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Editorial

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Forensic Toxicology: The Dangers of Chasing the Single Molecule

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On depictions dating from the Middle Ages, physicians are often shown holding a glass with a yellowish liquid: urine, the most accessible biological sample. Dipping their finger into the fluid and then licking it allowed them to taste sugar (if it was present) and therefore to diagnose diabetes. Mothers of babies with cystic fibrosis tasted the saltiness of their offspring on kissing them and knew that something was terribly wrong. The sweet smell of ketones would announce the imminent demise of patients in diabetic coma.

From the early primitive use of our senses to identify sweet, salty, or aromatic substances (and diagnose conditions), we have come quite a long way. Today, forensic toxicology – the analysis of biological samples for the presence of toxins, including drugs – is a recognized and respected discipline.

The progress made by technology in the area of forensic toxicology is truly amazing: the limits of detection are continuously being pushed lower and lower. Indeed, we are not far away from being able to identify the presence of single molecules in a sample. While such analytical miracles based on solid technology have robust and reproducible results (may be not if one indeed has single molecules in a sample), the interpretation of results is far from simple. At one period in my life, I had the opportunity to travel to exotic, faraway kingdoms; before entering the foreign places I had to fill out immigration forms where the warning "Death penalty for drug possession" was indeed printed in bold red letters. Not that I ever used opiates, but I was well aware that some of our potential intestinal parasites were able to produce traces of morphine in order to reduce our ability to get rid of them through defecation (i.e., to induce constipation).[1,2] Now imagine the following scenario: for whatever reason, a biological sample from your body is analyzed and found positive for traces of morphine. You are brought in front of a judge, and your defense line sounds like this: "*I didn't do it, your honor; it was my intestinal parasites*." To make things worse, the most efficient morphine producer is apparently the nematode Ascaris suum, the large roundworm of pigs (and people).

Not likely to elicit sympathy in the aforementioned judge. Start composing your obituary. Of course, such a thing could never happen to me, since I decided to use mebendazole prophylactically. But there are plenty of other options to get in trouble…..

While I am not a vegetarian, I could in theory consume large amounts of healthful foods not realizing the associated risks. Many vegetables and grains produce and therefore contain benzodiazepine sedative-hypnotics.[3,4] Now imagine the following scenario: after a car accident in which you are involved, a biological sample is analyzed and found positive for diazepam. The line "*I ate too many potatoes*" will not help you keep your driving license or your fortune (if you live in a society enamored of litigation and run by lawyers).

But that was only a benign scenario; let's figure out a way to get on the coveted "*No fly list*" that is regularly updated by Homeland Security. What about traces of organophosphates (cholinesterase inhibitors; ChE-I) in your luggage?

While non-organophosphate inhibitors of cholinesterase are very common in nature,

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their numbers running in the hundreds,[5] it has only recently been recognized that potent pesticide and nerve gas – like organophosphate inhibitors of cholinesterase also occur in nature, produced by bacteria, algae, and marine sponges, and possibly by many other creatures. Rainer Neumann and Heinrich H Peter, working in Switzerland for Ciba-Geigy, were the first to point out that "Nature made them first": they isolated two related furo-dioxa-phosphepin cholinesterase inhibitors from cultures of the soil microorganism Streptomyces antibioticus.[6] The identification of a closely related compound named cyclophostin, from Streptomyces lavendulae, was reported a few years later by a Japanese group.[7] A structurally different imidazole phosphor ester named anatoxin-A(s), produced by green-blue algae, was also reported.[8] Finally another imidazole phosphor ester, ulosantoin, was isolated and identified from a marine sponge named Ulosa ruetzleri (Orange Lumpy Encrusting Sponge); the compound has an ChE inhibitory potency comparable to that of paraoxon, in the low nanomolar range.[9]

In conclusion, while our ability to identify substances at lower and lower concentrations, virtually chasing the single molecule, is impressive to say the least, results need to be interpreted with caution and in context. Possible explanations for the presence of restricted substances in biological samples, although sometimes really weird and unlikely, nevertheless need to be explored. One must never forget that truth is, in the words of Lord Byron, "*stranger than fiction*".[10]

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Toxicity of Antioxidants

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The fundament of toxicology is the risk-benefit analysis. This means that the positive as well as the negative health effects of all compounds, including antioxidants, have to be assessed and related to each other. Only in this way the rational for the use of a compound can be evaluated.

The beneficial effects of antioxidants are due to their ability to protect against free radical damage. In the diseases and other pathological conditions where free radicals are implicated, the biochemistry may greatly vary, i.e. the radicals involved, their flux, the site where they are generated and the target they attack can differ.[1] Thus for each type of radical damage specific criteria need to be fulfilled by an antioxidant in order to be active. Free radicals of biological interest are often divided into oxygen centred and nitrogen centred radicals. Reactive Oxygen Species (ROS) is a collective term that includes both oxygen centred radicals and certain oxygen containing non-radicals that are oxidizing agents or easily converted into radicals. In the Reactive Nitrogen Species (RNS) the reactivity of the species is located on or near a nitrogen atom. The adjective "reactive" is not always appropriate; H_2O_2, O_2•- and NO• quickly react only with very few molecules. OH• reacts instantaneously with almost everything. RO_2•, RO•, HOCl, NO_2•, ONOOH, and O_3 have a reactivity that lies in between these extremes.

Regarding safety, in the 16th century Paracelsus already stated that all compounds are toxic, provided the dose is high enough. There is no reason to assume that antioxidants should be an exception to this rule. On the contrary, the mega-dosages sometimes recommended make safety a very relevant issue for antioxidants. The biological origin of most antioxidants helps to increase the acceptance of antioxidant supplementation by the general public. The perception is that the biological origin guarantees that antioxidants are not harmful; biological and natural is incorrectly thought to be synonymous to safe.[2]

The adage on the use of antioxidant supplementation seems to be: The more, the better. Indeed, antioxidant supplements are taken frequently by a great part of the public at a relatively high dose despite our limited knowledge on their beneficial health effect and safety. From a toxicological point of view, the free radical processes as well as the profiles of antioxidants have to be elucidated to a greater extend to be able to further rationalize and optimize antioxidant therapy.[3]

An important issue in the use of antioxidants is metabolism.[4] Like other bioactive compounds, metabolites formed by phase 1 and phase 2 enzymes can contribute to the beneficial and toxic effect of an antioxidant. More importantly, during the actual antioxidant activity of a free radical scavenging antioxidant, it is converted into a metabolite.[1] The effect of such a metabolite on a biological system is relevant since the metabolite usually contains some residual reactivity of the radical that has been scavenged.

According to 2013, Annual Report of the American Association of Poison Control Centers (AAPCC): National Poison Data System (NPDS), more than 65,000 instances of vitamin toxicity are reported annually to US poison control centers.[5] Unintentional and intentional exposures continue to be a significant cause of morbidity and mortality in the United States.[4] It

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is of importance to identify groups that are at risk. In spite of the focus on the adverse effects of antioxidants and vitamins, priority should be given to identify groups that are likely to benefit.[6]

The bottom line is that in the appraisal of antioxidants, their benefits must first be identified and substantiated by elucidating the molecular mechanism. The risks must then be identified the molecular mechanism. The optimal benefit-risk ratio has to be determined for each antioxidant and each individual separately, also taking into account, the dose.[7]

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interests.

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Editorial

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In Vitro Toxicology Testing: It's Time to Report the Sex of Cells

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In light of evidence taken from numerous fields indicating that males are routinely used more than females as test subjects,[1-3] and the resulting poor outcomes of such practice,[4,5] a conversation on balancing the sex of sample sets is not a new one. In a 2014 meta-analysis of nearly 300 studies, Prendergast and colleagues discovered that females have erroneously been considered more variable owing to their estrous cycle; instead, males were found to be more inconsistent under some experimental conditions.[6] These results were subsequently confirmed by meta-analysis of microarray datasets.[7] If we are to agree that sex of the subject (be it animal or human) is important to disclose, then the lack of representation to this day is troubling – and it is furthermore troubling that many studies fail even to report the sex of tissues or cells, meaning we have no idea as to the relevance of sex to measured outcomes. There have been several calls to action on this point in the biomedical field,[3] yet we as a toxicology community have not yet been fully persuaded of our failure.

In toxicological studies, cultured cells are routinely used to identify molecular mechanisms driving chemical actions in our environment, the results of which are foundational to designing whole-animal experiments (using so-called "tiered testing") and developing toxicological profiles that provide guidance by predicting harm.[8] As part of sound experimental design, researchers often report several specifics of cultured cells, such as species origin and media conditions, but regularly do not report the sex of their cells,[9] and are seemingly not challenged on this point by peer reviewers and publishing friends.

This invisibility cloak on sex reporting exists and extends beyond any semblance of welcome, despite the fact that every somatic cell indeed has a sex, and sex-specific cell behavior can occur without considering gonadal hormone exposure history. As such, sex chromosome complement, especially in cells studied in culture, has tremendous potential to impact gene expression and resulting mechanistic signaling pathways,[10] and therefore stands to have significant implications for risk assessment, disease susceptibility, and harm prevention.

The sex of cells can clearly matter, and matter in a big way, and the brain has been particularly well studied for its sexual dimorphism at this level. For example, isolated dopaminergic neurons display different morphologies, independent of hormonal status, depending on whether they come from males or females,[11] and male hippocampal neurons are more likely than female ones to be killed by the oxidizer peroxynitrite.[12] Still other studies using embryonic neurons from male and female rats have found males to be more sensitive to ischemic environments, while females have been found more sensitive to apoptotic agents.[13] Regarding exposure to potential toxicants, female murine cells have demonstrated greater sensitivity than their male counterparts to ethanol.[14] This partial list will no doubt continue to grow as more researchers seek to validate or eliminate sex as an experimental factor. Indeed, as others have suggested,[15] the identification of cell sex may explain why observed effects for "identical conditions" may result in dissimilar findings. Should we really expect a cell derived from a female to behave exactly the same as one derived from a male?

Sexual dimorphisms of course exist for cells beyond the brain, and dimorphic cell responses can occur through specific metabolic pathways. For example, researchers have found

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evidence that human cells display a wide variety of different metabolites across the sexes, such as the Carbamoyl-phosphate synthase 1 (CPS1) gene, involved in protein and nitrogen metabolism.[16] This is in keeping with other basic research that has repeatedly shown that males and females metabolize drugs differently.[17,18]

Moving forward, we should hold ourselves to a higher standard of awareness. In a 2015 report by Wan and colleagues,[19] researchers used quantitative Polymerase Chain Reaction (qPCR) – a very common molecular method – to newly identify NIH/3T3 (ATCC No.: CRL-1658) murine embryonic fibroblast cells as female; 3T3 cells have long been used in toxicological experiments, most recently to assess developmental toxicity to therapeutic drugs[20] and phototoxic potential,[21] but also to document cell behavior on nanotubes,[22] response to nanoparticles,[23] and antioxidant response.[24]

Importantly, sex determination using qPCR is now added to the list of several other available tools that can be used to determine the sex type of cultured cells, including immunodetection of H-Y antigen,[25] nested PCR,[26] Southern blot,[27] and enzymatic assays.[28] It should also be noted that it is likely that sex of a cell must be determined more than once over prolonged passage in culture – e.g., many "male" cells, such as the T-84 colonic carcinoma epithelial cell line, have been found to have lost the Y chromosome over time.[15]

Especially in this era of genomics, understanding sex and gender in science seems fundamental and relevant to basic and applied sciences. Enhanced reporting efforts that clearly identify the cell source as female or male would not only enrich our understanding of risk assessment and mechanisms of action for chemical toxicity, but might also contribute to building a new framework with which to understand complex chemical interactions. In the end, sex is a biological variable that could affect measured endpoints – and is just as relevant as other experimental features such as culture conditions.

In summary, there seems to exist a pervasive assumption that the sex of cells in culture in not important,[29] even in the face of mounting evidence that sex influences gene expression in cultured cells, tissues, and beyond.[30-32] Potential benefits of identifying cell sex have been raised for biomedical science writ large,[8,33] and here, now, for toxicology more pointedly. Yet, unless it is the focus of their research, most investigators rarely consider whether cells bearing an XY genotype will behave the same as an XX genotype. It is not expected that sex will play a role in every experimental outcome, but at the very least it should be documented as a biological factor and eliminated as having a significant influence on experimental data.

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Editorial

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Toxicology of Mixtures - Cd +PCBs Experimental Model

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Humans are exposed to hundreds of thousands of chemicals from very different sources and the presence of more than 200 xenobiotics has been so far determined in human blood or urine.[1] Hence, exposure assessment and toxicological evaluation should be focused on mixtures rather than on single chemicals. The importance of "cocktail effects" evaluation is summarized in European Commission statement that highlighted that even low level exposure to a complex cocktail of pollutants over decades can have significant effect on the health status of European citizens.[2] Although, toxicity studies and risk assessments are focused on single chemicals, research on the toxicology of mixtures have emerged decades ago. Actually, more than thirty years ago United States Environmental Protection Agency (EPA) published Guidelines for the Health Risk Assessment (HRA) of Chemical Mixtures[3] and after a few years Technical Support Documents and Guidance Manuals[4,5] followed by the efforts of Agency for Toxic Substances and Disease Registry (ATSDR)[6] and Organisation for Economic Cooperation and Development (OECD).[7] However, although a certain progress has been made, the toxicology of mixtures remains a matter of great concern and challenge for the scientific community.

Indeed, to study and assess the potential health risks of "cocktails of pollutants" properly, it is of paramount importance to understand the basic concepts of joint action and interactions of chemicals. Components of a mixture can act independently in the body leading to the addition of doses or responses, or their actions can combine thus leading to stronger - synergistic or weaker - antagonistic response.[6,8,9] These combined actions of mixture compounds resulting in response different than expected by additivity are defined as interactions and can be of toxicokinetic or toxicodynamic type. Furthermore, the investigations on the toxicity of chemical mixtures provide evidence that both chemicals with similar or dissimilar modes of action may produce combined effects at doses below their No Observed Effect Level/No Observed Effect Concentration (NOEL/NOEC), suggesting that a mixture can produce a toxic effect not observed for any component of the mixture.[10]

One of the biggest challenges that toxicology of mixtures is facing today, is to define adequate model for the mixture toxicity evaluation. The choice of the study design for chemical mixture toxicity assessment is influenced by the number of chemicals in the mixture, dose-response relationship for single chemicals and their mechanisms of toxicity i.e. ability to interact. Risk of chemical mixtures can be assessed by using whole mixture approach in which mixtures are evaluated as single entities or by using component-based approach such as dose addition, response addition or approach in which interactions between components are also considered. The concept of dose addition is used for chemicals with similar mode of action while response addition is used for dissimilarly acting chemicals. Up-to-date several types of models have been proposed for specific-interaction studies: isobolographic model, multifactorial analyses, fractionated factorial designs, effect/response-surface analysis, physiologically-based pharmacokinetics modeling, etc.[9,11-13] However, all these methodologies have certain limitations, are commonly extremely costly *in vivo* studies and are sometimes difficult to interpret.

Cadmium (Cd) and Polychlorinated biphenyls (PCBs) are widely spread persistent environmental pollutants that enter food chain and pose risk to human health. Therefore we investigated the effects of single exposure to different doses Cd or PCBs and the effects of co-

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exposure to these chemicals. During the experiment the effects on body weight gain, hematological parameters, liver and kidney function, as well as their thyroid disrupting effects were investigated in rats. Animals were treated orally for 28 days with six different doses of Cd or PCBs ranging from 0.3 to 10 mg /kg b.w./day or 0.5 to 16 mg /kg b.w./day, respectively. In order to investigate combined effects of Cd and PCBs, nine groups of animals were exposed to different dose combinations of Cd and PCBs (1.25, 2.5 or 5 mg Cd/kg b.w./day and 2,4 or 8 mg PCBs/kg b.w./day). Detailed data on the experiment, statistical methods and concept used for interaction assessment are given in our previously published paper.[14]

The study demonstrated significant effects on body weight gain suggesting possible developmental toxicity, and also confirmed hematotoxic, hepatotoxic and nephrotoxic effects of these toxic agents. The obtained results also gave the evidence of thyroid disrupting effects: cadmium mainly caused decrease in T3 hormone levels suggesting predominant disruption of extrathyroid processes, while PCBs showed more profound effect on T4 hormone levels presumably as the result of PCBs direct effect on thyroid gland.

Investigation on the effect of co-exposure to Cd and PCBs implicates different toxicological profile of mixtures if compared to single chemicals. Thus, regarding the effects on hematological parameters, the mixture produced decrease in red blood cells count and hemoglobin content, the effects that were not observed during single chemical treatment, while the effects on white blood cells count and platelets were shown to be additive. Mixture exerted more profound decrease in body weight gain i.e. additive effect of Cd and PCBs. Additive effects of these chemicals were also observed for investigated parameters of liver function indicating no toxicodynamic interactions between these chemicals in liver. On the other hand, synergistic interactions between Cd and PCBs were proved for the parameters of kidney function. As reported in our previous study,[14] alterations in thyroid function, i.e. levels of thyroid hormones in serum can be attributed to the synergism between these two chemicals.

Based on these results, it could be concluded that single agent toxicity studies cannot fully predict the toxicity of mixtures. Our findings implicate that toxicity of mixture can be more profound than the toxicity of its components, and furthermore that mixture of chemicals can produce toxicity although the same dose regime of single components induces no toxic response. This study contributes to better understanding of mixture toxicity and gives one more piece of evidence that exposure assessment and safety evaluation should focus on chemical mixtures rather than on single chemicals.

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Editorial

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Selective Targeting of Cancer Cells using Personalized Nanomedicine

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Cancer is mostly caused by genetic alteration in either of gain of function and/or loss of function in response to mutagens, ionizing radiations etc. Synthetic lethality raised as an exciting new avenue to kill cancer cells by identifying potential druggable targets. Synthetic lethality define as a lethal interaction between two separate viable mutations when present together within a cell results in cell death, while mutation is only in either of the genes alone, cell remains viable.[1] Therefore, synthetic lethality is a new pragmatic strategy for the selective killing of cancer cells by exploring and targeting the synthetic lethal interactors of cancer cell's specific vulnerabilities like chromosomal instability (CIN) phenotype. Mutation in CIN genes leads chromosomal aberrations (aneuploidy by gene amplification, chromosomal translocation) and deoxyribonucleic acid (DNA) mutations leads to single/double strand break that are repaired by non-homologous end joining (NHEJ) resulting in accumulation of errors, leads to genomic instability, the hallmark of cancer.[2]

The sequencing of first human genome in 2000 gave a new track to understand the differences arises among individuals in response to harmful agent's exposure and in treatment outcome called as Pharmacogenetics and Pharmacogenomics.[3,4] Personalized medicine is the tailored treatment based on genetic constitution of a person responsible for individual variably in drug response and treatment outcome.[5] Nanotechnology based approaches helped in delivering drugs emerged based on synthetic lethal interaction. Nanotechnology offered the advantage of targeted drug delivery, reducing drug dose and dosage frequency and reducing systemic drug exposure thus limiting side effects and overcoming drug resistance.[6,7] The major breakthrough in the development of personalized medicine is the application of nano-approach to synthetic lethality to target mutated cancer cells with no harm to normal cells. This approach will help in increasing the effectiveness of treatment, preventing the development of metastasis of cancer cells and reducing adverse effects to healthy tissues.

