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Research

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Hepatotoxic-Microcystins in Two Drinking Water Reservoirs in the Central Region of Ghana

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ABSTRACT

Background: Microcystins are cyclic peptides containing seven amino acids with the condensation of two terminal amino acids of the linear peptide to form a cyclic compound. The cyclic nature of microcystins suggests that they are highly stable in water across a wide range of pH and temperatures. Microcystins are produced by several blue-green algae (cyanobacteria) including *Microcystis*, *Anabaena*, *Planktothrix*, *Oscillatoria* and *Radiocystis* commonly found in freshwater reservoirs in Ghana. Microcystins have very serious health implications on both humans and animals. Known symptoms associated with microcystin poisoning include skin irritation, allergic responses, mucosa blistering, muscular and joint pains, gastroenteritis, pulmonary consolidation, liver and kidney damage and other neurological effects.
Methods: In this study, we present results of toxicological analysis conducted on water samples from the Brimsu and Kwanyarko Reservoirs used as sources of drinking water by some parts of the Central Region of Ghana in 2011. HPLC was used to measure microcystin and phytoplankton was identified using an inverted microscope.
Results: HPLC analyses of samples gave four variants of microcystin, MC-LR, MC-YR, MC-RR and MC-LA with microcystins ranging from 0.79 µg/L during the intake of water at the Brimsu treatment plant to 0.1 µg/L in the final drinking water products of both reservoirs. Microcystin-LA is a microcystin variant identified in Ghana for the first time. Cyanobacteria diversity was low in both reservoirs. However, biomass was very high and constituted about 84% and 93% of the total algal counts of the water intake for Kwanyarko and Brimsu Reservoirs respectively. Dominant cyanobacteria species found in these reservoirs are *Microcystis aeroginosa* and *Planktothrix agardhii*.
Conclusions: Due to the chronic effect of these toxins it is recommended that drinking reservoirs with low levels of microcystins must be regularly monitored to keep it free from microcystin to ensure safeguarding human health.

KEY WORDS: Cyanobacteria; Microcystin; Drinking water reservoirs; Toxins; HPLC.

ABBREVIATIONS: HPLC: High Pressure Liquid Chromatography; WHO: World Health Organization; PDA: Photodiode Array Detector;

INTRODUCTION

Microcystins are a large group of cyclic peptides produced by several groups of cyanobacteria (blue-green algae). These include *Microcystis*, *Planktothrix* and *Anabaena*,[1] *Oscillatoria*,[1,2] *Radiocystis*,[3] *Anabaenopsis* and *Cylindrospermopsis*,[1,4] *Aphanothece*,[5] *Aphanocapsa*,[6] *Arthrospira*,[7] *Synechocystis*,[8] and *Scytonema*.[9] Conditions that favour the growth of cyanobacteria are stable water column, warm water, high nutrient (phosphorus, nitrogen) concentrations and organic compounds such as humus and domestic waste, low concentration of nitrogen relative to phosphorus (N/P ratio) and high pH among others.[1,10]

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Cyanobacteria, once established may alter epilimnetic conditions such as elevated pH due to carbon di-oxide depletion, which reduce the availability of photosynthetically active radiation (PAR) to favour their own growth.[11] Bloom forming cyanobacteria such as *Microcystis*, *Anabaena* and *Aphanizomenon* are the common genera known to be capable of producing toxins.[1] In addition, they have adverse effects on water quality which include foul odours and fish kills due to oxygen depletion and ammonia release as the cyanobacteria decay.[12] The most hazardous consequence of cyanobacterial blooms is the production of potent toxins such as microcystins (liver toxins) and neurotoxins (nervous system).[12,13] Neurotoxins disrupt the normal propagation of neural impulses to muscles, thereby, causing paralysis and death through asphyxiation in animals.[14] Microcystins, the most widely studied and the focus of this paper, inhibit the synthesis of protein phosphatises 1 and 2A and are potent liver tumour initiators and promoters.[15] Microcystins are responsible for causing poisoning of the domestic livestock such as cattle, pigs, sheep, wildlife and humans worldwide.[12] Although, the primary target of microcystin poisoning is the liver, other organs such as the gastrointestinal tract and kidneys may be affected. Common human reactions associated with cyanobacteria poisoning are skin rash, fever and nausea.[16]

The risks to humans, associated with the intake of microcystins in drinking water was reported.[17,18] In China, the incidence of high primary liver cancer among the populace was attributed to increased microcystin concentration in the drinking water.[17] Zhou et al[19] also reported that human colorectal cancer rates were positively correlated with the microcystin concentrations in drinking water supplies in China. These studies demonstrate clearly the adverse effects of microcystin uptake through drinking water to human health and should be well monitored during the treatment of drinking water.

Addico et al[20] reported for the first time, the presence of microcystin in the intake of the Weija water treatment plant when they studied the cyanobacteria population associated with the Weija and Kpong Reservoirs in Ghana with respect to drinking water quality and microcystin. Six different microcystin variants were identified at the intake of the Weija water treatment plant. Addico et al[20] also studied the cyanobacterial diversity and biomass in relation to the nutrient regime of four freshwater reservoirs (Weija, Kpong, Barekese and Owabi) sourced for the production of drinking water in Ghana and concluded that cyanobacteria was the dominant phytoplankton in all the four reservoirs mentioned above.

Unfortunately, microcystin monitoring is not a quality criterion in most developing countries including Ghana. The World Health Organization (WHO) has standardized a guideline value for microcystin concentration ensuring human safety as 1 µg/L.[21] This project was set up to evaluate the quality of drinking water in the Kwanyarko and Brimsu drinking water treatment plants in the Central Region of Ghana with respect to the presence of microcystin and their levels in the water so as to safe guard human health.

MATERIALS AND METHODS

Study Area

Kakum (Brimsu Reservoir)

The Brimsu Reservoir is located on the Kakum River and lies on latitude 05°11'.714 N and longitude 01°17'.688 W (Figure 1) and the head waters are at the Kakum National Park and flows directly into the sea at Iture near Elmina. Rainfall is bimodal. The major season is between May to June and the minor season is between September to October. The climate of the study area is wet semi-equatorial with an annual rainfall of between 1250 and 2000 mm.[22,23] The natural vegetation of the study area is the moist semi-deciduous forest. However, human activities within the catchment area such as deforestation, shifting cultivation and bush burning have degraded the vegetation. The soil is porous, well drained, and loamy. The reservoir has an elevation of 150 to 300 m above sea level.[23] Agriculture is the main land use activity within the catchment area. Crops cultivated are cassava, maize, oil palm, citrus and plantain. Other purpose of the land use includes human settlements, transportation, timber harvesting, mining and quarrying.

Kwanyarko Reservoir

The Kwanyarko Reservoir commissioned in 1965 is located in the Agona District on the Ayensu River. The Kwanyarko Reservoir lies at latitude 5° 20' N and longitude 0° 30 W (Figure 2). The Ayensu River which is one of the rivers of the coastal rivers basin originates from the Atiwa range of hills in the Akim Abuakwa District.[24] It drains into the Gulf of Guinea at about 16 Km East of Winneba through the Oyibi Lagoon. Its main tributaries are Abukyen and Akora. The River Ayensu basin falls within two ecological zones, the Northern zone which is covered with the moist semi-deciduous forest and the Southern zone covered by the South-East coastal savanna consisting of coastal scrubs or thickets and grasslands with short strips of strands and mangroves. The two climatic regions have two rainfall maxima The wet semi-equatorial type has mean annual rainfall between 1200 mm and 2000 mm, while the dry equatorial region has mean annual rainfall less than 900 mm. Rainfall occurs within two phases in the basin, with the first phase occurring between March to July and the second phase in October. The Basin is underlain by rocks of the middle Precambrian age.[24] Human activities undertaken within the basin mostly includes agriculture and human settlement.

Sampling and Preparation of Samples

Samples for microcystin analyses were collected from the Brimsu and Kwanyarko Reservoirs. The Kwanyarko Reservoir supplies drinking water to about ten communities including Swedru, Agona Nyakrom and Budumburam, while the Brimsu Reservoir

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Figure 1: Map of the Catchment of Kakum River Showing the Brimsu Reservoir.

