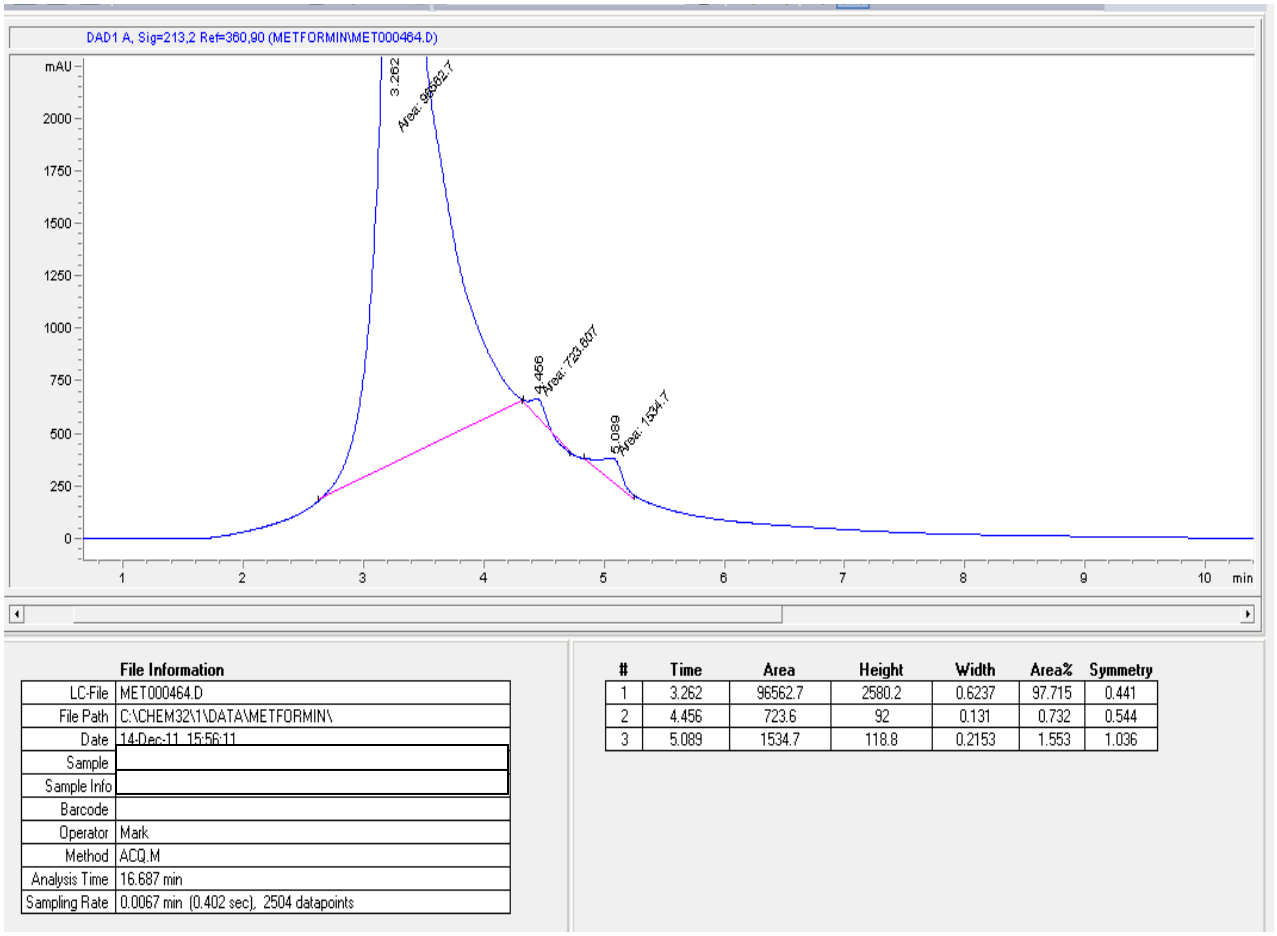


File Information	
LC-File	MET000463.D
File Path	C:\CHEM32\1\DATA\METFORMIN\
Date	14-Dec-11, 15:19:36
Sample	
Sample Info	
Barcode	
Operator	Mark
Method	ACQ.M
Analysis Time	32.26 min
Sampling Rate	0.0067 min (0.402 sec), 4840 datapoints

#	Time	Area	Height	Width	Area%	Symmetry
1	3.232	144271	3002.5	0.8008	100.000	0.384

In figure 21, graphic presentation showed that the detection of cisplatin and its suspected metabolites by measuring the peak time and area by using HPLC after 3 hours of cisplatin administration in a cancer patient. Here peak time, area and concentration of metabolite 1 (CM2) 3.232 min., 144271, 1363 µg/ml. Flow rate= 0.5 ml/min., Temp. = 40° C, solvent system=20% acetonitril: 80%MeOH, injected amount was 10 µl. WL=213

Figure 25 : RT and area of cisplain and its metabolites in urine after 12 hours.

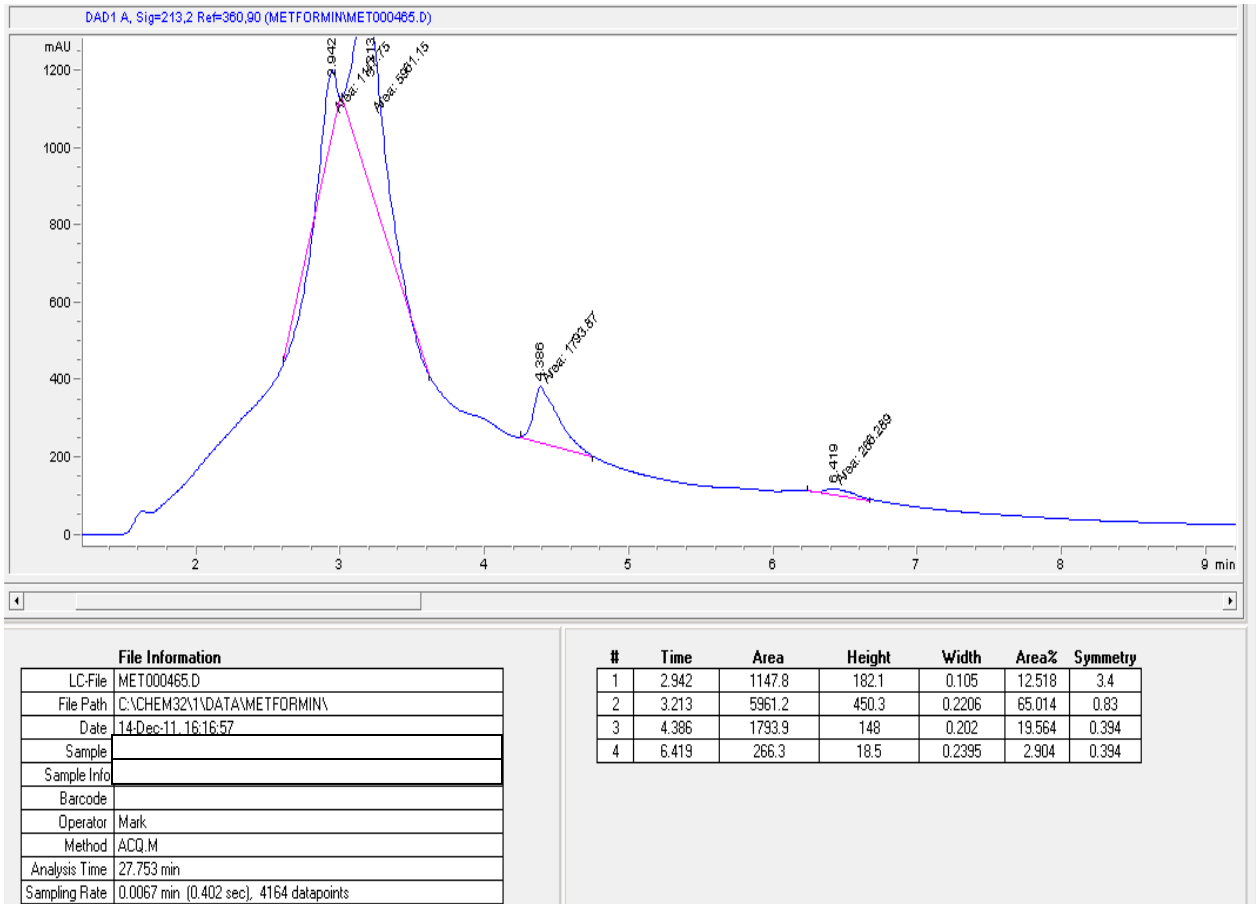


File Information	
LC-File	MET000464.D
File Path	C:\CHEM32\1\DATA\METFORMIN\
Date	14-Dec-11, 15:56:11
Sample	
Sample Info	
Barcode	
Operator	Mark
Method	ACQ.M
Analysis Time	16.687 min
Sampling Rate	0.0067 min (0.402 sec), 2504 datapoints

#	Time	Area	Height	Width	Area%	Symmetry
1	3.262	96562.7	2580.2	0.6237	97.715	0.441
2	4.456	723.6	92	0.131	0.732	0.544
3	5.089	1534.7	118.8	0.2153	1.553	1.036

In figure 22, graphic presentation showed that the detection of cisplatin and its suspected metabolites by measuring the peak time and area by using HPLC after 3 hours of cisplatin administration in a cancer patient. Here peak time, area and concentration of metabolite 1 (CM2), metabolite 2 (CM3) and metabolite 3 (CM4) were 3.262 min., 9656.2, 756.03 µg/ml, 4.456 min., 723.6, 106.66 µg/ml, 5.089 min., 1534.7, 235.78 µg/ml respectively. Flow rate= 0.5 ml/min., Temp. = 40° C, solvent system=20% acetonitril: 80%MeOH, injected amount was 10 µl, WL= 213.