To effectively kill the cancer cell, there is a need to refine and combine various therapeutic choices for the development of a personalized combination regimen depending upon the need of individual patient. To improve the clinical activity of dasatinib (tyrosine kinase inhibitor of src-family kinases) for epithelial ovarian cancer (EOC) it was given in combination with CX-4945 (CDK2 inhibitor) and increased apoptosis with reduced cell proliferation was observed across multiple EOC cell lines.[8] Three different block co-polymers, polycaprolactone, polyethylene glycol and poly-2-aminoethyl ethylene phosphate, self-assembled to formed nano-micelles for carrying PLK-1 siRNA (siPLK1) and paclitaxel and was given systemically to MDA-MB-435 induced tumor xenograft bearing mice for synergistic cancer cell killing requiring 1000-fold less paclitaxel compared to paclitaxel monotherapy, with no side effects.[9]

Synthetic lethal interactions mediated cancer cell killing is very promising and flourishing treatment strategy that exploits the tumor cell's vulnerabilities. Poor bioavailability, toxicity issues, emergence of drug resistant cases, and the presence of multiple survival pathways are the multi-factors that are leading to introduction of nanotechnology for synthetic lethal application and will lead to success as a targeted therapies. Identifying synergistic killing potential of SL interaction with radiotherapy and theranostics is also under progress. Overall,

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nanoformulations mediated synthetic lethal killing of cancer cells with siRNA or chemical inhibitor will lead to a way towards personalized nanomedicine with great success.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Editorial

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Overview on Progress in Forensic Deoxyribonucleic Acid Analysis

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Forensic science is "the application of *science* to criminal and civil laws".

The field is very extensive and cover different disciplines including medicine, biology, chemistry, physics.

Medical examination of bodies (forensic medicine) in order to determine cause and time of a suspicious death have been used for centuries, while the use of science to investigate crimes in order to identify criminals began in the mid to late 1800's.

The major advancement in forensic science occurred in the late 1980's when scientists were able to investigate human genome deoxyribonucleic acid (DNA).

Prof. Alec Jeffreys, from the University of Leicester identified in the human genome some sequences repeated in a variable number tandem repeats (VNTR).

He developed a procedure called "DNA fingerprinting" that was firstly used in a forensics in 1987 in UK for solving a double murder case occurred in Leicestershire. Colin Pitchfork is known to be the first person convicted of a crime based on DNA fingerprinting evidence.

Forensic DNA analysis was revolutionized by the introduction of the polymerase chain reaction (PCR), developed by Murris, that enables the production of millions of copies of a specific DNA sequence allowing the analysis also of DNA traces.

DNA analysis permits now-a-days the identification of a person through biological fluids, hair, human remains and also skin cells left on touched objects (contact trace).

Since its first application, DNA analysis has played a crucial role not only in the investigation of violent crimes but also in case of mass disasters victims identification (DVI), for the reconstruction of controversial biological relationships and for the creation of DNA databases useful in the fight against crime.

Short tandem repeats (STRs) are the most widely used markers for forensic DNA testing, but also other genetic polymorphisms, such as the ones in the mitochondrial DNA (mtDNA) genome or in sexual chromosomes (X-Y) provide useful results.

A common problem in forensics concerns the genetic identification of degraded biological samples such as the material collected at the crime scenes or recovered from mass disaster that may have been remained exposed to harsh environmental conditions (sunlight, humidity, etc.) damaging DNA structure.

In this perspective, not only the range of genetic markers used is increased with the introduction of mini-STR and SNPS analysis but new automated high-throughput analytical methods (i.e. next generation sequencing) have been developed in order to increase the ability to recover

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informations from biological samples even if degraded or in small quantity.

Recently, the analysis of SNPs useful for ancestry identification or for physical characteristics determination (i.e. hair, eye or skin colour) have been introduced: this offers a powerful new tool in forensics to connect a suspect with a crime scene or to identify human remains.

The availability of new procedures has also a great impact in "defrosting" unsolved cold cases: new sensitive testing methods are applied to the analysis of old samples often allowing the case resolution after a long time.

In addition to allow a rapid identification of suspects directly at crime scene or for DNA database purposes, portable rapid DNA devices have been developed, in order to obtain a full DNA profile anywhere in under 2 hours from a reference sample (buccal swab).

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Research

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Mycotoxin MT81 and Its Benzoylated Derivative Exhibit Potential Antisteroidogenic Activities In Prepubertal Female Wistar Rat

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ABSTRACT

MT81, a mycotoxin (polyhydroxyanthraquinone, Molecular formula $C_{22}H_{18}O_7$) was isolated and identified from *Penicillium nigricans.* It is highly toxic (LD_{50} value is 35.1 mg/kg body weight) and shows antimicrobial, antileishmanial activities and produces hepato-renal dysfunction and hematological disorders. The benzoylated derivative of MT81 was synthesized in our laboratory and its LD_{50} value is 87.1 mg/kg body weight. In spite of its reduced toxicity, the benzoylated derivative (BzMT81) shows potent antimicrobial effects. The present study was aimed to investigate the antifertility activities of Mycotoxin MT81 and its benzoylated derivative on the reproductive system of female prepubertal albino Wistar rats. MT81 and BzMT81 arrested vaginal opening, reduced body weight and the weights of ovaries and uterus. Total cholesterol and ascorbic acid content of the ovaries were elevated whereas the activities of Δ^5-3β-hydroxysteroid dehydrogenase and glucose-6-phosphate dehydrogenase were decreased in a dose-dependent manner. In the adrenal gland of rat these parameters showed opposite findings. The study reveals that MT81 and BzMT81 both inhibit ovarian steroidogenesis and causes ovarian sterility in female prepubertal rat but the analogue shows more potentiality.

KEYWORDS: MT81; Benzoylated MT81; Vaginal opening; Δ^5-3β-hydroxysteroid dehydrogenase; Ovarian steroidogenesis.

ABBREVIATIONS: HSD: Δ^5-3β-hydroxysteroid dehydrogenase; BSA: Bovine Serum Albumin; ANOVA: Analysis of variance.

INTRODUCTION

Mycotoxins are environmental pollutants present in virtually all parts of the world. More than 300 chemically different mycotoxins formed by more than 350 fungal species and causing diseases (mycotoxicoses) to living organisms are described.[1] As yet only a few mycotoxins have been related to important food- and feed-borne diseases, the potential impact on human and animal health of many of them remains to be elucidated. The most frequently toxinogenic fungal species found in food and feed commodities belong to the genera *Aspergillus*, *Fusarium* and *Penicillium*.

Reproductive failure and a drop in reproductive performances are brought on by mycotoxins. Zearalenone produces in farm animals a true estrus enlargement of both vulva and uterus and other general responses associated with estrogens.[2,3] Zearalenone has strong estrogenic effects, as well as haematotoxic and genotoxic properties.[4] Higher doses of zearalenone affect ovulation, conception, implantation, foetus development, and the newborn's viability.

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Prepubertal female pigs are the most affected farm animals by zearalenone. Zearalenone produced hyperestrogenism in young gilts and delayed cycling in prepuberal gilts.[5] Ochratoxin A causes a remarkable delay in sexual maturation in rats on account of suppressed ovarian steroidogenesis.[6,7]

Long-term exposure to low concentrations of zearalenone leads to impairment of proliferative activity of the follicle granulosa cells and connective tissues of the ovarian stroma in prepubertal gilts.[8] In gilts, zearalenone caused follicle atresia and apoptotic changes in granulosa cells.[9] In pregnant and lactating sows exposed to the action of zearalenone, the number of follicles with normal morphology was decreased. This reduction of reserves of the early stages of follicles can cause the premature depletion of healthy follicles and shorten the reproductive period in sows.[10] The derivatives of zearalenone, α- and β-zearalenol, inhibit progesterone synthesis in porcine granulosa cells.[11]

Many mycotoxins have direct effects on the fetus[12] and have teratogenic effects.[13] For the investigation of antifertility activity, various experimental parameters were reported earlier e.g., delay in sexual maturity,[14,15] resorption of fetuses in pregnant rats,[16] direct effects on fetuses.[17] At present it is supposed that among many other factors, gonadal steroids have an important role in the maintenance of oestrus cycle and thus ovulation.

The mycotoxin problem has attained considerable significance in terms of public health and animal husbandry. It is therefore a matter of any national interest to make appropriate studies to deal with the problem of isolation, identification and toxicological evaluation of mycotoxins as contaminants in human diet and their consequent effects on animals. From this standpoints, mycotoxin MT81 was isolated, purified and identified in our laboratory from a locally isolated fungal strain of *Penicillium nigricans*, (patent no. 156916 dated 15.2.82, Government of India). MT81 is a dextrorotatory polyhydroxyanthraquinone compound (molecular formula, $C_{22}H_{18}O_7$ and Molecular weight, 394) and its LD_{50} value is 35.1 mg/kg body weight in mice. MT81 is a good antimicrobial,[18] hyperglycemic,[19] and antileishmanial[20] agent. It produces liver,[21] brain[22] and kidney dysfunction[23] and massive bone marrow depression.[24]

Besides their toxicological effects, mycotoxins may possess good antibiotic activities. From this aspect, some mycotoxins can be used as medicine if their toxicity can be reduced. So, Benzoylated derivative of MT81 (Bz-MT81) was synthesized in our laboratory to generate a more potent and less toxic compound (LD_{50} values 87.1 mg/kg body weight in mice) in order to find out good therapeutic agent which may lead to more effective drug formulation. This derivative possesses antibacterial, antifungal[18] and antileishmanial[20] effects.

India is a country where population density is significantly higher. So this present study was an attempt to synthesize a less toxic compound and to search out its efficacy as an antifertility agent. At the same time, the present study was designed to investigate the comparative antifertility activities of mycotoxin MT81 and its benzoylated derivative on the reproductive system of prepubertal female albino Wistar rats by assessing the ovarian and adrenal activity as well as steroidogenesis in these organs.

MATERIALS AND METHODS

Chemicals and Reagents

MT81, Bz-MT81 were synthesized in our laboratory. Nicotinamide adenine dinucleotide, Dehydroepiandrosterone sulphate, Nicotinamide adenine dinucleotide phosphate, Glucose-6-phosphate, Bovine serum albumin were purchased from Sigma Aldrich Inc., USA. All other chemicals used were purchased from Himedia India Ltd. Merck India Ltd. etc.

Animals

Ninety closed colony, randomly breed albino Wistar prepubertal female rats were taken at 25 to 30 days of age (weighing 50 to 60 g). The rats were housed in cages under standard conditions (12:00 h light: 12:00 h dark, 25±2 °C) with a standard laboratory pellet food and drinking water *ad libitium*. The animals were acclimatized for 15 days before experimentation. The study was approved by the Institution's animal ethical committee.

Treatments

Ninety albino Wistar prepubertal female rats were equally divided into six groups, each group comprising of 15 rats. Treatments were done intraperitoneally with MT81 and benzoylated MT81 dissolved in propylene glycol and it was carried out on every alternate day for 14 days (total 7 doses). Alternate dosing is comparatively safe and tolerable for the treated animals as the LD_{50} value of MT81 is low. We selected the dose and experimental schedule according to our previous study.[25]

The groups and treatments were as follows:

Group-I : Saline control (0.1 ml of 0.9 % NaCl/ 20 g of body weight)
Group-II : Vehicle control (0.1 ml of propylene glycol/ 20 g of body weight)
Group-III : 5 mg /kg (0.1 ml MT81/ 20 g of body weight)
Group-IV : 7 mg/kg (0.1 ml of MT81/ 20 g of body weight)
Group -V : 7 mg/kg (0.1 ml of Bz-MT81/ 20 g of body weight)
Group-VI : 9 mg/kg (0.1 ml of Bz-MT81/ 20 g of body weight)

Total body weights of the rats in each group were taken before and after the treatment period. Sixty rats were sacrificed 24 hours after the last dose and food was withdrawn 18 hours before sacrifice though drinking water was supplied sufficiently. Five rats of each group were kept for the study of the age of vaginal opening and appearance of the first estrous.

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Biochemical Assays

Ovaries, uterus and adrenal glands were dissected out from each animal immediately after sacrifice and were done free from fat and the weights were recorded. The ovaries and adrenal glands were taken out for the estimation of cholesterol,[26] ascorbic acid,[25] glucose-6-phosphate dehydrogenase,[27] Δ^5-3-beta-hydroxyesteroid dehydrogenase.[25]

Estimation of Ovarian and Adrenal Cholesterol

Ovary and adrenal gland of each rat were homogenized in 0.25 sucrose solution for the estimation of cholesterol. 4.75 ml of ethanol and acetone mixture was added to 0.05 ml of homogenate (2%, w/v). The mixture was shaken well and kept for 10 min. The mixture was then centrifuged at 3000 rpm for 10 min. 1 ml of the supernatant was evaporated to dry in boiling water bath, then the residue was mixed with 3 ml of glacial acetic acid and warmed for 20 min. After addition of 2 ml color reagent (10% $FeCl_3$, 6 H_2O, 100 ml acetic acid, conc. H_2SO_4), the reading were taken at 560 nm within 10 min. The amount of cholesterol present is calculated by plotting the standard curve.[26]

Measurement of Ovarian and Adrenal Ascorbic Acid

For the estimation of ascorbic acid, the ovarian and adrenal tissues were homogenized using 2.5 ml of 5% metaphosphoric acid-10% acetic acid solutions. The mixture was centrifuged after extraction and a very small drop of concentrated bromine was added to the supernatant. Tube was shaken and kept for 10 min for complete oxidation. Excess liquid bromine was then removed. 0.5 ml of dinitrophenylhydrazine–thiourea reagent (2.2% 2, 4-DNPH in 100 of 10N H_2SO_4, 5% thiourea) was added with 2 ml of tissue extract and incubated at 37 °C for 3 h and then 2.5 ml of 85% H_2SO_4 was slowly added in ice-cool condition. It was mixed well for half an hour in room temperature for color development and optical density was observed at 540 nm.[25]

Assay of Ovarian and Adrenal Δ^5-3β-Hydroxysteroid Dehydrogenase Activity

For the estimation of ovarian and adrenal Δ^5-3β-hydroxysteroid dehydrogenase (HSD) activities[28,29] one ovary and adrenal gland from each animal were taken. The tissues were homogenized carefully at 4 °C in 20% glycerol containing 5 mM potassium phosphate and 1 mM EDTA to make a tissue concentration of 100 mg/ ml homogenizing mixture. Then the mixture was centrifuged at 10,000 *g* for 30 min at 4 °C. The supernatant (1 ml) was mixed with 100 µm sodium pyrophosphate buffer (pH 8.9), 40 µl of ethanol containing 30 µg dehydroepiandrosterone and 960 µl of 25 mg% Bovine Serum Albumin (BSA), to make the incubation mixture to a total of 3 ml. In spectrophotometer cuvette, after addition of 0.5 µM NAD to the tissue supernatant mixture, enzyme activity was measured at 340 nm against a blank (without NAD). One unit of enzyme activity was the amount causing a change in absorbance of 0.001 per minute at 340 nm.

Assay of Ovarian and Adrenal Glucose-6-Phosphate Dehydrogenase Activity

Glucose-6-phosphate dehydrogenase (G-6-P-D) activity was assayed biochemically by monitoring the formation of NADPH at 340 nm.[27] The tissue was homogenized with 0.5 ml of Tris buffer (pH 7.4). Then it was centrifuged at 7000 rpm for 15 min at -20 °C. In a cuvette, 0.02 ml of extract, 0.2 ml of 0.5 M Tris-HCl buffer (pH 8.3), 0.01 ml of 20 mM of NADP solution and 0.76 ml of glass distilled water were taken and mixed. Then 0.01 ml of the glucose-6-phosphate (substrate) was added to the cuvette and was mixed well. The reading was taken at 30 sec interval up to 5 min at 540 nm in spectrophotometer. Tissue protein was estimated by the method of Lowry et al.[30]

Statistical Analysis

The results were expressed as the Mean±Standard error of mean (SEM). Statistical analyses of the collected data were done by one-way Analysis of variance (ANOVA) followed by multiple comparison *t*-test. Difference was considered significant when $P<0.05$. *P* values are taken in respect of vehicle control in all cases of toxin-treated group.

RESULTS

Effect of MT81 and Bz- MT81 on age of vaginal opening and appearance of first estrous

Table 1 shows the effect of MT81 and Bz-MT81 on age of vaginal opening and appearance of first estrous in immature female rat. At the beginning of the experiment all the rats exhibited stage of diestrus (anestrus) at the study of vaginal smear as all of them were immature. Compared with the control group, MT81 (Groups III and IV) and Bz-MT81 (Groups V and VI) exposure delayed the age of vaginal opening and appearance of first estrous significantly ($p<0.001$) in a dose-dependent manner. The rats treated with MT81 and Bz-MT81 showed continuous diestrus stage throughout the period of treatment. On the other hand, saline and vehicle control group animals showed the age of vaginal opening and appearance of first estrous comparatively earlier.

Group	Vaginal opening (age in days)	First estrous (age in days)
Normal	47.28±0.53	63.3±0.55
Vehicle control	38.24±2.2	64.4±1.24
MT81(5mg/kg)	47.48±1.91*	71.12±1.73*
MT81(7mg/kg)	60.7±0.53*	79.62±1.56*
Bz-MT81(7mg/kg)	49.8±1.32*	73.2±0.94*
Bz-MT81(9mg/kg)	64.5±1.48*	81.8±0.37*

No. of animals per group =10. Results are Mean±SEM. Probability values are given in asterisks. *indicates $p<0.001$. *P* values are taken in respect of vehicle control in all cases of toxin-treated group.

Table 1: Effect of MT81 and Bz- MT81 on age of vaginal opening and appearance of first estrous in prepubertal female rat.

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Body weight and reproductive organ weights

The body weights of the prepubertal female albino rats were decreased significantly (p<0.001) in higher dose of MT81-treated (Group IV) and Bz-MT81-treated (Groups VI) rats. The weights of ovaries (both sides), uterus decreased (p<0.001) whereas that of adrenal glands increased in a dose-dependent manner (Table 2).

Effects of MT81, Bz-MT81 on ovarian and adrenal cholesterol and ascorbic acid content

MT81 and its benzoylated analogue caused an accumulation of total cholesterol (p<0.001) and ascorbic acid in the ovary (Figures 1 and 2) of female prepubertal rats, whereas the cholesterol and ascorbic acid content of adrenal gland (Figures 3 and 4) were decreased.[31]

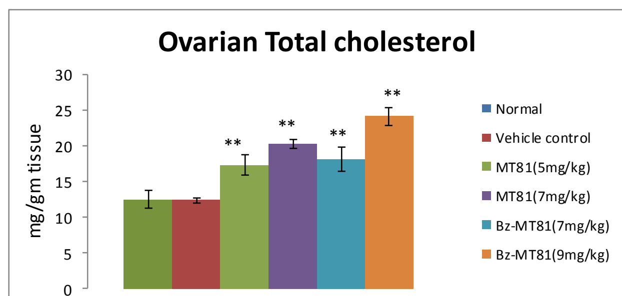

Figure 1: The bar diagram shows the effect of MT81 and benzoylated-MT81 on total cholesterol content in the ovaries of prepubertal female Wistar rat. No. of animals per group=10. Results are Mean±SEM. Probability values are given in asterisks. *indicates p<0.001. P values are taken in respect of vehicle control in all cases of toxin-treated group.

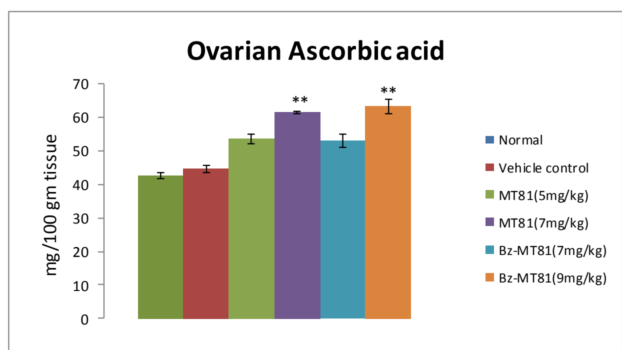

Figure 2: Shows the effect of MT81 and benzoylated-MT81 on total ascorbic acid content in the ovaries of prepubertal female Wistar rat. No. of animals per group=10. Results are Mean±SEM. Probability values are given in asterisks. **indicates p<0.001. P values are taken in respect of vehicle control in all cases of toxin-treated group.