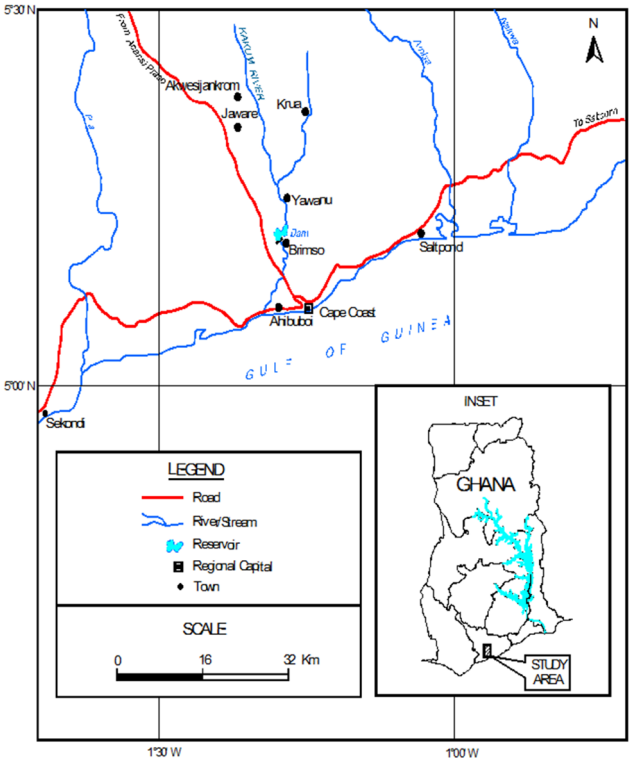
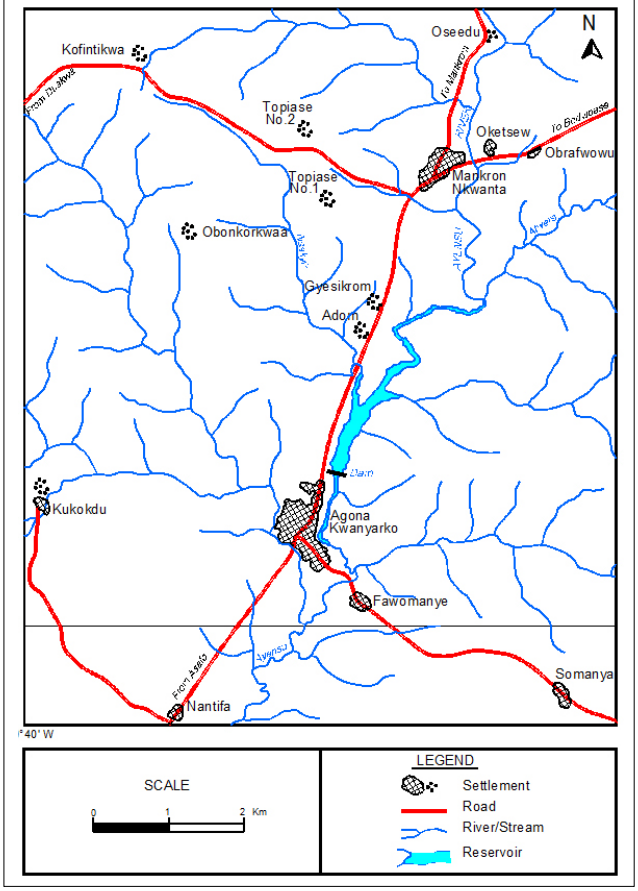


Figure 2: Map of the Catchment of Ayensu River Showing the Kwanyaku Reservoir.



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serves about 6 towns including Salt pond, Elmina and Abura Dunkwa all in the Central Region.

Water samples (1 litre) were collected at each stage of treatment (i.e., Intake, Flocculation, Sedimentation, Filtration and Chlorination) into clean plastic bottles. Five samples were collected from each reservoir during every visit (March and September). One set of samples were collected for phytoplankton analysis and another set for toxicological analyses. The samples were stored in a cooling bag on ice and transported to the laboratory.

A total of 40 samples were collected for toxicological analyses from the two reservoirs, covering a period of two years (January 2010 to December 2010). In the laboratory, the 1 litre samples were filtered through a Watman Glass Fiber filter (GF/C) of pore size 0.45 µm. The filters with the cells were carefully folded into two with the upper surface of the innermost filter, placed in a suitable container, frozen overnight and dried in an oven at 45 °C for 8 hrs. The dried filters were kept at -20 °C until needed.

Extraction of Intracellular Microcystin

Filter discs containing dried cells were placed in glass bottles containing 20 ml of 80% acidified methanol (0.1 % TFA, Fisons, Loughborough, Leicestershire, UK) and was subjected to extraction for 1 hr at room temperature with intermittent shaking using Fisons whirly mixer as described by Lawton et al.[25] A total number of 40 samples were extracted. The extraction procedure was repeated thrice and extracts pooled together and centrifuged using AFC refrigerated centrifuge at 3000 rpm for 20 min. The clear supernatant was decanted into a pear shaped flask (50 ml) and dried using a Buchi evaporator R-200 at 45 °C. The residue was resuspended in 2×250 µl of methanol prior to analysis by high pressure liquid chromatography (HPLC)/photodiode array detector (PDA).

Detection Limits and Calibration Curve

All chemicals and solvents used were of analytical grades. Microcystin-LR, MC-RR, MC-YR, MC-LW, MC-LY and nodularin (Alexis Corporation, Lausen, Switzerland) were stored at -20 °C until required. Methanol was purchased from Ruth burn (Walkerburn, Peeblesshire, UK). HPLC calibration of the six different microcystins (MC-LW, -YR, LR, LY and -RR) and nodularin standards of 100 µg each were dissolved in methanol to constitute the calibration solution. Injections of 100 µl in triplicate were used for the calibration curve. The calibration curves for the experiment were obtained over a range of six concentrations from 100 µg/ml to 0.001 µg/ml (Table 1). Analysis of standards was done using a Waters Acquity UPLC/MS with photodiode array detector. Microcystins were identified by their UV spectra at 238 nm and chromatograms extracted by their respective mass. Calibration curves were obtained by plotting area of absorbance (UV/Vis) against the concentration of standard (µg/ml). A coefficient of linearity (r^2) of about 1 was obtained (Figure 3). Figures 4a and 4b show a spectrum of MC-LR standard extracted at a molecular weight of 996 nm used for the analysis at low and high energy.

Table 1: Correlation Coefficients (r^2) of Microcystins and Nodularin Standards Used for the Detection of Microcystin in Water Samples from Ghana

Standards	Correlation coefficients (r^2)
MC-LR	1.000
MC-YR	0.999
MC-RR	0.997
MC-LW	0.993
MC-LY	0.991
Nodularin	0.999

Figure 3: Calibration Curves of Microcystins and Nodularin Standards used for Detection of Microcystins in Water Samples from Ghana Showing Linearity of the Method.

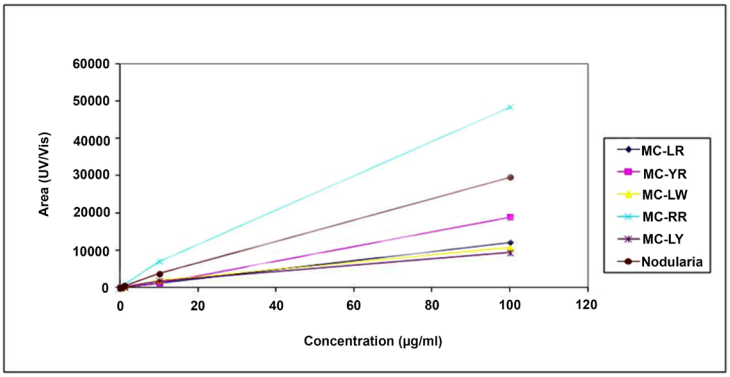

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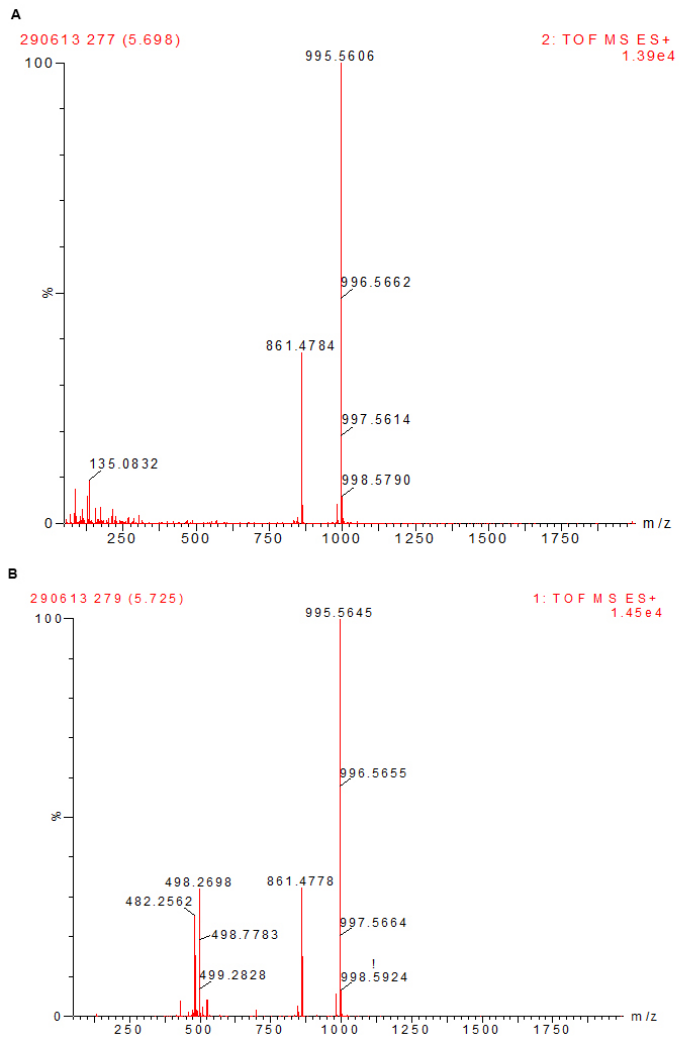
Figure 4: A Spectrum of MC-LR Standard Extracted at a Molecular Weight of 996 nm used for the Analysis at Low (top) and High (below) Energy.