Figure 26 : RT and area of cisplain and its metabolites in urine after 24 hours

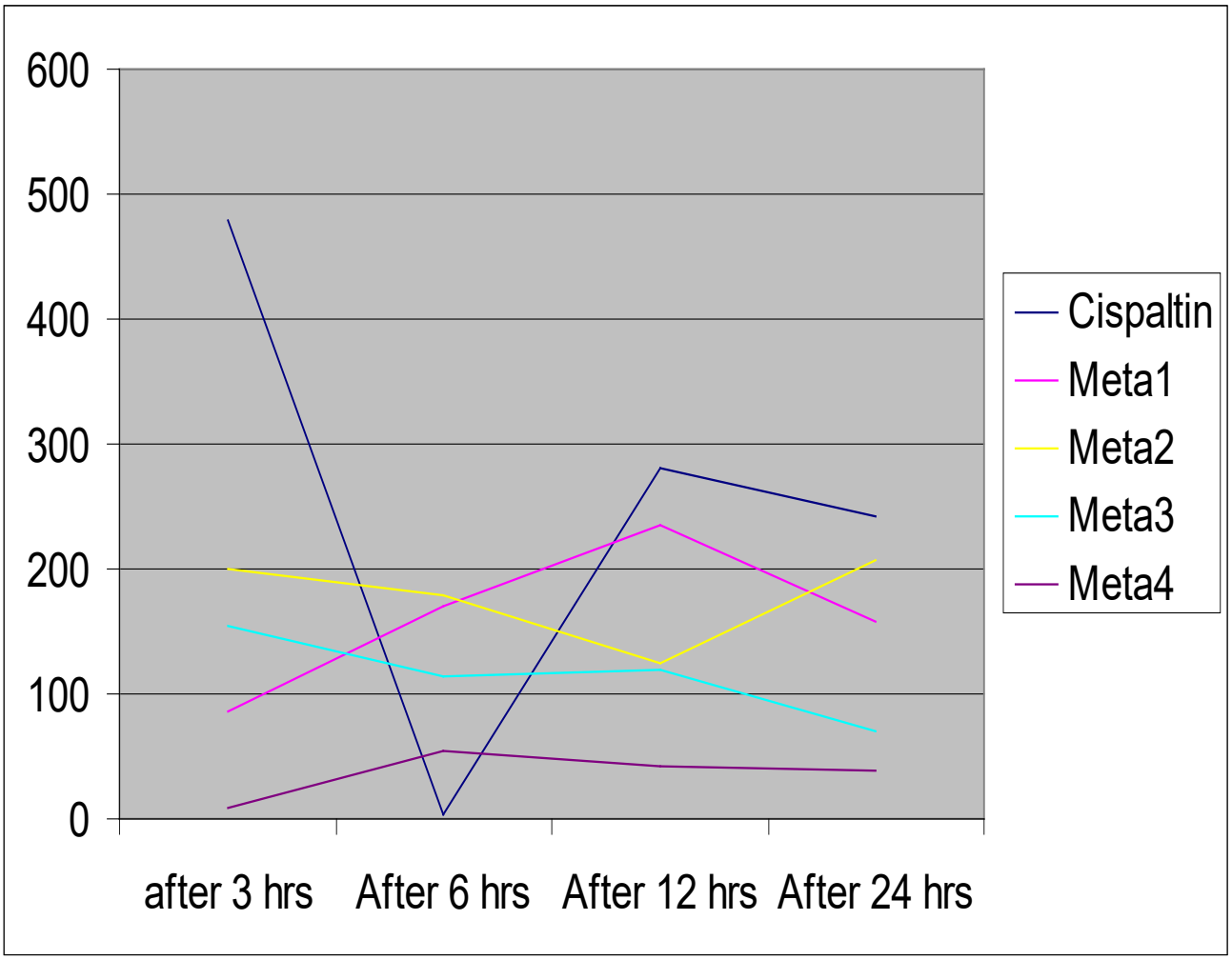


File Information	
LC-File	MET000465.D
File Path	C:\CHEM32\1\DATA\METFORMIN\
Date	14-Dec-11, 16:16:57
Sample	
Sample Info	
Barcode	
Operator	Mark
Method	ACQ.M
Analysis Time	27.753 min
Sampling Rate	0.0067 min (0.402 sec), 4164 datapoints

#	Time	Area	Height	Width	Area%	Symmetry
1	2.942	1147.8	182.1	0.105	12.518	3.4
2	3.213	5961.2	450.3	0.2206	65.014	0.83
3	4.386	1793.9	148	0.202	19.564	0.394
4	6.419	266.3	18.5	0.2395	2.904	0.394

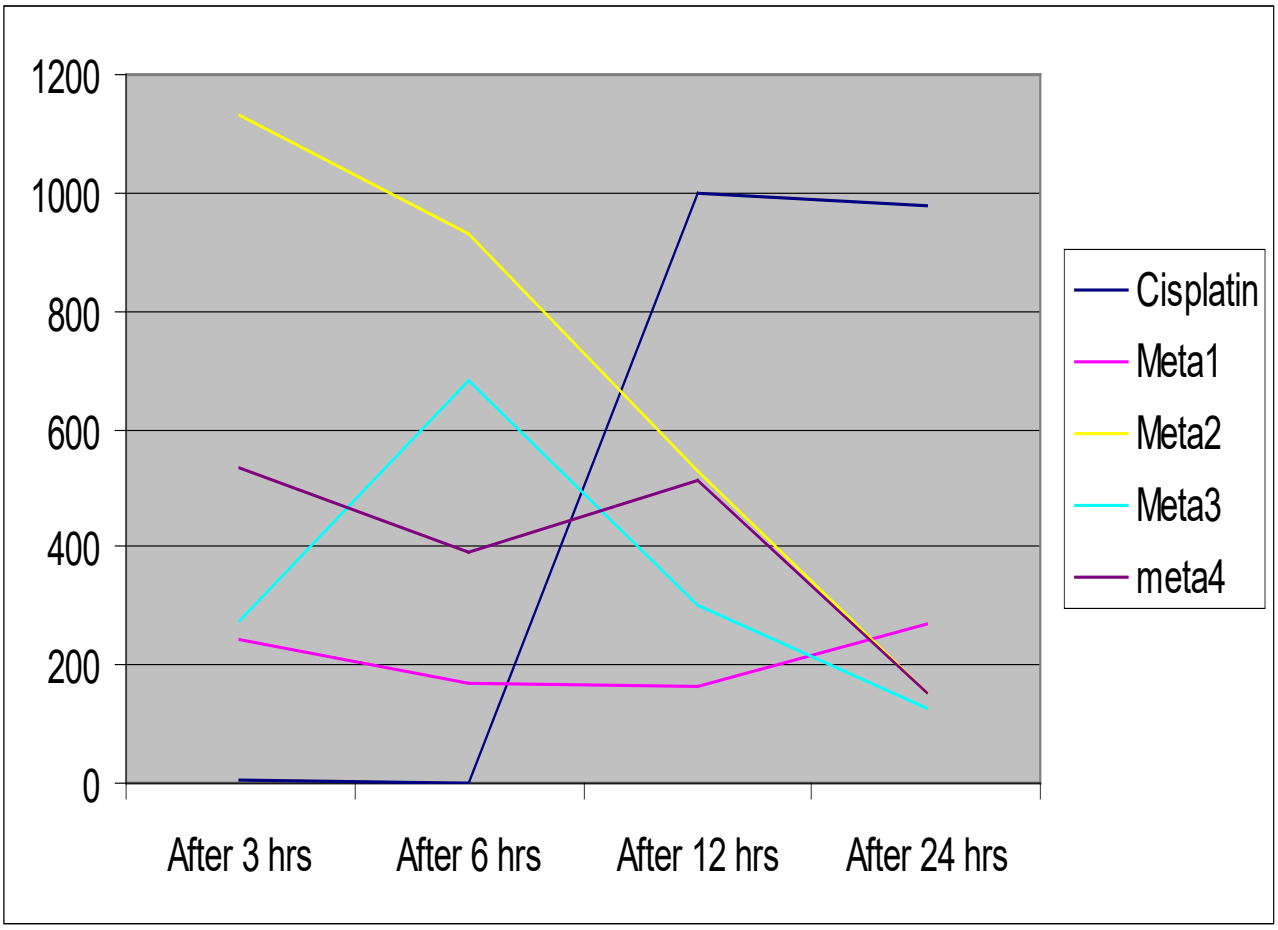
In figure 22, graphic presentation showed that the detection of cisplatin and its suspected metabolites by measuring the peak time and area by using HPLC after 3 hours of cisplatin administration in a cancer patient. Here peak time, area and concentration of cisplatin ©, metabolite 1 (CM2) and metabolite 2 (CM3) were 2.942 min., 1147.8, 437.12 µg/ml, 3.213 min., 5961.2, 964.53 µg/ml, 4.386 min., 1793.9, 644.76 µg/ml respectively. Flow rate= 0.5 ml/min., Temp. = 40° C, solvent system=20% acetonitril: 80%MeOH, injected amount was 10 µl, WL=213.

Graph 1 : Mean Plasma concentration of cisplatin & its metabolites in blood sample of 50 patients



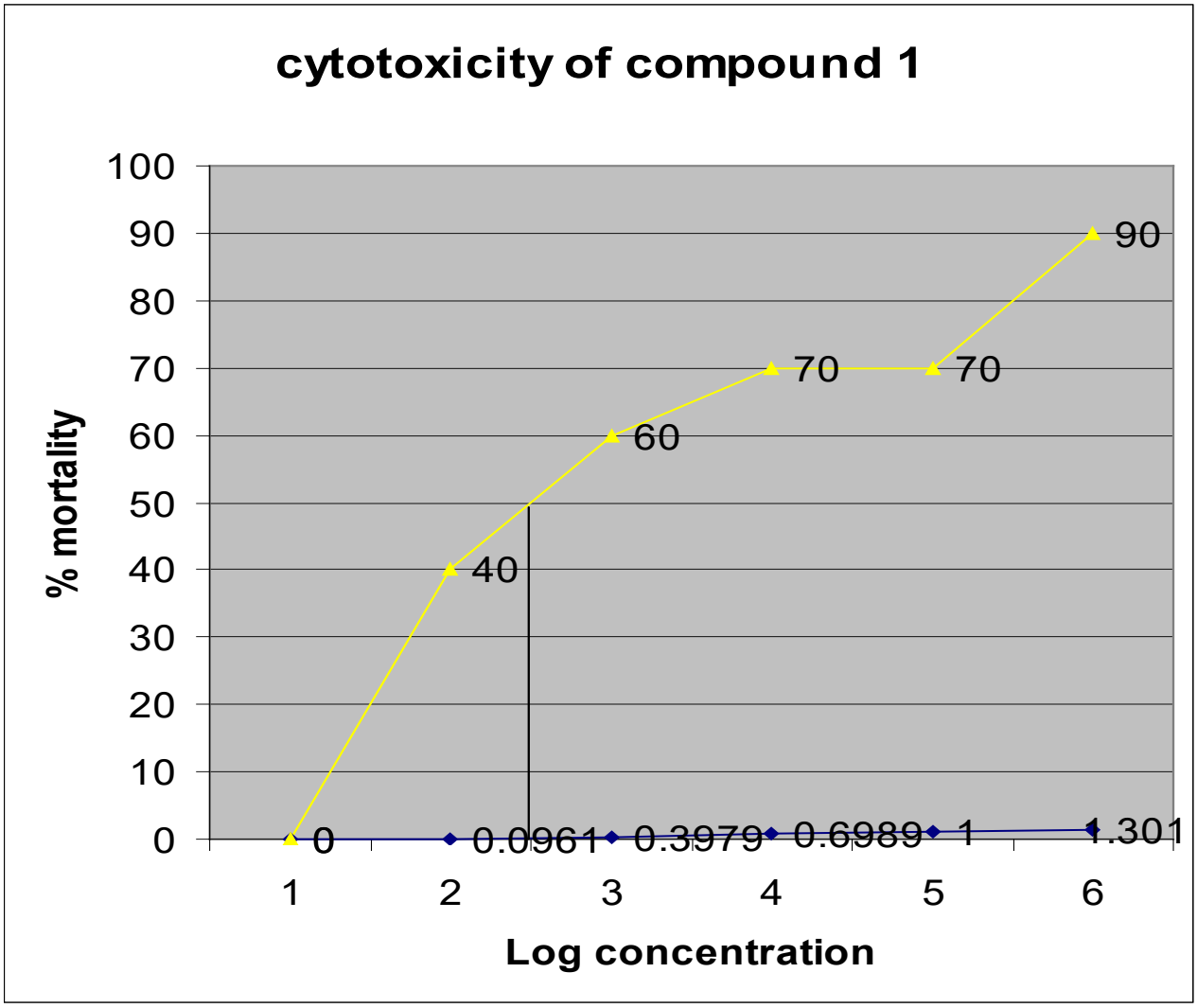
In graph 1, graph showed that the mean±SD plasma concentration of cisplatin © and its suspected metabolites M1 to M4 later named CM2, CM3, CM4 and CM5 at different time intervals (at 3, 6, 12 and 24 hour) of 50 cancer patients.

Graph 2 : Mean urinay concentration of cisplatin & its metabolites in urine sample of 50 patients.