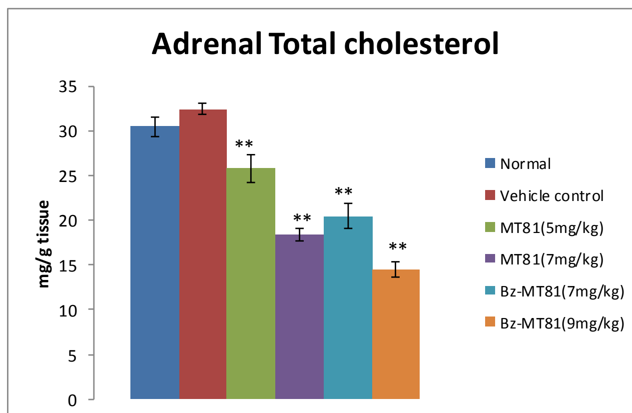

Figure 3: The bar diagram shows the effect of MT81 and benzoylated-MT81 on total cholesterol content in the adrenal glands of prepubertal female Wistar rat. No. of animals per group =10. Results are Mean±SEM. Probability values are given in asterisks. **indicates p<0.001. P values are taken in respect of vehicle control in all cases of toxin-treated group.

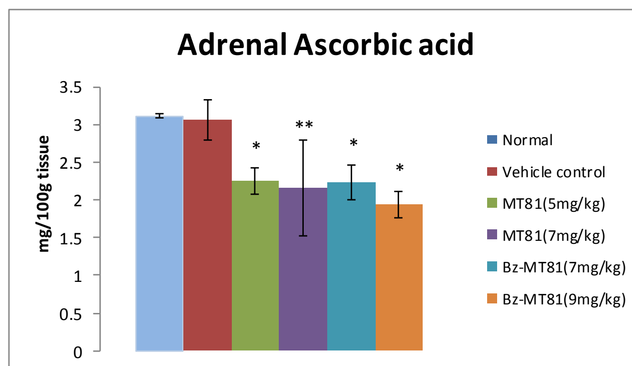

Figure 4: Shows the effect of MT81 and benzoylated-MT81 on total ascorbic acid content in the adrenal glands of prepubertal female Wistar rat. No. of animals per group=10. Results are Mean±SEM. Probability values are given in asterisks. *indicates p=0.001; **indicates p< 0.001. P values are taken in respect of vehicle control in all cases of toxin-treated group.

Effects of MT81, Bz-MT81 on ovarian and adrenal Δ-3β HSD and G-6-P-D activity

The treatment of prepubertal female albino rats with MT81, Bz-MT81 reduced the activities of Δ-3β HSD and G-6-P-D (Figures 5, 6) enzymes in ovary and increased the activities of these enzymes (Figures 7, 8) in the adrenal gland.[32]

DISCUSSION

In case of prepubertal rats, administration of MT81 and Bz-MT81 remarkably delayed the onset of sexual maturity as

Group	Difference in body weight(g)	Weight of ovaries (both sides) (mg/100g body wt.)	Weight of uterus (both sides) (mg/100g body wt.)	Weight of adrenal (both sides) (mg/100g body wt.)
Normal	40.17±0.32	30.30±1.33	78.46±0.25	15.16±0.97
Vehicle control	39.304±1.42	30.84±0.34	77.48±0.62	15.80±0.29
MT81(5mg/kg)	25.454±1.51*	24.66±1.96*	49.58±0.87*	17.10±1.53
MT81(7mg/kg)	16.50±0.37*	18.50±0.32*	37.12±1.07*	19.46±0.70*
Bz-MT81(7mg/kg)	29.664±1.48*	27.14±0.62*	52.72±0.64*	18.60±1.37*a
B Bz-MT81(9mg/kg)	18.556±1.56*	20.52±1.15*	39.36±1.14*	24.12±1.59*

No. of animals per group=10. Results are Mean±SEM. Probability values are given in asterisks. *a indicates 0.02>p>0.01; *indicates p<0.001. P values are taken in respect of vehicle control in all cases of toxin-treated group.

Table 2: Effect of MT81 and Bz- MT81 on body weight, weight of ovaries, uterus and adrenal gland prepubertal female rats.

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evidenced by the age of vaginal opening and appearance of first estrus (Table 1).

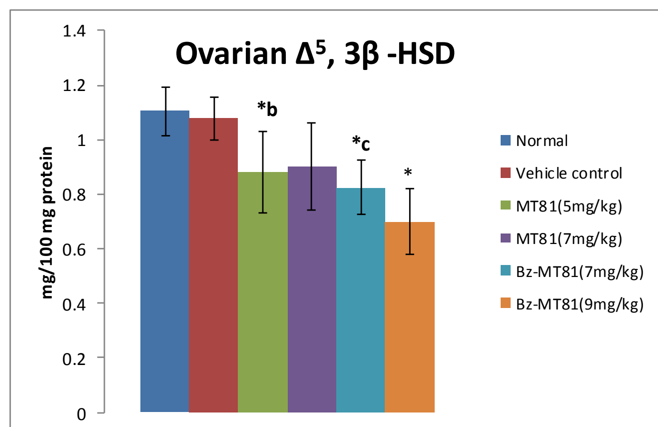

Figure 5: The bar diagram shows the effect of MT81 and benzoylated-MT81 on the activity of Δ5, 3β-hydroxy streroid dehydrogenase in the ovaries of prepubertal female Wistar rat. No. of animals per group =10. Results are Mean ± SEM. Probability values are given in asterisks. *b indicates 0.02> p>0.01; *c indicates 0.01> p>0.001; * indicates p=0.001. P values are taken in respect of vehicle control in all cases of toxin-treated group.

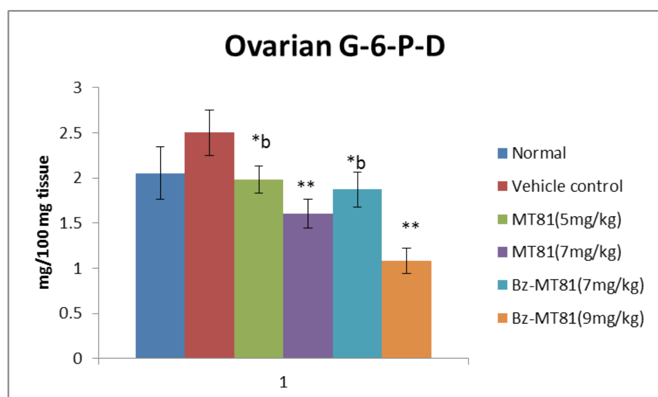

Figure 6: Shows the effect of MT81 and benzoylated-MT81 on the activity of glucose -6-phosphate dehydrogenase in the ovaries of prepubertal female Wistar rat. No. of animals per group =10. Results are Mean ± SEM. Probability values are given in asterisks. *b indicates 0.02> p>0.01; *indicates p=0.001; **indicates p<0.001. P values are taken in respect of vehicle control in all cases of toxin-treated group.

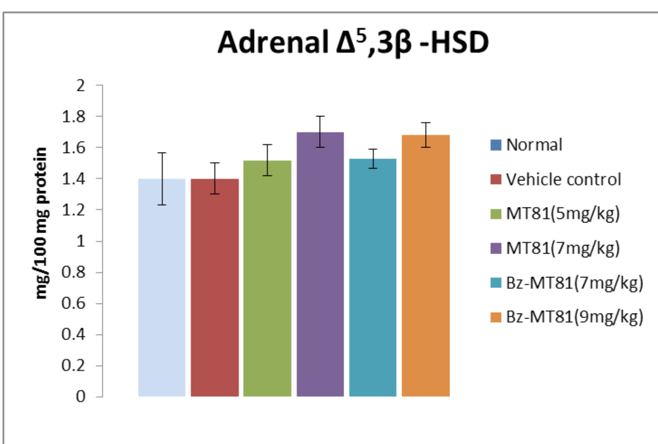

Figure 7: The bar diagram shows the effect of MT81 and benzoylated-MT81 on the activity of Δ5, 3β-hydroxy streroid dehydrogenase in the adrenal gland of prepubertal female Wistar rat. No. of animals per group=10. Results are Mean±SEM. Probability values are given in asterisks. *c indicates 0.01>p>0.001; *indicates p=0.001. P values are taken in respect of vehicle control in all cases of toxin-treated group.

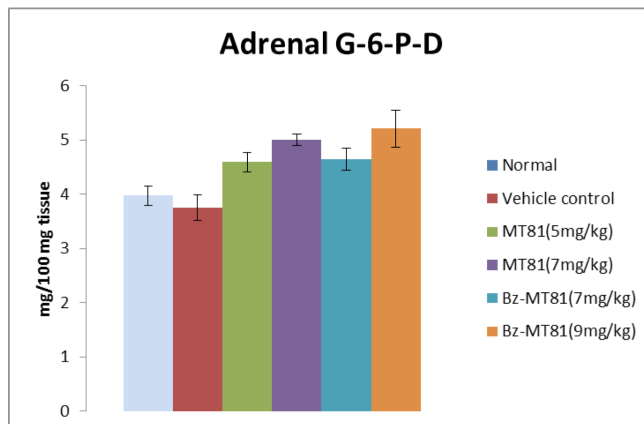

Figure 8: Shows the effect of MT81 and benzoylated-MT81 on the activity of glucose-6-phosphate dehydrogenase in the adrenal gland of prepubertal female Wistar rat. No. of animals per group=10. Results are Mean±SEM. Probability values are given in asterisks. *indicates p=0.001; **indicates p<0.001. P values are taken in respect of vehicle control in all cases of toxin-treated group.

The results of this investigation demonstrated the reduction in body weight and the wet weight of the ovary, uterus; that may be due to decreased anabolic role of estradiol on the body weight and wet weight of the ovary. This reduction may also be due to other toxic effects of MT81 and Bz-MT81 on other systems of the animal (Table 2). The disturbance in the reproductive cycle i.e. the delay in the age of vaginal opening and appearance of first estrus and the decrease in the weight of the ovary and uterus may be related with the reduction of ovarian steroidogenesis.

Cholesterol is as an obligatory precursor in progestin biosynthesis in rat, rabbit and bovine luteal tissues.[33] Thus, in present study, the significant elevation in cholesterol content of ovarian tissue of MT81 and Bz-MT81-treated rats suggest the non-utilization of cholesterol towards biosynthesis of hormone in ovaries. Thus it results the hypofunctioning of steroidogenic activity of the ovary of the toxin treated rats. The accumulation of ascorbic acid in the ovaries of treated rats gives additional support to the inhibition of steroidogenic activity.

It is well documented that Δ^5-3β HSD is an important key enzyme involved in steroid biosynthesis.[34] According to Mckerns[35,36] gonadotropins through the activation of glucose-6-phosphate metabolism in the pentose phosphate pathway increases the rate of production of NADPH essential for the hydroxylation reaction in the formation of steroid hormones from cholesterol. The reduction of cholesterol and ascorbic acid along with accompanying increase in the weight of the adrenal gland, the activities of the Δ^5-3β HSD and G-6-P-D indicates the increased steroidogenesis in adrenal gland.

The importance of G-6-P-D from pentose phosphate pathway for the synthesis of estrogen in the sexually immature animals has been reported earlier.[37,38]

Anestrus vaginal smears and ovarian hypofunction appear to be due to the absence of or decrease in circulating go-

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nadotropins. This is established by the regaining of the normal ovarian and uterine weights in starved animals following the injection of hypophyseal extract and chorionic gonadotrophin.[39,40] Maximum synthesis and secretion of ovarian steroids take place in proestrus stages. The disturbances in the reproductive cycle and the decrease in the weight of the ovary and uterus in the present investigation may be related to the diminution of ovarian steroidogenesis.

The administration of MT81 and Bz-MT81 in prepubertal female rats resulted in decreased Δ^5-3β HSD and G-6-P-D activities possibly due to the decrease in ovarian hormone production which was also seen in the effect of alpha and beta-zearalenol on the enzymatic activity of 3β HSD.[41]

Further support regarding the reduction of ovarian steroid hormone synthesis is evident from the accumulation of cholesterol and ascorbic acid in the ovary of toxin-treated rats.It is related to the hypofunctioning and non-functional ovary.[7,42] The abovesaid effect was reversed in case of adrenal gland.

Therefore, in the present investigation a fall of Δ^5-3β HSD and G-6-P-D of ovary after toxin treatment suggests that Bz-MT81 is less toxic than MT81 but its effect was more in case of the anti-fertility activity. From the above findings, it is evident that MT81 and Bz-MT81 cause prominent inhibition in ovarian steroidogenesis in a dose-dependent manner in prepubertal female Wistar rats and Bz-MT81 is more potent than its parent toxin MT81 as Bz-MT81 has less LD_{50} value compared to MT81.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Research

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In Vitro Cytotoxicity and Antioxidative Potential of *Nostoc Microscopicum* (Nostocales, Cyanobacteria)

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ABSTRACT

Many cyanobacterial species (cyanoprokaryotes, blue-green algae) are potent producers of various secondary metabolites with low molecular weight and diverse biological activities (antitumor, antimycotic, antiviral, antimicrobial, immunomodulatory, enzyme inhibition, cytotoxic activity). They have the potential to serve as convenient source of active substances for new medications and other commercial products. Therefore, cyanobacteria have become an object of intense scientific interest. New findings have shown that different cyanoprokaryotic species produce compounds with antioxidant activity. Also, there are reports for successful clinical trials using antitumor agents that contain active substances isolated from these organisms. On the other hand, it is well known that certain cyanobacteria produce toxins that could harm plants, animals and humans. Some cyanotoxins induce severe injuries even by contact with polluted water while washing or swimming. Hence, cyanotoxins production must be analyzed for all widely distributed cyanoprokaryotic species as well as those with potential application in medical practice. Certain species of the genus *Nostoc* are well known producers of substances with antitumor, enzyme inhibition, immunomodulatory, antioxidant or cytotoxic activities. However, little is known about the species *Nostoc microscopicum*. Therefore, the aim of the present study was to evaluate the antioxidant activity and potential cytotoxic effects of *Nostoc microscopicum* using extracts derived with different solvents. We show a moderate antioxidant activity of different *Nostoc microscopicum* extracts and prominent cytotoxic activity against several human cell lines (HeLa, FL, A549). Our results determine *Nostoc microscopicum* as interesting source of active compounds for pharmacology and biotechnology. In addition, this report presents for the first time an evidence for saxitoxins and microcystins production by *Nostoc microscopicum* and highlights the toxicological importance of this cyanobacterial species.

KEYWORDS: Cyanobacteria; *Nostoc microscopicum*; Cyanotoxins; Cytotoxicity; Antioxidant activity.

ABBREVIATIONS: DPPH: 2,2-diphenil-1-picrylhydrazyl; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC50: The concentration of a substance needed to induce 50% inhibition of a biological function; ELISA: Enzyme-linked immunosorbent assay; DMSO: Dimethyl sulfoxide; DMEM: Dulbecco's Modified Eagle's Medium; ATCC: American Type Culture Collection; NBIMCC: National Bank for Industrial Microorganisms and Cell Cultures; SE: Standard Error.

INTRODUCTION

Cyanobacteria are photosynthetic Gram-negative prokaryotes, which are considered as one of the most ancient autotrophic forms of life on the planet. They are traditionally used as dietary and therapeutic agents in different parts of the world. Recently, cyanoprokaryotes have gained more scientific interest concerning their ability to produce compounds with antioxidant activity.[1-3] As a phototrophic form of life, cyanoprokaryotes are under the influence of intensive oxi-

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dative stress caused by produced during photosynthesis free radicals. To prevent serious cellular damage these organisms have developed protective mechanisms based on the activity of antioxidative enzymes like superoxide dismutase and catalase, as well as low molecular weight antioxidants like glutathione, vitamins (ascorbic acid, tocopherols), phenols, microsporine amino acids and others. The antioxidant capacity of certain blue-green algae was compared with medicinal plants and it was found that cyanoprokaryotes are potent producers of different types of antioxidants.[3-5] Recent studies have demonstrated a strong antioxidant activity of scytonemin, a ultraviolet (UV)-absorbing pigment, isolated from *Nostoc commune*.[6] Antioxidative potential has been reported for extracts derived from different *Nostoc* species.[1,4,5,7] The number of cyanoprokaryotes with reported antioxidant activity is increasing and includes species from the genera *Nostoc*, *Spirulina*, *Fischerella*, *Phormidium*, *Anabaena*, *Oscillatoria*, *Calothrix*.[5,8] In addition, cyanobacteria produce a variety of low molecular weight compounds (lipopeptides, aminoacids, fatty acids, alkaloides, macrolides, phytosterols, amides, etc.) that have antitumor, antiviral, larvicidal, herbicidal, enzyme inhibition, antibacterial, antimycotic, cytotoxic or immunomodulatory activity.[9] The cryptophicines are a typical example. They were initially isolated from a lichen symbiont belonging to the genus *Nostoc*.[10] These compounds have a strong antiproliferative effect due to their ability to inhibit tubuline synthesis and induce mitotic spindle destruction. Other examples for biologically active substances derived from *Nostoc* species are the nostocyclopeptides with cytotoxic activity,[11] nostocycline A that has antimicrobial effect[12] or insulapeptolides,[13] nostopeptines,[14] microviridins G and H[15] with protease inhibitory role. Another type of cyanobacterial paracyclophanes (nostocyclophanes) exhibit antibacterial, fungicidal and cytotoxic activities.[16,17] Here we can include also nostocyclopeptide M1 that shows antitoxin activity against microcystins,[18] lipopeptides with antibiotic effects like muscoride (isolated from *Nostoc muscorum*) and nostocine A (derived from *Nostoc spongiaeforme*).[19,20] Indoles with antiviral and cytotoxic effects (for instance staurosporine and indolecarbazole), were isolated from *Nostoc sphaericum*.[21] The protein cyanovirin, which is derived from *Nostoc ellipsosporum*, has antiviral activity and it is specifically effective against all human immunodeficiency viruses.[22] It has been recently shown that an extract from *Nostoc commune* var. *sphaeroides* Kützing suppresses the expression and secretion of proinflammatory cytokines in murine macrophages and splenocytes and thus, exerts immunomodulatory activity.[23] The significance of cyanobacteria (*Nostoc* species in particular) as a source of antioxidants and compounds with other biological activity stems from their diversity, accessibility and the possibility for large-scale cultivation in bioreactors.[24]

More than 60 marine and 20 freshwater cyanoprokaryotic species are identified as producers of toxins. Most of them belong to the genera *Anabaena, Aphanizomenon, Nostoc, Microcystis, Nodularia, Cylindrospermopsis, Planktothrix, Oscillatoria, Raphidiopsis, Phormidium* and *Lyngbya*.[25,26] During the last two decades, many microcystin-producing *Nostoc* species have been identified.[27-30] It has been suggested that cyanobacteria growing on sediments in or around water basins could produce a significant amount of microcystins[31] and therefore their distribution should be monitored and routine tests for microcystins content should be performed for drinking water and water basins.[27] Certain lichens associated with *Nostoc sp.* strain IO-102-1 produce 6 rare forms of microcystins.[32] Except microcystins some *Nostoc* species produce other toxic compounds like nostocarboline and the neurotoxic aminoacid β-methylamino-L-alanine.[33]

To date, there is little information about the species *Nostoc microscopicum* concerning the production of biologically active compounds and cyanotoxins. This is the first report that demonstrates microcystins and saxitoxins production by *Nostoc microscopicum*. We also detected a moderate antioxidant activity of *Nostoc microscopicum* extracts and cytotoxic effect on three continuous human cell lines. These data provide a base for further investigations of *Nostoc microscopicum* aiming to identify novel biologically active compounds.

MATERIALS AND METHODS

Preparation of *Nostoc Microscopicum* Extracts

Four cyanobacterial extracts were obtained using different extracting agents (Table 1). *Nostoc microscopicum* Carmichael ex Bornet & Flahault (Culture Collection of Autotrophic Organisms (CCALA) #124) lyophilized dry mass was purchased from the CCALA, Institute of Botany, Academy of Sciences, Czech Republic. For the preparation of each extract 500 mg lyophilized material were mixed with 500 µL of the corresponding solvent. All four samples were incubated at room temperature in the dark for 24 h. After that the extracts were centrifuged for 15 minutes at 5000 rpm and the resulting supernatant was filtered through Whatman no 1 paper. Following filtration, the samples were freeze-dried and the obtained dry substances were dissolved in distilled water to a final concentration of 4 mg/mL. Distilled water or D-PBS (Gibco, Invitrogen, USA) were used for further dilution of the extracts during the experiments. All extract solutions were stored at 4 °C in a dark place.

