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Reducing the Negative Impact of Mercury Exposure Using Native Food Plants as Heavy Metal Scavengers

Final Report

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Background of the organizations executing the project

Institute for Aboriginal Health, University of British Columbia, Vancouver, Canada.

The Institute for Aboriginal Health (IAH) assists health and human service faculties, schools, and departments in program and planning development concerning Aboriginal health issues; and develops effective mechanisms that may connect with all Aboriginal communities. The Institute for Aboriginal Health (IAH) Division of Research and Development (R&D) purpose is to increase community-based research capacity within BC Aboriginal communities and in partnership with Canadian and international Indigenous peoples. Partnerships are collaboratively designed, developed, and implemented. The IAH Aboriginal community-based research development initiatives are each based on community developed research ethic protocols. The IAH endeavors to ensure that Aboriginal community based (CB) research follows the Tri-Council Policy Statement; the OCAP principles of: Ownership, Capacity, Access and Possession; and the BC ACADRE 4Rs of Research: Respect, Relevance, Reciprocity, and Responsibility. These guidelines form the basis for all the initiatives.

Experimentation was implemented in a Canadian laboratory (The University of British Columbia's Aboriginal Health and Natural Products Chemistry Laboratory, Institute for Aboriginal Health) where laboratory expertise and all equipment (e.g., cold vapor atomic fluorescence spectrophotometer, and portable Lumex) were available for our purposes. Some aspects of the proposed work plan were conducted at institutions of collaborating countries.

Fundación Smith-NAGAL, Quito, Ecuador.

La Fundación Smith-NAGEL is a non-governmental organization addressing nutrition, food and gastronomy in Latin America. This foundation is a legal entity that was created in Ecuador, and was approved by the Ministry of Welfare by Ministerial accord #2755. Objectives and activities are oriented towards the investigation and transmission of knowledge of traditional foods from the Americas. Its main focus is on crops and products from the Andean region, particularly those with high relevance to nutrition, high quality food, and gastronomic benefits.

LITERATURE REVIEW AND PROJECT BACKGROUND

Literature research was conducted using databases, including electronic library resources available. We used Napralert, an extensive database inclusive of chemistry, ethnobotany, and other disciplines. The literature review was helpful to gain a better understanding of the problem and to fine tune our experimental plan. A review of the topic was useful during the dissemination phase and for effective knowledge translation. .

Mercury is a widespread environmental and industrial pollutant that induces serious effects in both, humans and environment. Exposure to mercury vapours and to organic mercury affects specifically (Central nervous system) CNS, while the kidney is the target organ for inorganic mercury. It is postulated that the antioxidant glutathione (GSH) depletion by mercury may be a trigger for the production of reactive oxygen species (ROS) that induce lipid, protein and DNA oxidation. Generation of ROS in the cytoplasm of cells may increase the mitochondrial hydrogen peroxide production and lipid peroxidation of mitochondrial membrane, resulting in loss of membrane integrity and finally cell necrosis or apoptosis (Park et al, 2007).

In the case of metallic (liquid, elemental) mercury, only the inhalation route has proven to be biologically relevant. When taken orally, less than 0.01% is typically absorbed through the GI tract. Skin contact normally results in even less absorption in most instances. However, up to 80% of inhaled mercury vapour can be expected to be absorbed through the lungs into the blood. All of the absorbed metallic mercury is excreted in urine, with exhaled breath being a significant avenue of excretion only in extremely high exposures. Unlike inorganic mercury, organic mercurials are readily absorbed through the (Gastrointestinal) GI tract (~95%). For organomercurials, blood is a good indicator of exposure, and urine is a poor indicator due to the fact that only mercury oxidized to cationic form is eliminated in the urine. Hair is also suitable indicator, since it is one of the routes of organic mercury elimination (Risher et al, 2005).
Chelation therapy is presently the treatment of choice for reducing the body burden of mercury and other heavy metals. Chelation consists of the introduction of charged molecule (typically containing one or more sulfhydryl groups) into the body for the purpose of binding specific metal ions of opposite electrical charge, and facilitating the elimination of the formed complex from the body in the urine (Risher et al, 2005).

The profile of successful chelating agent includes high affinity for the toxic metal but low affinity for essential metals, minimal toxicity, lipid solubility and good absorbability from the gastrointestinal tact (Blanusa et al, 2005).
Presently, in intoxication with elemental mercury vapour and either with inorganic or organic mercury, DMPS (unithiol), meso-DMSA (succimer), DPA (D-penicillamine) or NAPA (N-acetyl D-penicillamine) are recommended (Blanusa et al, 2005). Only DMSA is FDA-approved for pediatric use in treating mercury poisoning (Risher et al, 2005). Due to adverse effects of these chelators alternative therapies are needed.

This report reviews literature on usage of plants as potential chelators for mercury and other heavy metals as well as possible biomarkers for *in vitro* and *in vivo* bioassays. The selection of primary screening "*in vitro*" assay will be based on the costs involved and availability of necessary equipment.

Cilantro (*Coriandrum sativum*) usage as chelator proof of concept in:

Humans

Omura, 1998, claims in his US patent that some resistant infections, cancer and other diseases which have infection and localized metal deposits in pathological areas can be treated by promoting removal of heavy metal (ex. Hg) and or Al. Heavy metals removal is obtained by ingestion of Cilantro coupled with antibiotics and/or antiviral agents intake enhancement methods: Shiatsu massage, mechanical and electromagnetic stimulation of organs etc. Patent does not limit treatment to Cilantro but includes also vegetables of other Umbelliferae family members like leaves of various types of parsley and green leaves of caraway, carrots, celery, dill, fennel and parsnips. Cilantro (Chinese parsley) is supposed to be the most effective in heavy metals (Hg, Pb Al, Cd and Ni) removal. Patent claim is not limited to usage of green plants but also Cilantro constituents like Bergapten, Umbelliferone, Scopoletin, Xanthotoxol etc. According to Omura, Cilantro active component is resistant to 100^0C heat and can be extracted with ethanol. Study included only 11 patients with *Chlamydia trachomatis*, *Herpes simplex* or *Cytomegalovirus* infections. Blood and organs levels of heavy metals were determined by non invasive, patented by him method, Bi-Digital O-Ring Test.

Coleman et al. (2006) in his US patent application includes Cilantro as a dietary supplement for promoting removal of heavy metals. He defines composition for assisting natural body functions in cleansing the body of heavy metals. It comprises of primary chelator like Cilantro or an

extract thereof; alpha-lipoic acid in amount sufficient to move amounts of heavy metal species from a user central nervous system into the user's vascular system. Optionally, a secondary chelator is added in an amount sufficient for capturing at least a portion of heavy metals species released from primary chelator to limit return of heavy metal species released back into the central nervous system. Finally, vascular system promoter for subsequent release from the body via an excretion pathway is proposed from the group consisting of L-arginine, *Rhodeola rosea* extract, citruline, *Ginco biloba* extract and combinations thereof. Number of primary and secondary chelators and their precursors are named. Replacement of essential heavy metals like Ca, Mg, Zn and addition of vitamins is encouraged in the treatment. No scientific data are presented in this patent application.

Rodents

The preventive effect of Cilantro on lead deposition was investigated in male ICR mice (Aga et al, 2001). Mice were given 1000ppm lead as lead acetate in drinking water for 32 days. Administration of Cilantro was performed for 25 days from day 7. The lead reached its highest concentration in the femur. Administration of Cilantro and positive control, DMSA, significantly: $p< 0.005$ and $p<0.001$, respectively, decreased lead deposition in femur. DMSA, but not Cilantro, was also effective in significant reduction ($p<0.005$) of lead accumulation in the kidneys. Calcium concentration in femur was not affected by DMSA or Cilantro. Both agents lowered urinary ∂-aminolevulinic acid (ALA is up regulated lead poisoning biomarker) excretion significantly in comparison to control groups. Results of this study suggest that Cilantro has suppressive activity on lead deposition. Authors were supposed to purify active components of Cilantro and follow it up by in vitro activity of ∂-ALA-D. No follow up was found in the literature since 2001.

The preventive effect of Cilantro on Al deposition was investigated in male ICR mice (Aga et al, 2002). Mice were given 1000ppm Al as Al chloride in drinking water for 39 days. Administration of Cilantro was performed for 25 days from day 14. It was found that Al was accumulated in the brain, kidney and femur but was unchanged in liver. Positive control used to validate this animal model: deferipone decreased localized Al depositions. Authors suggest that Cilantro has suppressive activity on Al deposition even though only ambiguous decreasing trend was observed.

Fish

Cilantro and chitosan (Ren et al, 2006)) inhibited accumulation of Cd in liver in 20-30% and 25-40%, respectively, in cultured rainbow trout. Fish were fed regular diet enriched either with 2% of lyophilized Cilantro or 3% chitosan with or without 10mg Cd/kg in test diet. Accumulation of Cd in muscle (just above detection limit), liver, and kidney was investigated for 12 weeks. Results suggest that the addition of Cilantro or chitosan to fish feed might be used to decrease toxic heavy metal accumulation in fish. Data obtained in this study (2006) were solid.

Other plants used to reduce burden of heavy metals

Two aqueous extracts from seaweeds (*Halimeda incrassata* and *Bryothamnion triquetrum*) reduced H_2O_2 and CH_3HgCl–induced oxidative stress in GT1-7 mouse hypothalamic immortalized cells (Fallarero et al, 2003, Fallarero et al, 2004). Methanolic extract of *Tribulus terrestris* fruit provided protection against mercury-intoxicated mice (Kavitha et al, 2006). *Mentha piperita* reduced the side effects of arsenic-induced hepatopathy in livers of Swiss albino mice (Sharma et al, 2007). Chelation properties of these plants were not studied.

Centella asiatica supplementation reduced oxidative stress in rats exposed to arsenic (Gupta et al, 2006a), aquaeos extract of *Hippophae rhamnoides* fruit protected against arsenic toxicity in Swiss albino mice (Gupta et al, 2006b), but neither of them displayed chelating properties.

Moringa oleifera lowered all oxidative stress markers in mice caused by arsenic (Gupta et al, 2007) and reduced uptake of this metal in blood, liver, kidneys and brain.