Microcystin and Nodularin Analysis Using UPLC/MS

Analyses of microcystins were performed at the Ecotoxicity Laboratory of the Faculty of Pharmacy and Life Sciences of the Robert Gordon University at Aberdeen, Scotland. This was based on the previously described method by Lawton et al.[25]

Identification and Analysis of Phytoplankton

The samples of phytoplankton were collected twice a year (March and October) in 2010 and 2011. Samples were collected using a 6 litre Van Dorn water sampler. This was emptied into a clean bucket, sub-sampled into 125 ml plastic bottles, preserved with Lugol's solution and kept on ice in an ice chest for analysis in the laboratory. In the laboratory 10 ml to 25 ml aliquots of samples were transferred into counting chambers as described by Falconer.[16] Sedimentation was carried out in counting chambers with a settling time of 4 h for every 1 cm of water column of the sample.[26] Identification and counts were done using a Carl Zeiss inverted microscope. All colonies and filaments were counted as individuals, the average number of cells determined for 20 individuals and cell concentration calculated. In order not to contaminate the samples, counting chambers were cleaned with detergent, rinsed with distilled water after each sample analysis and the cover slides were also changed. Identification was carried out as described by Cronberg and Annadotter.[27]

RESULTS AND DISCUSSION

Microcystins are a large group of toxic peptides with different hydrophobicity that can be readily and easily separated by reverse-phase HPLC. Microcystins have maximum absorption spectra at 238 nm, exhibited by all microcystins except those that contain tryptophan which has maximum absorption spectra at 222 nm.[25] These characteristics described were used to analyse water samples collected from Ghana. The linearity of the analytical method was achieved for all the standards used (Table 1, Figure 3), with linear coefficients (r^2) between 0.991

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to 1 for MC-LY and MC-LR respectively. The results of analyses of intracellular samples from Ghana during March 2010 (dry season) showed the identification of four different microcystins, MC-LR, MC-YR, MC-LA and MC-RR (Figures 5, 6, 7 and 8). Microcystin LA is a variant identified for the first time in Ghana. All these four microcystin variants were present at the intake of the Brimsu Reservoir. Only one type of microcystin (MC-LR) was identified at the intake of the Kwanyarko Reservoir. All four microcystins identified in this study are very toxic with microcystin-LR and YR being implicated as a cause of death for 76 dialysis patients in Caruara, Brazil. These toxins were found to be present in the patients' livers and blood samples.[28] All the other three sets of samples which were analysed were below the detection limit of 0.01 µg/L.

Quantification of four microcystins variants yielded

Figure 5: MC-LR Identified at the Raw Water Intake of the Brimsu Reservoir Treatment Plant extracted at a Molecular Weight of 996 nm with a Retention Time of 5.7 min.

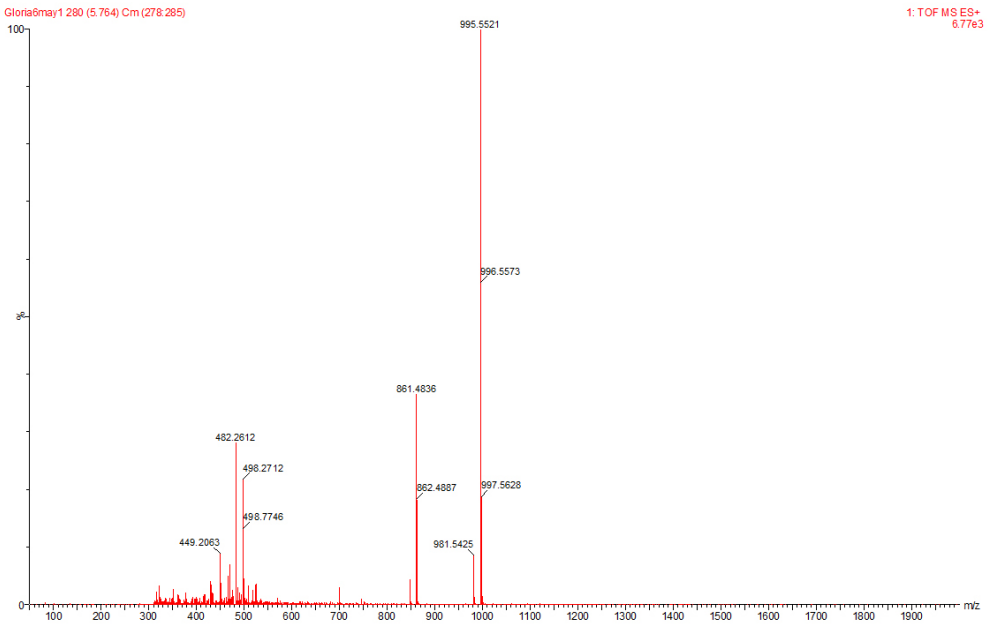

Figure 6: MC-YR Identified at the Raw Water Intake of the Brimsu Reservoir Treatment Plant Extracted at a Molecular Weight of 1045 nm with a Retention Time of 5.6 min.

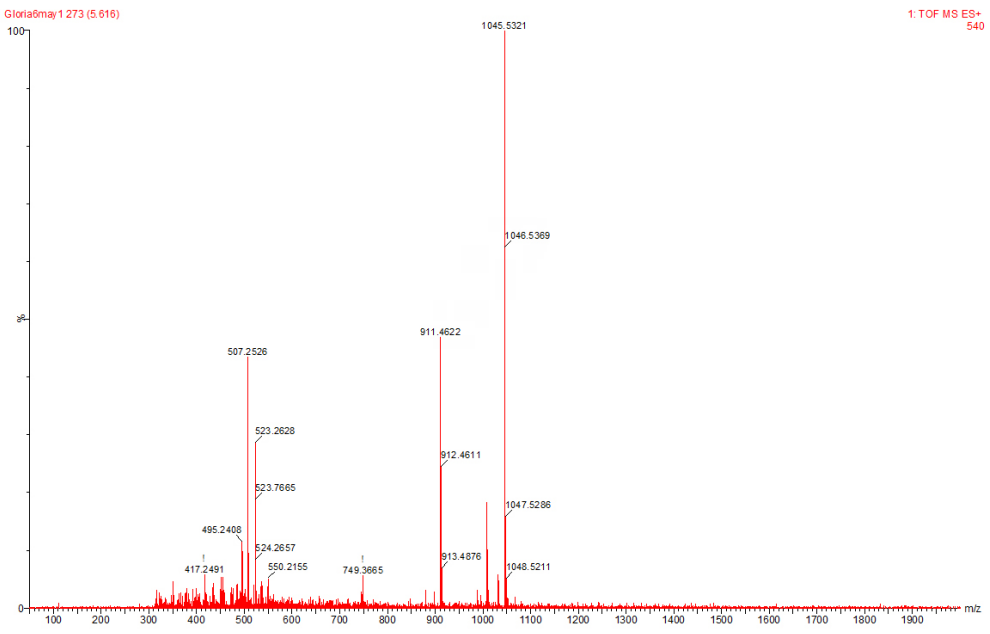

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Figure 7: MC-LA Identified at the Raw Water Intake of the Brimsu Reservoir Treatment Plant Extracted at a Molecular Weight of 910 nm with a Retention Time of 7.9 min.

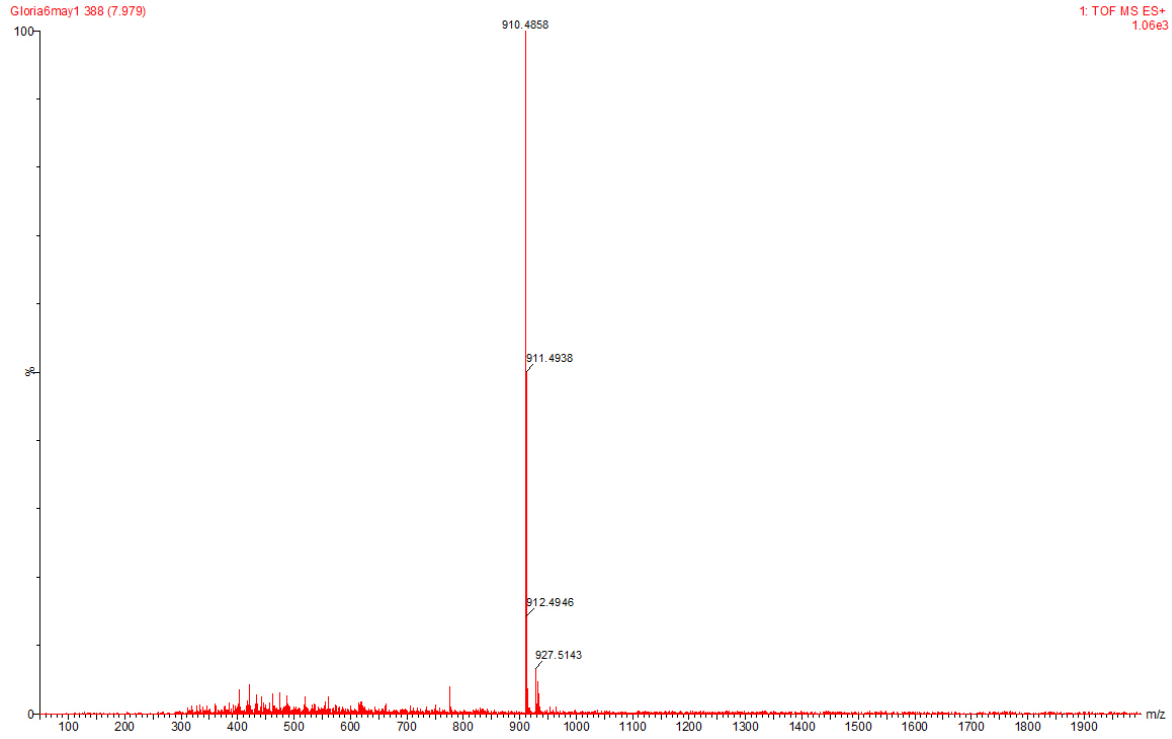
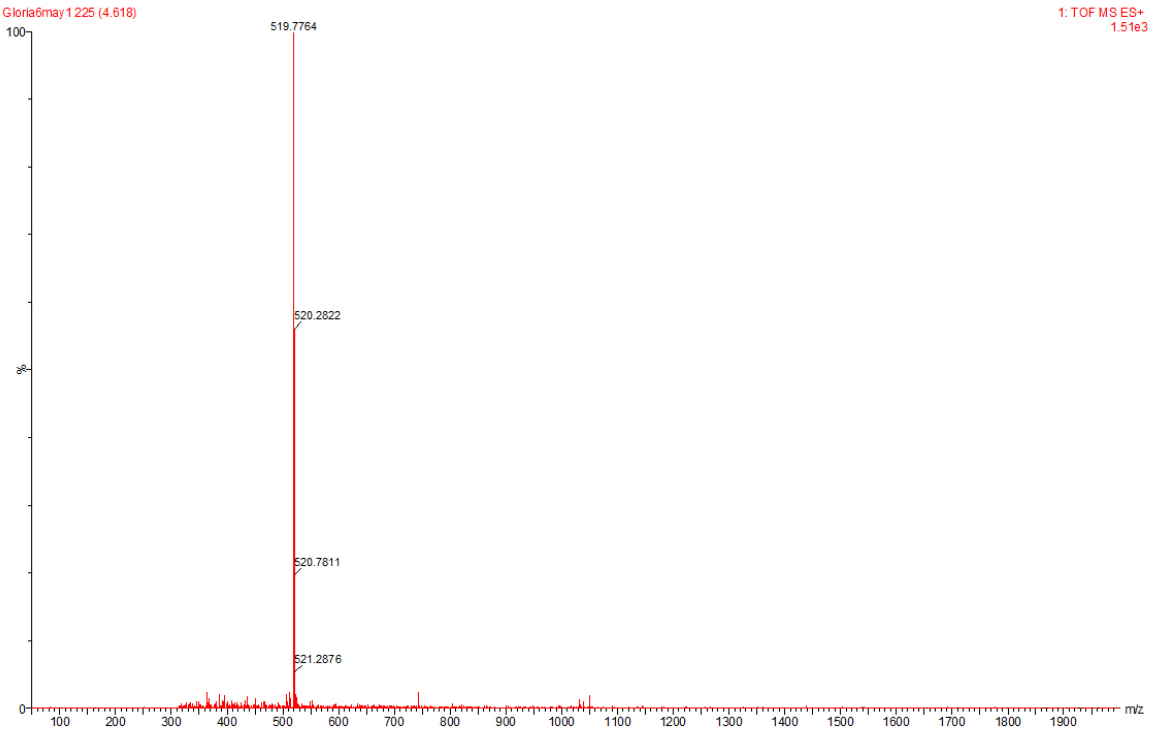