Extract	Abbreviation	Solvent
Extract-1	E-1	5% acetic acid in Milli-Q water
Extract-2	E-2	75% methanol in Milli-Q water
Extract-3	E-3	75% ethanol in Milli-Q water
Extract-4	E-4	Dimethyl sulfoxide (DMSO)

Table 1: *Nostoc microscopicum* extracts assayed.

Determination of DPPH Free Radical Scavenging Activity (DPPH assay)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is based on a spectrophotometric detection of the change in 520 nm absorbance due to 2, 2-diphenil-1-picrylhydrazyl radical scavenging by the test substance.[34] 50 µL of 200 µM DPPH (Sigma-Aldrich, Germany) methanolic solution was mixed with 50 µL of *Nostoc*

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microscopicum extract. The reaction mixture was incubated at room temperature for a period of 1 h in the dark. All extracts were assayed in 3 different concentrations - 200 μg/mL, 100 μg/mL and 50 μg/mL. Ascorbic acid solution in serial dilutions (600 μg/mL, 400 μg/mL, 200 μg/mL, 100 μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, 6.25 μg/mL, 3.125 μg/mL, 1.6 μg/mL) was used as a standard. All samples were tested in triplicates. Following incubation with DPPH radical absorption at 520 nm was detected using Synergy 2 microplate reader (Biotek, USA). The following formula was used to calculate the percent antioxidant activity (DPPH radical scavenging activity):

$$\% \text{ Antioxidant activity} = [(A_{blank}-A_{sample})/A_{blank}] \times 100$$

where A_{blank} is the absorbance of the control that contains all reagents except the *Nostoc microscopicum* extract, and A_{sample} is the absorbance of the sample that contains *Nostoc microscopicum* extract.

Total Phenolic Content

The total phenolic content of *Nostoc microscopicum* extracts was estimated using the Folin-Ciocalteu method.[35] 100 μL of each extract were mixed with 1.8 mL 2% water solution of sodium carbonate and incubated for 2 minutes at room temperature. After that 100 μL 50% water solution Folin-Ciocalteu reagent was added to the mixture and all samples were incubated for 0.5-2 h at room temperature in the dark. As a result, colored complexes form in the positive samples that contain phenols. Gallic acid (Sigma-Aldrich, Germany) in serial dilutions (500 μg/mL, 250 μg/mL, 125 μg/mL, 62.5 μg/mL, 31.25 μg/mL, 15.625 μg/mL, 7.8 μg/mL, 3.9 μg/mL, 1.95 μg/mL, 0.98 μg/mL) was used as a standard. The results were obtained based on spectrophotometric detection of absorption at 760 nm. The data was presented as μg equivalent gallic acid/mg extract (μg GAE/mg). All samples were analyzed in triplicates.

ELISA for Detection of Saxitoxins

The saxitoxins content of *N. microscopicum* methanolic extract was evaluated using Ridascreen™ kit (R-Bopharm, Germany) with a detection limit of 0.010 ppb (μg/L saxitoxins). The samples and the standards with known saxitoxins concentration were pipetted on a microtiter plate that contain coated with saxitoxins-specific antibodies wells. Then, they were mixed with enzyme-conjugated purified saxitoxins. Hence, the free saxitoxins in the samples and the enzyme-conjugated ones will compete for binding to the toxin-specific antibodies coated on the plate wells. Following 2 h incubation at room temperature, the plates were washed with buffer in order to remove the unbound enzyme-conjugated saxitoxins. A chromogenic substrate solution for the marker enzyme was added to the plate following washing. As a result, a colored product forms in the wells containing complexes of enzyme-conjugated saxitoxins and saxitoxins-specific antibodies. The reaction was detected spectrophotometrically, absorption at 450 nm wavelength was analyzed. The resulting values (absorption units) are reversely proportional to the concentration of saxitoxins in the sample.

Evaluation of Microcystins Concentration

Microcystins enzyme-linked immunosorbent assay (ELISA) kit (Abraxis LLC, USA) with detection limit 0.010 ppt (ng/L microcystins) was used to assess the microcystins content of *Nostoc microscopicum* methanolic extract. The assay is based on competitive ELISA and it is analogical to the described above saxitoxins ELISA.

In Vitro Cytotoxicity Assays

For evaluation of potential cytotoxic effects of *Nostoc microscopicum* extracts we used three continuous human cell lines: HeLa (ATCC CCL 2, NBIMCC 164) established from cervical adenocarcinoma cells; A549 (ATCC CCL 185, NBIMCC 2404) derived from lung carcinoma, and FL (ATCC CCL 62, NBIMCC 94) established from normal amniotic cells. The cells were expanded in 75 cm^2 culture flasks in Dulbecco's modified eagle's medium (DMEM) (Gibco, Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories, Austria) and antibiotics (100 U/mL penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, Germany)). The cells were cultivated at 37 °C, 5% CO_2 and high humidity until they reach confluence. Then, they were trypsinized and the concentration of viable cells was determined using the Trypan blue assay. A549, FL and HeLa cells were diluted to a concentration of 2×10^5 cells/ml and seeded in 96-well plates (200 μL cell suspension/well). After 24 h incubation, *Nostoc microscopicum* extracts in 4 different concentrations (400 μg/mL, 200 μg/mL, 100 μg/mL, 50 μg/mL) were added to the culture plates and the cells were cultured for 24 h, 48 h and 72 h. An equivalent amount of dulbecco's phosphate buffered saline (D-PBS) (Gibco, Invitrogen, USA) was added to the control wells with cells. All samples were tested in triplicates.

The cytotoxic effect of *Nostoc microscopicum* extracts was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT is a yellow colored tetrasolic salt that is reduced by mitochondrial dehydrogenases in viable cells to a purple colored formazan product, which accumulates in mitochondria. Dead and impaired cells cannot transform MTT. In our experiments, the MTT assay was performed according to the methodic of Edmondson.[36] At the end of each exposure period (24, 48 and 72 h) 20 μL 5 mg/ml MTT (Sigma-Aldrich, Germany) solution were added to the culture wells. Then, the plates were incubated for 3-4 hours in the dark at 37 °C, 5% CO_2 and high humidity. After that, the culture medium was discarded and the cells were washed with D-PBS (Gibco, Invitrogen, USA). The accumulated formazan was solubilized with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany): 100 μL DMSO per well were added and incubated with the cells at 37 °C for 15 minutes. The amount of formazan accumulated in the cells was measured photometrically at 570 nm on a syn-

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ergy 2 microplate reader (Biotek, USA). The mean absorbance measured for the cells without any extract addition was referred as 100% cell viability (control).

Percentage of inhibition (PI) was calculated as follows:

PI (%) = $[1-(A_{570\ nm}test)/(A_{570\ nm}control)]\times 100$

IC_{50} values for each extract were then calculated.

Statistical Analysis

Results are presented as mean±SE from individual determinations with at least three replicates. Statistical differences were analyzed by the Mann–Whitney *U*-test using the Statview programme (SAS Institute, Inc.). Values of $p<0.05$ were considered as significant. All results were compared to those from the control group.

RESULTS

Antioxidant Activity of *Nostoc Microscopicum* Extracts

To evaluate the antioxidant activity of N. *microscopicum* extracts we used two of the most commonly performed methods—the DPPH radical scavenging activity assay and the Folin-Ciocalteu method that measures total phenolic content. Both methods are based on spectrophotometric measurement of the interaction between an antioxidant and a substance that changes its color due to alteration in redox potential.[37] The DPPH assay detects changes in the absorption spectrum of the free radical DPPH when it is reduced by an antioxidant.[34] Figure 1A shows the results from the DPPH assay with *Nostoc microscopicum* extracts. A clear difference in the DPPH radical scavenging activity was found between the extracts obtained with different solvents. The DMSO extract showed the highest antioxidant activity (more than 10%), while the extracts obtained with acetic acid and methanol displayed two times lower DPPH radical scavenging activity. Extract-3, where we used ethanol as extracting agent, showed median antioxidant activity compared to the other extracts.

Recent studies have shown that phenolic compounds contribute to the antioxidant activity of microalgae, including cyanobacteria.[3,5] Therefore, in order to better characterize the antioxidant activity of N. *microscopicum* extracts we measured their total phenolic content. The results of this experiment are shown in Figure 1B. They demonstrate relatively low, but significant concentration of phenolic compounds in N. *microscopicum* extracts. Again, we found a difference between the four extracts. In this case, extract-1 showed the highest result with approximately 5 µg GAE/mL, while extract-4 had the lowest total phenolic content (lower than 2 µg GAE/mL). The level of phenolic compounds detected in the methanolic and ethanolic extracts was median compared to E-1 and E-4.

Nostoc Microscopicum Produces Cyanotoxins

For assessment of microcystins and saxitoxins production we used only extract-2 because it has been shown that cyanotoxins are most effectively extracted by methanol.[38] We detected presence of both microcystins and saxitoxins in the *Nostoc microscopicum* extract (Figures 2A and 2B). The measured microcystins concentration was 1.5 ppt (ng/L). Interestingly,

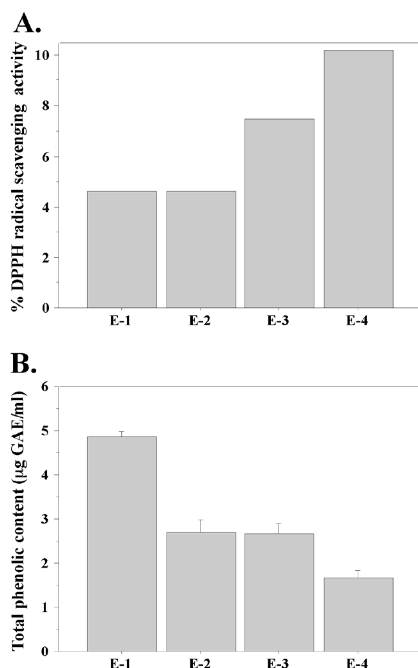

Figure 1: Antioxidative potential of *Nostoc microscopicum* extracts. A. Percentage (%) antioxidant activity based on measurement of DPPH free radical scavenging activity. B. Total phenolic content of *Nostoc microscopicum* extracts. Data represent mean ±SE.

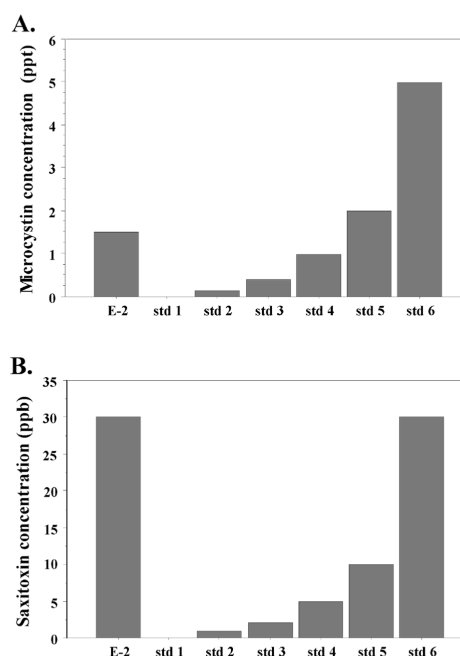

Figure 2: Cyanotoxins production by *Nostoc microscopicum*. A. Concentration of microcystins (ng/L). B. Concentraction of saxitoxins (µg/L). E-2: methanolic *Nostoc microscopicum* extract; std: standard.

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the saxitoxin content was very high, equal to the standard with the highest concentration in our assay—about 30 ppb (30 µg/L). This result demonstrates that *Nostoc microscopicum* is a potent producer of saxitoxins.

In Vitro Cytotoxicity of *Nostoc Microscopicum* Extracts

The potential cytotoxic effects of *Nostoc microscopicum* extracts were investigated using three human cell lines—two cancer cell lines (A549 and HeLa) and one cell line derived from normal amniotic cells (FL). The aim of our experiments was to determine whether *Nostoc microscopicum* extracts exert a common cytotoxic effect. The results from the MTT assays with FL cells are shown on Figure 3. They demonstrate that E-1 has the strongest cytotoxic effect with a mean IC_{50} value 253.1 µg/mL calculated for the 24 h test-period (Figure 4A). The extract obtained with ethanol (E-3) had the weakest effect on the cell viability. FL cells demonstrated clear dose-dependent response to all extracts with a prominent cytotoxic effect at the highest tested concentrations (200 and 400 µg/mL). The same tendency was determined for HeLa (Figure 4) and A549 cells (Figure 5).

The MTT assays with HeLa cells confirmed that N. *microscopicum* extract-1 has the highest cytotoxic activity (Figure 4A). The mean IC_{50} value for HeLa cell line treated with E-1 for 24 h is 269.7 µg/mL. Extract-2 also showed a prominent inhibition of the cell functions (Figure 4B) at the highest test-concentration (400 µg/mL).

Similar to HeLa and FL, A549 cells displayed the

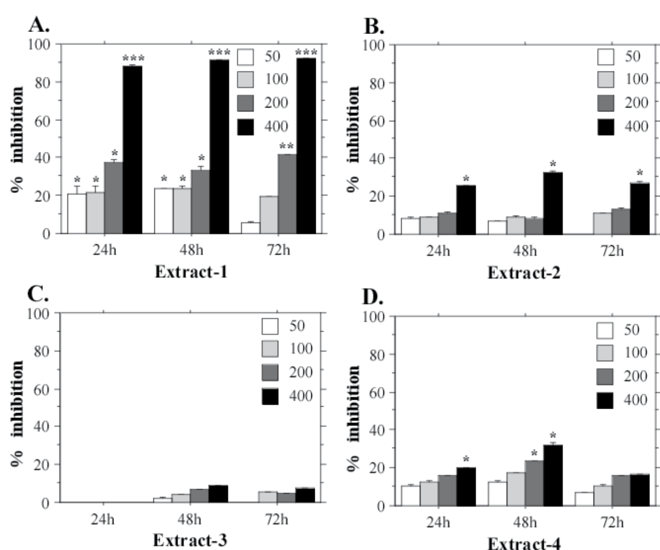

Figure 3: Cytotoxic effects of different concentrations (50, 100, 200, 400 µg/mL) of *Nostoc microscopicum* extracts on FL cells after exposure for 24, 48 and 72 h determined with the MTT assay. Data are reported as mean values±SE from individual determinations with at least three replicates. Asterisks indicate significant differences in percentage of inhibition compared to the control (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

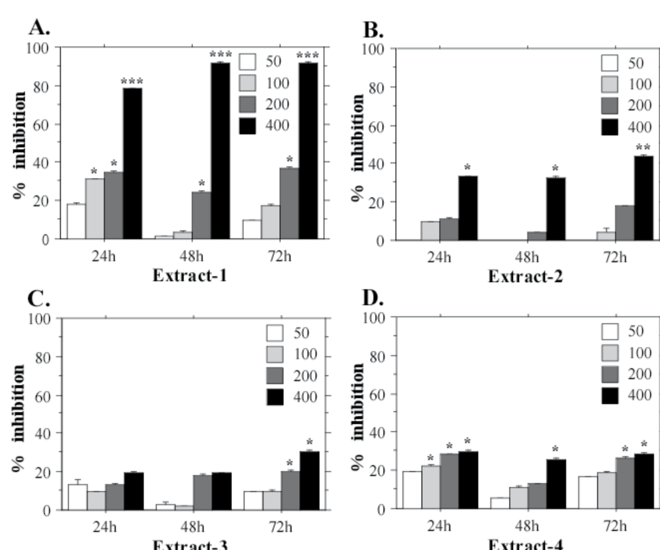

Figure 4: Cytotoxic effects of different concentrations (50, 100, 200, 400 µg/mL) of *Nostoc microscopicum* extracts on HeLa cells after exposure for 24, 48 and 72 h determined with the MTT assay. Data are reported as mean values±SE from individual determinations with at least three replicates. Asterisks indicate significant differences in percentage of inhibition compared to the control (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

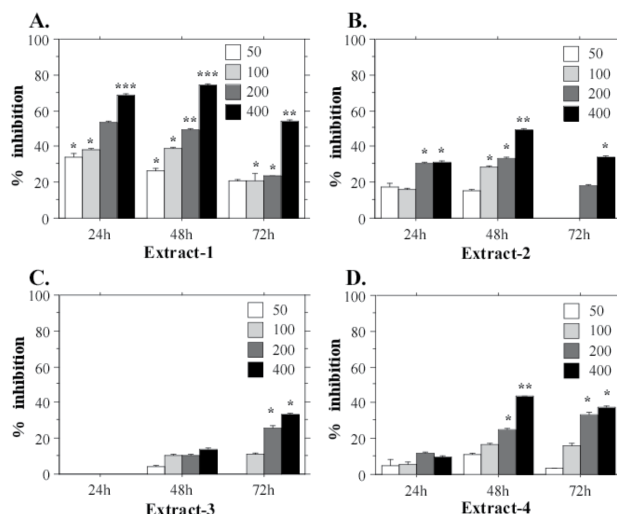

Figure 5: Cytotoxic effects of different concentrations (50, 100, 200, 400 µg/mL) of *Nostoc microscopicum* extracts on A549 cells after exposure for 24, 48 and 72 h determined with the MTT assay. Data are reported as mean values±SE from individual determinations with at least three replicates. Asterisks indicate significant differences in percentage of inhibition compared to the control (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

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strongest inhibition of the mitochondrial dehydrogenases when treated with extract-1 (Figure 5). This cell line also demonstrated highest sensitivity to E-1 with significantly lower IC_{50} value at the 24 h test-period (173.3 µg/mL) at 400 µg/mL. E-1 exhibited the highest cytotoxic effect during the first 24 h of exposition, while at the longer exposition times (48 h, and especially 72 h) the inhibition effect was weaker (Figure 5A). On the contrary, extract-3, which generally exhibits the weakest cytotoxic activity, shows its effect after a longer exposition period, i.e. 72 h (Figure 5C). This tendency is evident also for HeLa cells (Figure 4C), which suggests a specific mechanism of action for E-3 on the cancer cell lines. However, more experiments are needed to confirm this hypothesis.

DISCUSSION

Over recent decades scientists and industrialists have concentrated their interests on natural antioxidants. The reason is that synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ) and propyl gallate (PG) have been shown to manifest carcinogenic and toxic effects in experiments with laboratory animals.[39] Microalgae, in particular cyanoprokaryotes, have been suggested as a very convenient source of antioxidants.[9] They are phototrophic organisms and thus, they have developed effective antioxidant systems that protect them from the free radicals produced during photosynthesis. Microalgae could be easily grown in a laboratory and used for large-scale cultivation in bioreactors with the ability to control the quality of the cultures by providing purified culture medium that is free of pesticides, herbicides and other toxic substances. Therefore, microalgae provide a more accessible way to produce qualitative substances of interest.[24]

Several research groups have recently published data showing antioxidative potential of extracts derived from different *Nostoc* species,[5,7,40] as well as production of specific antioxidants.[6] To date, there are no reports on *Nostoc microscopicum* concerning antioxidative potential or manifestation of other biological activities. Ivanova et al[41] have demonstrated different genotoxic effects of *Nostoc microscopicum* extracts on *Allium cepa*. Hrouzek et al[42] report a weak cytotoxic effect of *Nostoc microscopicum* extract on mouse cell lines YAC-1 and Sp/2. However, there is no information about potential toxic effects of this cyanoprokaryotic species on human cell lines. The lack of data about *Nostoc microscopicum* and the numerous reports on diverse biological activities of extracts or purified substances from many *Nostoc* species stimulated our investigations on the antioxidative potential, cytotoxic effects and cyanotoxins content of N. *microscopicum* extracts.