Biomarkers of mercury toxicity and effect of chelators

This literature review proves that oxidative stress biomarkers are suitable to assess mercury toxicity since mercury disrupts the pro-oxidant/anti-oxidant balance of tissues leading to biochemical and physiological dysfunction. Reactive oxygen species (ROS) include oxygen ions, free radicals and peroxides both inorganic and organic and they increase dramatically upon exposure to mercury and heavy metals. On the other hand, naturally occurring, anti-oxidative stress defence molecules like intracellular glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (Gpx) or glutathione-S-transferase (GST) are significantly diminished with mercury and other heavy metals exposure. Successful plant derived chelator should reverse these effects within affected cells and tissues. Lipid oxidation markers like thiobarbiturate reactive substances (TBARS) are up regulated by

heavy metals. Oxidative stress markers can be measured either spectrophotometrically or fluorometrically in relevant cells and tissues.

Endogenous metals are essential components of many enzyme systems. ∂-aminolevulinate dehydratase (∂-ALA-D) is a metalloenzyme requiring zinc ions for activity. ∂-ALA-D catalyses the asymmetric condensation of two molecules of ∂-ALA to porphobilinogen in the initial steps of heme biosynthesis. ∂-ALA-D is a sulfhydryl containing enzyme and numerous metals such as mercury and lead which compete with zinc and/or oxidize the thiol groups, inhibit it's activity (Nogueira et al, 2006). ∂-ALA-D activity can be measured biochemically as well as in the blood or hepatic tissue/cells, exposed to mercury. Successful plant derived chelator should restore ∂-ALA-D activity to pre-mercury treatment.

Metallothioneins (MTs) is a family of cysteine-rich (30% of total aminoacids), low molecular weight proteins. MTs have the capacity to bind both physiological and xenobiotic heavy metals through the thiol group of its cysteine residues. There is statistically significant correlation between mercury exposure and depletion of MTs in blood and tissues. MTs can be determined by ELISA in blood or relevant tissues.

Cell viability determination is essential for mercury toxicity and evaluation of chelating properties of tested plants. Cytotoxicity assays include: neutral red uptake, mitochondrial dehydrogenase activity (MTT conversion), thymidyne incorporation and protein-content (SRB). Spectrophotometric, fluorometric or radioisotope methods can be used.

Since potential plant chelators can display protective effects against mercury toxicity through for example regulation of oxidative stress, results have to be confirmed by alterations of mercury uptake. It is well established that mercury toxicity depends on the type of tissues and cells. Primary cells for example are less sensitive, probably due to the slower mercury uptake. Methods used for mercury uptake and uptake inhibition include: radioisotope, cold vapour atomic absorption spectrometry (CV-AAS), and spectrophotometric.

Methods used to determine toxicity of heavy metals and chelating properties of agents

Chemical

Cilantro sorbent (Karuasagar et al, 2005) was found to sorb organic and inorganic mercury from aqueous solutions with good efficiency in both batch and column (immobilized) conditions. The degree of removal was found to be greater then 95% in pH range of 4-10 within optimal time of

45min. In these experiments, 250mg of the sorbent (dried plant particles) was shaken with 20ml of Hg solution containing 0.5mg absolute Hg. Both, solution and the sorbent were analyzed for Hg content by cold vapour atomic absorption spectrometry (CV-AAS) using mercury analyzer. This method could be used for initial screening and comparison of several plants for their sorption capacity and Hg binding specificity. Disadvantage would be high cost of Hg analyses. Possibly, determination of Hg in the sample could be achieved by new, simpler, but also not high-through method (Khan et al, 2005): A simple spectrophotometric determination of trace level mercury using 1,5 Diphenylthiocarbazone solubilized in micelle.

(Ramadan et al, 2003) compared Radical Scavenging Activity (RSA) of Cilantro, Black Cumin (*Nigella sativa*) and Niger (*Guizotia abyssinica*) crude seed oils and oil fractions. Oils were investigated for their RSA toward the stable galvinoxyl radical by electron spin resonance (ESR) spectrometry and toward 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical by spectrophotometric method. Oils were extracted with hexane and testing was performed in toluene. Cilantro showed the highest scavenging effect (~30% in 60min). Only spectrophotometric method could be used in our laboratory.

In vitro

Erythrocytes

(Aaseth et al, 1981) determined effect of chelating agents on CH_3HgCl inhibition and removal in RBC in the absence of extracellular thiols. DMSA at 1mM proved to be the most effective in ^{203}Hg uptake inhibition (>90%) in human erythrocytes and removal (>80%) from pre-loaded RBC. Uptake was very rapid ~10min. Removal was followed for 3h after 1h pre-loading. Data presented in this paper were solid, but method requires usage of isotopes and Beta/gamma counter. Chelating capacity in RBC does not implicate clinical applicability of the drugs but could be a good tool for potential plants screening.

(Bohets et al, 1995) uses human blood and pure ∂-ALA-D enzyme, purchased from Sigma to determine effect of DMSA and other thiols used in clinics, on enzyme toxicity. Blood is incubated for 90min at 37^0C with tested compounds. Assay is quick and would require plate reader with 555nm filter, but some optimization and adaptation would be necessary.

Liver cells

In (Aaseth et al, 1981), isolated rat hepatocytes were exposed to ^{203}Hg for 6h and binding was determined in the absence of extracellular thiols. Almost 50% of ^{203}Hg was taken up by the cells

scavenger enzyme. Treatment with Hg resulted in a significant increase in MDA and Hsp70 and reduction in cellular GSH and GPx activity.

(Aleo et al, 2002) proved that subcytotoxic (0.1-10μM) $HgCL_2$ affect eiher the antioxidant defences of MDCK cells or their gap-junctional (GJIC) functions. MDCK cells in logarithmic phase were exposed for different periods of time (from 4-72h) to $HgCl_2$ (0.1-100μM). Cell proliferation was determined by direct counting in hemocytometer, cell viability by Vybrant Apoptosis Assay kit and FACS. Total GSH and the activities of Glutathione peroxidase (Gpx) and catalase (Cat) were determined by spectrophotometric or colorimetric methods. MDCK cells were tested for their capacity to establish functional gap junctions by micro-injection dye transfer assay.

Neuronal cells

(Yin et al, 2007) Studies on primary cultured astrocytes demonstrated that CH_3HgCl exposure was associated with increased mitochondrial membrane permeability (assessed by potentiometric dye), alterations in glutamine/glutamate cycling (assessed by ^{3}H-glutamine uptake) and increased ROS formation (lipid peroxidation biomarkers).

(Gasso et al, 2001) data showed that disruption of redox equilibrium and Ca2+ homeostasis contribute equally to $HgCl_2$-mediated toxicity, whereas oxidative stress is the main cause of CH_3HgCl neurotoxicity. Studies were performed in rat cerebellar granule neuron cultures using fluorescent methods. CH_3HgCl exhibited an LC_{50} (2.47μM) tenfold lower than that of $HgCl_2$ (26.4μM). Antioxidant, propyl gallate abolished ROS generation and partially inhibited the increase of Ca2+ induced by both compounds.

Lymphocytes

(Hemdan et al, 2007) demonstrated that low-level exposures to Hg (up to 0.5μg/ml), in the absence of inflammation (anti-CD_3/-CD_{28}/-CD_{40} stimulation), polarizes the immune response toward Th2, but *Salmonella* antigens promote TH-1 polarization. Testing was performed in human PBMC's. Cell vitality was assessed by MTT and modulation of cytokines profile (IFN-γ, TNF-α, IL-6, IL-10 and IL-4) by ELISA .

(Yole et al, 2007) compared effects of organic and inorganic mercury, 1min-4h exposure, in human YAC-1 lymphoma cells, by flow cytometry. Cell death, microtubules, actin, CD_3 receptor expression, protein tyrosine phosphorylation and intracellular Ca2+ levels were investigated. It was concluded that two groups of organic and inorganic Hg compounds may induce cell death by distinct pathways.

(Silva-Perira, 2005) showed synergistic effect of CH_3HgCl and $HgCl_2$ in their cytotoxic/genotoxic effect. Genotoxicity was assessed by chromosome aberrations and

during incubation. DMSA at 1mM almost totally inhibited binding of ^{203}Hg to hepatocytes. Method requires access to rat perfused livers, usage of isotopes and Beta/gamma counter.

(Korashy et al, 2006) established that induction of Glutathione transferase (GST) by heavy metals (Hg2+, Pb2+ and Cu2+) was strongly correlated with an increase in the ROS production in murine hepatoma Hepa 1c1c7 cells. GST was determined colorimetrically (340nm) and ROS fluorometrically (485 excitation and 535 emmission).

Biomarkers of sensitivity and effect associated with Cd and Hg toxicity were studied (Tchounwou et al, 2002) in human liver carcinoma cells (HepG2). Gene profile (CAT-Tox) assay was performed to measure the transcriptional activation of stress genes in HepG2 cells and results indicated that matallothioneins and heat shock proteins appear to be excellent candidates for biomarkers for detecting metal-induced proteotoxic effects at the molecular and cellular levels. A dose response relationship was recorded with respect to both cytotoxicity (MTT assay) and gene expression.

(Tchounwou et al, 2004) assessed potential of Hg to induce early-stage apoptosis in HepG2 cells. Annexin-V assay was performed by flow cytometry and the data indicated a dose response relationship between Hg exposure and the degree of early apoptosis.

Microarray analysis of Hg-induced changes in gene expression in HepG2 cells (Ayensu et al, 2006) showed importance of immune responses. 20,000 genes were analyzed and 2,211 target genes were identified. These genes are categorized as control and regulatory genes for metabolic pathways involving the cell cycle, apoptosis, cytokine expression, Na+/K+ ATPase, stress responses, G-protein signal transduction, transcription factors, DNA repair as well metal-regulatory transcription factors etc.

Renal cells

(Bohets et al, 1995) determined 24h cytotoxicity of several Hg compounds in renal cells: LLC-PK (pig), MCDK (dog) and human primary (hPTC), by neutral red uptake, mitochondrial dehydrogenase activity (MTT conversion), thymidyne incorporation, protein content as well as Hg uptake. The endogenous content of Glutathione (GSH) was studied under normal and Hg exposure conditions as well with addition of L-Buthionine sulfoximine (BSO, specific inhibitor of GSH synthesis) or 2-oxo-4-thiazolidine carboxylic acid (OTC, a substrate for GSH synthesis). MTT could be easily performed in high-throughput manner if plate reader with 570-600nm filter would be available.