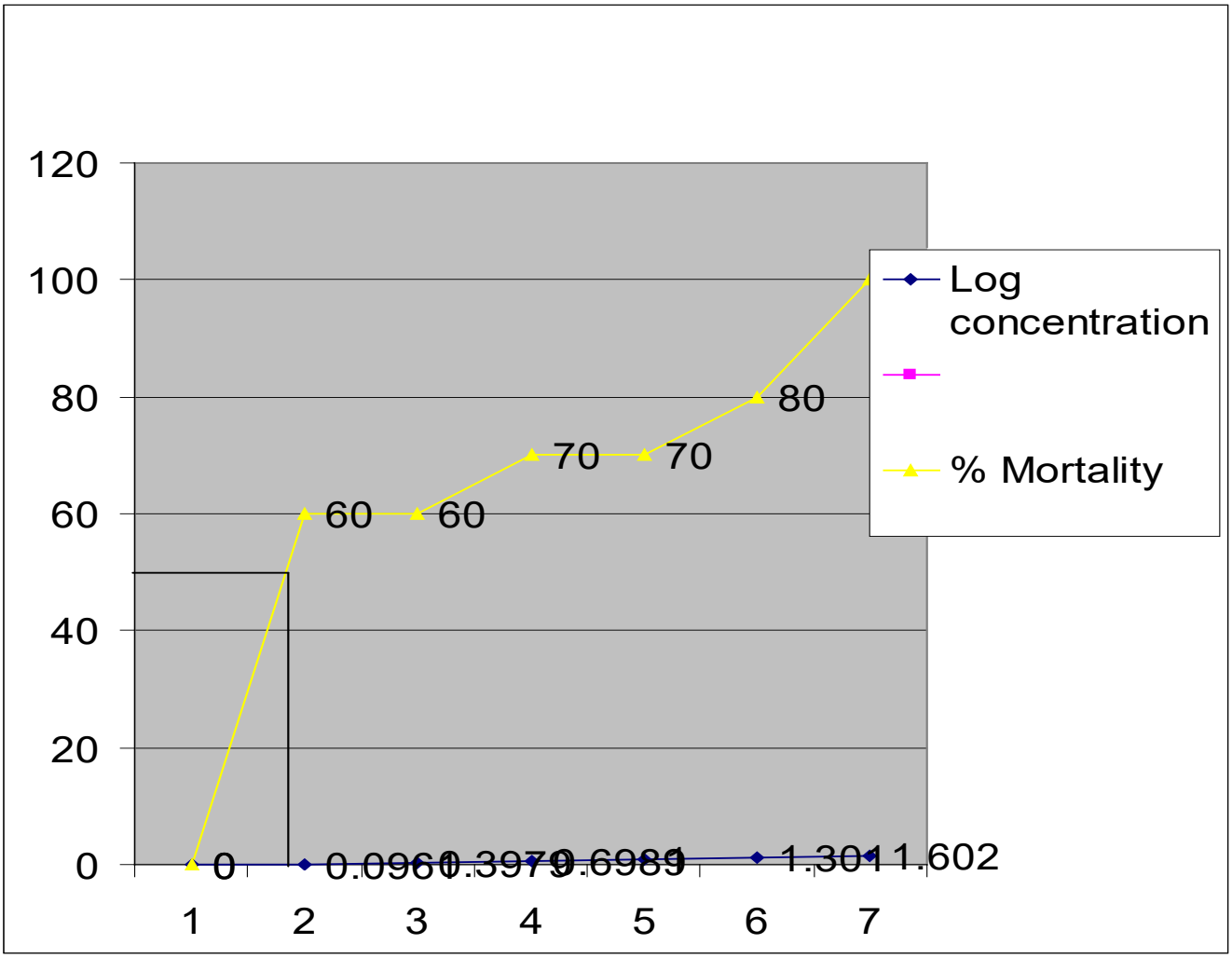
In graph 2, graph showed that the mean±SD urine concentration of cisplatin © and its suspected metabolites M1 to M4 named CM2, CM3, CM4 and CM5 at different time intervals (at 3,6,12 and 24 hour) of 50 cancer patients.

Graph 3 : Cytotoxicity test of compound 1 ©.



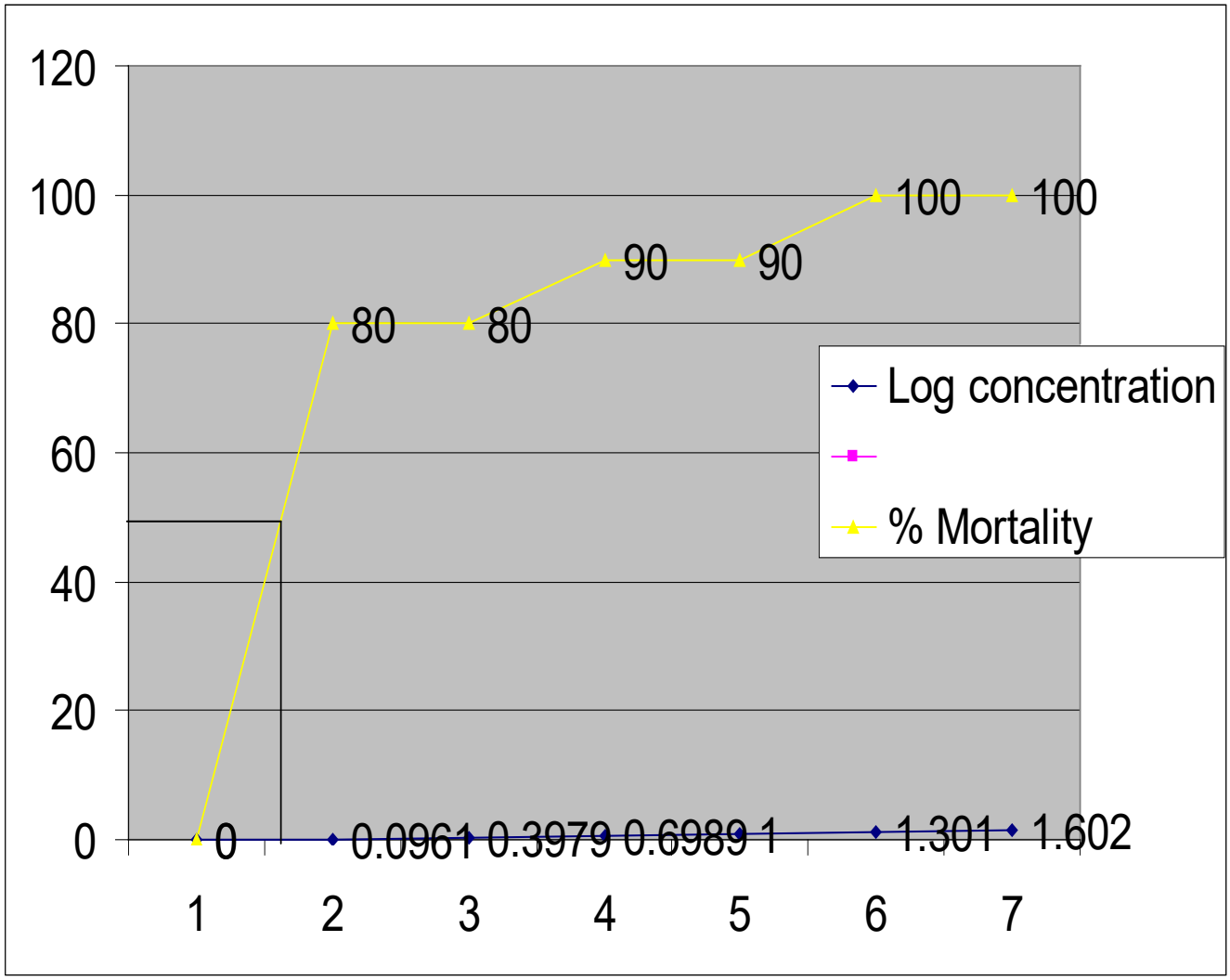
Cytotoxicity test of cisplatin was done by using brine shrimp lethality bioassay by plotting the different log concentration of cisplatin (0.0961, 0.3979, 0.6989, 1, 1.3010) against % mortality (0 to 100%) and LC_{50} of cisplatin was calculated 1.8µg/ml (table 3)

Graph 4 : Cytotoxicity test of CM2.



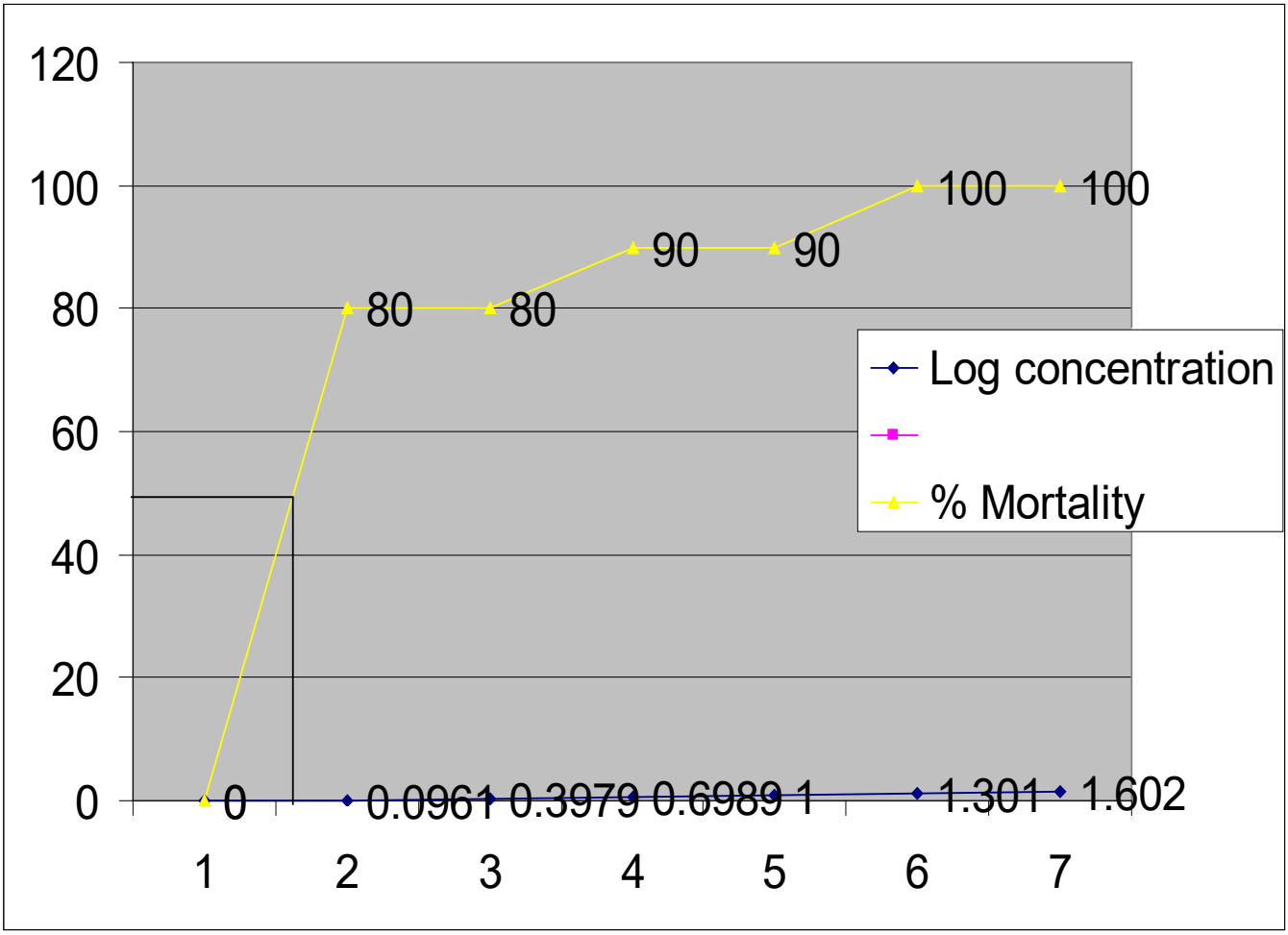
Cytotoxicity test of Metabolite 1 (CM2) was done by using brine shrimp lethality bioassay by plotting the different log concentration of cisplatin (0.0961, 0.3979, 0.6989, 1, 1.3010) against % mortality (0 to 100%) and LC_{50} of cisplatin was calculated 1.2 µg/ml (table 4)