The examined N. *microscopicum* extracts showed differences in antioxidant activity. They differed in DPPH free radical scavenging activity and in total phenolic content. Presumably, the main reason for this variation is the type of solvent used for extraction. All extracting agents, namely acetic acid, methanol, ethanol and DMSO belong to the group of polar solvents. Among them, only DMSO is aprotic polar solvent, while the others are protic solvents. Protic and aprotic polar substances differ in mechanism of action. Aprotic solvents do not form hydrogen bonds between dissolved molecules and thus, provide relatively free and more reactive nucleophiles in the solution. Protic solvents like water form hydrogen bonds. They could play a role of weak nucleophiles that form bonds with weak electrophiles.[43] Our results indicate that protic polar solvents, in particular DMSO, effectively extract non-phenolic compounds with antioxidant activity.

It has been suggested that phenolic compounds display antioxidant activity in microalgae. Recently, it was found that total phenolic content in microalgae biomass increases after exposition to UV light, which shows that these compounds participate in the antioxidant response against the stress induced by UV light.[44] In addition, there are reports that compared the antioxidant activity and total phenolic content of extracts from different microalgal species. They clearly demonstrate that the extracts with high phenolic content have also high antioxidant activity.[45,46] Goiris et al[3] demonstrated that phenolic compounds play a significant role for the antioxidant activity of microalgae. Interestingly, in our experiments we found a reversed correlation between the total phenolic content of *Nostoc microscopicum* extracts and the results from the DPPH assay. The extract with the lowest DPPH radical scavenging activity displayed the highest total phenolic content compared to the other *Nostoc microscopicum* extracts. On the other hand, the extract with highest DPPH radical scavenging activity had the lowest total phenolic content. Probably, these surprising results are based on the characteristics of the assays we used. The Folin-Ciocalteu method detects only a particular group of compounds, i.e. phenolic compounds, while the DPPH assay measures the antioxidant activity of a broader spectrum of substances, including phenols, carotenoids, polysaccharides, polyvalent unsaturated fatty acids, tocopherols and others.[47] Another possible explanation is that the DMSO used as solvent for preparation of extract-4 does not extract effectively phenolic compounds.

Numerous reports have shown that certain widely distributed members of genus *Nostoc* produce microcystins.[27-30,32] Sivonen et al[48] reported that *Nostoc sp*. strain 152 produces 9 hepatotoxic peptides with similar toxicological properties to the hepatotoxic hepta- and pentapeptides produced by other cyanobacterial species. Five of these peptides were identified as novel types of microcystins – LR homologues. Some lichens associated with terrestrial *Nostoc sp.* strain IO-102-1 also produce six rare forms of microcystins.[32] *Nostoc* species produce other toxic compounds except microcystins. Some *Nostoc* root symbionts of sago palms (*Cycas micronesica*) produce β-methylamino-L-alanine[33] – a neurotoxic amino acid, which plays a role in amyotrophic lateral sclerosis/parkinsonism-dementia. These data stimulate the studies on microcystins production by *Nostoc microscopicum*.

Saxitoxins are the main component of a paralytic poi-

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son, known as paralytic shellfish poison (PSP). They block the Na^+ channels in the membranes of nerve and muscle cells resulting in paralyses. A dose of 1-3 mg saxitoxins is lethal for the human and ingestion of 0.5-1 µg could induce deafness and suffocation. Therefore, it is necessary to routinely monitor water basins and foods for saxitoxins content. Unlike microcystins, the information about saxitoxins production by *Nostoc* species is scarce. Teneva et al[40] reported that *Nostoc linkia* and *Nostoc punctiforme* synthesize low amounts of saxitoxins. The present report demonstrates for the first time significant production of saxitoxins by *Nostoc microscopicum*.

The cytotoxic effect of *Nostoc microscopicum* extracts was examined at three exposure periods – 24, 48 and 72 h. The results we obtained show clear dose-dependent responses. The viability of the cells and their functions were inhibited with increasing the concentration of *Nostoc microscopicum* extract. This trend was evident for all three human cell lines treated with cyanobacterial extract – a result that shows a common cytotoxic effect, but not selective antitumor growth inhibition. This common cytotoxic effect could be due in part to the cyanotoxins content in the extracts. We cannot exclude that the toxic effect is induced by novel unidentified compounds, specifically produced by *Nostoc microscopicum*.

CONCLUSIONS

This is the first report that demonstrates cyanotoxins production by *Nostoc microscopicum*. In particular, we show a significantly high content of saxitoxins in *Nostoc microscopicum* extracts. These data warns for special attention and careful purification if this cyanobacterial species is used for manufacture of pharmaceutical and other industrial products. In addition, we report a moderate antioxidative potential of *Nostoc microscopicum* extracts, as well as cytotoxic activity against human cell lines. Collectively, our data contribute for a more detailed characterization of *Nostoc microscopicum* and stimulates further toxicological research on this cyanoprokaryote and research aiming to identify novel biologically active compounds with potential biotechnological applications.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

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Case Report

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Mercury Exposure Through Gold Extraction: Varied Signs and Symptoms of Toxicity

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CASE REPORT

A 48-year-old male called the poison control center noting his "skin is starting to peel off". He stated ten days prior, a container fell from a shelf and spilled approximately 100 milliliters of elemental mercury used for gold extraction. Because it was felt there was a still valuable amount of gold in the mercury, he explored the silvery material for any recoverable specs of gold. Once complete, he used a vacuum to remove the mercury, and swept the remainder under a rug. He noted it was a cold January day, and turned the heat on, also noting there was no exhaust ventilation in this room. He felt he might be coming down with influenza-like-illness (ILI), and spent the next week in bed (in this same room).

The patient was referred to the local emergency department (ED), and instructed to notify the county hazardous materials agency of the spill. In the ED, his vital signs were within normal limits. He was noted to have a desquamating rash of his upper and lower extremities, with mild erythema (Figures 1 and 2). He complained of numbness of his fingers and lower extremities. Laboratory tests including a complete metabolic panel and complete blood count were within normal limits. An electrocardiogram and chest x-ray was normal. A whole blood mercury test and spot urine mercury test were obtained and returned (one week later) with values of 262 mcg/L (reference range; <10 mcg/L) and 144 mcg/g creatinine (reference range; non-exposed adult <4 mcg/g creatinine) respectively. The patient was referred to the Occupational and Environmental Clinic, where he was seen four weeks after the initial event. His county public health agency was notified of the event. He denied any tremor, but noted recent personality changes including depression and decreased interest in social interaction. He noted his rash had resolved, but he continued to have a sensory neuropathy, complaining of worsening balance, dizziness, cold sensitivity and numbness of his arms and legs. These neurologic symptoms limited his occupational and recreational activities, including woodworking, playing a musical instrument and dancing. He noted he had relocated from the location of the mercury spill. A repeat whole blood mercury level returned at 13 mcg/L and a twenty-four hour urine collection returned at 44 mcg/L (reference range; <20 mcg/L). Formal neurologic testing was offered, but due to the patient's lack of health insurance, he was unable to complete this testing. He was lost to follow-up following his clinic visit. The county department of public health was informed about a potential mercury spill at the residence but they were unable to make contact with the patient and the residence was not evaluated.

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Signs of mercury toxicity

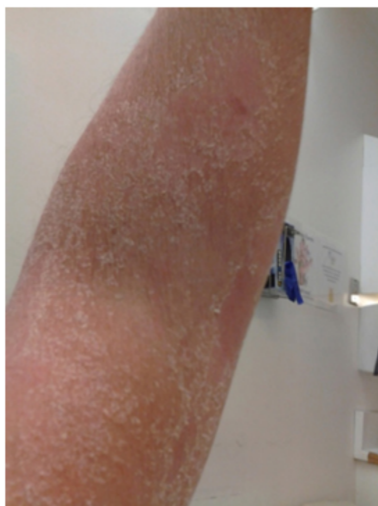

Figure 1: Left arm.

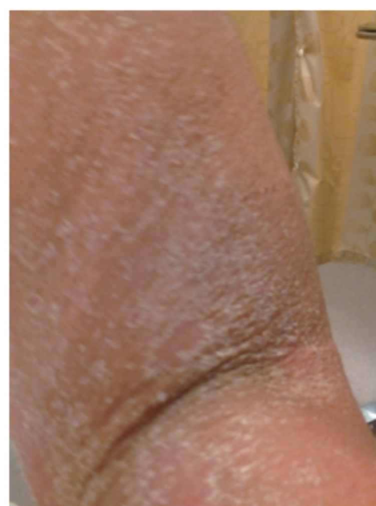

Figure 2: Left arm, closeup.

DISCUSSION

Small-scale mining of gold using amalgamation continues to occur because it efficiently captures gold and doesn't require more costly equipment or processes. Once the gold amalgam is collected, it can be heated to distill off the mercury. Often the mercury fumes are not processed appropriately and are discharged into the environment where they can be inhaled by individuals and cause toxicity.[1] Our patient did not process the mercury with heat. He had a large spill that was improperly cleaned up. Mercury will volatilize into the air at room temperature and vacuuming will facilitate dispersion. He was further exposed to the mercury vapors because he swept the remaining mercury under a rug, did not have ventilation to the outside and spent a prolonged period in that environment.

Elemental mercury exposure *via* inhalation has reported neurotoxic effects. Mercury's purported mechanism of toxicity includes reaction with sulfhydryl groups on proteins of cellular membranes resulting in inhibition of cellular enzymes.[2] Because sulfhydryl groups are ubiquitous, mercury can have effects on every organ system. Neurotoxicity due to inhalation of elemental mercury has been described as affecting the central nervous system (CNS) resulting in neuropsychiatric disturbances and movement disorders. Because the nervous system lacks significant detoxification mechanisms (such as glutathione stores or reduction-oxidation activity), it has been theorized that it is particularly sensitive to mercury. Following acute central nervous system manifestations, rarely peripheral nervous effects have been reported. These reports include both motor and sensory neuropathy.

Dermal effects are also described, including a mercury exanthem with diffuse and symmetric erythematous maculopapular eruptions beginning in the flexural areas and proximal extremities within a few days of exposure.[3] Fever, malaise, and thirst can accompany this, mimicking an infectious illness. The condition clears with extensive desquamation, particularly on the hands and feet, at around 14 days. Diagnosis depends on integrating characteristic findings with a history of known or potential exposure, along with elevated mercury blood or urinary excretion levels. Our patient was clearly exposed to mercury fumes. Unfortunately, the county department of public health was unable to make contact to evaluate the residence and document air levels. He presented with a desquamating rash and numbness of his fingers and lower extremities. The rash had resolved on follow-up four weeks later. He experienced depression, anhedonia, diminished balance, dizziness, sensory neuropathy with cold sensitivity with numbness of his arms and legs. Whole blood mercury and urinary excretion levels were elevated 10 days after the initial mercury spill and remained elevated four weeks later. All symptoms consistent with a diagnosis of mercury toxicity.

With the increases in the price of gold, artisanal gold mining has experienced resurgence, both in the United States and Internationally. The risk for mercury exposure is significant.[4] Elemental mercury exposure can be toxic to multiple organ systems, including the nervous and dermatologic systems. Clinical manifestations can vary. The index of suspicion must be raised in people working with jewelry or gold recovery. The risk is increased when patients are handling seemingly innocuous materials. Public health authorities should be notified when suspicious cases arise, as they may result in multiple exposures.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Opinion

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The Role of Industry in the Modern Opioid Crisis

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In March 2016, the Centers for Disease Control and Prevention (CDC) issued newly updated guidelines to address the growing opiate epidemic in the United States.[1] The long-awaited issuance from the CDC has not been without detractors, however, with many criticizing its scope as short-sighted and advantageous to corporate interests.[2] The backlash is not unexpected, particularly when contextualized alongside the alarming growth in the rate of addiction and mortality associated with opioid and opiate-derivative prescription in contemporary society.

In 2008, prescription painkillers were estimated to have been responsible for approximately 14,800 overdose deaths: a figure which rose above deaths attributed to cocaine and heroin combined.[3] In 2014, the number reached 28,657 in the United States.[4] This number includes overdoses due to heroin, a type of opiate. However, heroin is not without significance in this topic given that four out of five individuals addicted to heroin began with prescription opioid painkillers.[5] Europe is an interesting comparison, demonstrating a decline in overdose deaths: 7,100 to 6,100 in 2009 and 2013 respectively.[6] According Robert Anderson, the Chief of Mortality Statistics at the CDC, the situation has grown comparable to the HIV epidemic in the late 1980's and 1990's.[7] Opioids contributed to more than 61% of overdose-related deaths in 2014 (increases in drug and opioid deaths).

Drug overdose, the leading cause of accidental death in the United States, has grown concomitantly with prescription pain reliever sales and admissions related to substance use disorder treatment.[8] From 1999 to 2008, the overdose death rate grew by 400%, sales of prescription painkillers grew by 400% from 1999 to 2010, and admissions for substance use disorder grew by 600% from 1999 to 2009.[3] Correspondingly, deaths due to heroin overdose quadrupled from 2000 to 2013.[5]

The growth in sales of prescription painkillers is partially explained by sordid marketing tactics commonly practiced by pharmaceutical manufacturers.[9] In 2007, Purdue Pharma, the manufacturer of narcotic painkiller Oxycontin, pleaded guilty to illegally promoting the drug as less subject to abuse and addiction than alternatives on the market.[10] The company admitted to instructing subordinates to describe the drug as safe to health care professionals and to promote the drug for indications not approved by the U.S Food and Drug Administration (FDA). It was fined $600 million by the Department of Justice: more a cost of doing business than a punitive deterrent considering the $31 billion in revenue generated by Oxycontin.

An investigation by the Los Angeles Times revealed that Purdue possessed but withheld from law-enforcement information suggesting that its drug was being trafficked illegally. Indeed, the company was monitoring an illegal distribution operation in a California district under the auspices of Representative Judy Chu (D-Monterey Park). Illegal distribution took place for years unchecked while Chu received over $31,000 in contributions from the pharmaceutical industry, according to the Center for Responsive Politics.[11]

In addition to the CDC draft guidelines, high level officials, including members of Congress and The White House, have responded to increasing pressure in recent years to en-

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act change by enacting legislation to expand grants for prescription drug abuse and treatment.[12] The President asked congress for $1.1 billion in fiscal 2017 to fight prescription opioid and heroin abuse. The Comprehensive Addiction and Recovery Act (CARA) was recently passed in the Senate to combat drug abuse. Not unsurprisingly, the pharmaceutical industry has mounted its own opposition against perceived threats to the $9 billion opioid market.[13]

To take one example, The Washington Legal Foundation (WLF), an industry-supported non-profit organization, criticized the new guidelines through a series of tortured legalisms. They argued that the CDC, a public institution, should have been more inclusive of external parties during the drafting process. The WLF describes itself as a "public-interest law firm and policy center" which devotes a substantial portion of its resources to defending free-enterprise principles.[14] Their clients have included Johnson & Johnson and Purdue Pharma, the manufacturer of Oxycontin. The group has accepted over $1 million in donations from Charles and David Koch, the billionaire financiers known for supporting ultra-conservative causes politically and financially.[15] One can only surmise which external parties the group had in mind.

Although political support for the treatment of addiction has been generally positive, it has not gone unnoticed that serious efforts to curtail access to these drugs, i.e the source of the problem, have not taken place in legislative chambers: an obvious victory for industry groups.[16] Indeed, at the very same time that lawmakers were proposing measures to treat addiction and drug abuse, Congress and the Executive Branch signed measures to curtail the Drug Enforcement Agency's (DEA) abilities to take action against manufacturers, pharmacies, and wholesalers suspected of distributing narcotics inappropriately.[17] While evidently another boon for the pharmaceutical sector, supporters claim that the Ensuring Patient Access and Effective Drug Enforcement Act of 2016 will facilitate cooperation between industry and law-enforcement. Not unsurprisingly, the top DEA official for the regulation of drug manufacturers resigned in protest.

The evidence outlined above suggests a role for industry brokers that extend beyond garden variety market participants. It is easy to carry on, in fact, as misbehavior in the pharmaceutical industry makes a regular appearance in the major headlines around the world: one only need to pay attention. Primary care physicians, including emergency medicine providers, ought to remain especially vigilant, as we provide nearly half of the opioid painkillers consumed in the United States.[18]

It is widely recognized among medical professionals and laypersons alike that opioid analgesic overdose is a potentially lethal, but wholly preventable, condition that results from prescribing practices, inadequate understanding on the patient's part of the risks of medication misuse, errors in drug administration, and pharmaceutical abuse.[19] However, the downstream effects which devastate contemporary society are only recently being elucidated and examined by mainstream scholars featured in the toxicology and forensic medicine literature. It goes without saying that we have a moral and professional duty to treat pain when we see our patients suffering. Nevertheless, if we want to get serious about preventing opioid toxicity for the benefit of society, we must recognize our moral and professional responsibility as academics and privileged intellectuals to investigate the power dynamics which govern in the background of our clinical practice. It is the author's opinion that this interpretation of the physician's duty generalizes to all of medicine, the opioid epidemic notwithstanding.

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Research

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Adverse Consequences of Alcohol Consumption: A Preliminary Study

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ABSTRACT

Alcohol is a depressant drug which slows down the activity of the central nervous system (CNS) and cause health related problems associated with various positive and negative social experiences. It has both pharmacological and toxic effects on mind and other organs of the human body. In spite of the fact that alcohol is addictive and injurious to health, more than 2 billion chronic alcoholics prevail throughout the world and the number is alarmingly increasing with the addition of new drinkers including teenagers every year. At the same time, young women are found to be more vulnerable towards the adverse consequences of alcohol consumption due to their intrinsic physical differences than men. In recent years, a drinking culture has been socially accepted among the women and it is a matter of great concern among the health scientists. Alcoholism sees as the world's highly prevalent public health problem and therefore it is a serious issue, not confined to any group, culture or country. In view of increasing incidences of alcohol consumption in India and other countries associated with health related and social problems, the present study is carried out to assess the consumption pattern of alcohol in a selected population and try to understand the ill-effects of this social malice on the drinker's social behavior.

KEYWORDS: Alcohol; Health effects; Social problems; Criminal behavior; Addiction.