(Sargazi et al, 2006) used Hg as positive control for testing Al-induced injury to porcine kidney LLC-PK1 cells. Effects on markers of oxidative damage were studied: Malondialdehyde (MDA), a marker of lipid peroxidation, cellular GSH as the intracellular sulphydryl compound, heat shock protein 70 (Hsp70) and glutathione peroxidase (Gpx) as a reactive oxygen species

polyploidy cells and mitotic index was used as measure of cytotoxicity. Cultures of human blood were treated with low doses (0.1-1000μg/L) of Hg compounds for 48h.
(Colombo et al, 2004) showed differential effects of Hg, Pb and Cd on IL-2 production by Jurkat T cells. When Jurkat cells were stimulated with anti-CD_3/CD_{28}, mercury and lead (1μM) inhibited IL-2 production regardless of the state of T cell activation. Cadmium stimulated IL-2 production only in preactivated T cells. Mixture of three metals had no effect. Activation with PMA/calcium ionophores indicated that the target of heavy metals is located downstream from PKC and calcium mobilization. The results suggest that the state and mode of T cell activation are important parameters in heavy metal toxicity.

Epithelial cells

(Park et al, 2007) investigated production of ROS by $HgCL_2$ (2,4,6 and 8ppm as of mercury) in human bronchial epithelial cells (BEAS-2B cell line). Exposure of cells to $HgCL_2$ led to cell death, ROS increase and apoptosis.

In vivo

(Aaseth et al, 1981) determined chelating activity of DMSA and other thiols on ^{203}Hg concentration in organs of female NMRI mice. Methyl mercury was either injected (5μmol/kg), 4 days prior oral chelator (1mmol/kg daily) treatment for 8 days or injection of chelating thiol and CH_3HgCl were combined and the treatment consisted of 4 days. Blood, liver, brain and kidney were analysed and in both scenarios treatment with DMSA reduced concentration of mercury in all organs tested. This method requires Core facilities, usage of isotopes, Beta/gamma counter and would be very expensive.
(Brandao et al, 2006) evaluated acute effects of $HgCl_2$ in mice. Mice received single dose of $HgCL_2$ (4.6mg/kg) for 3 consecutive days. 30min after last injection mice received one single injection (subcutaneously) of DMPS, NAC or $(PhSe)_2$. 24h after latest $HgCl_2$ injection, blood, liver and kidney samples were collected, δ-ALA-D, Na+,K+ ATPase activities, TBARS, NPSH and ascorbic acid concentrations were evaluated. Plasma aspartate (AST) and alanine aminotransferase (ALT) activities, as well as urea and creatinine levels were determined. The group of mice exposed to Hg and (PhSe)2 presented 100% lethality. Exposure to Hg caused decrease of the body weight gain and treatments did not modify that. δ-ALA-D, AST and ALT activities, TBARS, ascorbic levels and NPSH levels were not changed after $HgCl_2$ exposure. Therapies with chelating agents may damage renal tissue by formation of more toxic complexes.
(Pingree et al, 2001) used urinary porphyrin concentration in rats as biochemical measure of renal mercury content. Rats exposed to CH_3HgOH at 10ppm in drinking water for 6 weeks were

treated with up to 3 consecutive doses of DMPS (100mg/kg,ip) at 72h intervals. Consecutive DMPS treatments of CH_3HgOH-exposed rats significantly decreased kidney concentrations of total, as well as Hg2+ and CH_3Hg+ species.

Literature review gives strong support for studying beneficial effect of cilantro and other plants on mercury toxicity. Lack of simple medium-throughput "*in vitro*" assay to screen extracts and pure compounds for protective activity against mercury toxicity, makes it necessary to undertake such assay development. It was decided to use MTT assay as primary bio-assay to measure protective effect of selected plants on acute toxicity of mercury. Mammalian liver HepG2 cells were selected as relevant model for mercury chloride toxicity. Effect of plant material will be compared to that of DMSA.

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Chemical analysis of *Coriander sativum* (Cilantro) crude extract

Introduction

Using column chromatography fractionation the following compound were isolated from a methanolic cilantro extract: the volatile oil linalool the monoterpene hydrocarbons *a*- and *b*-pinene and limonene, anethole, and camphor; oleic and linolenic fatty acids; flavonoid glycosides (quercetin, isoquercitin, and rutin). Other compounds have been reported in the literature including chlorogenic and caffeic acids; tannins; sugars, coumarins; mucilage; and starch (Budavari, 1996; Hansel et al., 1992; Leung and Foster, 1996; List and Harhammer, 1973; Wichtl and Bisset, 1994). Understanding chemical interactions between mercury and crude plant extracts and isolated chemicals, coupled with effective assays for detection of heavy metal chelating agents should provide innovative and practical ways to address heavy metal poisoning. Resources and time limitation did not permit the assay implementation of individual isolated compound, and this should be conducted in the next phase of the project.

Material and methods

The coriander (*Coriandrum sativum*) fresh material was obtained from a certified organic supplier. The fresh material was washed several times using destilled water, the roots were removed, and leaves and shoots were frozen and freeze dried for 48 h. The freeze dried material was ground into a fine power and kept frozen at −18°C in polyethylene bags until needed.

Crude extract preparation

The powdered coriander l(10 g) was submitted to extraction with methanol (100 mL), for 60 min, under agitation at room temperature (25°C±2°C), and then the mixture was centrifuged at 3000 g for 10 min. After transferring the supernatant into the flask, the residue was resuspended in methanol (100 mL) and again submitted to the same extraction process. The supernatants were combined and transferred to amber flasks, flushed with nitrogen and stored in a freezer at -18°C until used for analysis.

Extract fractionation by column chromatography

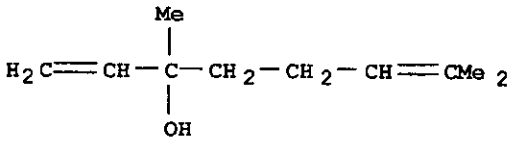
The methanolic extract was submitted to saponification with 10% KOH in methanol and left in the dark at room temperature overnight to remove the lipids and chlorophyll, according to the analytical procedure described by Rodriguez-Amaya (1999b). Then, the mixture was exhaustively washed with distilled water in a separatory funnel until free of alkali. The pigments were then concentrated in a rotary evaporator under reduced pressure at 35°C and submitted to column chromatography. A 10 mL sample of extract was applied to a glass column (1.5×21 cm), packed with sephadex 20 of approximately 30 cm. A methanolic gradient was used to elute the fractions. The fractions obtained were taken to dryness and some were further fractionation.

Identification of chemical constituents

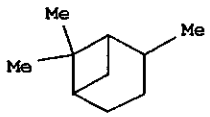
Identification was undertaken on the basis of the visible absorption spectra, thin-layer chromatography (TLC) and specific chemical reactions. The fractions were adequately diluted in methanol and the visible absorption spectra (350–550 nm) were obtained using the Hitachi U 3200 spectrophotometer and compared with the values obtained by from the literature (Rodriguez-Amaya 1999b; Britton 1991; and Davies 1976). The fractions obtained from the column were concentrated in a rotary evaporator under reduced pressure of 35°C and applied on silica gel thin layer (TLC-60-F254, 20×20 cm with thickness 0.25 mm—Merck), using 3% methanol in benzene as the mobile phase. The Rf values were calculated for each stain. The plate was then exposed to hydrochloric acid vapour in order to detect the presence of epoxy carotenoids. The test is considered positive when the yellow stains of the chromatogram turn blue or green.

Few drops of 0.1 N hydrochloric acid were then added to the alcoholic pigment solution and after 3 min, the spectrum was recorded in order to verify the presence of the 5,6 epoxide group in the carotenoids' structure. Other compounds were compared to pure standards using High Phase Column Chromatography (HPLC).

Structures of these compounds are shown in table No. 1 and 2



Linalool (1)



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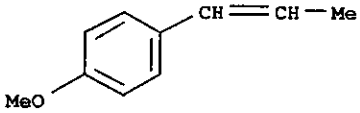
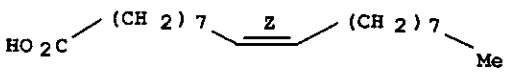
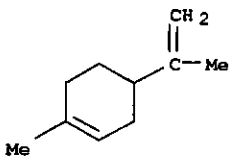


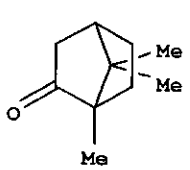
Anethole (3)



Oleic acid (4)

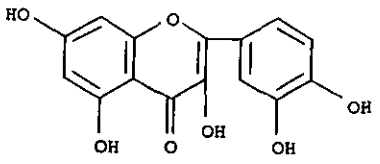


Limonene (5)

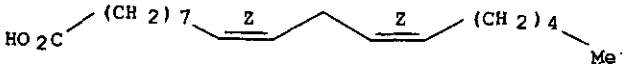


Camphor (6)

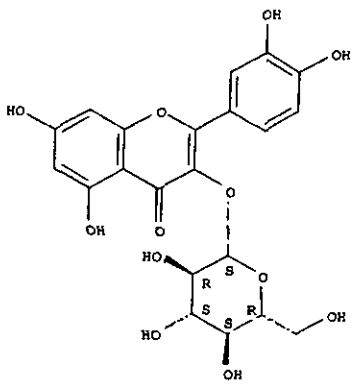
Table 1. Compounds identified from *Coriander sativum* (Cilantro) using chromatographic analysis, and spectral and chemical evidences. Linalool (1), Pinene (2), Anethole(3), Limonene (4), Camphor (5), Quercetin (6), Isoquercetin (7), Rutin (8), Oleic acid (9), Linoleic acid (10).



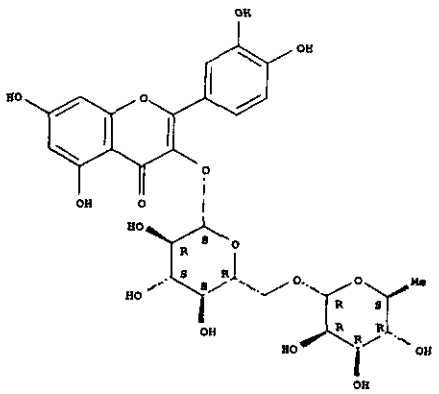
Querceting (7)



Linoleic acid (8)



Isoquercetin (9)



Rutin (10)

Table 1 (Cont.) Compounds identified from *Coriander sativum* (Cilantro) using chromatographic analysis, and spectral and chemical evidences. Linalool (1), Pinene (2), Anethole(3), Limonene (4), Camphor (5), Quercetin (6), Isoquercetin (7), Rutin (8), Oleic acid (9), Linoleic acid (10).