Figure 8: MC-RR Identified at the Raw Water Intake of the Brimsu Reservoir Treatment Plant Extracted at a Molecular Weight of 520 nm with a Retention Time of 4.6 min



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a total intracellular microcystin concentration of 0.79 µg/L at the intake of the Brimsu Reservoir (Table 2), which was below the WHO safety guideline value of 1 µg/L (WHO, 2004, WHO, 2016). The Italian Government has instituted a law setting 0.84 µg/L total microcystin as the tolerable limit in freshwater to prevent possible intoxication.[29] Addico et al[30] have earlier recorded a total intracellular microcystin concentration of 3.21 µg/L at the intake of the Weija Reservoir. This value is much higher than the 0.79 µg/L obtained at the Brimsu Reservoir. However, the risk of human exposure to microcystin through drinking water is still relevant due to the tumour promoting activity of microcystin at very low concentrations. Microcystin as a toxin or cyanobacteria are not considered significant as a water quality criterion in Ghana. It must be noted that over 40% of the Ghanaian population has no access to pipe-borne water and raw water is their sole source of drinking water.

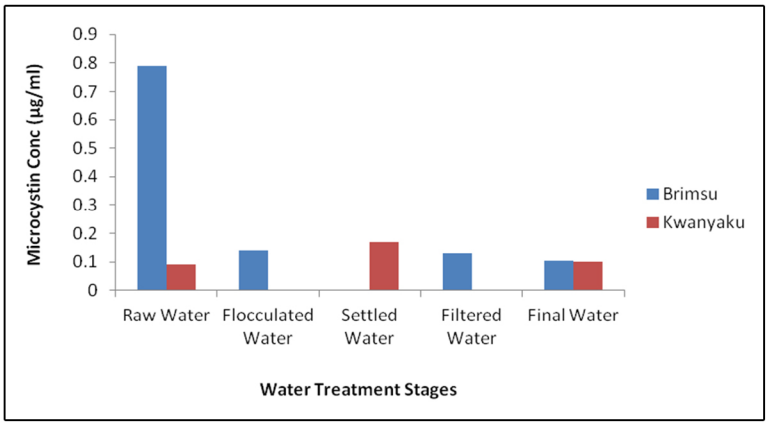
The results of our study show a decreasing concentration of intracellular microcystin as water treatment progresses down the treatment chain from the intake to the final drinking water product, with the concentrations of the final drinking water product of both the Brimsu and the Kwanyarko Reservoirs having concentrations of 0.10 µg/L (Table 2, Figure 9). This reduction in microcystin concentrations during treatment is expected due to the process of flocculation, sedimentation and filtration which reduces the algal cell biomass in the water and hence decreases the intracellular microcystin level. The effect of water purification by Coagulation/flocculation process causing cyanobacterial removal has been reported by Falconer.[16]

In this study, intracellular microcystin reduced from 0.79 µg/L at the point of intake to 0.10 µg/L in the final drinking water of the Brimsu Reservoir. This indicates a total elimination of over 68% concentration of microcystin. It also implies that the treatment process employed in Brimsu Reservoir is more efficient in mediating the removal of cells than that of the Kwanyarko Reservoir. Unfortunately, with the studies conducted on the Weija and Kpong Reservoirs only intake samples were anal-

Table 2: Concentration of Microcystin and Microcystin Type Detected in the Reservoirs.

Reservoirs / Stage of Treatment	Microcystin Concentration (µg) / Date of Sampling
Brimsu Reservoir	March 2010
Raw Water (Intake)	MC-YR (0.29 µg) MC-LR (0.50 µg) MC-AR (< LOQ) MC-RR (< LOQ) Total Microcystin (0.79 µg/l)
Flocculated water	MC-LR (0.14 µg)
Settled water	Lost Sample
Filtered water	MC-LR (0.13 µg)
Final Water	MC-LR (0.105 µg)
Kwayaku Reservoir	
Raw water	MC-LR (0.09 µg)
Flocculated water	Lost Sample
Settled water	MC-LR (0.17 µg)
Final water	MC-LR (0.10 µg)

Figure 8: Changes in Microcystin Concentration During Drinking Water Treatment in the Brimsu and Kwanyaku Reservoirs. Samples from Settled Water and Flocculated Water from Brimsu Reservoir and Kwanyaku Reservoir Respectively were Lost not Zero.



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ysed making it impossible to determine the microcystin elimination efficiency in these two reservoirs.[20]

Indications are that exposure to low concentrations of microcystin in drinking water can cause chronic effects in mammals due to their potent tumour promoting activity.[15,16] An example has been observed in Southern China, where the incidence of primary liver cancer was reported to be very high among the populace and this was correlated with the use of water from ditches and ponds as drinking water,[31,32] together with other potent liver carcinogens such as aflatoxin B_1 in food and hepatitis B virus.[33] Quantification of microcystins in these water supplies showed relatively low concentrations at about 0.46 µg/L[17] and suggested that people living in that region may have ingested 0.19 pg (0.0019 µg) of microcystin per day during the four summer months over 40-50 years of their life span. The value of 0.10 µg/L in the final drinking water product of the Brimsu and Kwanyarko treatment plants is much higher than the value found in the drinking water of Southern China that was associated with the high incidence of liver cancer among the population. Again, in Finland, an outbreak of gastroenteritis was associated with a mass development of microcystin-producing *Planktothrix agardhii* in the raw water supply.[28] A microcystin concentration of 0.1-0.5 µg/L and filaments of *Planktothrix* was detected in the drinking water associated with the outbreak.[28] *Planktothrix agardhii* together with *Anabaena flos-aquae*, *Microcystis aeruginosa* and *Pseudanabaena recta* were the main cyanobacterial species found in these two reservoirs (Tables 3 and 4). These species of cyanobacteria are known to be very common in the Ghanaian drinking water reservoirs[20,30] and are considered very toxic and implicated in many poisoning episodes.[1,12,14] Hoeger et al[34] cautioned that due to tumour promoting activities of microcystin, chronic exposure of populations to a concentration of around 0.1 µg/L should be avoided.

Table 3: Phytoplankton Species Composition and Biomass Obtained at the Five Treatment Stages of the Brimsu Water Treatment Plant in the Central Region of Ghana. (Counts/ml)

Species	Intake	Flocculation	Sedimentation	Filtration	Chlorination
Green Algae					
Ankistrodesmus falculatus	-	77	46	12	-
Staurastrum gracile	3	-	6	2	-
Scenedesmus *dimorphus*	12	20	24	-	-
Ulothrix tenuissima	44	-	-	-	-
Blue-green Algae					
Anabaena flos-aquae	546	208	191	50	32
Merismopedia punctata	91	84	70	9	-
Microcystis aeruginosa	300	171	86	53	40
Planktothrix agardhii	693	253	153	68	31
Pseudanabaena recta	553	79	-	-	-
Diatoms					
Synura sp.	89	18	39	5	-

Table 4: Phytoplankton Species Composition and Biomass Obtained at the Five Treatment Stages of the Kwanyaku Water Treatment Plant in the Central Region of Ghana. (Counts/ml)

Species	Intake	Flocculation	Sedimentation	Filtration	Chlorination
Green Algae					
Ankistrodesmus falculatus	-	51	-		9
Chlorella vulgaris	12	-	2		14
Staurastrum gracile	-	8	11		4
Scenedesmus *dimorphus*	-	-	-		-
Ulothrix tenuissima	185	-	-		-
Blue-green Algae					
Anabaena flos-aquae	123	112	95		38
Chlorococcus cronbergae	8	-	-		-
Merismopedia punctata	175	218	-		-
Microcystis aeruginosa	603	202	75		23
Planktothrix agardhii	244	90	78		24
Pseudanabaena recta	133	54	32		-
Diatoms					
Gyrosigma sp.	3	-	-		-
Navicula graciloides	12	-	9		-
Synura sp.	3	-	-		-
Synedra acus	13	88	-		2