Graph 5 : Cytotoxicity test of CM3



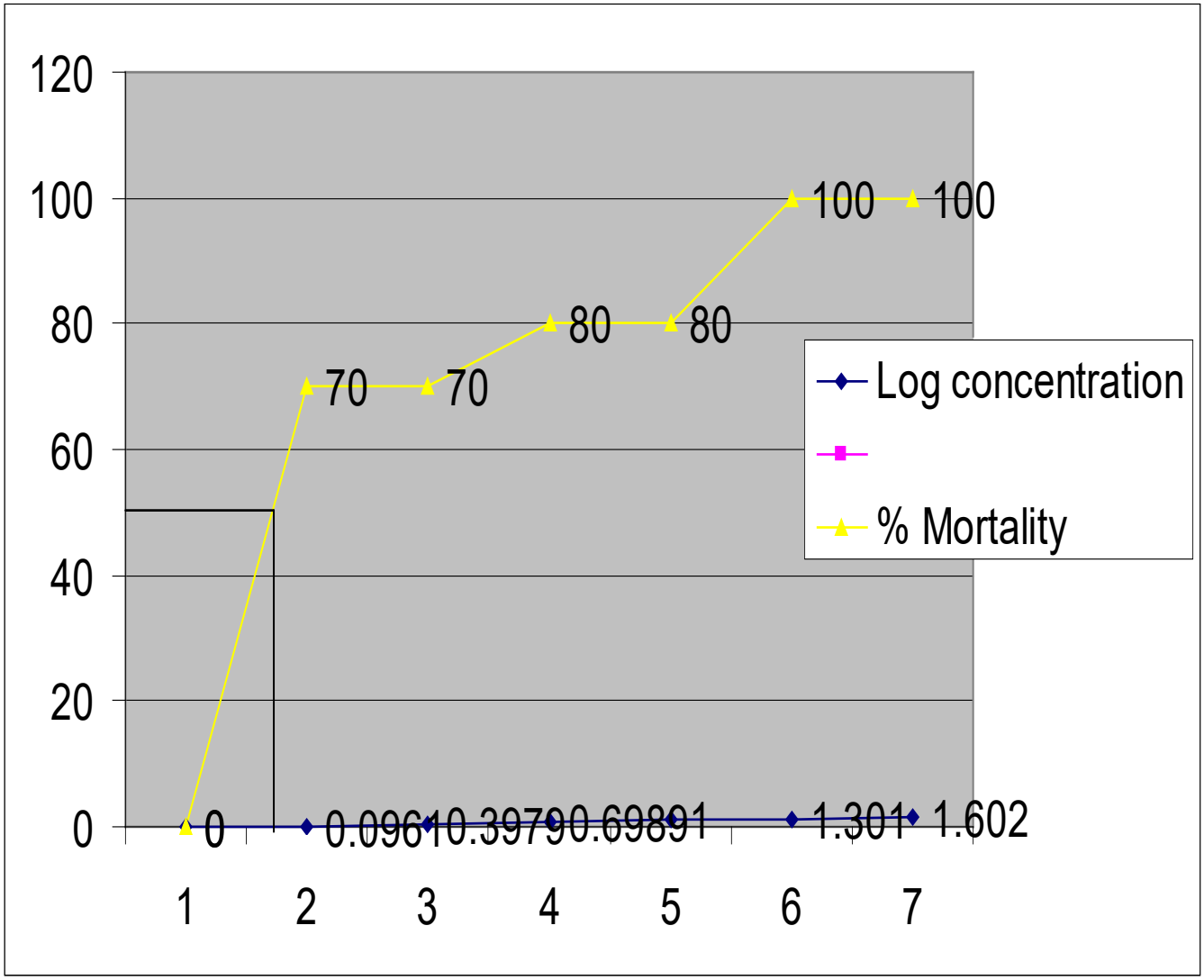
Cytotoxicity test of Metabolite 2 (CM3) was done by using brine shrimp lethality bioassay by plotting the different log concentration of cisplatin (0.0961, 0.3979, 0.6989, 1, 1.3010) against % mortality (0 to 100%) and LC_{50} of cisplatin was calculated 1.08 (table 5).

Graph 6: Cytotoxicity test of CM4



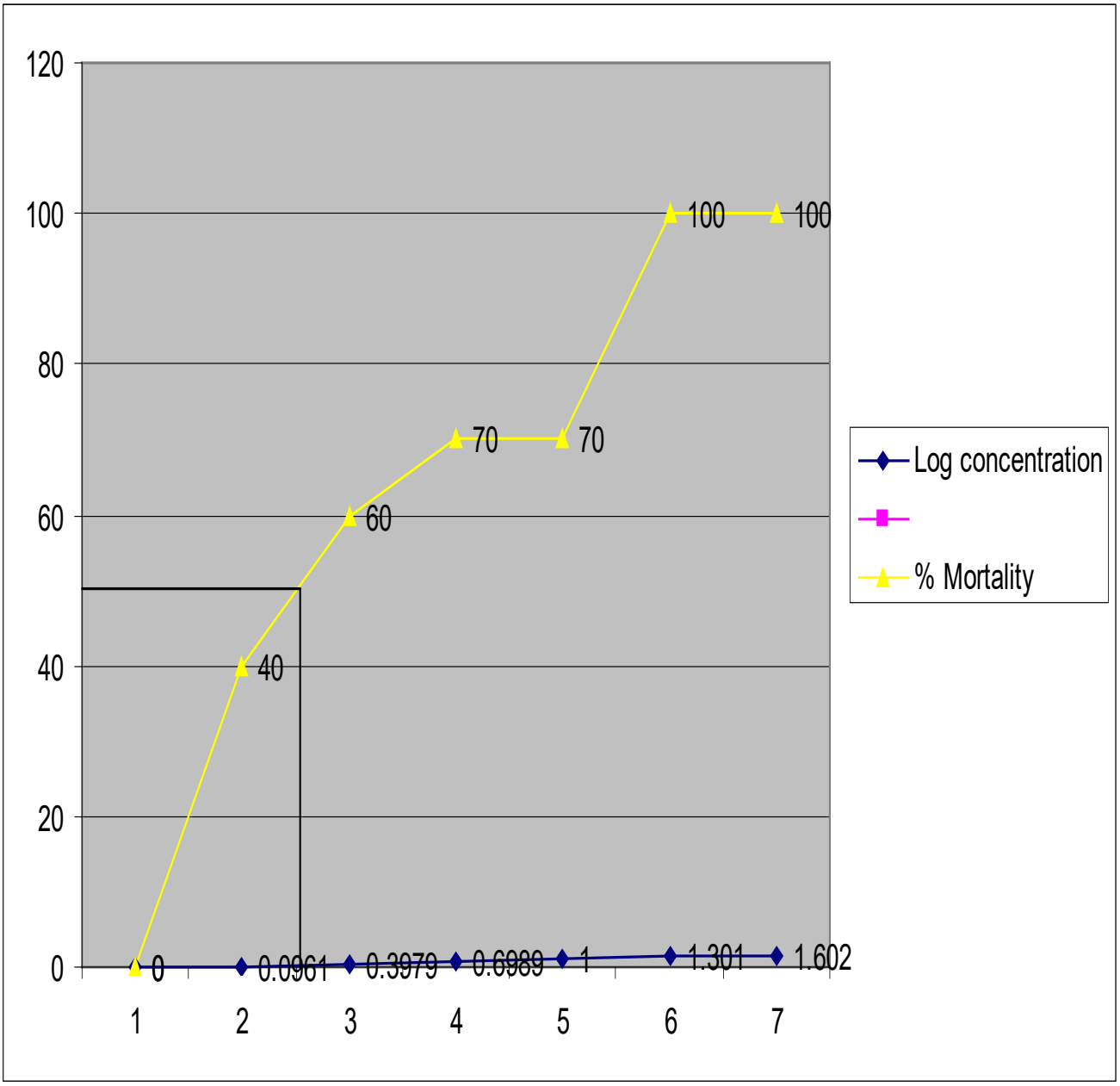
Cytotoxicity test of Metabolite 3 (CM4) was done by using brine shrimp lethality bioassay by plotting the different log concentration of cisplatin (0.0961, 0.3979, 0.6989, 1, 1.3010) against % mortality (0 to 100%) and LC_{50} of cisplatin was calculated 1.18 µg/ml (table 6).

Graph 7: Cytotoxicity test of CM5



Cytotoxicity test of Metabolite 4 (CM5) was done by using brine shrimp lethality bioassay by plotting the different log concentration of cisplatin (0.0961, 0.3979, 0.6989, 1, 1.3010) against % mortality (0 to 100%) and LC_{50} of cisplatin was calculated 1.25 µg/ml (table7).

Graph 8: Cytotoxicity test of Cisplatin



Cytotoxicity test of cisplatin (standard solution) was done by using brine shrimp lethality bioassay by plotting the different log concentration of cisplatin (0.0961, 0.3979, 0.6989, 1, 1.3010) against % mortality (0 to 100%) and LC_{50} of cisplatin was calculated 1.75 (table 8).

Table 3: Results of brine shrimp lethality bioassay

Concentration © (µg/ml)	Log C	% Mortality				LC50 (µg/ml)
		Compound 1(*C)				
		Group A	Group B	Group C	Average	
1.25	0.0969	30	50	40	40	1.8
2.5	0.3979	50	70	60	60	
5	0.6989	60	80	70	70	
10	1	70	80	60	70	
20	1.3010	80	100	90	90	
40	1.6020	100	100	100	100	

LC_{50} of administered cisplatin was 1.8 µg/ml

*C=Cisplatin

Table 4: Results of brine shrimp lethality bioassay

Concentration © (µg/ml)	Log C	% Mortality				LC50 (µg/ml)
		Compound 2 (*CM2)				
		Group A	Group B	Group C	Average	
1.25	0.0969	70	60	50	60	1.2
2.5	0.3979	70	60	50	60	
5	0.6989	80	60	70	70	
10	1	80	70	60	70	
20	1.3010	90	70	80	80	
40	1.6020	100	100	90	97	

LC_{50} of metabolite 1, CM2 was 1.2 µg/ml

*CM= Cisplatin metabolite

Table 5: Results of brine shrimp lethality bioassay

Concentration (C) (µg/ml)	Log C	% Mortality				LC50 (µg/ml)
		Compound 3 (*CM3)				
		Group A	Group B	Group C	Average	
1.25	0.0969	90	80	70	80	1.08
2.5	0.3979	90	80	70	80	
5	0.6989	100	90	80	90	
10	1	100	100	70	90	
20	1.3010	100	100	100	100	
40	1.6020	100	100	100	100	

LC_{50} of metabolite 2, CM3 was 1.08 µg/ml which was lowest among all compounds.