INTRODUCTION

Alcohol is generally known as ethyl alcohol which is a low molecular weight hydrocarbon derived from the fermentation of sugars and cereals. The term toxic alcohol referred to isopropanol, methanol and ethylene glycol. It is a sedative hypnotic drug which affects the CNS to cause depression and alter the mood and behavior. Alcohol intoxication is also called as drunkenness which is induced by the ingestion of alcohol and it causes a feeling of well-being, self-confidence, talkativeness and excitement.[1,2] Addiction is a progressive and incurable disease, which affects physical, mental and spheres of human beings, characterized by inability to control their actions over the use of alcohol and other sedatives. The factors including psychological, genetic and behavior can all contribute to having this disease. There is no such effective remedy for this ever growing and frightening disease.[3,4] The major forms of this dependence are either inability to stop drinking before drunkenness is achieved, or inability to abstain from drinking because of the withdrawal symptoms.[5] Alcohol is remained to be one of the most common psychoactive substances which have been used for recreational, medicinal and ceremonial purposes for at least 10 thousand years.[6] Earlier, the intake of alcohol was believed to relief from stress but now it is widely accepted fact that alcohol consumption, even at low amount is associated with heart problems and mortality in middle aged and older adults. The involvement of alcohol in numerous premature deaths due to disease, accidents and violence has been reported every year. World Health Organization (WHO)[7] reported that 4% of the global burden of disease is attributable to alcohol and it is the third leading cause of death and disability in developed countries, after tobacco and hypertension. More than 8 million Ameri-

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cans are believed to be dependent on alcohol and up to 15% of the population is considered at risk. Studies have also revealed that more than half of all trauma patients are intoxicated with ethanol at the time of admitted to the hospital. In a report from US Poison Control Centers, 219 major outcomes and 15 deaths were reported from 6026 single exposures to ethanol in beverages in 2014.[8] The harmful consequences of alcohol, including injury to physical health, psychological well-being and relationships cause an impact on all facets of society. At the same time, moderate alcohol consumption has certain health benefits like beneficial influence on coronary heart disease, stroke and diabetes mellitus[9] while excessive chronic drinking resulting in alcohol toxicity including mild to moderate tremors, irritability, anxiety and agitation.[10,11] There has been a marked increase of female alcoholics worldwide including India in recent years.[7] It acts as a teratogen and the consumption of heavy alcohol during pregnancy may lead to fetal alcoholic syndrome.[11] Behavioral and cognitive deficits have also been reported even at low-levels of prenatal alcohol exposure which further suggested that consumption of alcohol is not safe in pregnancy.[7] Hangover generally reported by the drinkers, can be characterized by the assemblage of unpleasant physical and mental symptoms, including headache, fatigue, redness of the eyes, thirst, rapid heartbeat, tremor, sweating, dizziness, vertigo, depression, anxiety and irritability. Multiple factors such as the effects on urine production, gastrointestinal, blood sugar concentration, sleep pattern, biological rhythms have been suggested for the onset of hangover.[12]

Alcohol is rapidly absorbed from the gastric mucosa (20%) and small intestine (80%) and reached at a peak concentration 20-60 minutes after ingestion into the bloodstream where it affects almost every organ in the body, but the brain is quite vulnerable. In the liver, it is oxidized to acetaldehyde by alcohol dehydrogenase and coenzyme nicotinamide adenine dinucleotide. This acetaldehyde converted into acetic acid, which is further undergoes oxidation to carbon dioxide and water.[13] Experimental studies have shown the importance of situational factors, setting and social context in determining the adverse health effects and social consequences of drinking. The social consequences, including family problems, stranger violence, non-traffic injuries, social interaction and attempted suicide have not received greater attention as problems associated with alcohol. The issues, mainly related with the long-term drinking and driving have dominated public discourse on alcohol related problems. A positive association has been observed in chronic consumption of alcohol and suicide.[14] There are various ways through which alcohol consumption may lead to suicide as it may develop suicidal ideation into action and also provide motivation to carry out a suicidal attempt.[15] Further, impaired decision making and difficulties in problem solving ability may trigger a person to commit suicide as they think that it is the only solution of their problems. There are about an estimated 2 billion alcoholics worldwide and this number is increasing with added new drinkers every year including teenagers. Due to the consumption of alcohol and associated adverse health effects, it ranks among the world's major threats to human health and safety.[7] According to WHO data, alcohol abuse leads to the death of 3.3 million people every year and the death of 4.9 million people were reported in 2010.[7] It also leads to 25 percent of the road accidents and is a major contributor in domestic violence and family disputes.[16] Due to increasing incidences of alcohol intoxications and adverse health effects associated with it, the present study is carried out to assess the consumption pattern of alcohol in a selected population and try to understand the ill effects due to the alcohol consumption associated with abnormal social behavior and problems.

MATERIALS AND METHODS

The consumption of alcohol has been considered as social evil and there should be some measures to eradicate this evil. In the forensic science, the knowledge of alcohol and its adverse effects on body and collection and preservation of samples related with alcohol intoxication will be important for law enforcement agencies for the effective implementation of law and order. Alcohol intoxication and addiction can have adverse health and socio-economic effects on the individual drinker and also the whole environment. At the same time, the individuals other than the drinkers can also get affected by drinker's unwarranted acts like traffic accidents, aggression and violent behavior. These undesirable acts create unnecessary pressure on public resources *viz* criminal justice, health care and other social institutions. The study pertaining to the pattern of alcohol consumption and associated adverse social consequences is needed, especially when the consumption of alcohol is increasing in the women and teenagers. It is creating an alarming situation in the public sphere. Hence, it is desirable to regulate the consumption of alcohol in our youngsters to prevent the alcohol dependency and protect our society from this malice. Although the analysis of alcohol in a biological sample is necessary at the molecular level, but the preliminary studies, related to its intoxication and consumption pattern also useful in disseminating the information about its harmful impact onto the society.

To fulfill the aforesaid objectives, an empirical study was carried out in Sagar District of Madhya Pradesh, India. A questionnaire was prepared with 28 items, in which individual's details including age, sex, socio-economic profile along with the background, consumption pattern of different alcoholic drinks and associated ailments were in-listed. The questions related to the behavior and other experiences during and after intoxication and adverse health effects in term of disease or toxicity were also included in the questionnaire. The random sampling method was employed to collect quantitative data pertaining to the alcohol consumption and associated consequences in the studied population.

The study was conducted between the month of August and November, 2015 with the sample size of 150 individuals. The questionnaire was distributed among the selected volunteers and they were asked to fill the answer without disclosing their identity. The volunteers were from age groups of 10-50 years

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and from different socio-economic background. Most of the volunteers were from male category and literate. Total 150 personal interviews were conducted in which questions related to individual habits, experiences, causes of alcoholism, associated diseases, weak performances on occupations, interventions in this regard were discussed. Questions related to the domestic violence, involvement in illegal activities and withdrawal symptoms were also discussed thoroughly. Data were collected and scores of individual questionnaire were tabulated for the analysis. After analyzing the data of percentage changes, specific inferences were made and presented in the study.

RESULTS

Socio-Economic Background of the Drinkers

The socio-economic background of the participants has been analyzed and represented in Table 1. In the present study out of total sample collected, 91% participants were male, while 9% participants were female. Forty-three percent of the participants were from rural backgrounds and 57% were from urban background. Those who participated in the study, 48% were from the joint family and 52%, were from nuclear family system. The economic status of the person has been represented in lower income, middle income and higher income class where we have found that the largest drinkers were from lower income group (55%) while educational background represents that the literate have the highest percentage of alcohol consumed (81%).

Reasons for alcohol consumption: There is no specific reason which can be assigned why people start drinking. There are a variety of situations in which people start consuming alcohol. In the present study, we have reported that the most of the people consumed alcohol because of stress (46%), happiness (62%), depression (17%) and other reasons (4%). The details are represented in Figure 1.

Initial age of alcohol consumption: The maximum unsafe use of alcohol has been reported in the populations from the age of 15-29 years. The study has been carried out on 150 persons of different age group such as 10-11, 11-15, 16 and above, in which 3% were in the age between 10-11, 30% in between 11-15 and 66% were in the age of between 16 and above in which they started consuming alcohol. The details are represented in Figure 2.

Adverse consequences of intoxication: The persons other than the drinkers also get affected from adverse consequences of alcohol through the traffic accident and violent behavior. Adverse health effects due to consumption of alcohol has been observed and presented in Figure 3. The study revealed that there were various situations in which drinkers mainly involved after the intoxication. These consequences include traffic accidents (34%), law and order situation (23%), admitted to hospital due to injury or accident (8%) and business problem (22%).

S. No.	Participant details	Class	Percentage
1	Gender	Male	91%
		Female	9%
2	Place	Rural	43%
		Urban	57%
3	Family type	Nuclear	52%
		Joint	48%
4	Economic status	Lower	55%
		Middle	17%
		Higher	28%
5	Education	Literate	81%
		Illiterate	19%

Table 1: Details of participants represented from various classes and background.

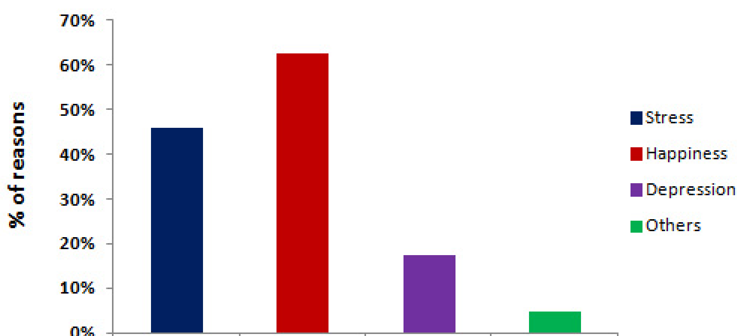

Figure 1: Various factors of alcohol consumption including stress, happiness, depression etc.

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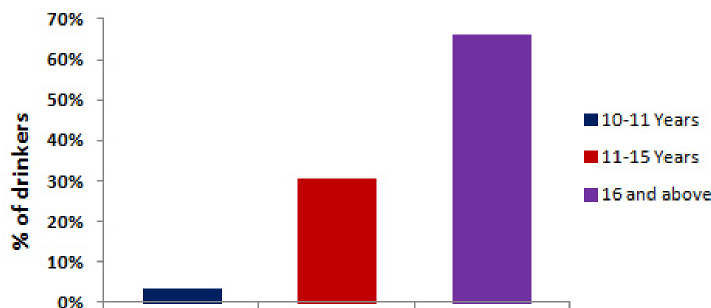

Figure 2: Initial age of starting alcohol consumption revealed the pattern of alcohol consumption and age of maximum adverse consequences.

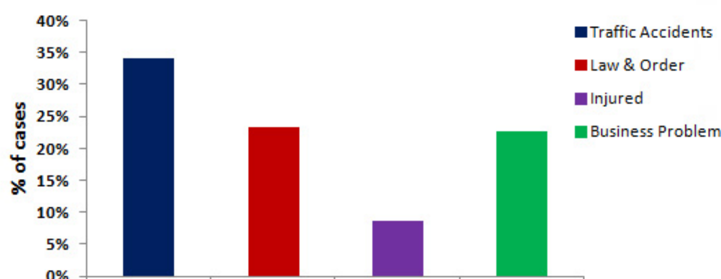

Figure 3: Adverse consequences of intoxication resulted in traffic accident and various other problems.

Impaired behavior during intoxication: The study revealed that the behavior of peoples during intoxication significantly modified. People have reported that they have been feeling bad (22%), active in illegal activities (14%), aggression (26%), domestic violence (19%) and involved in suicidal attempts (9%). The details are represented in Figure 4.

Withdrawal symptoms of alcohol: The consumption of alcohol is addictive and it results the dependency. If drinkers will not get the alcohol at the time of their need or desire, altered mood and behavior may be shown in terms of withdrawal symptoms. The study has been revealed that the condition of the person during need or absence of alcohol such as anxiety (34%), aggression (32%), stealing money (13%) and ready to perform illegal activities (8%). The details are represented in Figure 5.

Adverse effects on health after intoxication: Alcohol intoxication leads to various effects on human health. On the basis of self assessment and questionnaire based study, it has been observed that people showed a number of effects during alcohol intoxication such as insomnia (11%), slurred speech (46%), vomiting (10%), diarrhea (9%), stomach upset (10%), headache (17%), breathing difficulties (9%), distorted hearing (16%), impaired judgment (7%), anemia (11%), coma (2%) and black out (19%). The details are represented in Figure 6.

Disease due to heavy and regular intake of alcohol: Acute and chronic alcohol consumption has been found to be largely asso-

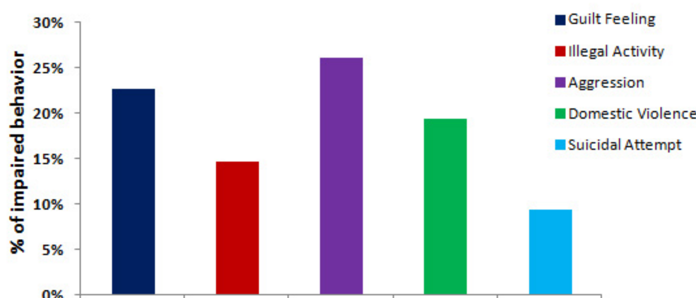

Figure 4: Behavior of persons during intoxication found to be impaired as abnormal behavior including aggression, violence and suicidal attempts.

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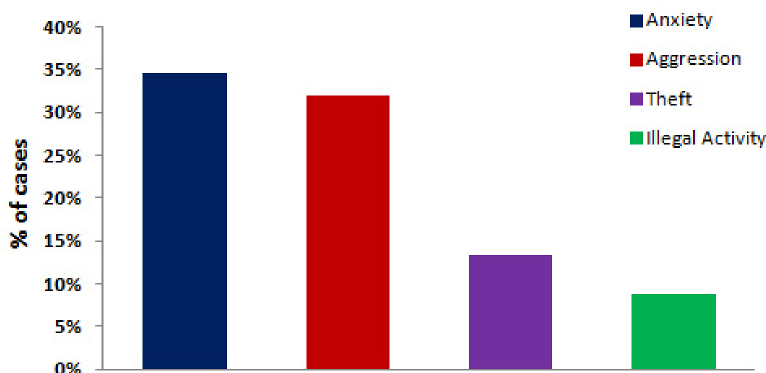

Figure 5: Withdrawal symptoms of alcohol due to lack of alcohol may resulted in altered mood and behavior conditions.

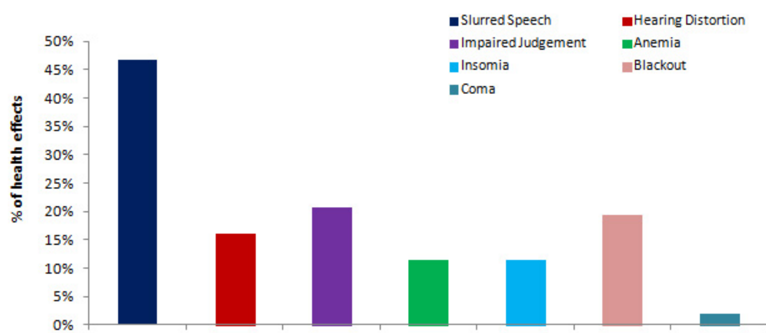

Figure 6: Adverse health effects of alcohol intoxication includes various symptomatic effects including slurred speech, insomnia, breathing difficulties, distorted hearing, anemia and others.

ciated with the various diseased conditions. In the present study, people have reported that they suffered various disease conditions, including liver problems (16%), brain related problems (16%), mouth or throat problem (8%), heart related problems (6%), blood pressure (14%) and other problems (12%). The details are represented in Figure 7.

Direct effects of alcohol: Direct effects of alcohol may occur in the form of hangover which may include dehydration, gastrointestinal disturbances, low blood sugar and altered sleep pattern. In the present study the direct effects of alcohol are seen in the form of diarrhea (9%), headache (17%), dizziness (16%), sweating (22%), vomiting (10%), gastritis (6%) and fatigue (8%). The problem of sweating, diarrhea, dizziness and vomiting are commonly associated with dehydration while gastrointestinal disturbances include gastritis and abdominal pain. Symptoms of fatigue are generally linked with the low blood sugar levels and altered sleep pattern. The details are represented in Figure 8.

DISCUSSION

The chronic consumption of large amount of alcohol may be a risk factor for the development of peripheral neuropathy with the sensory symptoms including numbness, paresthesias, dysesthesias, allodynia, and loss of vibration and position sense mainly in the distal lower extremities.[17] It is a primary axonal neuropa-

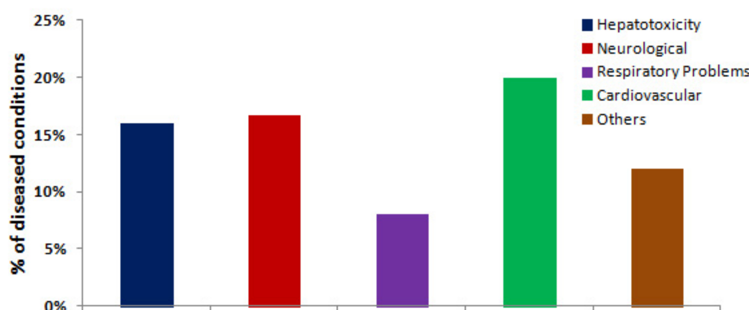

Figure 7: Diseased conditions due to chronic and acute alcohol consumption leading to the serious health related issues.

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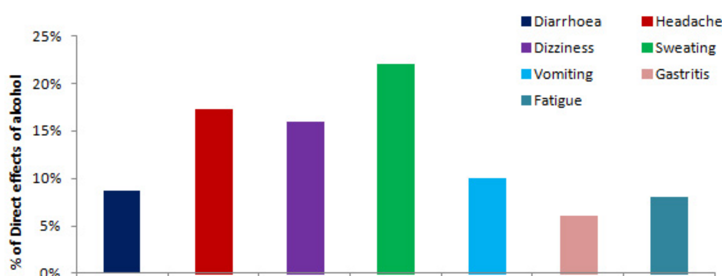

Figure 8: Direct effect of alcohol due to dehydration/electrolytic imbalance, gastrointestinal disturbances and low blood sugar levels.

thy marked by wallerian degeneration of the axons and myelin sheath neural fibers.[18] Studies have also suggested that nutritional deficiencies including thiamine may be linked with the development of neuropathy in patients. The implicated mechanisms behind the neuropathy have found to link with the increased oxidative stress leading to free radical damage to the nerves.[19,20] Besides, direct effects to the human body, alcohol intoxication is also associated with the social problems, including domestic violence, traffic accidents, child neglect, abuse, absenteeism in the workplace and others.[21] The harmful use of alcohol becomes the leading risk factor for death in male ages from 15-59 due to the injuries, violence and diseases.[22] Further, its consumption is an important cause of mortality, morbidity and social damage worldwide. The cases of drunken driving and road rage have been well established through experimental and epidemiological studies.[9,23] There are various aspects of drinking through which household functioning of a family member becomes impaired. In our society, people usually drink outside the family and home and thus, affect the family duties of an individual. It also costs money and hampers economic resources to leave the other family members on the breadline. White and Chen,[24] reported the linkage between drinking patterns and partner violence. They further explained that persons who heavily consumed alcohol and develop dependency are more likely to show violent behavior with their partners. In the present study, the behavior of persons after intoxication has been found to be impaired and involved in illegal activities, aggressive behavior and domestic violence.

The adverse health effects on children are also found to be associated with the alcohol use by the family member or caretaker. Youngster has been found to have at a greater risk to develop alcohol dependence due to their immature brain as compared to adult.[25] Few drinkers have been reported suicidal tendencies, as they thought that it is the only solution of their problems. It is further associated with the damage to the brain regions involved in learning and memory, decision making and reasoning.[26] An injury in the brain parts may lead to the functional abnormalities which can further associated with the lower academic performance and affect the learning and cognitive functions.[27,28] Toumbourou et al[29] reported that unsafe use of alcohol caused approximate 31.5% of deaths among 15-29 year persons worldwide. In the present study, we have found that the maximum drinkers started drinking at the age of 16 years or above but the percentage of drinkers under the age of 15 are also remarkable and indicates an alarming situation. The adverse consequences of alcohol intoxication such as slurred speech, vomiting, diarrhea, stomach upset, headache, breathing difficulties, distorted hearing, impaired judgment, anemia, insomnia, coma and blackout have been observed in the present study. Further, withdrawal symptoms in the absence of alcohol, including anxiety, aggression, stealing money and involvement in illegal activities have also been observed.

The consumption of alcohol in women is reported less frequently than men and therefore women are at lower risks of adverse health consequences than male drinkers. Due to the physical difference, the metabolism of alcohol in women occurs differently than men and hence the risks including liver cirrhosis, alcohol dependence linked with alcohol consumption are often augmented for women.[30] The incidences of breast cancer have been reported in drinkers who consumed moderate to heavy drink.[31] Smith-Warner et al[32] suggested that low consumption of alcohol (one drink per day) is associated with a 9% increase in the risk of developing breast cancer relative to non-drinkers, while consuming higher quantity may lead to risk of breast cancer to 41%. Consumption of alcohol during pregnancy may cause complication of pregnancy and delivery associated with spontaneous abortion and stillbirth.[33] It may also affect the developing fetus and cause neurological disorders in the child.[34,35] In the present study, the ratio of consumption of alcohol in women was found less compared to men probably because of the patriarchal setup in which women are supposed to be inside the house and their freedom is restricted in many ways. Adverse consequences of alcohol in terms of disease conditions including hepatotoxicity, neurological problems, anxiety, cardiovascular diseases and others have also been observed in the present study indicating the adverse health effects of alcohol consumption.