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Identification of plants with potential chelating properties and determination of their cytotoxicity

Literature review supports hypothesis that Mercury induces oxidative stress in mammalian cells. Therefore, the plants for this study were selected on the basis of their antioxidant properties. Total antioxidant activity assay of essential oils from selected plants was performed "in house" using Thiocyanate method. Additionally, several known compounds were isolated from cilantro. They were not put through the bioassay process, due to the time constricts. Also, screening assay to identify plants with protective activity against acute Mercury Chloride toxicity was successfully developed in human liver HepG2 cells. Initially, cytotoxicity of selected agents to HepG2 cells was determined. Non-toxic doses of materials were chosen as well as sub-cytotoxic concentrations of Mecrury Chloride for the screening. Effect of plant materials was compared to that of 2,3-dimercaptosuccinic acid (DMSA).

Test for anti-oxidative properties and cytotoxicity to mammalian HepG2 cell lines, and isolation of pure compounds from selected plants

Plant oils were extracted with hexane for later testing to determine scavenging effects of these samples. Radical Scavenging Activity (RSA) of cilantro and other selected plants and their oils was investigated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical by spectrophotometric method.

Cilantro seeds have been used for dyspeptic complaints and loss of appetite. Due to its essential oil content cilantro acts as a stomachic and spasmolytic (Wichtl and Bisset, 1994). Cilantro also has been reported to have strong lipolytic activity (Leung and Foster, 1996). Modern clinical studies have not been conducted on cilantro.

From the water-soluble portion of the methanol extract of cilantro seeds, 33 compounds have been isolated, including two new monoterpenoids, four new monoterpenoid glycosides, two new

monoterpenoid glucoside sulfates and two new aromatic glycosides compound. Their structural determination has been clarified by spectral investigation (Ishikawa et al. 2003).

Total antioxidant activity assay of essential oils from selected plants.

The total antioxidant activity of selected extracts was determined according to the Thiocyanate method (Osawa and Namiki, 1981). Twenty microliters of extracts were added to the screw-cap vials containing 0.13ml linoleic acid (approximately 60%) mixed with 5.0ml of 0.02 M phosphate buffer pH 7.0 and 5.0 ml of 99.5%(w/v) ethanol. These were mixed thoroughly and kept in the dark at 45^0C. The mixture prepared as above but without the extract served as the control. Aliquots (0.1ml) were drawn from the incubation mixture and mixed with 3.0ml of 75%(v/v) ethanol and 0.1ml of 30%(w/v) ammonium thiocyanate. Precisely 3min after the addition of 0.1 ml of 20m M ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance of the resulting red color was measured at 500 nm using a spectrophotometer (UNICAM8700 UV/VIS Spectrophotometer; Unicom Ltd, Cambridge, UK). α-Tocopherol (100mg/ml) was included as a natural antioxidant control for comparison. All data are the average of triplicate analysis. The inhibition of lipid peroxidation as a percentage was calculated using the following equation: % Inhibition= $(A_t-A_0)x100$ where A_0 is the absorbance of the control reaction and A_t is the absorbance in the presence of the extract sample (Dhuet et al. 1999).

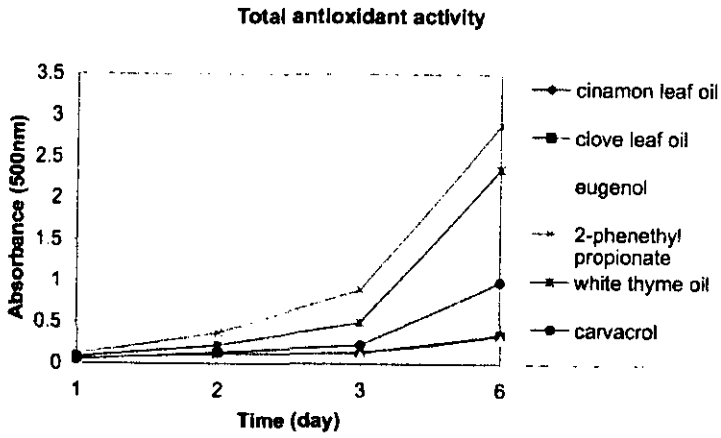

Fig 1. Essential oils and pure compounds tested for antioxidant activity included cinnamon oil

leaf, cloverleaf, white thyme oil, and pure compounds eugenol, 2-phenethyl propionate, and carvacrol. Among the essential oil tested, white thyme oil was displayed the highest antioxidant properties. The tested pure compounds are present in thyme oil.

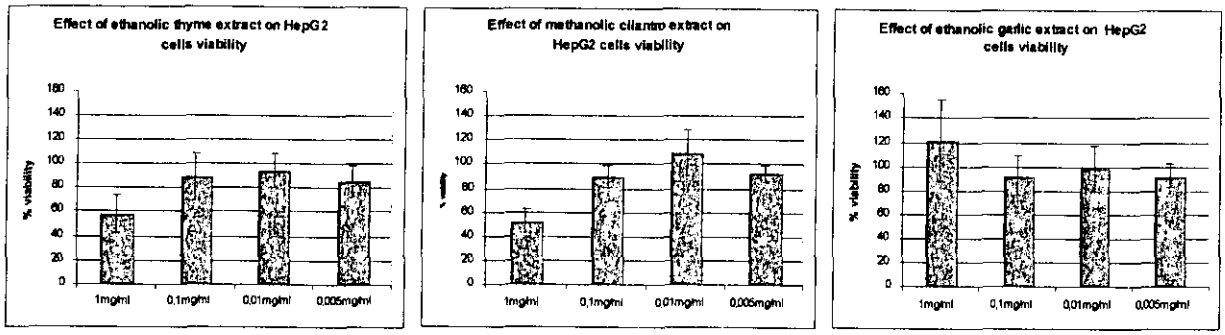

Fig 2. Determination of cytotoxicity of cilantro, thyme, and garlic alcoholic extracts using a HepG2 cell line. Toxicity to HepG2 cells is expressed as percentage of cells viability.

Possible side effects and dosage of selected plants

There are no known contraindications, interactions with other drugs, side effects, or restrictions on the use of cilantro, thyme, or garlic during pregnancy and lactation. Also, previous studies have shown cilantro's oxidative properties. Cilantro seed oil and its fractions exhibited the strongest radical scavenger activity compared to black cumin and niger seed oil (Ramadan et al., 2003).

Development of screening assay to identify plants with protective activity against mercury toxicity

HepG2 cells (ATCC No. HB-8065) were gift from UBC Chemistry Department Cell Culture collection (No.399). Mercury Chloride, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO and DMSA were purchased from Sigma, Tannic Acid from C.P.Bakers Analyzed. All cell culture media and supplements were obtained from Invitrogen.

Preparation of Cilantro extract

Fresh Cilantro was purchased from local organic market. 223g fresh material was freeze dried with the aid of Modulyo 4K freeze dryer (Edwards High Vaccum Ltd, Ontario, Canada). Obtained dry material (~20g) was extracted with the total of 1.5L HPLC grade methanol at room temperature. Solvent volume was reduced with Buchi Rotavapor R110 and finally, extract was freeze dried to yield ~4g of crude dry material.

Cell culture and cytotoxicity assay

HepG2 cells were grown in MEM-Alpha medium, containing 10% FBS and 1/100 Pen-Strep in T25 tissue culture flasks. Cells were harvested by trypsinization. After centrifugation and cell count they were diluted to 2x10^5 in the same medium and 100µl of cell suspension was distributed to each well of 96-well plates. Following morning medium was aspirated and replaced with 150µl of fresh medium to which 50µl of 4x concentrated extract, pure compound or vehicle were added. Cells were incubated for 24h in the presence of test agents prior addition of 50µl of 2.5mg MTT/ml PBS for the final MTT concentration of 0.5mg/ml. 4h incubation followed after which, cells were washed with 200µl PBS and purple Formazan was solubilized with 150µl of DMSO. Absorbance was determined at 570nM for each treatment against DMSO blank using DTX880 Multimode detector (Beckman Coulter).

The mean absorbance value for the negative control (vehicle) was calculated and used to represent 100% cell viability. The individual absorbance values from the cells undergoing the treatments with extracts, compounds or vehicle were then divided by the mean value for the negative control cells and expressed as a percent to determine the change in cell viability caused by each treatment. Cytotoxicicty assay performed in such manner allowed to identify non-toxic doses of DMSA, Tannic Acid and cilantro extract as 50µM, 50µM and 80µg/ml, respectively and sub-cytotoxic doses of Mercury Chloride ranging from 58 to 60µg/ml. Final concentration of Ethanol in the medium did not exceed 0.5% and proved to have no effect on HepG2 cells viability.

Effect of DMSA, Tannic Acid and Cilantro on acute Mercury toxicity in HepG2 cells

HepG2 cells were propagated and assayed as described above. Similar format of MTT assay was used to determine effect of tested materials on acute toxicity of Mercury Chloride. 2x10^4 cells were seeded in 100µl of medium of 96-well plates. After overnight incubation, culture medium was aspirated and replaced with 100µl of fresh medium to which 50µl of 4x concentrated extract, pure compound or vehicle were added, prior the placement of 50µl of 4x concentrated mercury solution or vehicle. DMSA, Tannic Acid (TA) and cilantro extract were dissolved in ethanol, Mercury Chloride in water. Final concentration of ethanol in the medium did not exceed 0.5%. Cells were incubated for 24h in the presence of test agents. MTT detection and calculations took place as described above. Positive control (100% cell viability) consisted of cells with vehicle only. Effect of tested materials was compared to the treatment of cells with Mercury only. Photos of individual wells representing different treatments were taken using Olympus 1X70 microscope equipped with DC330 digital camera (DAGE MTI Inc.)