*CM= Cisplatin metabolite

Table 6: Results of brine shrimp lethality bioassay

Concentration (C) (µg/ml)	Log C	% Mortality				LC50 (µg/ml)
		Compound 4 (*CM4)				
		Group A	Group B	Group C	Average	
1.25	0.0969	80	80	50	70	1.18
2.5	0.3979	80	70	60	70	
5	0.6989	90	90	60	80	
10	1	80	90	70	80	
20	1.3010	100	100	100	100	
40	1.6020	100	100	100	100	

LC_{50} of metabolite 3, CM4 was 1.18 µg/ml

*CM=Cisplatin metabolites

Table 7: Results of brine shrimp lethality bioassay

Concentration (C) (µg/ml)	Log C	% Mortality				LC50 (µg/ml)
		Compound 5 (*CM5)				
		Group A	Group B	Group C	Average	
1.25	0.0969	50	60	40	50	1.25
2.5	0.3979	50	70	60	60	
5	0.6989	60	90	90	80	
10	1	70	90	80	80	
20	1.3010	100	100	100	100	
40	1.6020	100	100	100	100	

LC_{50} of metabolite 4, CM5 was 1.25 µg/ml

*CM= Cisplatin metabolites

Table 8 : Results of brine shrimp lethality bioassay

Concentration (C) (µg/ml)	Log C	% Mortality				LC50 (µg/ml)
		Cisplatin (standard solution)				
		Group A	Group B	Group C	Average	
1.25	0.0969	30	40	50	40	1.75
2.5	0.3979	50	60	70	60	
5	0.6989	70	80	60	70	
10	1	70	80	60	70	
20	1.3010	100	100	100	90	
40	1.6020	100	100	100	100	

LC_{50} of cisplatin standard solution was 1.25 µg/ml

Table 9 : Serum creatinine and urea level in normal, cisplatin (16 mg/kg body wt., i.p), Cisplatin + methanolic extract of DAME, cisplatin+ MOME, Cisplatin metabolite3, Cisplatin metabolite3+ DAME, Cisplatin metabolite3+ MOME, (250 mg/kg body wt, p.o) treated mice.

Group	**Treatment (mg/kg)**	**Urea (mg/dl)**	**Creatinine (mg/dl)**
Normal	-	52.51±3.8	0.408 ±0.039
Cisplatin	16	300.49 ±28.51a	2.98 ±0.290a
Cisplatin metabolite 3	16	311.34±27.22a	3.09±0.88a
Cisplatin +DAME	250	137.5 ±5.38b	1.18 ±0.090b
Cisplatin +MOME	250	108.15 ±10.98b	0.535 ±0.054b
Cisplatin metabolite 3+ DAME	250	161.33± 09.54b	0.83± 0.076b
Cisplatin metabolite 3+ MOME	250	151.33± 05.54b	0.78± 0.096b

Values are mean ± SD; $n = 5$. All values are significant. a$P < 0.001$ with respect to normal; b$P < 0.001$ with respect to cisplatin and Cisplatin metabolite 3 group. DAME= Methanolic extract of *D. alata,* MOME= Methanolic extract of *M. olifera.*

In Table 9, it showed that the blood urea and serum creatinine level were found significantly decreased in mouse treated with methanolic extract of *D. alata and M. olifera,* 1 hour before administration of Cisplatin and Cisplatin metabolite 3 (CM3) respectively. All values are statistically significant. Serum creatinine and urea levels were significantly elevated ($P < 0.001$) in the cisplatin-treated animals compared to the normal group. The increase of serum creatinine and urea levels was 7 and 5.7-fold, respectively.

Treatment of animals with methanolic extract of methanolic extract of *D. alata and M. olifera* significantly reduced the elevated levels of serum creatinine and urea. The extract treatment was able to lower the serum creatinine and urea to almost normal level (Table 1)

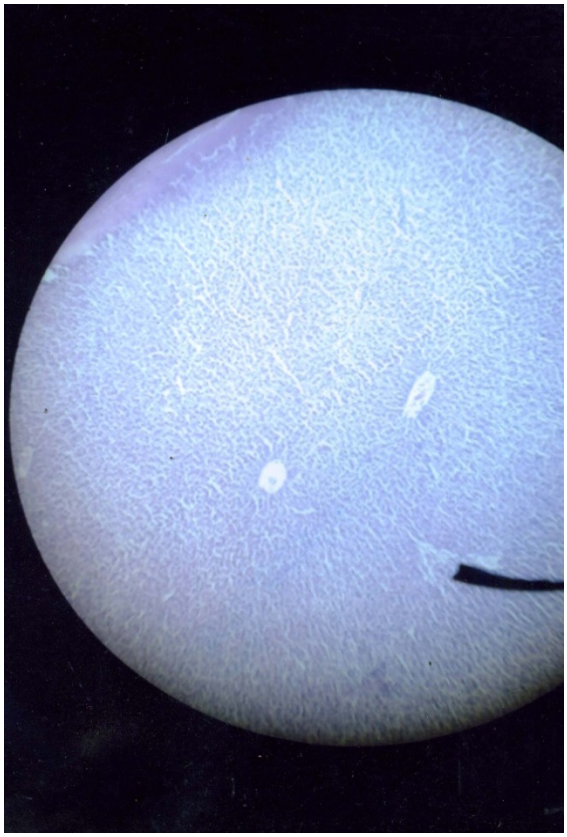
Table 10 : Level of GSH in the kidney of mice treated with normal, cisplatin (16 mg/kg body wt., i.p), cisplatin + methanolic extract of DAME, cisplatin+ MOME, Cisplatin metabolite3, Cisplatin metabolite3+ DAME, Cisplatin metabolite3+ MOME , (250 mg/kg body wt, p.o) treated mice

Group	Treatment (mg/kg)	GHS (nmol/mg protein)
Normal	-	12.4 ±0.88
Cisplatin	16	7.3 ±1.32a
Cisplatin + DAME	250	10.6 ±0.28b
Cisplatin + MOME	250	12.3 ±2.25b
Cisplatin metabolite3	16	6.9±1.78b
Cisplatin metabolite3+DAME	250	13.02± 0.13a
Cisplatin metabolite3+MOME	250	13.09± 0.55a

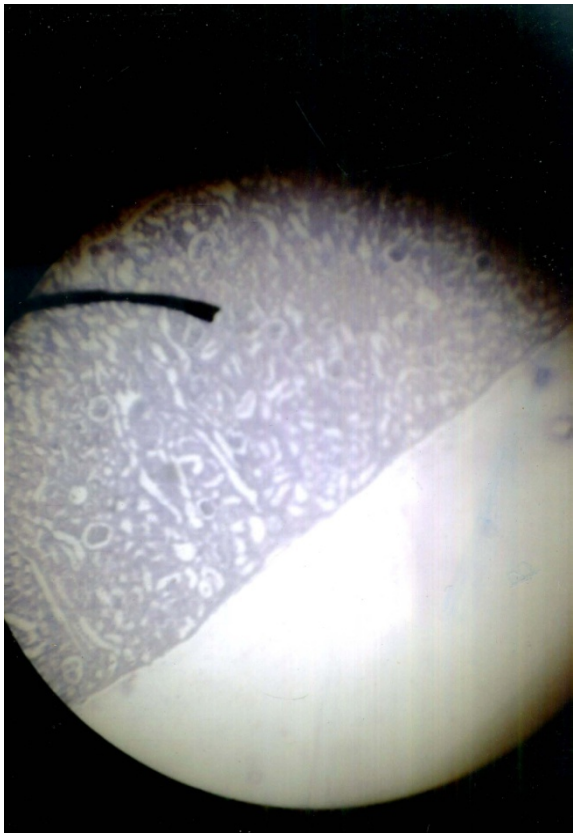
Values are mean ± SD; $n = 5$. All values are significant. a$P < 0.001$ with respect to normal group; b$P < 0.001$ with respect to cisplatin and cisplatin metabolite 3 group.

In Table 11, The renal GSH concentration decreased by over 40% ($P < 0.001$) in cisplatin-treated animals compared to the normal group. However, the administration of methanolic extract of *D. alata* and *M. olifera* at a concentration of 250 mg/kg body wt prior to cisplatin treatment increased the renal GSH concentration to the normal level (Table 2)

Photograph 1: Histopathology of liver and kidney of mouse after cisplain administration.



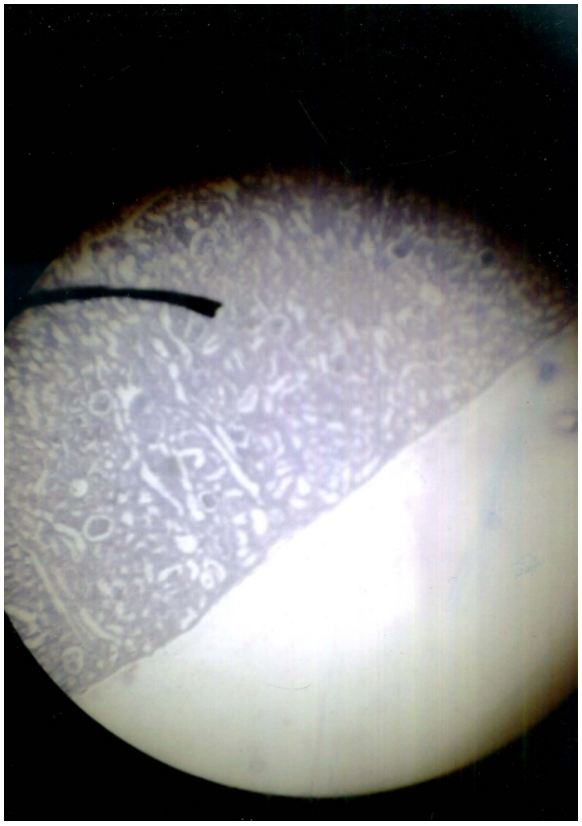
Liver



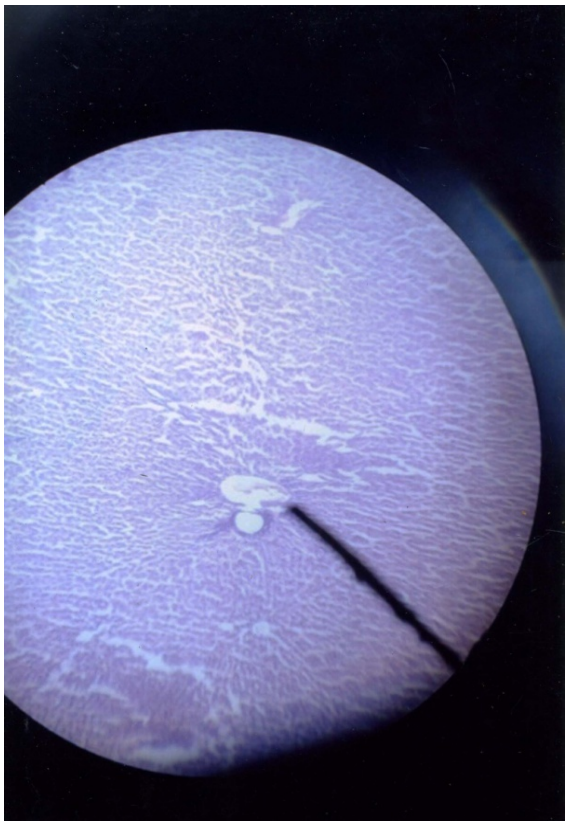
Kidney

Results of Histo-Pathological changes in Cisplatin induced liver and kidney of mice group A showed that normal findings of liver architecture and in kidney Proximal tubular necrosis –some proximal tubular cells became swollen and some of the cells nucleus became shrinkage (picnoticc cell)

Photograph 2 : Histopathology of liver and kidney of mouse after Cisplatin + extract of *D. alata*a administration .