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CONCLUSION

Microcystin-LA found in the intake of the Brimsu Reservoir for the first time in Ghana is very significant as those found earlier in the Weija Reservoir. This implies that microcystin is widespread in Ghana and government agencies concerned with potable water production and health provision must consider taking into account cyanobacteria and cyanotoxins levels into the drinking water criteria. Concentrations of intracellular microcystin detected in drinking water samples from the Brimsu and Kwanyarko Reservoirs are below the WHO guideline, however, people using the raw water as their source of drinking water may be at risk due to the tumour promoting activity of microcystin. The Brimsu Reservoir treatment plant was found to be more efficient in removal of cyanobacteria cells than that of the Kwanyarko Reservoir. It is recommended that drinking water in Ghana should be regularly monitored for the levels of cyanobacteria and microcystin to protect human health.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Case Report

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Acute Myocarditis: A Rare Complication after Black Widow Spider Bite

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ABSTRACT

Black widow spider bite can be a rare condition causing various symptoms from mild to very dramatic ones. In this article, we describe the case of a young girl bitten by a black spider whose evaluation was complicated by fulminant myocarditis, fortunately with a good prognosis. Our case study brings light to the various effects that toxins released from the black widow spider can have on the body, especially on the heart. Myocarditis can be one such clinical complication caused due to the effect of the toxic agent on the heart muscle.

KEYWORDS: Black widow spider; Myocarditis; Troponin level; Toxin.

ABBREVIATIONS: AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; INR: International Normalized Ratio; ECG: Electrocardiogram.

INTRODUCTION

Black widow spider is a rare type of spider that lives in moderate climatic conditions and is found in the rural area. *Latrodectus Tredecimguttatu*s, sometimes known as the Mediterranean black widow, the European widow spider, or the steppe spider, is a species belonging to the genus Latrodectus. It is commonly found throughout the Mediterranean region.[1]

Widow spiders are shy and nocturnal. They usually bite when their web is disturbed or upon inadvertent exposure to shoes or clothing.[2]

In Albania, poisoning due to the bite of this specific type of spider was first observed in the early 19th century. The first reported cases of patients experiencing acute poisoning due to the bite of this spider were restricted to a limited area in the country including the western lowlands specifically the Kavaja, Durrës and Myzeqe areas. The cases of poisonous spider bites were more often reported from the month of July to September. The period over which the cases of spider bites were reported, was related to the life cycle of the spider and the increased human agricultural activities during this period resulting in a greater contact with the external environment. The map indicating the distribution of affected patients extended across the northern and southern regions of the country during the upcoming years, but was predominant in the plain and hilly areas. The natural origin of this causative organism is attributed to the transportation of the spider from the neighboring countries together with various goods, especially with vehicle tires.[3]

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The clinical manifestations observed after a spider bite are variable and include abdominal pain, vomiting-nausea, headache, anxiety, itching, palpitations, and high blood pressure; however, pericarditis and myocarditis occur very rarely.[4] The grading system classifies the severity of envenomation into three categories. The characteristics of Grade 1 envenomations range from no symptoms to local pain at the envenomation site with normal vital signs. Grade 2 envenomations involve muscular pain at the affected site causing a gradual migration of the pain towards the trunk, diaphoresis at the bite site, and normal vital signs. Grade 3 envenomations include the grade 2 symptoms with abnormal vital signs; diaphoresis at the bite site; generalized myalgias to the back, chest, and abdomen; and nausea, vomiting, and headache.[2] Our patient experienced myocarditis resulting in pulmonary edema, hemoptysis as well as muscle cramps, itching, anxiety, headache, palpitations, and high blood pressure.

CASE REPORT

A healthy 17-year-old girl while moving the billowy bundle of straw was bitten by a spider in the middle anterolateral region of the right thigh. The patient lived in the village, Darëzezë, Fier region, Albania. In her family history, there were evidence suggesting that two adult men were bitten by black widow spiders in the previous years and were hospitalized (Map 1).

Ten minutes after the bite, the patient experienced severe pain in the lumbar and abdominal region and numbness in both the feet. She was taken to the emergency department of the regional hospital and was hospitalized immediately. After 24 hours, her condition deteriorated and her vital signs altered. The aggravation of her symptoms indicating generalized pain, acute respiratory distress, vomiting, hemoptysis, tremor and agitation led her to be transferred to the university hospital.

Clinical examination of the patient indicated pale skin, diaphoresis, oral cyanosis, orthopneic position, pulmonary auscultation characterized by crepitations in the base of the lungs, rhythmic cardiac tones, and normal abdominal palpation.

Vital signs were as follows: blood pressure recordings at 110/60 mmHg: Pulse rate as 110 beats/min, respiratory rate of 21 breaths/min, oxygen saturation at 75%, and a physical state of consciousness, alertness, cooperativeness and agitation.

Laboratory findings were: White Blood Cell count of $31.8\times10^3/mm^3$ (Lymphocytes 3.4%, Monocytes 2.7%, Granulocytes 93.9%), Red Blood Cell count of $4.78\times10^6/mm^3$, Hemoglobin 13.8g/dL, Platelet $275\times10^3/mm^3$, Hematocrit value of 45.0%, Glucose level of 103 mg/dl, Urea level of 27 mg/dL, Creatinine value of 0.6 mg/dL, (ALT) alanine aminotransferase activity of 22 U/L, (AST) aspartate aminotransferase activity of 75 U/L, Amylase activity of 127 U/L, Lactat dehydrogenase activity of 426 U/L, Creatine kinase activity of 286 U/L, CK-MB range of 110 mg/dL, Troponin level of 31, 3 ng/mL, International Normalized Ratio 1.339, Potassium level of 3.6 mmol/L, Sodium level of 132 mmol/L. PCO_2 of 44.8 mmHg, PO_2 of 24.5 mmHg, pH 7.334, O_2Hb of 47.0%, BE of -2.7 mmol/L, BE_{ecf} of -2.6 mmol/L, BB of 45.4 mmol/L, Osm of 293 mOsm/kg, H^+ concentration of 46.4 nmol/L and PAO_2 of 88.8 mmHg (Table 1).

Map 1: The Geographical Location of the Fier Region, Albania.

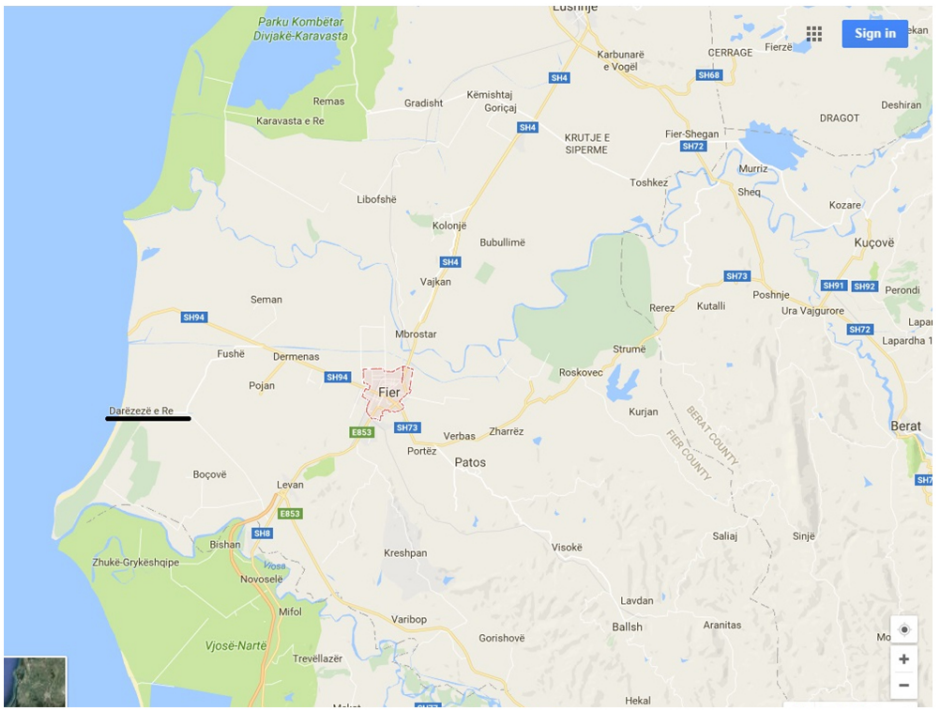

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Table 1: Hematological and Biochemical Recordings of the Patient from Day 1 to Day 8.

Hospital day	Normal range	Day 1	Day 2	Day 3	Day 4	Day 5	Day 8
White Blood Cell (×10³/mm³)	4.0-10.0		31.8	27.7	17.2	12.8	7.3
Red Cell (×10³/mm³)	4.20-6.10	4.15	4.78	4.75	4.18	4.25	5.29
Hemoglobin (g/dL)	13.8	11.6	13.8	13.9	12.3	12.9	14.1
Hematocrit (%)	35.0-50.0		45	44.8	39.6	38.1	42.3
Platelet (×10³/mm³)	150-390	361	275	267	237	243	257
Glucose level (mg/dL)	74-106	110	103	164	121	110	114
Urea (mg/dL)		28	27	28	30	38	35
Creatinine (mg/dL)	0.6-1.4	0.8	0.6	0.7	0.6	0.7	0.7
Aspartate aminotransferase (U/l)	0-35	13	75	82	71	51	42
Alanine aminotransferase (U/l)	0-45	21	22	24	23	21	22
Lactate dehydrogenase (U/l)	125-250		426				
Creatine kinase (U/l)	0-171		286				142
Creatine kinase-MB fraction	0-24		110				36
Total Bilirubin (mg/dL)	0.3-1.2	0.78	0.6	0.6	0.6	0.9	0.4
Total Protein (mg/dL)	6.2-8.3		6.7	6.4	6.1	6.6	
Sodium (mmol/L)	136-146		132	137			
Potassium (mmol/L)	3.5-5.1		3.6	3.9			
Chloride (mmol/L)	98-106		95	97			
Troponin I (ng/mL)	0,000-1,00		31.3	23		3.9	0.841
INR	0.720-1.200		1.339		1.339	1.386	1.154

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; INR: International Normalized Ratio.