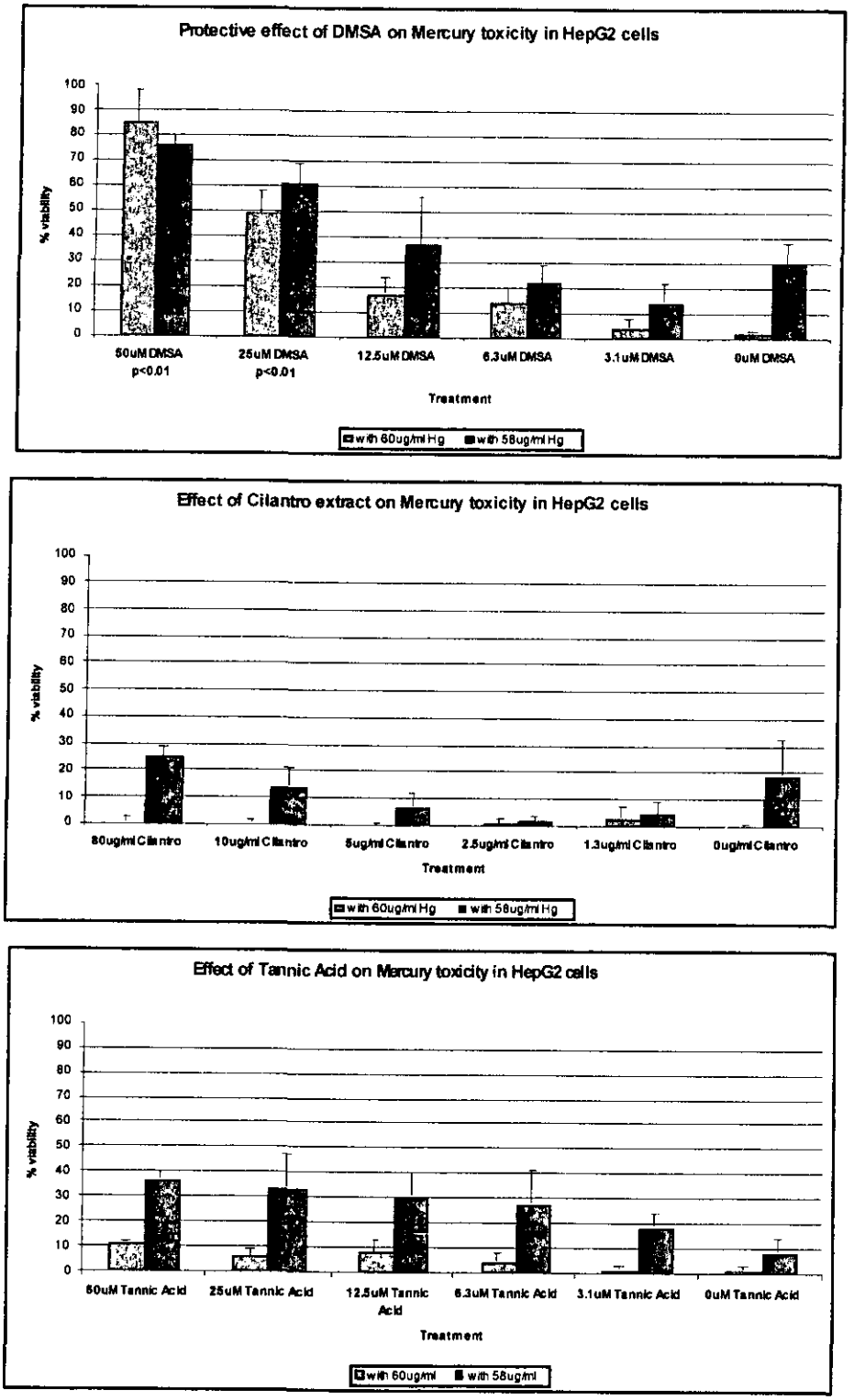

Fig 3. Effect of DMSA, Tannic Acid and Cilantro extract on acute toxicity of mercury

Figures 3 show clearly the protective effect of positive control, DMSA on acute toxicity of mercury in HepG2 cells. Two tested concentrations of DMSA: 50 and 25μM reached significance ($p<0.01$) for two sub-cytotoxic concentrations of mercury tested: 58 and 60μg/ml, which validates this bioassay. Tannic acid displayed only the beneficial trend without reaching significance. Unfortunately, the results for crude extract of cilantro are not conclusive and will require further investigation.

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The following figures show the effect of DMSA, Tannic acid, and cilantro crude extract and their protection against mercury toxicity.

List of Plates:

Plate 1. *Protective effect of DMSA on Mercury Chloride (60μg/ml) toxicity to HepG2 cells.* A: HepG2 cells without Hg and DMSA; B: HepG2 cells with 60μg/ml of Hg and without DMSA; C: HepG2 cells with 60μg/ml of Hg and 12.5μM DMSA; D: HepG2 cells with 60μg/ml of Hg and 25μM DMSA; E: HepG2 cells with 60ug/ml of Hg and 50μM DMSA; F: HepG2 cells without Hg and 50μM DMSA

Plate 2. *Protective effect of DMSA on Mercury Chloride (58μg/ml) toxicity to HepG2 cells.* A: HepG2 cells without Hg and DMSA; B: HepG2 cells with 60μg/ml of Hg and without DMSA; C: HepG2 cells with 60μg/ml of Hg and 12.5μM DMSA; D: HepG2 cells with 60μg/ml of Hg and 25μM DMSA; E: HepG2 cells with 60ug/ml of Hg and 50μM DMSA; F: HepG2 cells without Hg and 50μM DMSA

Plate 3. *Protective effect of Tannic acid on Mercury Chloride (60μg/ml) toxicity to HepG2 cells.* A: HepG2 cells with 60μg/ml of Hg and without TA ; B HepG2 cells without Hg and TA:; C: HepG2 cells with 60μg/ml of Hg and 12.5μM TA; D: HepG2 cells with 60μg/ml of Hg and 25μM TA; E: HepG2 cells with 60ug/ml of Hg and 50μM TA; F: HepG2 cells without Hg and 50μM TA

Plate 4. *Protective effect of Tannic acid on Mercury Chloride (58μg/ml) toxicity to HepG2 cells.* A: HepG2 cells without Hg and TA; B: HepG2 cells with 58μg/ml of Hg and without TA; C: HepG2 cells with 58μg/ml of Hg and 12.5μM TA; D: HepG2 cells with 58μg/ml of Hg and 25μM TA; E: HepG2 cells with 58ug/ml of Hg and 50μM TA; F: HepG2 cells without Hg and 50μM TA

Plate 5. *Protective effect of Cilantro on Mercury Chloride (60μg/ml) toxicity to HepG2 cells.* A: HepG2 cells without Hg and Cilantro; B: HepG2 cells with 60μg/ml of Hg and without Cilantro; C: HepG2 cells with 60μg/ml of Hg and 10μM Cilantro; D: HepG2 cells with 60μg/ml of Hg and 80μM Cilantro; E: HepG2 cells without Hg and 10μM Cilantro F: HepG2 cells without Hg and 80μM Cilantro

Plate 6. *Protective effect of Cilantro on Mercury Chloride (58μg/ml) toxicity to HepG2 cells.* A: HepG2 cells without Hg and Cilantro; B: HepG2 cells with 58μg/ml of Hg and without Cilantro; C: HepG2 cells with 658μg/ml of Hg and 10μM Cilantro; D: HepG2 cells with 58μg/ml of Hg and 80μM Cilantro; E: HepG2 cells without Hg and 10μM Cilantro F: HepG2 cells without Hg and 80μM Cilantro

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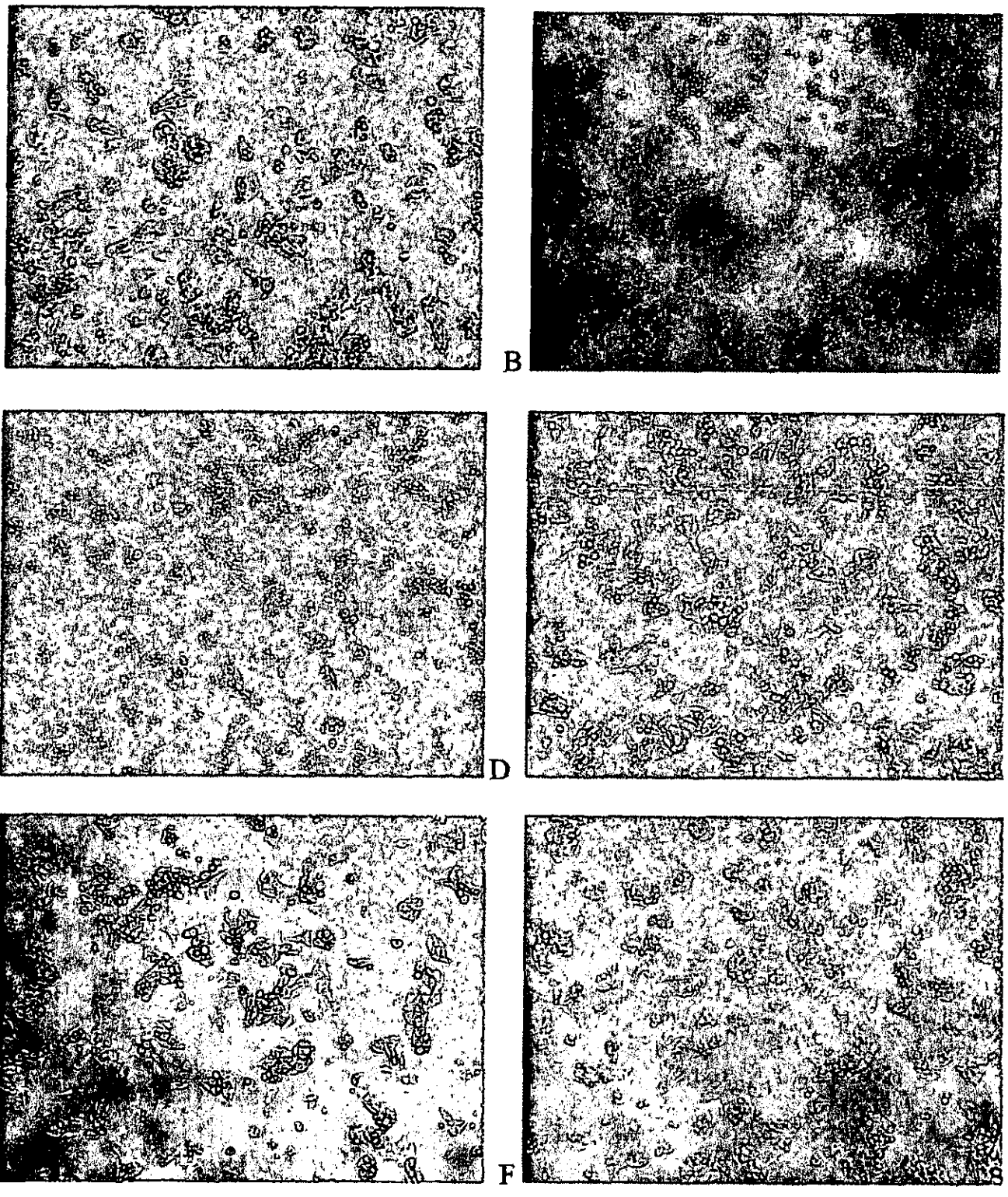