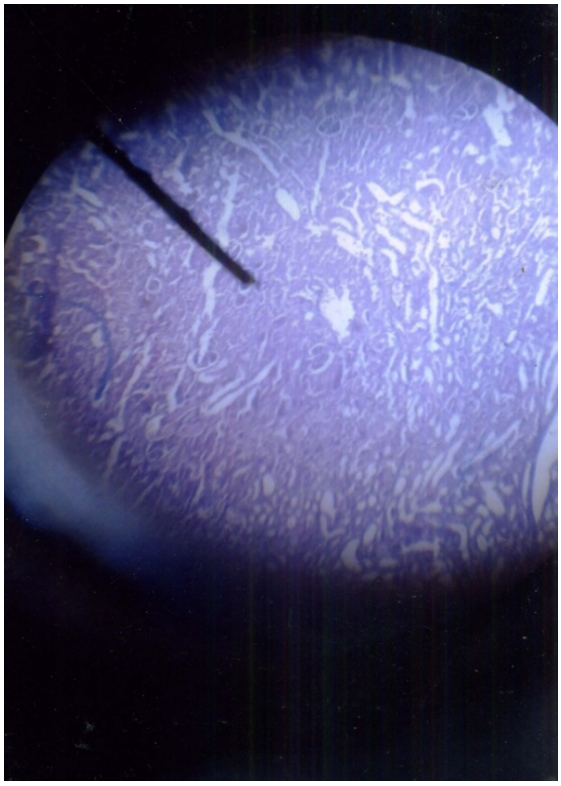
Kidney



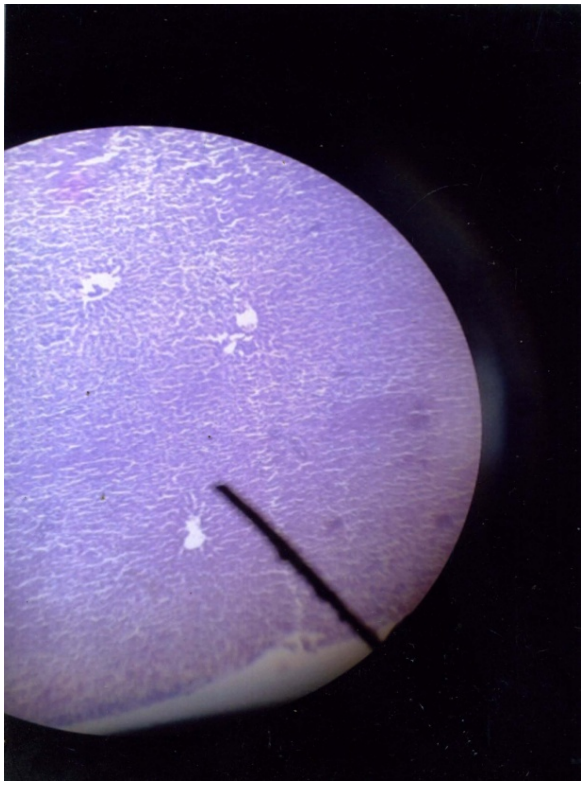
Liver

Results of Histo-Pathological changes in Cisplatin & *D. alata extract* induced liver and kidney of mice groupn B showed No changes were found in liver and in kidney a) Moderate amount of tubular necrosis were found. Rest of the tubular proximal cells became normal. b) Some of the proximal tubules shows picnotic cells and some are enucleated cells, some were swollen**.**

Photograph 3: Histopathology of liver and kidney of mouse after Cisplatin + extract of *Moringa oleifera* administration .



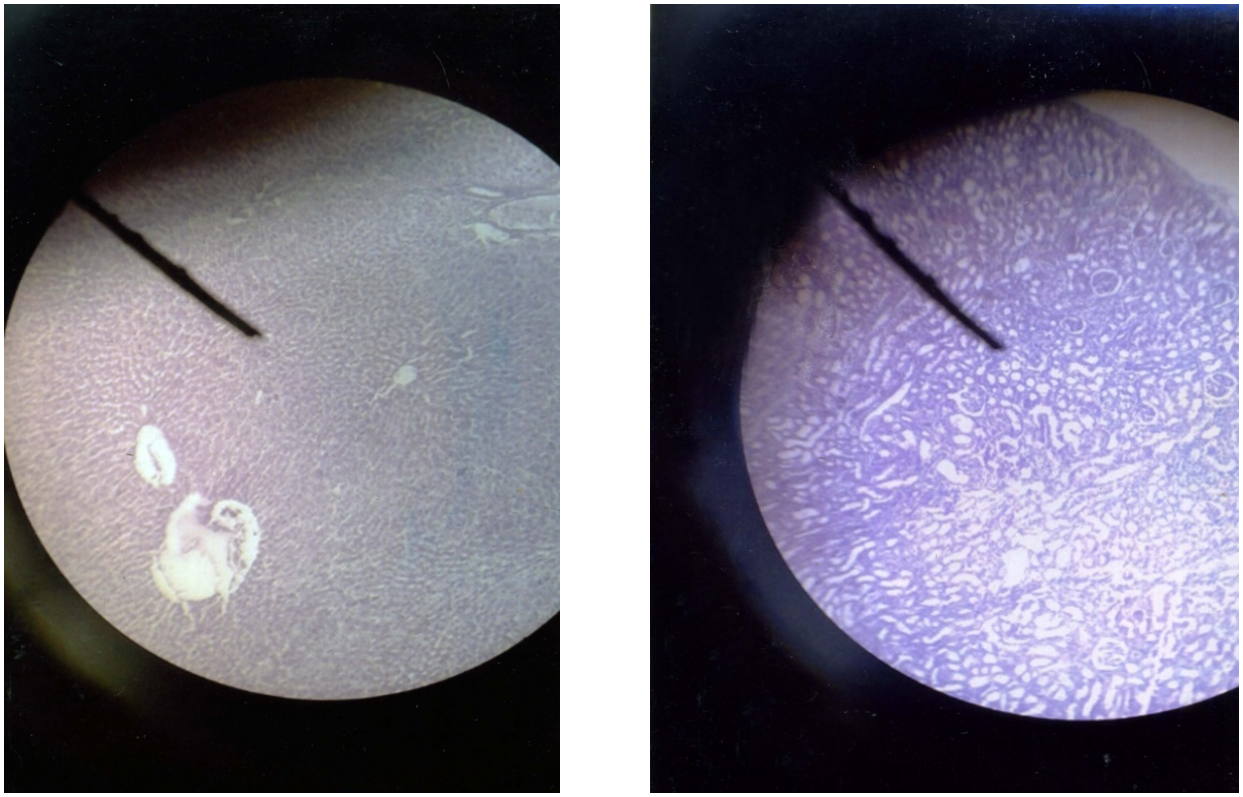
Kidney



Liver

Results of Histo-Pathological changes in Cisplatin & *Moringa oleifera extract* induced liver and kidney of mice groupn C showed normal hepatocytes and in kidney a) moderate amount of tubular necrosis were found. Rest of the tubular proximal cells became normal. b) Some of the proximal tubules shows picnotic cells and some are enucleated cells, some were swollen

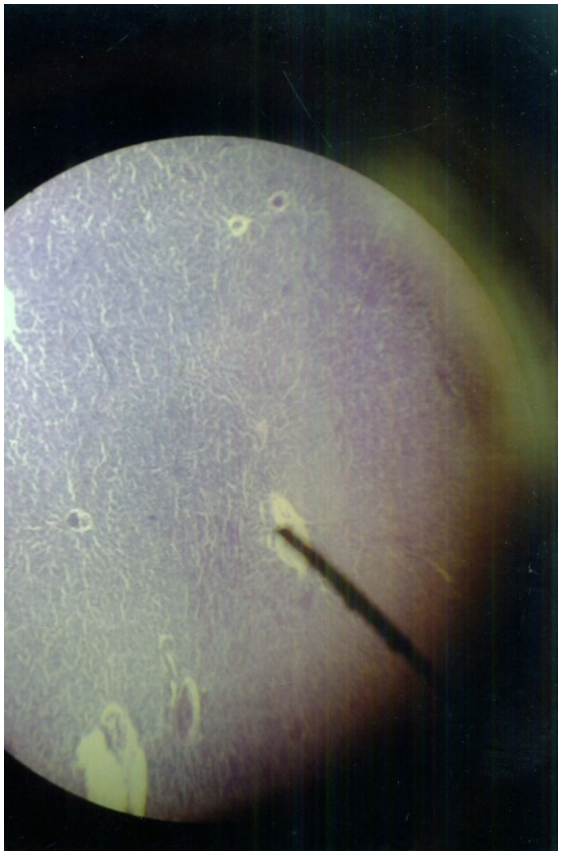
Photograph 4 : Histopathology of liver and kidney of mouse after Metabolite 1 administration .



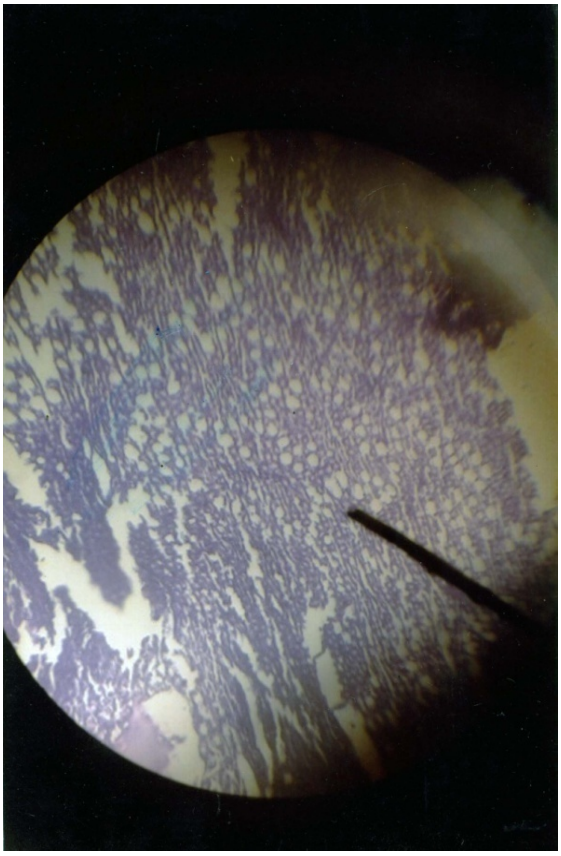
Liver Kidney

Results of Histo-Pathological changes in metabolite 2 induced liver and kidney of mice group D showed a little bit hepatocellular swelling and in kidney Massive necrosis of the Proximal con. Tubules with distorted cellular architecture along with other distal tubular abnormalities.

Photograph 5 : Histopathology of liver and kidney of mouse after Metabolite 1+ extract of *D. alata* administration .