Electrocardiography results implicated modifications that mimic anteroseptal infarction and transitory changes of the repolarization phase. The electrocardiographic recordings of the patient showed a normal sinus rhythm, normal QRS axis, cardiac frequency of about 90 beats/minute, qS V1,V2,V3, subdenivelation of ST segment 2-3 mm V2,V3,V4,V5,V6 (Figure 1).

Echocardiography results showed a normal left ventricle, modification in the segmental kinetics, hypokinesia of the medial and basal segments of the anterior wall.

The medical condition of the patient improved following the administration of diuretics, anti-inflammatory corticosteroids, analgesic opioid drugs, electrolytes, intravenous perfusions, vitamins, antibiotics, hypnotic sedatives drugs and gastric protections. The normal troponin level was established six days following the spider bite (Figures 2 and 3).

The patient was dismissed from the hospital after 10 days. The patient underwent routine checkup and echography conducted by the cardiologist.

DISCUSSION

Firstly, we discuss the kind of insect that bit the patient and its medical implications in the patient. Addressing this particular aspect of the study, we possess a clear anamnesis suggesting that the poisoning results from the black widow spider bite. In this particular case, the patient's condition has been treated in the clinical context but the question arises as to whether the use of anti-venom could reduce pain and suffering, shorten the duration of envenomation, and reduce or eliminate the need for hospitalization.[5]

We strongly suspect that myocarditis was caused by the toxins and with the treatment of the clinical symptoms, the condition was reversed. The electrocardiogram (ECG) recordings signify the need of a differential diagnosis for the ischemic conditions of the heart, such as myocardial infarction or pericarditis. But the medical history of the patient, her young age, the increase in troponin levels and the echocardial findings relevant to her condition indicated impaired functions of the left ventricle function thus, resulting in myocarditis.

The differential diagnosis of myocarditis was suggestive of acute coronary syndrome, congestive heart failure, pulmonary edema and pulmonary embolism. Severe myocarditis in the present study was further complicated by pulmonary edema on account of low cardiac output. Prognosis for acute myocarditis can be accurate depending on the clinical presentation of the left ventricle ejection fraction and the pulmonary artery pressure. Our case study describes the whole chain of events occurring in the affected patient with a favorable diagnosis and cure.

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Figure 1: ECG 24 Hours after Spider Bite.

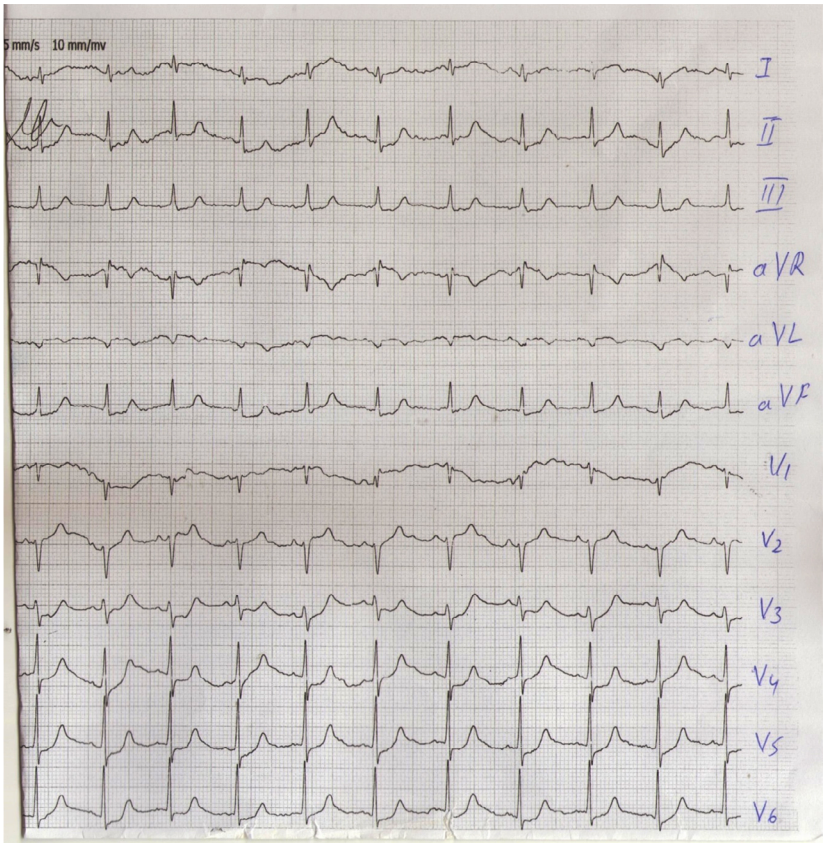


Figure 2: ECG 48 Hours after Spider Bite.

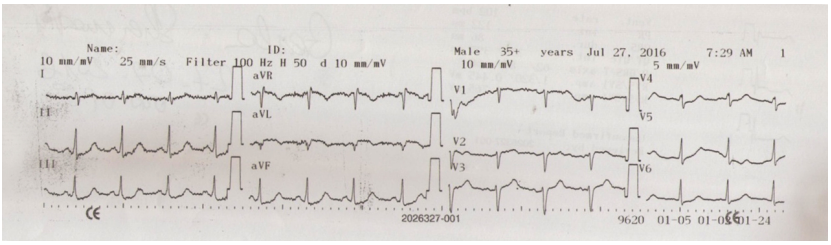
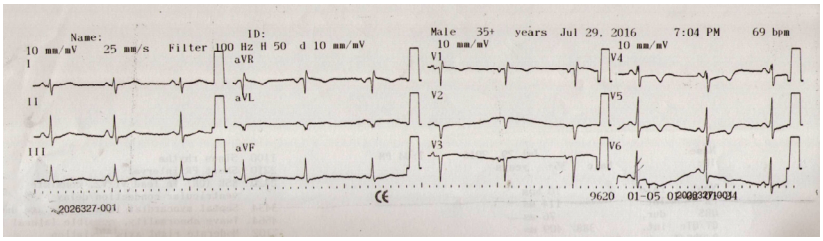


Figure 3: ECG 4 Days after Spider Bite.



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CONCLUSION

Acute myocarditis can occur very rarely after a spider bite. The ECG changes are a modification of the Quality Real Service (QRS) complex mimicking acute myocardial infarction and transitory changes of the repolarization phase. The elevation of myocardial injury markers [CkMb, troponin] as well as echocardiographic findings of segmental hypokinesia and low ejection fraction is compatible with acute/fulminant myocarditis. The aim of the pharmacological treatment of fulminant myocarditis complicated by acute pulmonary edema was to reduce the intensity of symptoms of cardiac insufficiency and neutralize the effects of the toxins released.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Research

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Coupling Microextraction With Thin Layer Chromatography-Image Processing Analysis: A New Analytical Platform for Drug Analysis

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ABSTRACT

Aim: A new analytical platform combining ultrasound assisted-dispersive liquid-liquid microextraction (US-DLLME) with thin layer chromatography (TLC)-image processing analysis has been proposed.
Materials and Methods: Acetylsalicylic acid (ASA) was selected as the model compound to demonstrate the applicability of the proposed method. The complete analysis comprises of three steps: (i) US-DLLME (injection of extraction and disperser solvent into an aqueous sample, ultrasonication and centrifugation), (ii) TLC of sedimented phase, and (iii) photography of TLC plate followed by quantification of ASA spots using freely available imageJ software (National Institute of Health (NIH), Bethesda, MD, USA).
Results: The newly developed method is simple, environmentally benign and does not require any special instrument and handling skills. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.1 and 0.35 µg/spot, respectively. The assay was found to be linear in the range of 1-250 µg/spot with a square of correlation coefficient (R^2) of 0.999.
Conclusion: The developed method was successfully applied for the analysis of ASA in urine and pharmaceutical formulations. Results of analysis of ASA in commercial aspirin tablets obtained by the proposed method were in close agreement with the results of the UV-Visible spectrophotometric analysis.

KEY WORDS: Dispersive liquid-liquid microextraction; Thin-layer chromatography-image processing analysis; Quantitative-TLC, ImageJ.

ABBREVIATIONS: ASA: Acetylsalicylic acid; US-DLLME: Ultrasound assisted-dispersive liquid-liquid microextraction; TLC: Thin Layer Chromatography; LOD: Limit of Detection.