Plate 2. Protective effect of DMSA on Mercury Chloride (58µg/ml) toxicity to HepG2 cells
A: HepG2 cells without Hg and DMSA; B: HepG2 cells with 60µg/ml of Hg and without DMSA; C: HepG2 cells with 60µg/ml of Hg and 12.5µM DMSA; D: HepG2 cells with 60µg/ml of Hg and 25µM DMSA; E: HepG2 cells with 60ug/ml of Hg and 50µM DMSA; F: HepG2 cells without Hg and 50µM DMSA

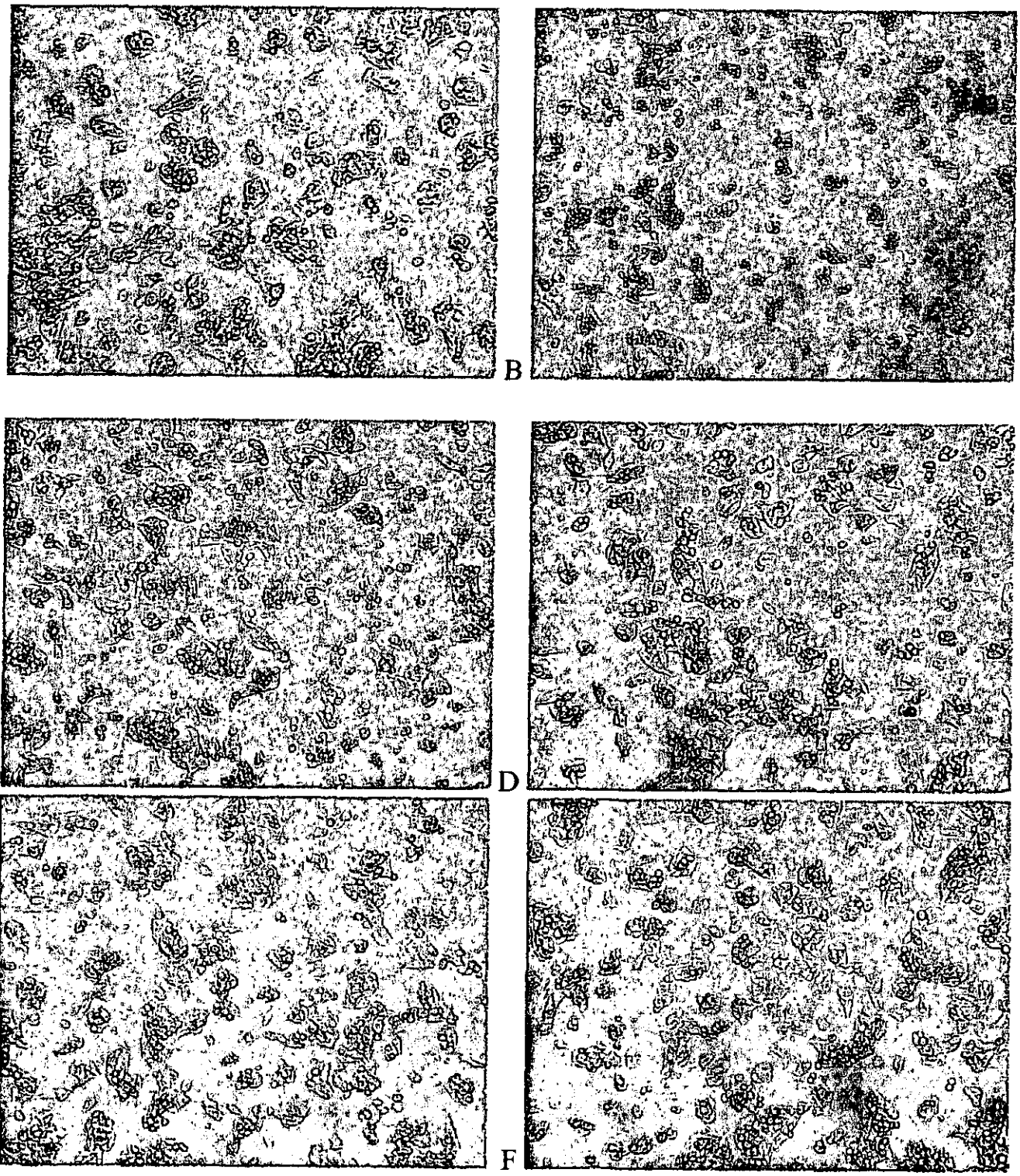


Plate 3. Protective effect of Tannic acid on Mercury Chloride (60µg/ml) toxicity to HepG2 cells
A: HepG2 cells with 60µg/ml of Hg and without TA ; B HepG2 cells without Hg and TA:; C: HepG2 cells with 60µg/ml of Hg and 12.5µM TA; D: HepG2 cells with 60µg/ml of Hg and 25µM TA; E: HepG2 cells with 60ug/ml of Hg and 50µM TA; F: HepG2 cells without Hg and 50µM TA

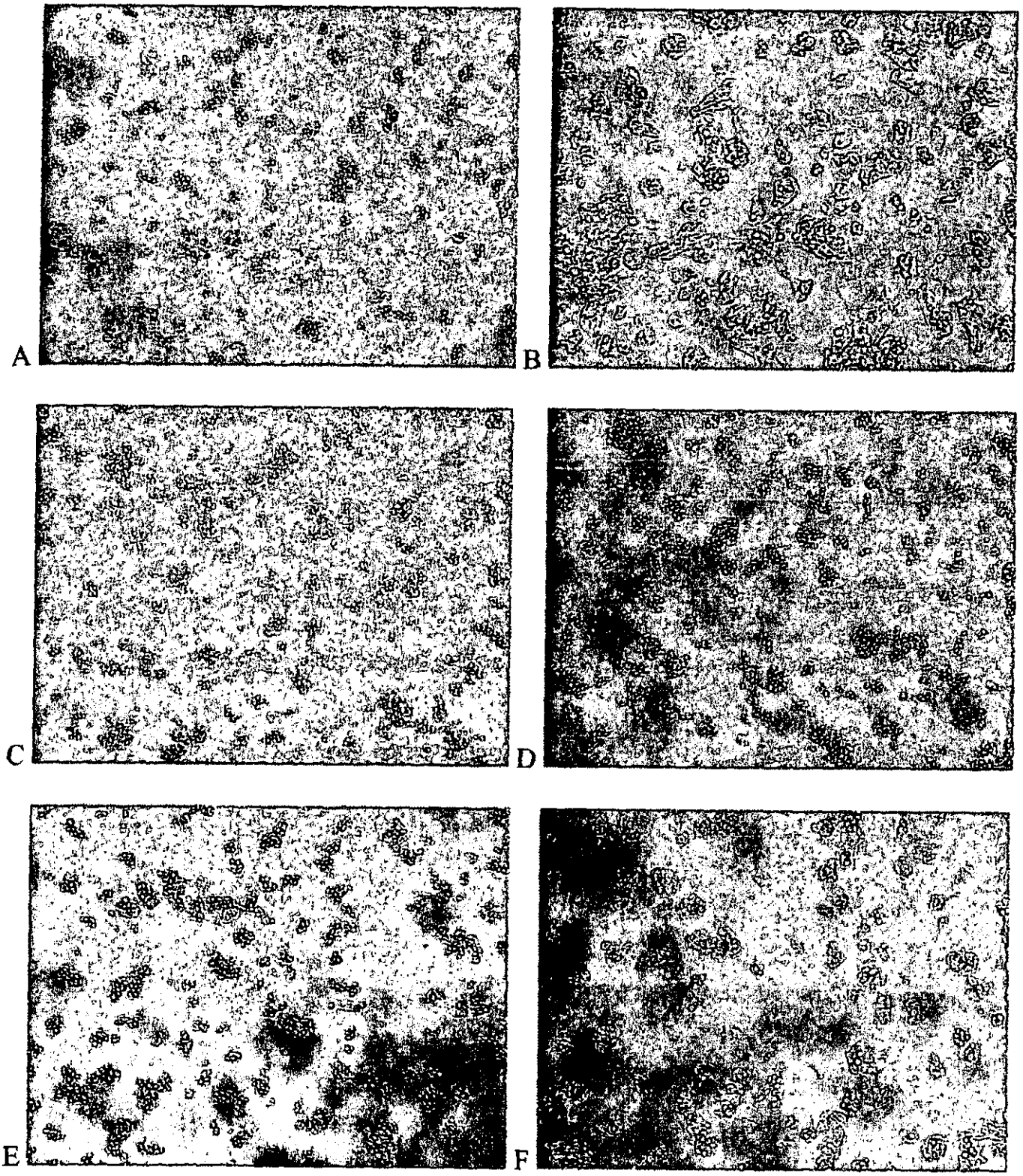


Plate 4. Protective effect of Tannic acid on Mercury Chloride (58µg/ml) toxicity to HepG2 cells
A: HepG2 cells without Hg and TA; B: HepG2 cells with 58µg/ml of Hg and without TA; C: HepG2 cells with 58µg/ml of Hg and 12.5µM TA; D: HepG2 cells with 58µg/ml of Hg and 25µM TA; E: HepG2 cells with 58ug/ml of Hg and 50µM TA; F: HepG2 cells without Hg and 50µM TA