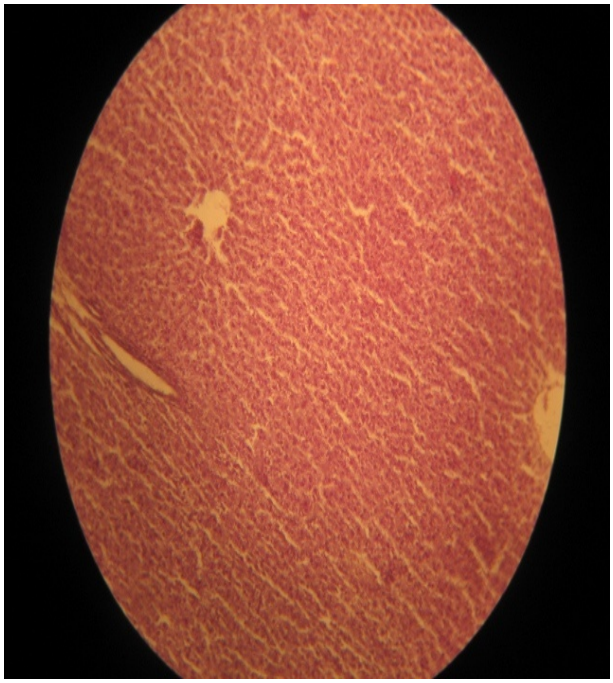
Liver



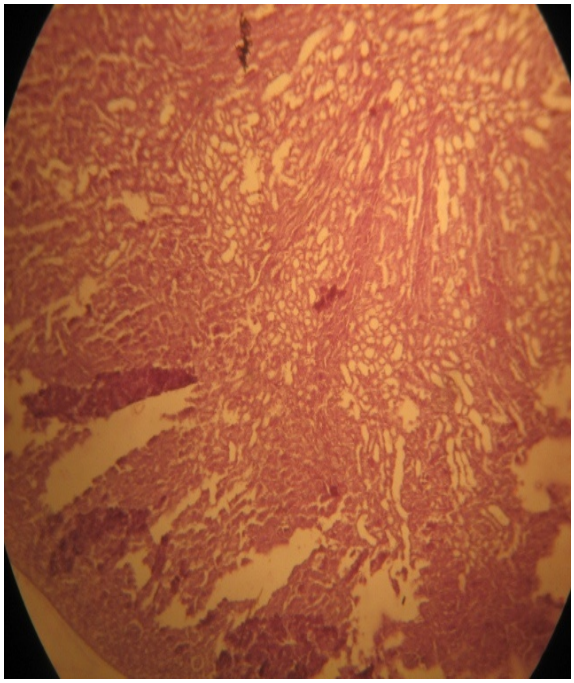
Kidney

Results of Histo-Pathological changes in metabolite 4 induced liver and kidney of mice group E showed A little bit swollen of hepatic cells. Massive necrosis of the Proximal con. Tubules with distorted cellular architecture along with other distal tubular abnormalities.

Photograph 6 : Histopathology of liver and kidney of mouse after Metabolite 1 + extract of *Moringa oleifera* administration .



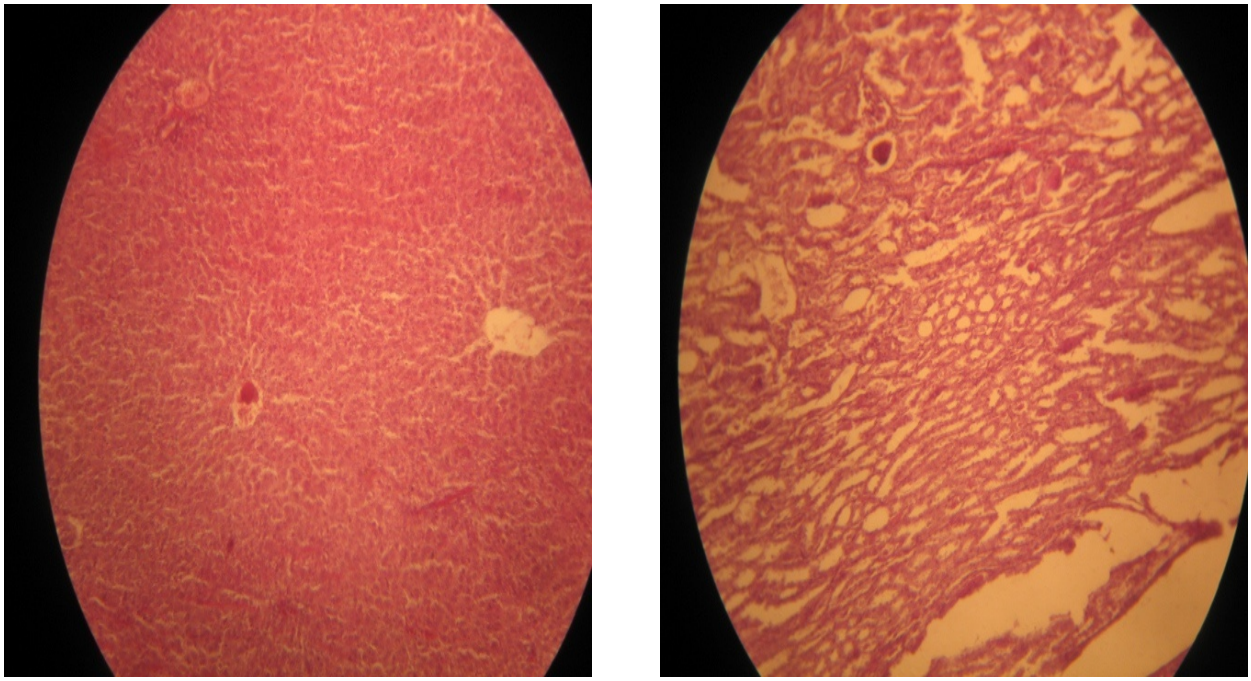
Liver



Kidney

Results of Histo-Pathological changes in Metabolite 2 + extract of *D. alata* induced liver and kidney of mice group E showed A little bit hepatocellular swelling and in kidney a) Mild to moderate increase in the restoration of normal physiological renal proximal tubules . b) Good numbers of prox. Con. Tubular necrosis remain unchanged.

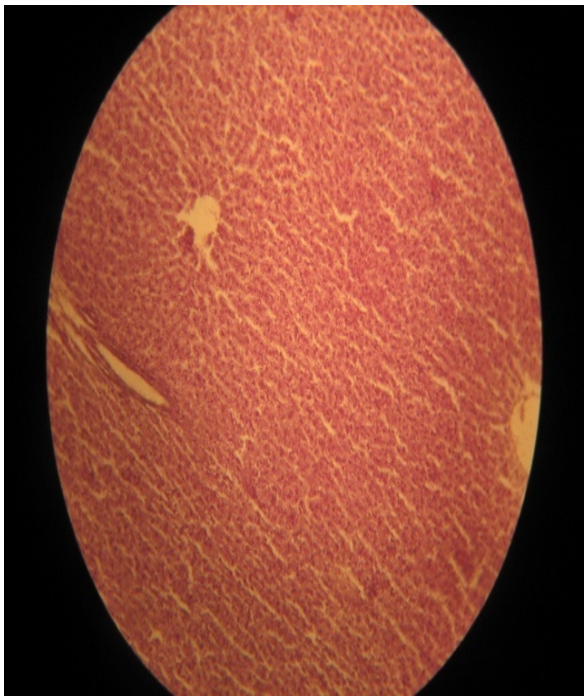
Photograph 7 : Histopathology of liver and kidney of mouse after Metabolite 2 administration .



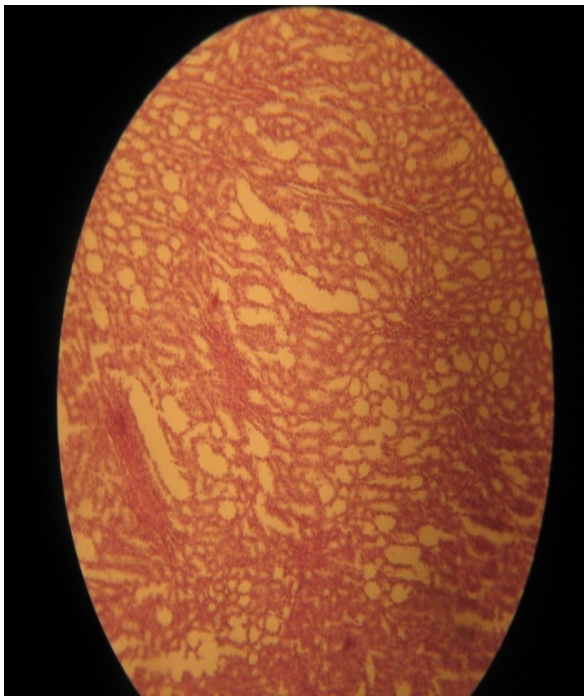
Liver Kidney

Results of Histo-Pathological changes in Metabolite 2 + *Moringa oleifera* induced liver and kidney of mice group F showed A little bit hepatocellular swelling. a) Mild increase in the restoration of normal physiological renal proximal tubules. b) A numbers of prox. Con. Tubular necrosis remain unchanged.

Photograph 8 : Histopathology of liver and kidney of mouse after Metabolite 2 + extract of *D. alata* administration .



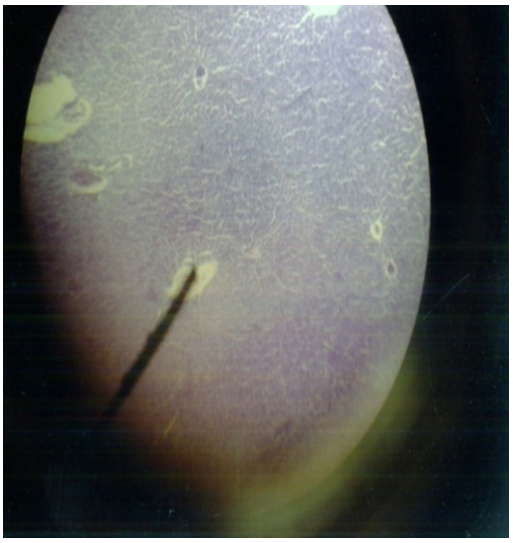
Liver



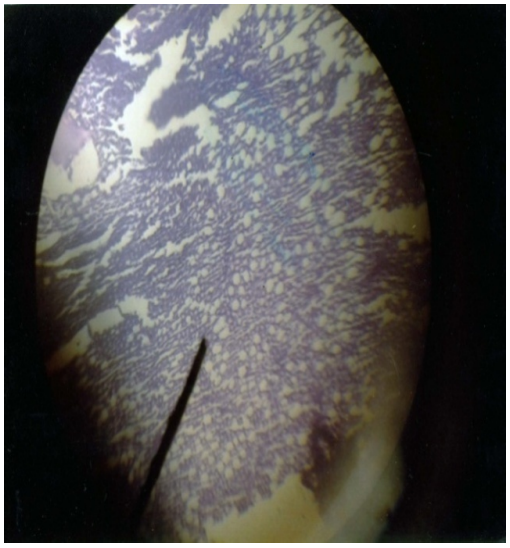
Kidney

Results of Histo-Pathological changes in Metabolite 4 + *D. alata* induced liver and kidney of mice group F showed No changes were found in liver. Normal hepatocytes . a) Mild increase in the restoration of normal physiological renal proximal tubules b) A numbers of prox. Con. Tubular necrosis remain unchanged.

Photograph 9: Histopathology of liver and kidney of mouse after Metabolite 2 + extract of *Moringa oleifera* administration .



Liver



Kidney

Results of Histo-Pathological changes in Metabolite 4 + *Moringa oleifera* induced liver and kidney of mice group F showed normal hepatocytes. A) mild increase in the restoration of normal physiological renal proximal tubules. b) numbers of prox. Con. Tubular necrosis remain unchanged.

Chapter 6

Discussion

Discussion

Cisplatin, the most widely used chemotherapeutic agents for the treatment of several human malignancies. The efficacy of cisplatin is dose dependent, but the significant risk of nephrotoxicity frequently hinders the use of higher doses to maximize its antineoplastic effects. Several advances in our understanding of the biochemical and molecular mechanisms underlying cisplatin nephrotoxicity have recently emerged and are reviewed in this article.