INTRODUCTION

Thin layer chromatography (TLC) has found wide applications for the separation and identification of both known and unknown substances in biological materials since the early 1960s.[1] Some of the well-known advantages of TLC technique include its simplicity and easy operation, high sample throughput, easy sample preparation, low cost of analysis, versatile visual detection and its equal applicability to pure form of drugs, pharmaceutical formulations and illicit drug preparations.[2,3] In the available literature, several authors have reviewed wide array of TLC applications ranging from toxicological drug screening, food and agriculture analysis, bio-

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logical sample analysis, systematic toxicological analysis (STA) in clinical and forensic toxicology, to the analysis of purity of pharmaceuticals and analysis of pesticides.[2,4-8] Quantitative TLC is carried out using high-performance TLC equipment costing thousands of dollars. An economic and more convenient alternative to this technique is capturing TLC plates images followed by their processing using commercially (e.g. Just TLC-Sweday, Sweden, TLSee -Alfatech, Italy) or freely (e.g. ImageJ, National Institute of Health (NIH), Bethesda, MD, USA) available software.[9] This software converts the spots in TLC plates into peaks and then the relationship between the peak area and spot density (i.e., amount of analyte) can be determined easily.[10-12] Quantitative analysis is also possible by analyzing the red, green, blue (RGB) values of TLC spots in Microsoft Paint program.[13]

Liquid-liquid extraction (LLE) is a widely used sample preparation and pre-concentration method for the analysis of various analytes by TLC. However, this popular extraction method suffers from few drawbacks such as: (i) Consumption of large volumes of toxic organic solvents, (ii) Time consuming, (iii) Involves multi-step procedure, (iv) Laborious, etc.[14]

To overcome the aforementioned drawbacks of LLE, Assadi and Co-workers developed an environmentally friendly, rapid, economical and easy to perform liquid-phase microextraction technique termed as dispersive liquid-liquid microextraction (DLLME), which offers high enrichment factors and extraction efficiency.[15] DLLME is based on the ternary component solvent system in which an extraction solvent (generally heavier than water) is rapidly injected into an aqueous phase along with a disperser solvent (miscible in both the aqueous phase and the extraction solvent). These results in the formation of a cloudy solution which comprises of tiny droplets of extraction solvent dispersed throughout the aqueous phase. Upon centrifugation, the dense extraction solvent, thus obtained, settles down as the sedimented phase and is used for analysis.[16]

So far, DLLME has been coupled with various analytical instruments such as gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), ultraviolet-visible spectroscopy, atomic absorption spectroscopy (AAS), etc., and has found widespread applications for the analysis of a variety of analytes in different matrices ranging from drugs of abuse, pesticides and metals to pharmaceutical formulations and food preservatives, etc.[14,17-21] However, no report is available in the literature related to the coupling of DLLME with TLC.

Among all non-steroidal anti-inflammatory drug (NSAIDs), Aspirin or ASA is the most widely used drug to treat pain, fever and inflammation.[22] However, due to the ease of the availability of aspirin at local drug stores, sometimes it may be used for suicidal purposes also.[23] In such cases, the undissolved tablet or capsule may be discovered in the stomach contents of the victim. Analysis of these undissolved tablets or capsules allows for a simpler identification of the drug.[24]

In the present study, for the first time, the coupling of a rapid, economical and environmentally benign DLLME technique with TLC has been reported, which is one of the most popular and widely used separation techniques. The results will enable the researchers to perform an analysis of the drugs in a simple manner without the need of any sophisticated instrument. Successful application of the developed method has been demonstrated for the analysis of ASA in pharmaceutical formulations and urine.

EXPERIMENTAL DESIGN

Chemicals and Reagents

All the reagents used were of analytical grade unless otherwise stated. The reference material of ASA was obtained from Hi-Media Laboratories Pvt. Ltd. (India). Silica gel 60F-254 pre-coated TLC aluminum plates (20×20 cm, 0.25 mm layer thickness) were obtained from Merck (Darmstadt, Germany). Acetone (ACE), acetonitrile (ACN), methanol (MeOH) and ethanol (EtOH) which were used as disperser solvent and dichloromethane (DCM), chloroform (CF), trichloroethylene (TCE), chlorobenzene (CB), carbon tetrachloride (CCl_4) which were used as extraction solvent were procured from Qualigens (Thermo Fisher Scientific, Mumbai, India). Hydrochloric acid (HCl), sodium hydroxide (NaOH) and sodium chloride (NaCl) were purchased from Hi-Media Laboratories Pvt. Ltd. (India). The stock solution of ASA was prepared in ACN at a concentration of 10 mg/ml and stored at 4 °C. Working standard solutions were prepared daily by diluting the stock solution. Three different brands of commercially available tablets of aspirin were procured from the local market and classified as class 1, 2 and 3 for this study.

Ultrasound Assisted-Dispersive Liquid-liquid Microextraction (US-DLLME) Procedure

Ten tablets were weighed and the average weight determined prior to crushing the tablets into a fine powder. These fine powdered contents equivalent to the weight of one tablet was transferred into a volumetric flask and dissolved in ACN to prepare a solution with a final concentration of 10 mg/ml. This solution was then sonicated for 10 min followed by centrifugation. The supernatant solution was used for spiking ultrapure water in the working concentration range. A mixture of CF (extraction solvent, 100 µl) and ACN (disperser solvent, 800 µl) was rapidly injected into 5 ml of the aqueous sample followed by ultrasonication for 3 min. Following the step of injection and ultrasonication, a cloudy solution was observed which comprised of tiny droplets of CF dispersed throughout the aqueous phase. This mixture was then subjected to centrifugation at 3000 rpm for 5 min. The resultant supernatant was discarded and 10 µl of the sedimented phase (CF) was subjected to TLC analysis. Schematic representation of US-DLLME-TLC-Image processing method is shown in Figure 1.

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An appropriate amount of ASA was spiked in the drug free urine sample collected from a 30 year old healthy volunteer. The urine sample was initially centrifuged and the supernatant was diluted with ultrapure water (1:1 *v/v*). Five ml of this urine sample was subjected to the above mentioned US-DLLME-TLC-Image processing procedure.

Thin Layer Chromatography (TLC) Procedure

TLC analysis of the sedimented phase obtained from US-DLLME was performed using 20×20 cm pre-coated silica gel 60 F-254 aluminum backed TLC plates (Merck, Darmstadt, Germany). 10 µl of the sample (2×5 µl) was applied to the marked start edge of the TLC plate of 1 cm height using a micropipette of 2-20 µl capacity. The plate was developed till not less than 7 cm of the migration distance of the solvent front from the starting point in a chamber which was pre-saturated with the solvent vapors of the mobile phase consisting of n-hexane: Ethyl acetate: Acetic acid (65:30:5 *v/v/v*) in ascending mode. The plate was then allowed to air dry for 10 min and kept in the development tank. Mobile phase was prepared daily in a volume adequate to supply the development chamber. The hRf value of ASA was found to be 71.4.

Image Processing Methodology

The developed TLC plates were then photographed using digital camera under ultraviolet light of wavelength 254 nm in a UV cabinet. The image taken from a smart phone camera was saved in the JPEG format, cropped into a desired size and opened with ImageJ, a free and open source image processing program (Wayne Rasband, National Institute of Health, MD, USA; https://imagej.nih.gov/ij/). The image was then split into the red, blue and green channel (Image>Color>Split Channel) and the green channel was used for further processing since it offered the best results. A median filter with a resolution of 5-10 pixel was applied to this image for noise removal (process>filter>median). Furthermore, the rectangular selection tool was used to select equal height and width of spots on the image of the TLC plate and 'select first lane/next lane' tool was applied to designate the lanes (Analyze>Gels>Select first lane). This was followed by the application of a plot lane tool to generate the line profile plots. 'Line tool' was then used to draw a straight line at the bottom of each peak in order to enclose the area under the peak. The peak area under the individual peak was then obtained by clicking inside the peak with the 'magic wand tool'. The TLC densitogram of ASA is shown in Figure 2.

UV-Visible Spectrophotometric Analysis

Agilent Cary Win UV-Visible Spectrophotometer with 1 cm quartz cells was used for recording the spectra. After US-DLLME, the CF extract was diluted up to 1 ml and subjected to UV-Visible spectrophotometric analysis. A blank solution of CF was used for all the spectra measurements. The standard solution of ASA was scanned between 200 to 300 nm and maximum

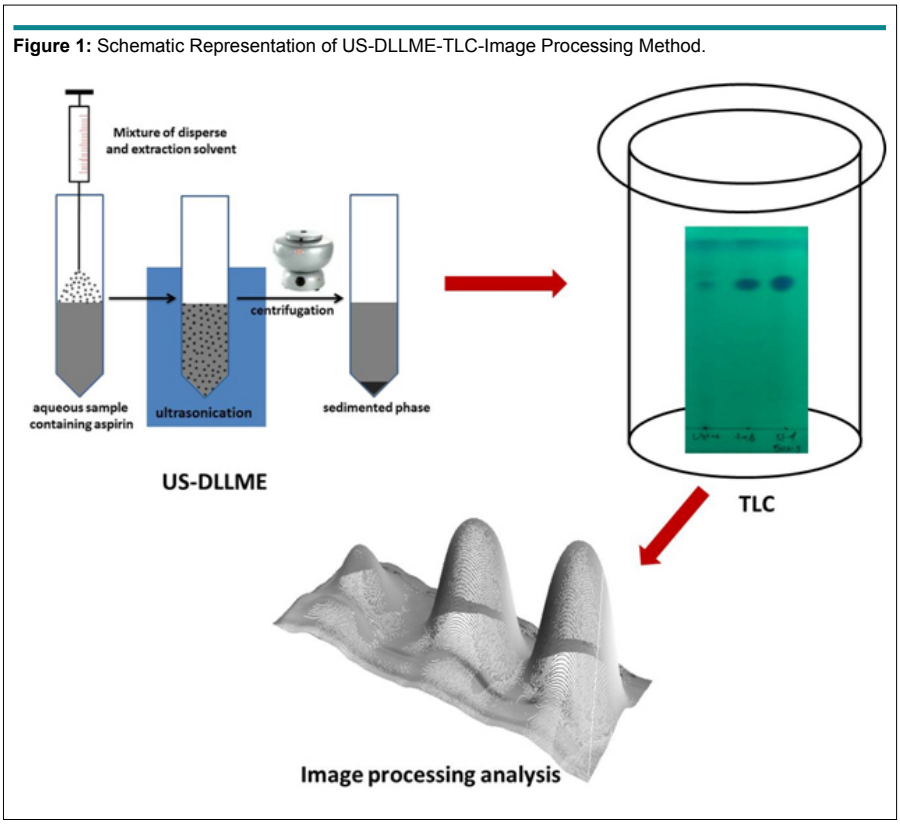

Figure 1: Schematic Representation of US-DLLME-TLC-Image Processing Method.