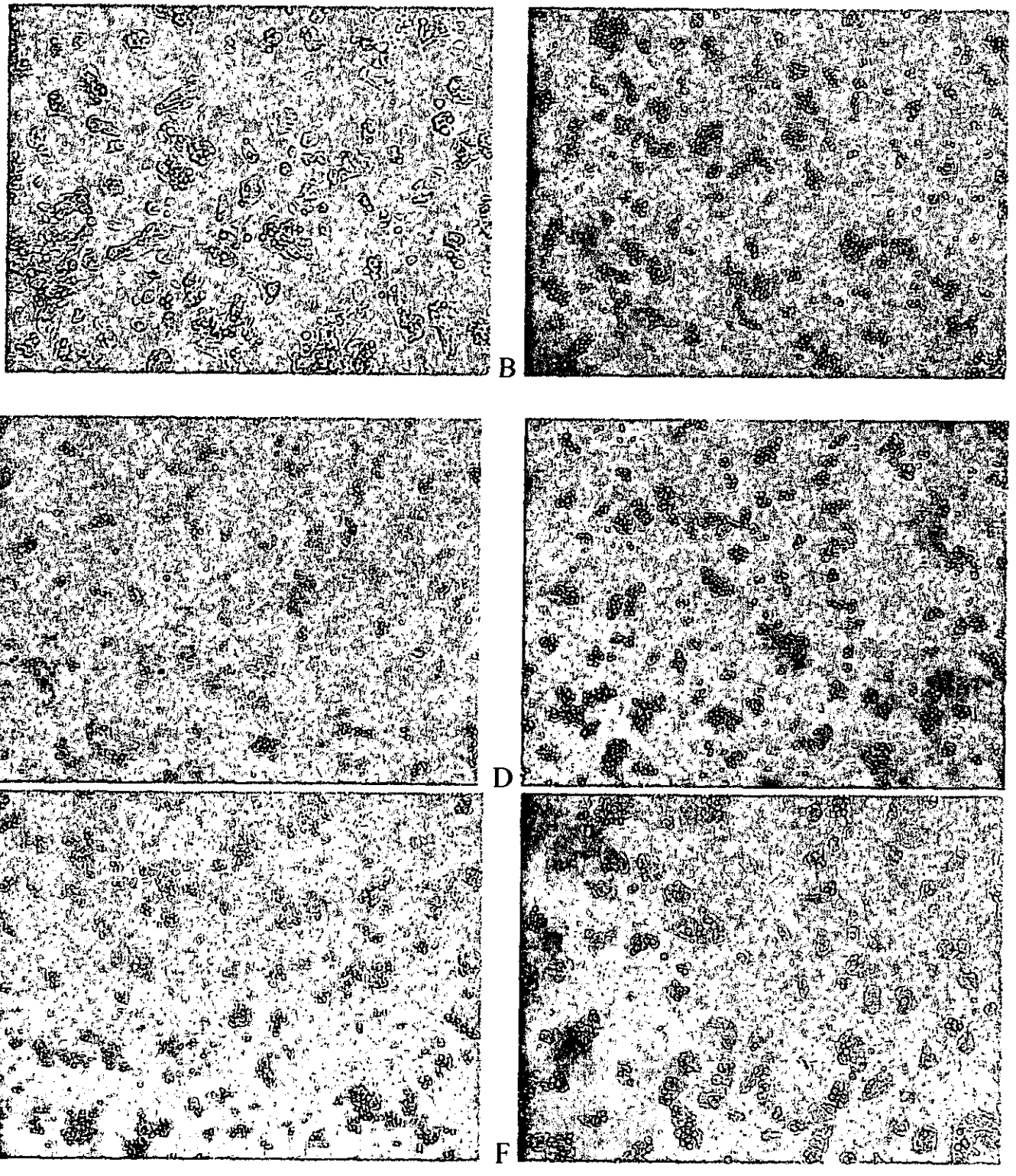


Plate 5. Protective effect of Cilantro on Mercury Chloride (60μg/ml) toxicity to HepG2 cells
A: HepG2 cells without Hg and Cilantro; B: HepG2 cells with 60μg/ml of Hg and without Cilantro; C: HepG2 cells with 60μg/ml of Hg and 10μM Cilantro; D: HepG2 cells with 60μg/ml of Hg and 80μM Cilantro; E: HepG2 cells without Hg and 10μM Cilantro F: HepG2 cells without Hg and 80μM Cilantro

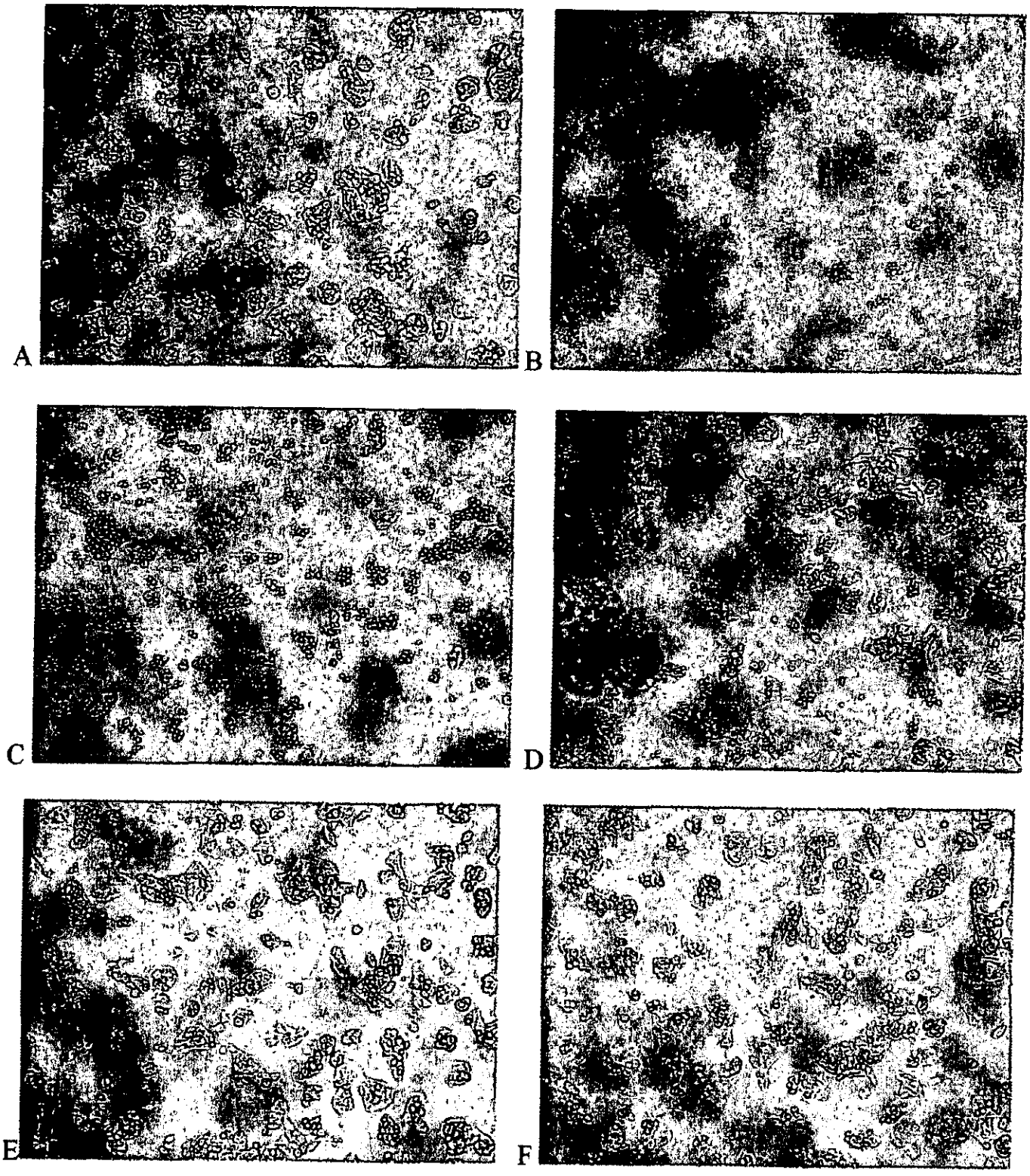
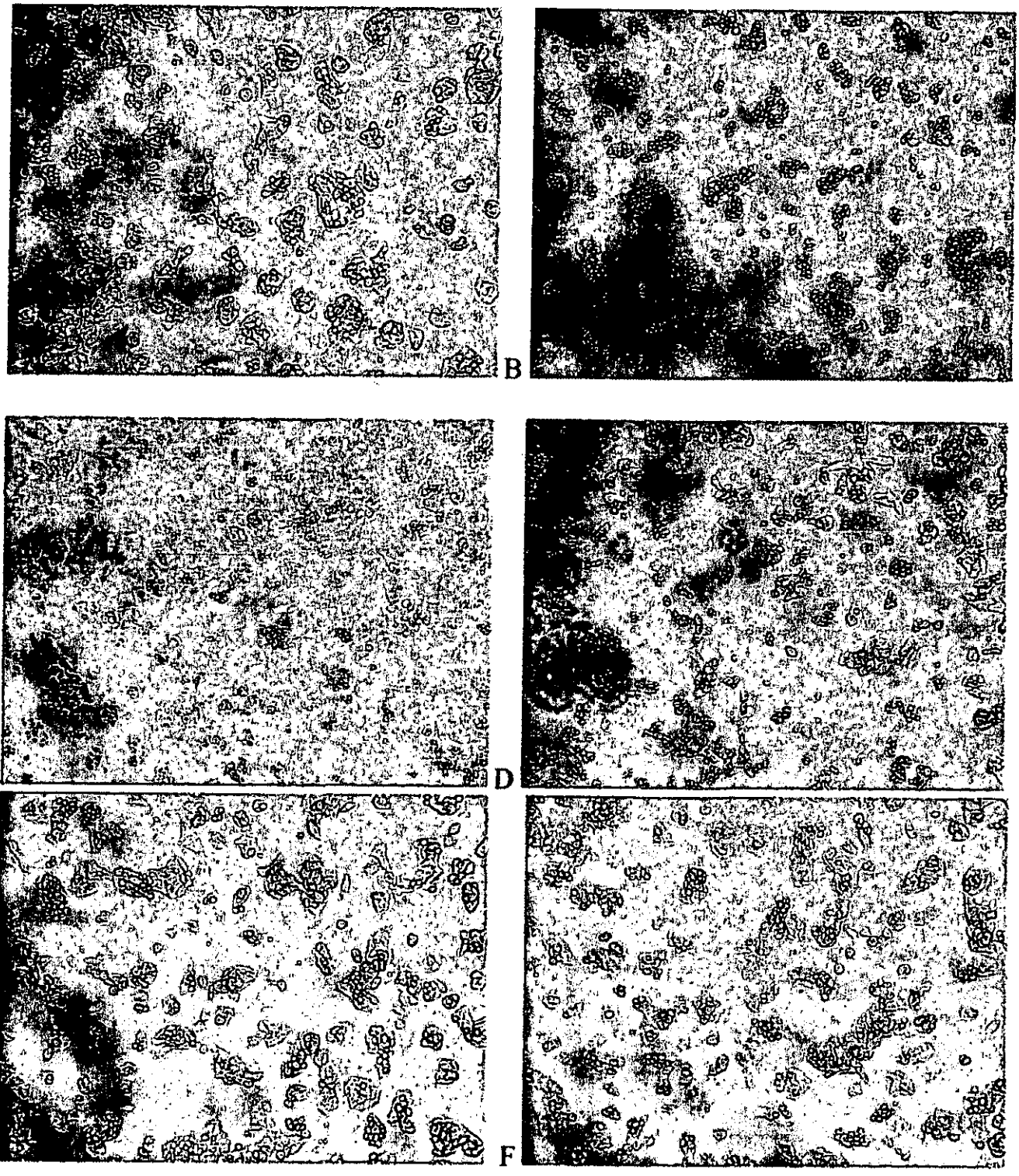