Cisplatin treated total 50 cancer patients those who were admitted in the inpatient department of Oncology, KYAMC, Sirajgonj were studied in this research.

This study revealed that about 56% (n=28) of the total population were placed in 53-60 age group while among the elder population where they found less suffered in malignancy denoted to only 8% (n=4) is shown in the graph 8. The incidence of malignant condition with the increases of the age of the patients. Though some study revealed that cisplatin induced severe transient anemia were found[159,160], but in our study we found only 2% of the patients (n=1) suffered from severe anemia, 48% (n=24) were found non-anemic (figure 1). Serum bilirubin level of 80% (n=40) of the patients were found within normal limit where only 4% (n=2) of the patients were found severe jaundice which is suggested the less involvement of hepato-cellular toxicities (figure 2). This study support the previous work done by Pollera CF and his colleagues in 1987, where they said that a small amounts of platinum are present in the bile and large intestine after administration of Cisplatin, the fecal excretion of platinum appears to be insignificant. Transient elevations of liver enzymes, especially SGOT, as well as bilirubin, have been reported to be associated with Cisplatin administration at the recommended doses[161]. Only 8% (n=4) were found edematous condition among the patients (figure 3).

Lymph node enlargement among the 50 patients were found 24% (n=12).

Moderate increased of serum creatinine (µg/dl), blood urea (µg/dl) and albuminurea levels were found as 28% (n=14), 20% (n=10) and 32% (n=16) of the total patients respectively, strongly suggested that cisplatin or its metabolites produced nephrotoxicities (*p<0.001***) (figure 6,7 & 26) that correlated to the previous study[162]. To prevent nephrotoxicities adequate hydration were assured before starting cisplatin treatment, though some nephrotoxicities were developed which was revealed in previous works also[163].

Total 14 (28%) out of 50 patients suffered from DM, among them 24% (n=12) was suffered from NIDDM and 4% (n=2) from IDDM (figure 4-8).

From the evidence of HPLC data sheet, four suspected metabolites along with Cisplatin (C, CM2, CM3, CM4 and CM5) were identified with their specific RT (retention time) and Area (figure 18-19). Total 500 samples (250 blood and their corresponding 250 urine samples) from different time interval (0, 3, 6, 12, 24 hour) were placed in HPLC for evaluate their retention time (RT) and Area, and finally their mean concentrations in both serum and urine. From HPLC data sheet, the statistical analysis reported that the mean plasma and corresponding urine concentration of cisplatin (as mg/dL) in 50 cancer patients at different time (0, 3, 6, 12, 24 hour) interval were measured and calculated as 478.3165 (±4.5643), 3.598 (±1.632), 281.4552 (±9.377), 241.9782 (±4.0988), 2.8988 (±0.5643), 1.6779 (±0.5453), 996.559 (±8.4365), 978.9265 (±7.6435) respectively (*p< 0.05*) (figure 18-19) where there was a sharp increase and highest concentration were found after 3 hour of infusion and gradually decreased the concentration which was supported by previous work[164], on the other hand mean plasma

concentration of one suspected metabolite CM2 was gradually increased highest level to 234.64 µg/dl (±6.30) after 12 hours (*p>0.01**) while corresponding mean urinary concentration increased to highest level to 269.43 (±6.98) after 24 hours (*p< 0.001***) (figure 18-19). The mean plasma concentration of second suspected metabolite CM3 was found increased to 154.5345 (±3.8876) after 3 hours and gradually decreased to 69.892 (±4.6651) (*p<0.01**) where as corresponding mean plasma concentration in urine was also found increased to 1129.04 (±6.3452) after 3 hour and gradually lowest to 154.0328 (±2.5648) (*p> 0.05*). The mean plasma concentration of another metabolites CM4 was found to 154.5345 (±3.4324) after 3 hour with corresponding urine concentration were found 272.478 (±5.5478) (*p >0.05*)

The mean plasma concentration of last suspected metabolite CM5 were analyzed. The mean plasma concentration of CM5 after 3 hours was found to minimum to 8.02 µg/dl (±8.75) which was sharply increased to 53. 86 (±2.73) after 12 hours then gradually decreased to 39.06 µg/dl (±5.44) after 24 hours (*p>0.01**).

On the other hand, the mean urinary concentration of CM5 reached maximum level to 533.38 µg/dl (±0.48) after 3 hours then 513.81 µg/dl (±9.39) after 12 hours and then finally decreased to 153.37 µg/dl (±4.35) after 24 hours (figure 18-19).

Though a proper hydration were assured before giving cisplatin injection, a marked number of 50 patients were suffered from moderate to severe nephrotoxicities (increased serum creatinine level in 28% of the patients, albuminurea in 16% patients and increased blood urea levels in 20%) which correlate the previous research work[11-13].

Previous several studies have attempted to contribute the potential approaches to prevention of cisplatin-induced nephrotoxicity[30-34]. Some of these strategies are very promising specially herbal medicine that were proved to decrease cisplatin induced nephrotoxicities[35-38].

Further studies will be performed on the potential approaches of some medicinal plants as *Dioscorea alata* and *Morienga olifera* to prevention cisplatin induced nephrotoxicities on cisplatin treated mice. Structure of suspected metabolites will be elucided by using HPLC-mass spectrometry.

Separation of cisplatin and its possible metabolites from urine samples of 5 patients was done by using column chromatography, glass chromatography, PTLC using a range of solvent systems with different polarity and finally their *Rf* values were done (table 3 & photograph 1).

Primary screening for cytotoxicity of these five compounds (C, CM2, CM3, CM4 and CM5) along with cisplatin (as standard solution) were done by using brine shrimp lethality bioassay (figure 20-25 & table 4-9). Among them, comparative to cisplatin, its metabolites are more toxic, specially CM2 (Meta 2, LC_{50}=1.2µgm/ml) and CM3 (1.08 µgm/ml), CM4 (Meta 4, LC_{50}=1.182µgm/ml) which was correlate to the previous study (*p<0.01**).

In vivo animal histo-pathology study of five compounds including cisplatin to see the Histo-Pathological changes in liver and kidney of mice. Total number of test animal (Mouse) : 50. Average wt of mouse: 40 g, total number of groups =13. Group A was treated with Cisplatin, Group B with Cisplatin and extract of *D. alata*, Group C with Cisplatin and extract *Morienga olifera ,* Group D with Metabolite 2 (meta2), Group E with Metabolite 2 and extract of *D. alata,*

Group F with Metabolite 2 and extract of *Morienga olifera,* Group G with Metabolite 3 (meta3), Group H with Metabolite 3 and extract of *D. alata,* Group I with Metabolite 3 and extract of *Morienga olifera* Group J with Metabolite 4 (meta 4), Group K with Metabolite 4 and extract of *D. alata*, Group L with Metabolite 4 and extract of *M. olifera*, and Placebo control Group M.

Massive necrosis of the Proximal con. Tubules with distorted cellular architecture along with other distal tubular abnormalities were seen the mice groups those are was treated with both metabolite 2 and metabolite 4, on the other hand mild to moderate changes in the kidneys of mice those treated with cisplatin showed proximal tubular necrosis –some proximal tubular cells became swollen and some of the cells nucleus became shrinkage (picnoticc cell). A little abnormalities in liver was seen only treated with metabolite 4, otherwise normal in rest of the test animal group.

A promising results showed that the use of some medicinal plant extracts (*D. alata and Morienga olifera*) gave rise to moderate restoration of nomal physiology of kidney and liver of mice.

The experimental results reveal that the of methanolic extract of *D. alata* and *M. olifera* could help prevent nephrotoxicity manifested consequent to cisplatin chemotherapy. The effect is mainly due to the capacity of the extract to restore renal antioxidant defence system. Our earlier investigations have shown that of methanolic extract of *D. alata* and *M. olifera* occurring in South India possessed significant antioxidant and antitumour activities[185].

Further study concerning the structure of suspected metabolites will be elucided by using HPLC-mass spectrometry.

Chapter 7

Conclusion

Conclusion

Cisplatin-induced nephrotoxicity was confirmed in our study. Nephrotoxicity is a poisonous effect of some substances, both toxic chemicals and medication, on the kidneys. Drugs are a common source of acute kidney injury. Drugs shown to cause nephrotoxicity exert their toxic effects by one or more common pathogenic mechanisms. Drug-induced nephrotoxicity tends to be more common among certain patients and in specific clinical situations. The essential values of some plants have long been published; however a number of them remain unexplored yet, therefore there is a necessity to explore their uses and to conduct pharmacognostic and pharmacological studies to ascertain their therapeutic properties.

Although extracts of *D. alata and Morienga olifera* had some effects on biochemical parameters; its effect on renal histology in injured mice kidney was very promising and significant. Results suggest that Portulaca oleracea extract may be used to cure or prevent cisplatin-induced renal toxicity without any adverse effect; hence it can serve as a novel combination agent with cisplatin to limit renal injury.

Chapter 8

Recommendation

Recommendation

Cisplatin (cisplatinum (II) diamine dichloride) is an anticancer drug extensively used against a variety of cancers. Cisplatin chemotherapy is found to manifest dose-dependent nephrotoxicity Cisplatin is nephrotoxic, but the mechanism by which cisplatin kills renal proximal tubule cells is not well defined. Some author suggested that the depletion of renal antioxidant defence system has been suggested to be the main cause of cisplatin-induced nephrotoxicity. The final step in this bioactivation is the conversion of a platinum-cysteine *S*-conjugate to a reactive thiol by a PLP-dependent cysteine *S*-conjugate β-lyase. LLC-PK_1 cells, a proximal tubule cell line with low cysteine *S*-conjugate β-lyase activity, are used to study cisplatin nephrotoxicity.

In previous experiences that demonstrated that many strategies have attempted to prevent or reduce its nephrotoxicity[85, 86]. Early experience suggested that the administration of cisplatin by prolonged continuous infusion and saline hyperhydration, with or without frusemide or mannitol osmotic diuresis, reduces nephrotoxicity.[85] Although contemporary protocols invariably employ hyperhydration and many use low-dose rates of cisplatin, it is clear that nephroprotection is not complete[87]. Hydration reduces the risk of nephrotoxicity with high-dose carboplatin[88].

In our study we found that with adequate precaution and proper hydration of the patient before and during cisplatin administration about 26% of the patients serum creatinine were high level and 12% of patients were found moderate to severe increase blood urea level (figure 13 & 14) that indicate nephrotoxicity.

Several medicinal plants extract were used to prevent cisplatin induced nephrotoxicity[113-135]. In this contest, we have found that nephro-protective effect of *Dioscorea alata* and *Moringa olifera* plants material. It reduces the nephrotoxicity of mice and also reduces the serum creatinine and blood urea in mice significantly.

Different parts *Dioscorea alata* and *Moringa olifera* plants were taken by peoples of the various parts of the world specially Bangladesh, India, China and Sri Lanka as vegetable with food in their meal. These two plants are popular vegetable in these countries. So it is easy to apply the different parts of these plant in a cancer patients receiving cisplatin.

In our study, separation of four suspected metabolites eluted by using different chromatographic techniques, HPLC and UV spectrophometry. Structure of these compounds will be elucided by mass spectrometry in future

Chapter 9
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Annexure 1

Annexure 1

Photograph 10 : *Dioscorea alata* tuber, the edible part of the plant

Photograph 11 : a) Seed pods of *Moringa oleifera,* b) *Moringa oleifera* tree

a) Seeds pods of *Moringa oleifera* with leaves.

Photograph 11 : a) Seed pods of *Moringa oleifera,* b) *Moringa oleifera* tree

b) *Moringa oleifera* tree along with Seed pods and leaves.

Annexure 2

Questionnaire