CONCLUSIONS

We can conclude from the present study that in the studied population group the persons were found consuming alcohol. Due to the consumption of such products their overall performance was also affected. There was the high number of cases reported re-

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lated to the road accident and disputes in the family. The middle class population was found to be largely consumed alcohol, primarily for pleasure and euphoria. There is a need for awareness regarding the ill-effects of alcohol consumption and people should be made aware about the social, economical and health consequences of alcoholism. It is needed to survey the general population with specific groups in a systematic manner in order to monitor changes in levels of alcohol consumption and drinking patterns. The monitoring of early alcohol use can help to prevent the development of alcohol dependence and protect our society from its adverse consequences.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Research

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Effects of Exposure to Cypermethrin on the Onset of Puberty and Ovarian Biomarkers in Female Prepubertal Rat: Attenuating Role of Zinc

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ABSTRACT

Cypermethrin, a synthetic pyrethyroid pesticide, is used for more than one decade to control a wide variety of pests in agriculture. The present study designed to evaluate the protective role of zinc in attenuating cypermethrin induced reproductive toxicity in female prepubertal rat. Female prepubertal rat received oral cypermethrin alone at two dose levels and zinc alone or combined with cypermethrin for consecutive 14 days. Cypermethrin arrested vaginal opening, reduced the weights of ovaries and uterus. Total cholesterol and ascorbic acid content of the ovaries were elevated whereas the activities of Δ^5,3β-hydroxysteroid dehydrogenase and 17 β-hydroxysteroid dehydrogenase were decreased in a dose-dependent manner. In the adrenal gland of rat these parameters showed opposite findings. The levels of serum LH, FSH and estradiol were also decreased. Cypermethrin treatment also produced oxidative stress in ovary by significant increase in malondialdehyde level, accompanied by a reduction in reduced glutathione and antioxidant enzymes. From the results, we may conclude that cypermethrin suppresses the female reproductive functions in rat by disrupting the estrous cycle and ovarian biomarkers by increasing oxidative stress and zinc attenuates the cypermethrin-induced toxicity.

KEYWORDS: Cypermethrin; Zinc; Vaginal opening; Δ^5,3β-hydroxysteroid dehydrogenase; Ovarian steroidogenesis.

ABBREVIATIONS: GnRH: Gonadotropin Releasing Hormone; HSD: Hydroxysteroid Dehydrogenase; ANOVA: Analysis of Variance.

INTRODUCTION

Pesticides are used worldwide to control both agricultural and household pests. In 2001, United States used approximately 122 million pounds of insecticides, and 12% of those compounds were for home and garden use.[1] One of the most frequently used classes of pesticides is the pyrethroids,[2] the synthetic analogs of the naturally occurring toxin pyrethrin, which is derived from the flowers of *Chrysanthemum cinerariafolium*. They reported approximately one fourth of the worldwide market for insecticides in 1998, and their use is continually growing.[3] Pyrethroids exhibit their toxic action by modifying the “gating” characteristics of neuronal voltage-sensitive sodium channels to delay their closure, thereby prolonging neuronal excitation.[4,5] Pyrethroids are considerably less toxic to mammals than organochlorines, organophosphates and carbamates. Cypermethrin is a synthetic pyrethroid used not only as ectoparasiticide in animals but also used widely as an insecticide in agriculture and public health programmes. In recent years, much attention is being focused on the possible role of essential trace elements in providing the necessary preventive efficacy with least toxicity and side effects.[6-8] Zinc, a key constituent or cofactor of many mammalian proteins, is intensively being studied for its protective efficiency in various models of animal toxicity.[8] A number of studies have strongly

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suggested zinc to be a beneficial agent in mitigating the damage arising in the setting of increased oxidative stress.[8-10]

The reproductive functions are maintained in vertebrates by hormones of the hypothalamic-pituitary-gonadal axis. Steroids from the gonads are influenced by the hypothalamic-pituitary (HP) axis and send feedback signals to the HP axis. Puberty can be divided into central and the peripheral puberty. Central puberty involves the onset of GnRH and gonadotropin secretions as the HP axis matures.The gonads are stimulated, the HP axis induces gonadal secretion of sex steroid hormones, and the steroids then send feedback to the HP axis. Peripheral puberty includes the processes other than that of hypothalamic-pituitary-gonadal axis. In the female, secondary sex characteristics such as the development of mammary glands, the vaginal opening, and uterine hypertrophy[11] are included under this peripheral puberty. Though the onset of puberty is a genetically driven event,[12] it can be changed by environmental factors,[13] nutritional states, metabolic status, phytoestrogens[14] and pesticides.[15]

The present study was aimed to investigate cypermethrin mediated changes in reproductive functions and also to find out the attenuating role of zinc on cypermethrin induced reproductive toxicity in female prepubertal rat.

MATERIALS AND METHODS

Chemicals and Reagents

A commercial formulation of cypermethrin [(RS)-α -cyano-3-phenoxybenzyl (1RS)-cis-trans-3-(2, 2-dichlorovinyl) 2,2-dimethylcyclopropanecarboxylate] 10% emulsifiable concentrate (EC), named "Ustad" (United Phosphorus Limited, Hyderabad, TS, India) was used in the experiments. Nicotinamide adenine dinucleotide (NAD), dehydroepiandrosterone sulphate, nicotinamide adenine dinucleotide phosphate, glucose- 6-phosphate, bovine serum albumin were purchased from Sigma Aldrich Inc., USA, LH ELISA Kit (Catalog No. CSB-E12654r), FSH ELISA Kit (Catalog No. CSB-E06869r), Estradiol ELISA Kit (Catalog No. CAYMAN 582251) were used all other chemicals used were purchased from Himedia India Ltd., Merck India Ltd., etc.

Animal Care and Treatment

Sixty-six Wistar female prepubertal rats at 25 to 30 days of age (weighing 50 to 60 g) were taken and acclimatized for 10 days before the start of the experimental procedure. The animals were housed in labelled cages with solid plastic sides and stainless-steel grid tops and floors, in a room designed for control of temperature (approximately 25±2 °C), and light cycle (12 h light, 12 h dark). Animals were fed standard laboratory pellets diet and water *ad libitum*. The experiment was conducted strictly in accordance to the Institution's Animal Ethical Committee. After 10 days of acclimatization, the animals were randomly divided to the control and experimental groups, each containing 11 rats. Groups were designed as:

1. Group I: Control (5 ml/kg body wt.)
2. Group II: Zinc (227 mg/l in drinking water) control
3. Group III: Cypermethrin-treated (34.33 mg/kg body wt., Low dose) group
4. Group IV: Zinc+Cypermethrin-treated (34.33 mg/kg body wt., Low dose) group
5. Group V: Cypermethrin-treated (51.5 mg/kg body wt., High dose) group
6. Group VI: Zinc+Cypermethrin-treated (51.5 mg/kg body wt., High dose) group

A commercial formulation of cypermethrin 10% Emulsifiable Concentrate (EC) was used in this study. Adequate dilutions were done with distilled water to get test concentrations (34.33 and 51.5 mg/kg body wt.). Solutions were freshly prepared immediately before usage. The doses were selected on the basis of acute toxicity dose of cypermethrin.[16] Acute oral LD_{50} of female rat (albino) is 309 mg/kg body wt. We have considered the $1/9^{th}$ LD_{50} and $1/6^{th}$ LD_{50} doses from our dose selection study. Control rats received 5 ml of distilled water /kg body wt.

Body weights of the rats in each group were taken before and after the treatment period. All rats were euthanized 24 hours after the last dose. Five rats of each group were kept for the study of the age of vaginal opening and appearance of the first estrous.

Study of Puberty Onset and Estrous Cycle

To determine the onset of puberty for nine days from postnatal day 25, the vaginal opening of female prepubertal rats in each group was checked at 10 AM daily. The estrous cycle was examined daily by the modified method of Marcondeset et al[17] with slight modifications and identified under a microscope (×100) using a vaginal smear flushed with physiological saline for 20 days from vaginal opening.

Measurement of Ovarian and Uterine Indices

Ovaries, uterus and adrenal glands were dissected out from each rat immediately after sacrifice and were done free from adherent tissue and the organ-weights were recorded.[18] The ovaries and adrenal glands were taken out for the estimation of cholesterol, Δ^5,3β-HSD, 17β-Hydroxysteroid dehydrogenase and oxidative stress parameters. Organ weight was expressed as organ index.

Ovarian index=(ovarian wt. (g))/(body wt. (g)) × 100
Uterine index=(uterus wt. (g))/ (body wt. (g)) × 100

Estimation of Ovarian and Adrenal Cholesterol

Ovary and adrenal gland of each rat were homogenized in 0.5% $FeCl_3$ solution (20 mg/ml) for the estimation of cholesterol. The homogenate was then centrifuged at 2000 rpm for 10 min. Then 0.1 ml of the supernatant was mixed with 6 ml of glacial ace-

tic acid.[19] After addition of 4 ml of color reagent (1 ml of 10% $FeCl_3$, 6 H_2O, 15 ml of conc. H_2SO_4) it was mixed vigorously and allowed to stand for 20 min. The reading was taken at 570 nm. The amount of cholesterol present is calculated by plotting the standard curve.

Measurement of Ovarian and Adrenal Ascorbic Acid

For the estimation of ascorbic acid, the ovarian and adrenal tissues were homogenized using 2.5 ml of 5% metaphosphoric acid-10% acetic acid solutions. The mixture was centrifuged after extraction and a very small drop of concentrated bromine was added to the supernatant.[20] Tube was shaken and kept for 10 min for complete oxidation. Excess liquid bromine was then removed. 0.5 ml of dinitrophenylhydrazine-thiourea reagent (2.2% 2, 4-DNPH in of 10N H_2SO_4, 5%) was added with 2 ml of tissue extract and incubated at 37 °C for 3 h and then 2.5 ml of 85% H_2SO_4 was slowly added in ice-cool condition. It was mixed well for half an hour in room temperature for color development and optical density was observed at 540 nm.

Ovarian and Adrenal Δ^5,3β-HSD and 17β-HSD Activity

The tissues (ovary and adrenal gland) were homogenized carefully at 4 °C in 20% glycerol containing 0.01 M EDTA in 0.05 M phosphate buffer.[21] Then the mixture was centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatant (1 ml) was mixed with 1 ml of sodium pyrophosphate buffer (pH-8), 40 µl of dehydroepiandrosterone (DHEA) and finally after the addition of 0.1 ml of NAD, the activity Δ^5,3β-HSD was measured at 340 nm against a blank containing no NAD.

One ml supernatant was added with 1 ml of 440 µM sodium pyrophosphate buffer, 960 µl of bovine serum albumin and 40 µl of ethanol containing testosterone. The activity of 17β-HSD was measured after the addition of NAD to the tissue supernatant mixture in a spectrophotometer at 340 nm against a blank (without NAD). One unit of enzyme activity was the amount causing a change in absorbance of 0.001 per minute at 340 nm.

Estimation of Luteinizing Hormone (LH), Follicle-Stimulating Hormone (FSH) and Estrogen

For the quantitative determination of luteinizing hormone, follicle-stimulating hormone and estradiol ELISA Kit (Catalog No. CSB-E12654r), ELISA Kit (Catalog No. CSB-E06869r) and ELISA kit (Catalog No. CAYMAN 582251) were used respectively. Assays were done according to the instruction of the manufacturers.

Estimation of Oxidative Stress Parameters

Ovarian Malondialdehyde (MDA)

Briefly 1 ml of ovarian homogenate (20 mg/ml) was mixed with 0.2 ml of 8.1 % sodium dodecyl sulfate, 1.5 ml of acetate buffer (20%, pH-3.5) and 1.5 ml of aqueous solution of (0.8%) thiobarbituric acid.[23] After heating the mixture at 95 ˚C for 60 min, obtained red pigment was extracted with 5 ml of n-butanol-pyridine (15:1). Then it was centrifuged at 5000 rpm for 10 min at room temperature and the absorbance of supernatants was noted at 535 nm in spectrophotometer (UV-245 Shimadzu, Japan).

Ovarian Reduced Glutathione (GSH)

Briefly 200 µl of ovarian homogenate (20 mg/ml) was mixed with 100 µl of sulfosalicylic acid and the mixture was centrifuged for 10 min at 3000 rpm. Then in 200 µl of supernatant, 1.8 ml of DTNB was added and shaken well.[24] The reading was taken at 412-420 nm.

Ovarian Superoxide Dismutase (SOD)

Superoxide dismutase was measured by the method of Marklund and Marklund. The SOD activity of the supernatant was estimated by measuring the percentage of inhibition of the pyragallol-auto oxidation by SOD.[25] The buffer contains 50 mM Tris HCl, 10 mM hydrochloric acid (HCl) in the presence of 1 mM EDTA. Then the buffer mixture (2 ml), 100 µl of 2 mM pyrogallol and 10 µl of ovarian homogenate were poured in a spectrophotometric cuvette and the reading was measured in the spectrophotometer (UV-245 Shimadzu, Japan) at 420 nm for 3 min.

Ovarian Catalase (CAT)

In a spectrophotometric cuvette, 1 ml of 30 mM H_2O_2, 1.9 ml of 50 mM phosphate buffer and 0.1 ml of ovarian homogenate (in 0.05M Tris HCl) were taken.[26] After mixing, readings were noted at 240 nm at 30 sec interval.

Ovarian Glutathione-S-Transferase (GST)

In a spectrophotometric cuvette, 0.1 ml of ovarian homogenate, 0.2 ml 100 mM PBS, 0.05 ml of 1 mM GSH and 0.02 ml of 60 mM CDNB was taken in a cuvette and reading was noted at 340 nm. The values were expressed in µmol CDNB conjugate formed/min/ mg protein.[27]

Ovarian Glutathione Peroxidase (GPx)

At first 0.2 ml of 0.4 M phosphate buffer (pH-7), 0.1 ml of 10 mM sodium azide, 0.2 ml of ovarian homogenate (20 mg/ml) in phosphate buffer (pH-7), 0.2ml of 4 mM reduced glutathione and 0.1 ml of 2.5 mM H_2O_2 were taken, mixed and incubated for 10 min at 37 ºC.[28] Then 0.4 ml of 10% TCA was added to stop the reaction and centrifuged at 3200 rpm for 20 min. Then 1 ml of 5, 5′-dithiobisnitrobenzoic acid (DTNB) and 3 ml of disodium hydrogen phosphate (Na_2HPO_4) were added to 0.5 ml of supernatant. The absorbance was measured at 420 nm.

Ovarian Glutathione Reductase (GR)

Briefly 2 ml of oxidized glutathione (GSSG), 20 µl of 12 mM

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NADPH.Na_4 and 2.68 ml of phosphate buffer (PBS) were added with 100 μl of ovarian homogenate and the reading was taken at 340 nm.[29]

Statistical Analysis

The results were expressed as the Mean±SEM. Statistical analyses of the collected data were done by one-way analysis of variance (ANOVA) followed by multiple comparison *t*-tests. Difference was considered significant when $p<0.05$.

RESULTS

Effects on the Age of Vaginal Opening and Appearance of First Estrous

Table 1 shows the effect of cypermethrin on vaginal opening and appearance of first estrous in female prepubertal rat. Compared with the control group, cypermethrin (low and high dose) exposure delayed the age of vaginal opening and appearance of first estrous significantly ($p<0.01$) in a dose-dependent manner. On the other hand, pretreatment of zinc showed comparatively earlier age of vaginal opening and appearance of first estrous.

Reproductive Organ Weights

In cypermethrin treated high dose group ovarian index, uterine index, adrenal index were decreased significantly compared to the control group. Pre-treatment of zinc improved these indices in cypermethrin-induced rat (Table 2).

Ovarian and Adrenal Cholesterol Content

Cypermethrin caused an accumulation of cholesterol ($p<0.01$) in the ovary (Figure 1) of female prepubertal rats, whereas the adrenal cholesterol (Figure 2) in cypermethrin exposed groups were decreased ($p<0.01$). Zinc ameliorated cypermethrin-induced cholesterol accumulation in rat.

Ovarian and Adrenal Ascorbic Acid Content

Cypermethrin (high dose) caused an accumulation of ascorbic acid content ($p<0.01$) in the ovary (Figure 3) of female prepubertal albino rats, whereas the ascorbic acid of adrenal gland (Figure 4) were decreased ($p<0.01$). Pre-treatment of zinc restored the status in cypermethrin-induced rat.

Ovarian and Adrenal Δ^5,3β-HSD and 17β-HSD Activity

The treatment of prepubertal female albino rats with cypermethrin (high dose) reduced the activities of Δ5,3β-HSD and 17β-HSD (Figures 5, 6) enzymes in ovary and increased the activities of these enzymes (Figures 7 and 8) in the adrenal gland. Pre-treatment of zinc restored the activities of these enzymes in cypermethrin-induced rats.

Effect on LH, FSH and Estrogen

The levels of luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol (E2) were reduced significantly

	First estrous (age in days)	Vaginal opening (age in days)
Group-I (5 ml /kg body wt.)	65.06±0.42	49.42±0.561
Group-II [Zinc (227 mg/l in drinking water control]	64.28±0.283	47.42±0.814
Group-III [Cypermethrin-treated (34.33mg/ kg body wt., Low dose) group]	73.46±0373 a*	53.3±0.478 a**
Group-IV [Zinc+cypermethrin-treated (34.33mg/ kg body wt., Low dose) group]	68.51±0.402 b*	50.43±0.4 a*
Group-V [Cypermethrin-treated (51.5mg/ kg body wt., High dose) group]	80.32±0.645 a***	66.71±0.586 a***
Group-VI [Zinc+Cypermethrin-treated (51.5mg/kg body wt., High dose) group]	75.15±0.485 a* c**	54.4±0.822 a**c**

Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (*indicates $p<0.05$, ** indicates $p<0.01$ and *** represents $p<0.001$).

Table 1: The effect of zinc on average duration of first estrous cycle phases and vaginal opening in cypermethrin induced female prepubertalrats

	Ovarian index	Uterine index	Adrenal index
Group-I (5 ml /kg body wt)	32.33±0.071	79.66±0.05	16.65±0.059
Group-II [Zinc (227 mg/l in drinking water control]	30.058±0.052	77.78±0.035	15.25±0.044
Group-III [Cypermethrin-treated (34.33 mg/ kg body wt.,Low dose) group]	24.138±0.041 a**	59.63±0.045 a**	15.01±0.049
Group-IV [Zinc+Cypermethrin-treated (34.33 mg/ kg body wt., Low dose) group]	28.1±0.07 b*	66.64±0.043 a* b*	15.11±0.043
Group-V [Cypermethrin-treated (51.5 mg/ kg body wt.,High dose) group]	18.641±0.06 a***	49.14±0.039 a***	12.34±0.04 a*
Group-VI [Zinc+Cypermethrin-treated (51.5 mg/ kg body wt., High dose) group]	22.25±0.06 a** c*	53.15±0.039 a**c*	13.25±0.045 a*

Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (*indicates $p<0.05$, ** indicates $p<0.01$ and *** represents $p<0.001$).

Table 2: Illustrates the effect of zinc on ovarian, uterine and adrenal indicesin cypermethrin induced female prepubertal rats.

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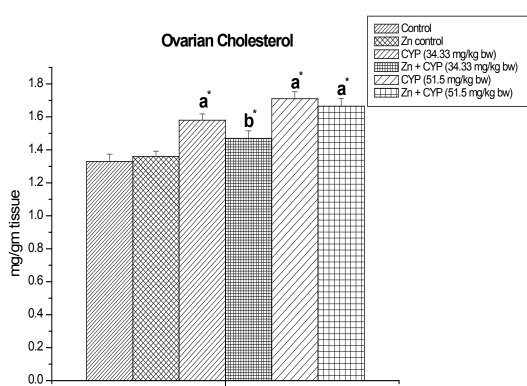

Figure 1: The effect of zinc on ovarian cholesterol content in cypermethrin induced female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV (*indicates $p<0.05$).