Plate 6. Protective effect of Cilantro on Mercury Chloride (58μg/ml) toxicity to HepG2 cells
A: HepG2 cells without Hg and Cilantro; B: HepG2 cells with 58μg/ml of Hg and without Cilantro; C: HepG2 cells with 658μg/ml of Hg and 10μM Cilantro; D: HepG2 cells with 58μg/ml of Hg and 80μM Cilantro; E: HepG2 cells without Hg and 10μM Cilantro F: HepG2 cells without Hg and 80μM Cilantro



Lessons Learned and Final Recommendations

We investigated cilantro and other selected plants, for their chemical composition and potential use in assisting mercury-exposed individuals. The conducted work contributed to the objectives of the UNIDO Global Mercury Project (GMP) seeking to introduce affordable and accessible solutions to reduce the negative health impacts of mercury exposure. Our work aimed to provide effective, affordable and culturally relevant methods to detoxify mercury-poisoned individuals, while mitigating the often-inaccessible solutions developed by pharmaceutical companies. As the poverty in the developing world increases and international actions to address it lacks coordination, the amount of mercury released into the environment from artisanal gold mining activities is expected to continue increasing.

Our pilot project was limited by time, resources, and technical difficulties of the problem, which impacted the production of proposed educational materials; on the other hand, even though public dissemination is essential, our findings are preliminary and may not be recommended for public at this time. Further investigation will be required to determine the full potential of selected plants, particularly cilantro, as a source of mercury chelating agents. Our preliminary results are encouraging and significant and further research in this area is recommended.

As previously described in our reports, we developed a reproducible bioassay using DMSA as a control capable to offer protection against acute mercury toxicity to mammalian cells cultures. This was the very first step required fro the implementation of a reliable method that allows for the testing of botanicals for potential chelating properties. Our approach focused on traditional foods consumed by a large population in the world. We used is a strategy as we tried to identified plants with high cultural acceptance and significant in the diet of the addressed populations. Furthermore, the selected plants are easy to cultivate, transport, and most of them are essential part of contemporary and traditional diets. Our preliminary study demonstrates that some traditional plant foods may effectively protect from mercury exposure and improve overall health. Particularly useful are those plants having antioxidant properties and as it seem to correlate to chelating activity. While some previous work has addressed the use of cilantro in mice, more detailed analysis is required using human cell parameters. Experimental work with other plants may also assist in our understanding of the mechanisms of mercury-binding molecules and their potential health benefits.

Our study focused mainly on cilantro, garlic, and thyme. Most of the work was conducted using cilantro, which is used traditionally as a carminative or digestive component of compounds in confection, infusion, syrup, and tincture dosage forms.

The evaluation of the gastronomic and nutritional ways to stimulate consumption of mercury-chelating food plants may be required to promote wide consumption. There are a wide number of recipes available, from different countries, and in combination of foods. The effect of cooking or food preparation will required further examination, as factor including temperature, fresh or dried, synergistic effect with other foods, and others may impact the chelating biological activities. These areas have not been examined yet as this will require time and resources for further investigation. However, from our preliminary study?, fresh consumption of cilantro, garlic, and thyme? Is there at least support in literature for thyme?, maybe recommended.

Fortunately, some of these plants are already incorporated in a fresh from in the preparation of many dishes. The main factor influencing the potential beneficial effect as heavy metal chelators may be related to the antioxidant content. Many of these antioxidants could be destabilized when exposed to temperature, extreme pH changes, and other factor related to cooking procedures.

The following technical recommendations are incorporated to this final report for consideration when conducting further research related in botanical Hg-chelating agents.

Technical recommendation for further development of biological assays to evaluate Hg-chelating agent from food plants:

- Whole blood, liver cells (HepG2) or renal cells (MDCK) are recommended as in vitro model. MTT and/or SRB assay should be used for cytotoxicity screening. For MTT assay plate reader with a 570-600nm filter is necessary. For SRB assay plate reader with 565nm filter is required.

- Oxidative stress of the cells can be determined either by ROS, Gpx or CAT. ROS is determined by fluorometric reaction (excitation=485nm, emission=535), for Gpx and CAT commercial kits are available. Plate readers with filters 340 and 240nm are required.

- Effect of potential plant chelators on ∂-ALA-D activity can be determined enzymatically. ∂-ALA-D. Plate reader with 535nm filter is required. Alternatively, the protective effect of plants against mercury damage can be assesed by following ∂-ALA-D activity in human Whole Blood.

- MDCK cells would be a perfect nephrotoxicity model for testing plant extracts for their capacity to reverse mercury interference in establishing functional gap junctions of renal monolayers. Fluorometer (excitation=485nm, emission=530nm) and costly Transwell-COL membrane culture inserts is required.

- Initially, evaluation of Hg-chelating properties from plant sources could also be done by chemical method by testing it's "sorbing" properties in comparison to randomly selected plants. Influence of potential plant chelator on essential heavy metals should be investigated as well (Ca, Zn, Mg and Cu).

- Quantification of mercuric ions should be examined by cold vapour atomic absorption spectrometry or spectrophotometrically to confirm chelating properties of tested agents.

- DMSA should be used as positive control, as demonstrated in our experimental phase. Antioxidant properties and high content of tannic acid in plants is a good indicator and should be used as one of the criteria when selecting plants for further studies. Equipment required for experimental: CO_2 incubators, Biosafety cabinet, inverted microscope, hematocytometer, centrifuges, calibrated single and multichannel pipettes, freezer and refrigerator, 96-well plate reader and/or fluorometer, spectrophotometer, heavy metal analyzer.

Lesson learned from our study and final considerations:

We hope our contributions can stimulate further research on Hg-chelating agents from botanicals. In spite of the limitations of our study, we are encouraged to continue seeking for practical solutions to health problems faced by vulnerable populations, particularly artisanal miners. Furthermore, we wish to continue supporting the dialogue among researchers, policy-makers, educators, and other stakeholders interested in assisting initiatives implemented by the Global Mercury Project, UNIDO. To this aim, the following considerations and lessons learned from our study are presented here:

- Meso-2,3-dimercaptosuccinic acid (DMSA) is a sulfur-containing organic compound that is FDA approved for the treatment of lead and mercury toxicity both in children and adults. Although it is sold as a prescription drug, it is also available as a dietary supplement. However, due the high cost, this is a not practical solution for artisanal miners and other populations exposed to mercury in poor countries, including those considered under the Global Mercury Project, UNIDO, other alternatives remain necessary.

- It has long been recognized that sulfur-containing compounds have the ability to chelate heavy metals. Within the normal operation of the human body, there are natural sulfur-containing compounds that perform this service, and these include N-acetyl cysteine (NAC), R-lipoic acid, S-adenysyl methionine (SAMe), and glutathione (GSH). Focusing in plant containing sulfur compounds is recommended for future studies.

- Meso-2,3-dimercaptosuccinic acid (DMSA), does not occur naturally in the human body, nor is it a constituent of food. In our experimental design we selected DMSA as the control compound for the development of a suitable bioassay to asses the potential of selected plants as sources of mercury chelating agents. From our results we recommend the use of DMSA as a control, even if other control compounds are selected as alternatives.

- It can be said that DMSA is the least toxic of the dithiol compounds. Numerous animal and human studies have shown DMSA administration increases urinary mercury excretion and reduces blood and tissue mercury concentration. Animal studies may be used for the evaluation of chelation and excretion

- Protection against mercury toxicity can be determined quantitatively by measuring the amount of mercury excreted in the urine after a challenge dose of a potential botanical Hg-chelating agent. A baseline 24-hour urine is collected before the challenge with a selected plant, then again on day three of a three-day dosing of 200 mg three times a day.

- Plant proteins containing a high amount of cysteine and cysteine residues could be of benefit as chelating agents and should be considered in future studies. For example, garlic and crucifers (e.g. broccoli, cabbage, cauliflower).

- We used hepatic cell (HepG2) in our experimental, which allowed us to determine if a selected plant may offer some protection to the liver. A decrease in hepatic glutathione

resulting from mercury excretion can compromise other hepatic functions dependent upon glutathione.

- The organic mercury species with greatest toxicity are methylmercury compounds, which have a high affinity for the brain and nervous system. Further studies should include brain cells (e.g. oligodendrocyte cell lines and neonatal rat brain oligodendrocytes) to determine the protective effect of botanical with potential mercury chelating properties.

- If animal or human studies could be designed in further research a complete blood count should be included to analyze the different components of blood: white blood cell count (WBC), red cell count (RBC), hemoglobin (Hb), mean cell hemoglobin concentration (MCHC), and red cell distribution width (RDW). These parameters will be good indicators of the protective effect of chelating agents and overall health of tested animals.

- Hg accumulation in plants has been studied in several species. Pea and spearmint absorb Hg from solution, and roots accumulate much greater amounts of Hg than shoots. Similar results have been found in Norway spruce, willow, and aquatic plants. These plants could also be good candidates for future studies in the search of mercury chelating agent for human consumption.

- Microscopic studies using fluorescent probes may give an indication of the mechanisms of action of plants with potential chelating activity.

- The evaluation of extract containing a combination of food plant extracts should be performed to identify potential synergistic effects.

- Limitations 1: Laboratory-based studies are costly and more resources need to be allocated for this type of the project. It should be mentioned that the Aboriginal Health and Natural Product Chemistry Laboratory provided in-kind contributions to the completion of this project..

- Limitation 2: mammalian cell lines are good indicators of potential heavy metal chelating agents; however, do not allow the evaluation of excretion of chelating complexes formed between plant constituents and mercury. This problem could be resolve by using animal models as previously suggested.

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