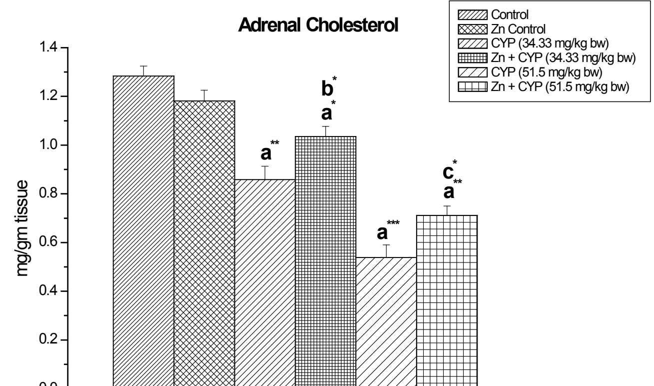

Figure 2: The effect of zinc on adrenal cholesterol content in cypermethrin induced female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (*indicates $p<0.05$, ** indicates $p<0.01$ and *** represents $p<0.001$).

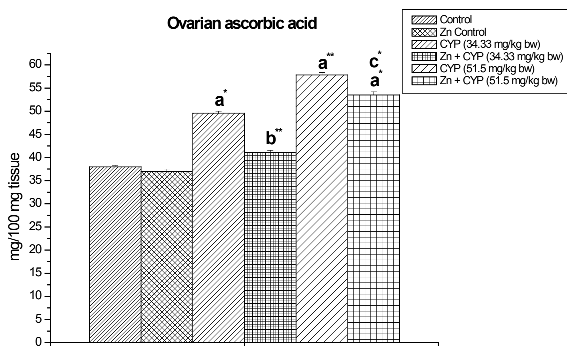

Figure 3: Illustrates the effect of zinc on ovarian ascorbic acid content in cypermethrin-exposed female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (*indicates $p<0.05$, ** indicates $p<0.01$).

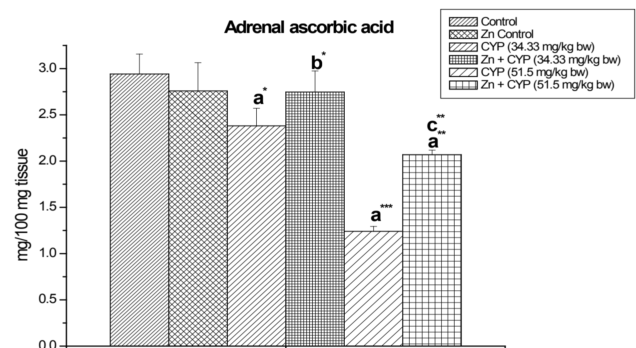

Figure 4: Effect of zinc on adrenal ascorbic acid content in cypermethrin induced female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (*indicates $p<0.05$, ** indicates $p<0.01$ and *** represents $p<0.001$).

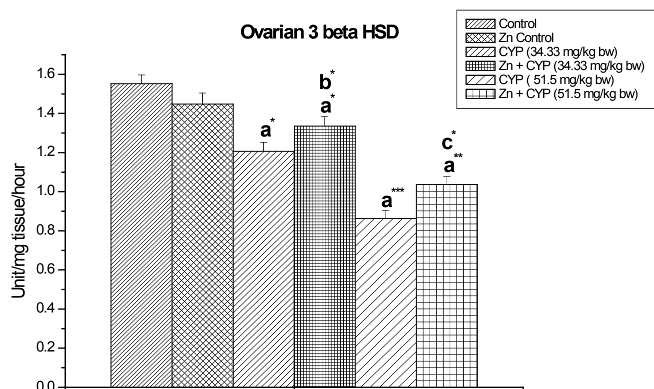

Figure 5: Illustrates the effect of zinc on ovarian Δ^5,3β-HSD in cypermethrin induced female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (*indicates $p<0.05$, ** indicates $p<0.01$ and *** represents $p<0.001$).

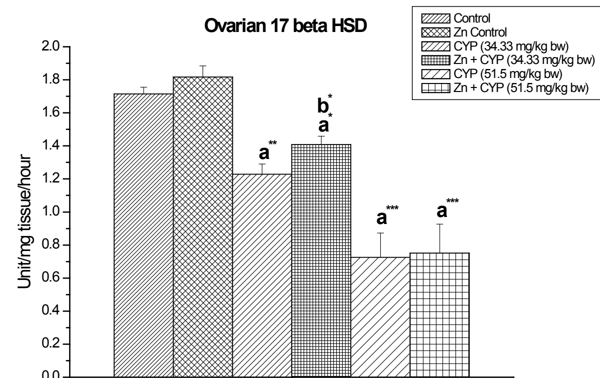

Figure 6: Effect of zinc on ovarian 17β-HSD in cypermethrin-exposed female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV (*indicates $p<0.05$, ** indicates $p<0.01$ and *** represents $p<0.001$).

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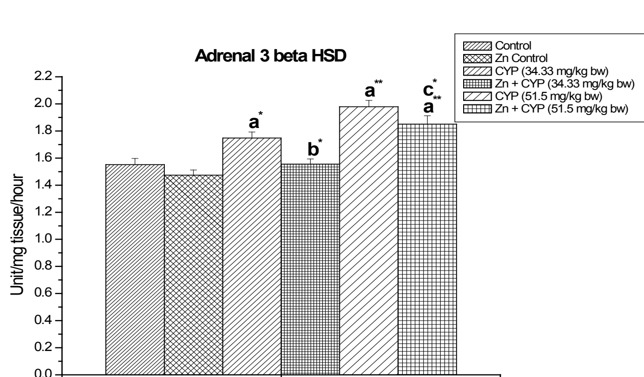

Figure 7: The effect of zinc on adrenal Δ^5,3β-HSD in cypermethrin induced female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (*indicates $p<0.05$, ** indicates $p<0.01$).

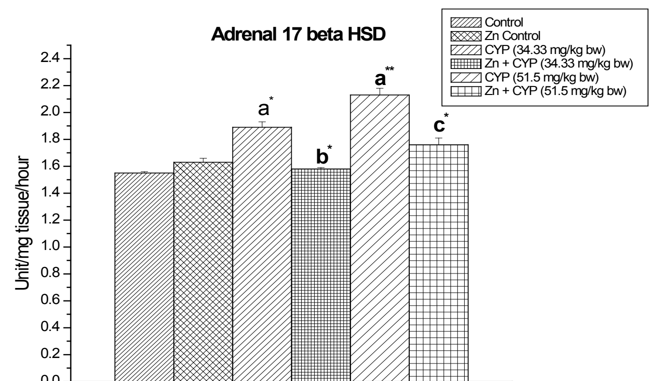

Figure 8: Illustrates the effect of zinc on adrenal 17β-HSD in cypermethrin-exposed female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (*indicates $p<0.05$, ** indicates $p<0.01$).

in rats of cypermethrin (high dose) treated group (Table 3). Pre-treatment of zinc restored these hormones towards normal status in cypermethrin-induced rat.

Effects on Oxidative Stress

The effect of zinc on ovarian malon-di-aldehyde (MDA) in cypermethrin exposed female albino prepubertal rat is shown in Figure 9. MDA content increased significantly ($p<0.01$) in cypermethrin treated high dose group compared to the control group whereas pre-treatment of zinc decreased the cypermethrin toxicity and normalized the oxidative status of the ovary.

Figure 10 shows the ovarian GSH in cypermethrin exposed female albino prepubertal rat. From this figure it is observed that GSH were decreased significantly ($p<0.05$) in cypermethrin treated groups compared to control.

As presented in Figure 11, the activity of SOD in the cypermethrin treated low and high dose were significantly de-

	LH (mIU/ml)	FSH (mIU/ml)	Estrogen (pg/ml)
Group-I (5 ml /kg body wt)	0.556±0.006	0.625±0.045	37.5±0.763
Group-II Zinc (227 mg/l in drinking water control)	0.561±0.006	0.7±0.005	37.16±0.703
Group-III Cypermethrin-treated (34.33mg/ kg body wt.,Low dose) group	0.44±0.005 a**	0.446±0.006a**	29.5±0.763a**
Group-IV Zinc + Cypermethrin-treated (34.33mg/ kg body wt., Low dose) group	0.513±0.006a*b*	0.608±0.006b**	34.33±0.881a*b*
Group-V Cypermethrin-treated (51.5mg/ kg body wt.,High dose) group	0.235±0.007a***	0.286±0.006a***	18.66±0.666a***
Group-VI Zinc + Cypermethrin-treated (51.5mg/ kg body wt., High dose) group	0.395±0.007a**c**	0.415±0.007a**c***	27±0.577a**c**

Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (*indicates $p<0.05$, ** indicates $p<0.01$ and *** represents $p<0.001$).

Table 3: The effect of zinc on reproductive hormone levels in cypermethrin induced female prepubertalrats.

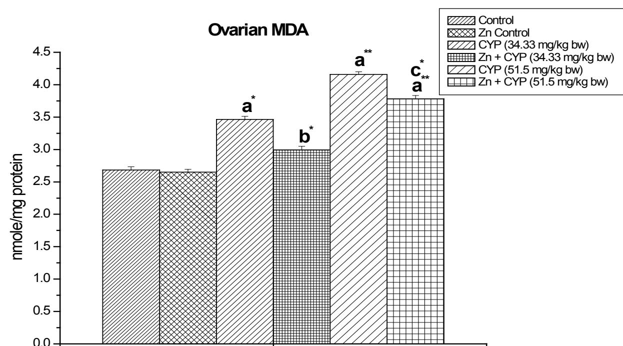

Figure 9: The effect of zinc on ovarian malon-di-aldehyde (MDA) level in cypermethrin induced female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (*indicates $p<0.05$, ** indicates $p<0.01$).

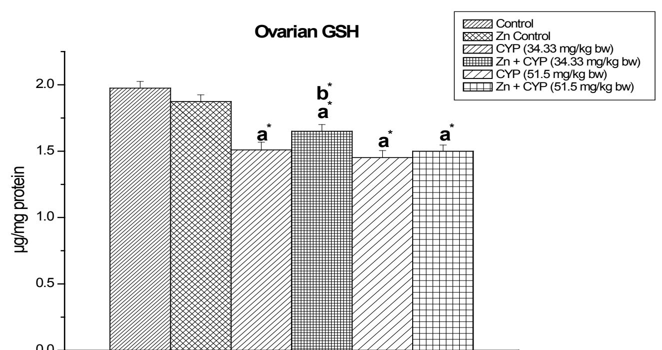

Figure 10: Effect of zinc on ovarian reduced glutathione (GSH) content in cypermethrin-exposed female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV (*indicates $p<0.05$).

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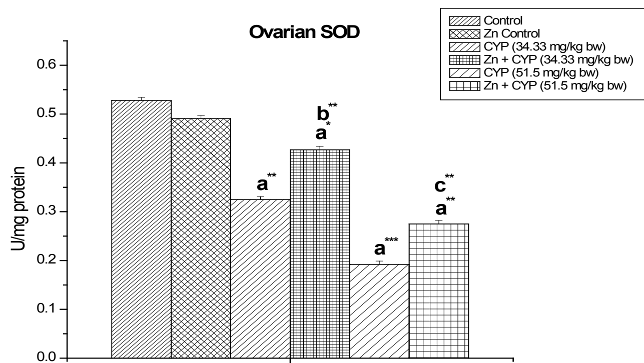

Figure 11: The effect of zinc on ovarian superoxide dismutase (SOD) activity in cypermethrin induced female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (*indicates *p*<0.05, ** indicates *p*<0.01 and *** represents *p*<0.001).

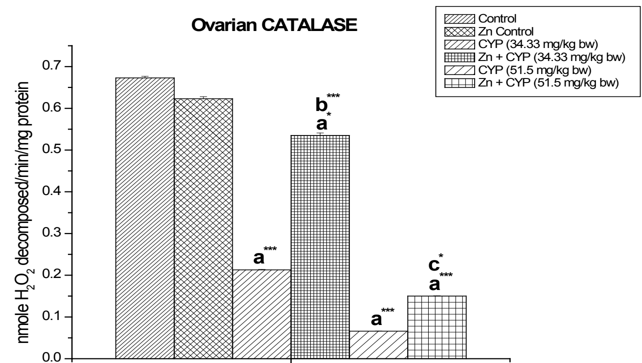

Figure 12: Effect of zinc on ovarian catalase (CAT) activity in cypermethrin-exposed female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (*indicates *p*<0.05 and *** represents *p*<0.001).

creased (p<0.01) and (p<0.001) compared to the control group. The activity of CAT (Figure 12) in the cypermethrin exposed low and high dose groups were significantly (p<0.001) decreased compared to the control group. However, pre-treatment with zinc with cypermethrin resulted in a significant increase in the activity of SOD, CAT. There was no significant changes found in GST level in cypermethrin treated low dose group but slight alteration has been found in cypermethrin treated high dose group (Figure 13).

The activity of glutathione peroxides and glutathione reeducates (Figures 14 and 15) in the cypermethrin treated group was decreased in ovary whereas zinc resulted in a significant increase in the activity of these two enzymes.

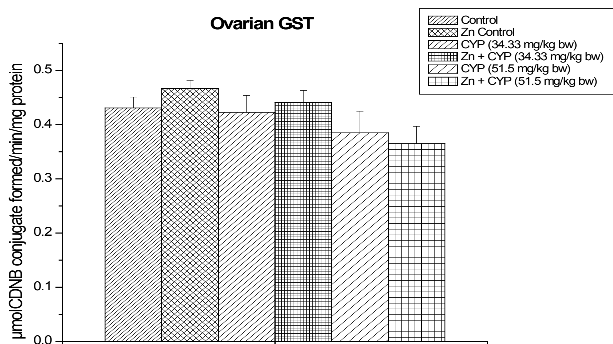

Figure 13: The role of zinc on ovarian glutathione-s-transferase (GST) activity in cypermethrin induced female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests.

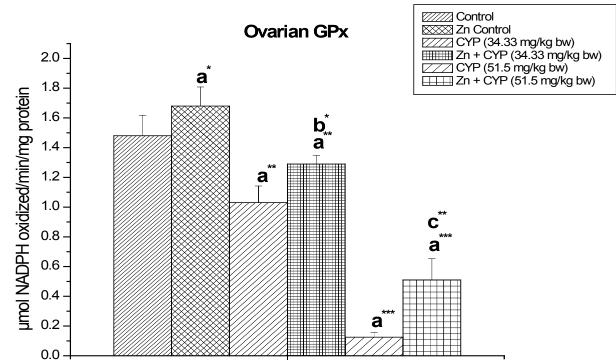

Figure 14: The effect of zinc on ovarian glutathione peroxidase (GPx) activity in cypermethrin-exposed female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (*indicates *p*<0.05, **indicates *p*<0.01 and ***represents *p*<0.001).

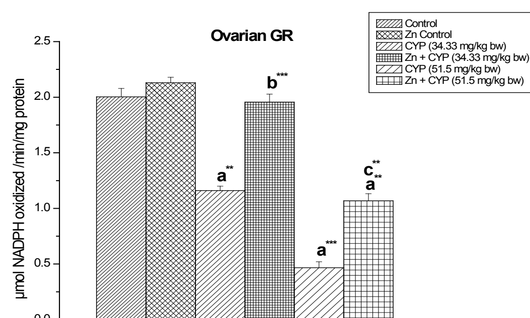

Figure 15: Effect of zinc on ovarian glutathione reductase (GR) activity in cypermethrin induced female prepubertal rats. Results are expressed as Mean±SEM. Analysis is done by ANOVA followed by multiple comparison two-tail *t*-tests. Superscript a Group I *versus* all other groups; superscript b Group III *versus* Group IV and superscript c Group V *versus* Group VI (** indicates *p*<0.01 and *** represents *p*<0.001).

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DISCUSSION

In this study decrease in ovarian index in case of cypermethrin treated rats compared to control may be due to decreased number of ovarian germ cells. Rat is a spontaneously ovulating species with estrous cycles and exhibits regular endocrine events. In this study, estrous cycle have been examined in the cypermethrin exposed female prepubertal rats as an indicator about the toxic effects of cypermethrin on the female reproductive function involving the component roles of hypothalamus, pituitary, ovary and uterus. The reduction in ovarian and uterine indices that may be due to reduced anabolic role of estradiol on the weight of the ovary and uterus.[30] This decrease may also be due to other toxic effects of cypermethrin another systems of the animal (Table 2). In case of female prepubertal rats, exposure to cypermethrin remarkably delayed the onset of puberty as evidenced by the age of vaginal opening and appearance of first estrous (Table 1). The delay in the age of vaginal opening and appearance of first estrous may be related with the decline in the ovarian steroid genesis process.[30]

In the present study, the significant rise in ovarian cholesterol content of cypermethrin treated rats advocates the less utilization of cholesterol towards the biosynthesis of ovarian steroid hormones. Thus it outcomes the malfunctioning of ovarian steroidogenic activity of the cypermethrin treated rats. Ascorbic acid accumulates in the ovaries of treated rats which gives another support to the inhibition of steroidogenic activity.[30] Pre-treatment of zinc decreased the cypermethrin toxicity and normalized the steroidogenic status of the ovary.

The low steroidogenic activity was also confirmed by the decreased activity of steroidogenic enzymes (Δ^5,3β-HSD, 17β-HSD). A reduced level of adrenal cholesterol and an increased activity of steroidogenic enzymes (Δ^5,3β-HSD, 17β-HSD) in adrenal cortex indicate the improvement of steroidogenes is by the adrenal gland.

Our studies stated that when cypermethrin was administered, serum. LH, FSH, and estrogen level significantly diminished. Zinc pre-treatment was able to restore the hormonal levels towards normal. From the results it may be considered that cypermethrin affected the set-point that regulate the secretion and function of some reproductive hormones secreted from the anterior pituitary and ovary that control the estrous cycle function in female rat.[31] The significant reduction in the level of LH, FSH and estradiol (Table 3) in cypermethrin treated rats indicates that cypermethrin may inhibit the function of ovary and uterus. This is also supported by the results from estrous cycle study. Most probably this is accomplished by inhibition of the secretion of LH and FSH from anterior pituitary and estradiol from ovary. The depression of the secretion of LH and FSH might be due to direct action of cypermethrin on anterior pituitary gonadotrophs, responsible for the secretion of LH and FSH; or hypothalamic neurons, responsible for the secretion of gonadotropin-releasing hormone (GnRH) that exercises tropic action on anterior pituitary gonadotrophs.[32]

Diminution in GSH levels in ovary after cypermethrin treatment may be the indication of oxidative stress, whereas GSH is utilized for the detoxification of reactive toxic substances. As one of the most essential biological molecules, GSH play a key role in the detoxification of the reactive toxic metabolites. Normal cellular function is executed through a balance between ROS production and antioxidant defense mechanisms existing in the cell.[33] There was no significant changes found in GST level in cypermethrin treated low dose group but slight change has been found in cypermethrin treated high dose group. From the results, the activities of ovarian SOD, CAT, glutathione peroxides and glutathione reductase of cypermethrin treated rats were significantly decreased. These results suggested that cypermethrin has the capability to persuade free radicals and oxidative insult as demonstrated by alterations in various antioxidant enzymes in ovary of prepubertal rats.

Zinc pre-treatment restored all these ovarian parameters towards normal level to a better extent. By scavenging or quenching free radicals, hydrogen peroxide or hypochlorous acid directly, or by binding free metal ion species like Fe^{2+} or Cu^{2+} by its sulfonic acid group, zinc reduced lipid peroxidation. By diminishing oxidative stress, it safeguards the tissue damage, as well as the ovarian toxicity.[34,35]

Thus, from the above mentioned results we may conclude that cypermethrin suppresses the female prepubertal reproductive function in rat probably by impairing the estrous cycle; inhibiting the secretion of female reproductive hormones and promoting the oxidative stress of the tissues of ovary. From the above findings, it is evident that cypermethrin cause prominent inhibition in ovarian steroidogenesis in a dose-dependent manner in prepubertal female Wistar rats and zinc has more potent ameliorative role on cypermethrin induced reproductive toxicity.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

CONSENT

The study was approved by the Institutional Animal Ethical Committee (IAEC), registered under Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests & Climate Change, Govt. of India and performed in compliance with the relevant laws and guidelines of the CPCSEA.

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