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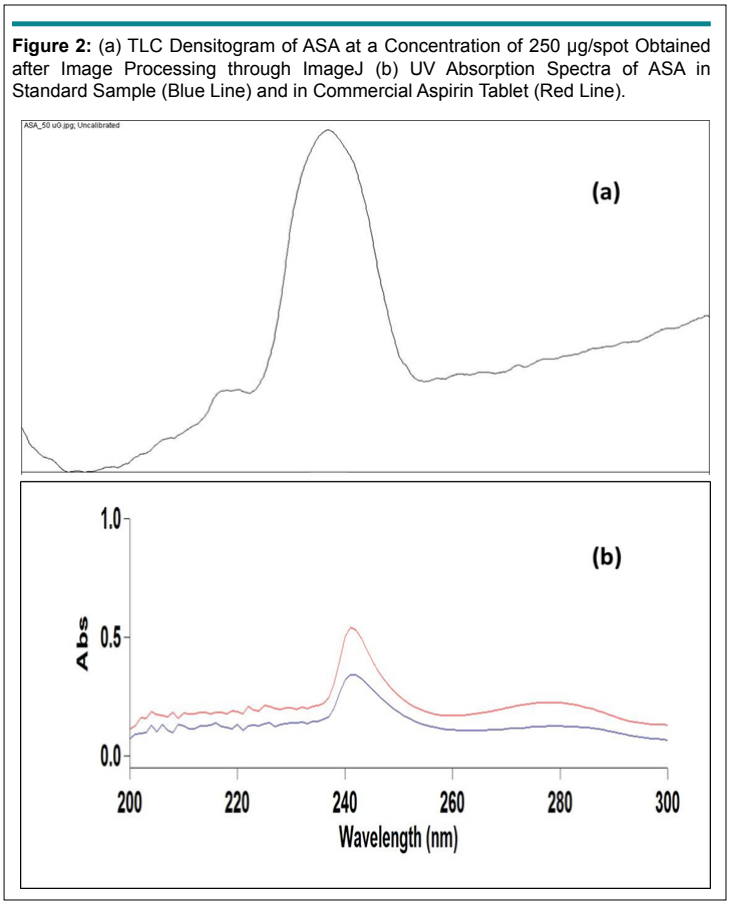

Figure 2: (a) TLC Densitogram of ASA at a Concentration of 250 µg/spot Obtained after Image Processing through ImageJ (b) UV Absorption Spectra of ASA in Standard Sample (Blue Line) and in Commercial Aspirin Tablet (Red Line).

absorbance was obtained at 276 nm.

RESULTS AND DISCUSSION

In resource limited countries, TLC is still a widely used analytical method for product quality assessment of pharmaceutical drugs. Although, planar chromatography does not have a separation power at par with column chromatography such as high performance liquid chromatography (HPLC) and gas chromatography (GC), however, it is still applicable for the analysis of finished pharmaceutical products which have lesser active ingredients.[25] Herein, an alternative analytical platform for the determination of ASA based on the use of DLLME combined with TLC-image processing method is presented.

In order to get an optimum extraction efficiency of the DLLME procedure, parameters affecting its extraction yield such as type and volume of extraction solvent, type and volume of disperser solvent, pH of the sample solution, ionic strength and sonication time, have been thoroughly studied and optimized using one-variable-at-a-time (OVAT) approach. All experiments were performed in triplicates.

Selection of Extraction Solvent and its Volume

Classically, extraction solvent has higher density than aqueous phase which facilitates its sedimentation following centrifugation. In the present study, various organic solvents having higher density (d) than water such as TCE (d=1.46), CF (d=1.48), DCM (d=1.33), CB (d=1.11) and CCl_4 (d=1.59) were screened as extraction solvents for ASA. A series of experiments were performed by taking a constant volume of ACE (disperser solvent, 500 µl) along with the extraction solvent (200 µl) at ASA concentrations of 10 µg/ml. As evident from Figure 3a, CF has the highest extraction efficiency for ASA followed by DCM and TCE.

In the further experiments, volume of CF was optimized in the range of 50-200 µl. It is clear from Figure 3b, that the peak area of ASA increases from 50 to 100 µl but tends to decrease further due to an increasing dilution of the analyte in the extraction solvent. Hence, 100 µl of CF was used as the extraction solvent for further experiments.

Selection of Disperser Solvent and its Volume

Disperser solvent, being miscible in both extraction solvent and aqueous phase, facilitates the dispersion of extraction solvent throughout the aqueous phase, thus, achieving a rapid equilibrium. Four widely used disperser solvents viz. ACE, ACN, MeOH and EtOH were screened in this study.[26-28] Experiments were performed taking 500 µl of each disperser solvent with 100

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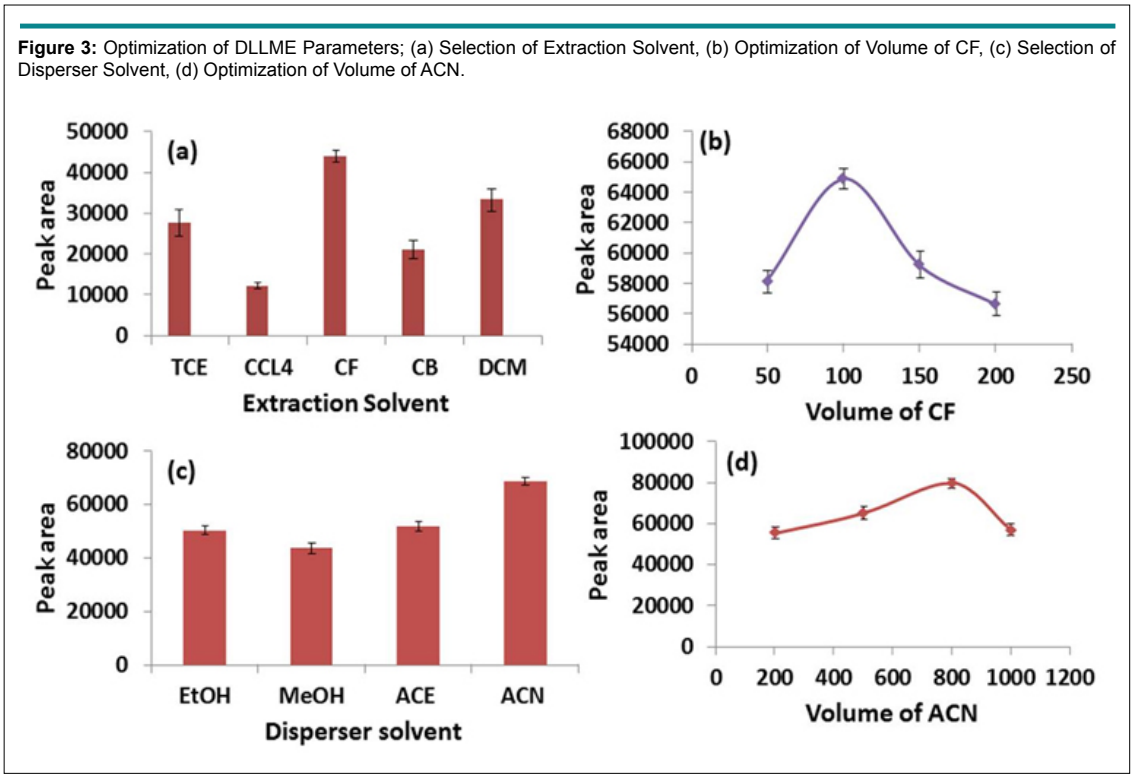

Figure 3: Optimization of DLLME Parameters; (a) Selection of Extraction Solvent, (b) Optimization of Volume of CF, (c) Selection of Disperser Solvent, (d) Optimization of Volume of ACN.

µl of CF. Figure 3c clearly shows that ACN has the highest peak area followed by ACE, EtOH and MeOH. Therefore, ACN was selected as the optimum disperser solvent.

The volume of ACN was then optimized in the range of 200-1000 µl (i.e., 200, 400, 600, 800 and 1000 µl). As the volume of ACN increases from 200 to 800 µl, the peak area of ASA also increases, however a further increase from this point results in a decreased peak area of ASA. This can be explained by the fact that at lower volumes, ACN does not disperse CF in the aqueous phase completely, and at higher volumes, the solubility of ASA increases in the aqueous phase, which results in a declining peak area (Figure 3d).[29] Therefore, 800 µl of ACN and 100 µl of CF were used as disperser and extraction solvents, respectively for further studies.

Effect of Ultrasonication Time

To evaluate the effect of ultrasonication time on extraction efficiency of DLLME, a set of experiments were conducted for screening the ultrasonication time in the range of 0-5 min (i.e., 0, 1, 3, 5 and 7 min). Ultrasonication facilitates the emulsification of extraction solvent in the aqueous phase and subsequently mass transfer of the analyte from the aqueous phase to the extraction solvent. Extraction efficiency of DLLME was found to be the highest at 1 min of ultrasonication and showed a declining trend beyond this point (Figure 4a). This can be explained by the fact that, as sonication time increases, the local temperature of the solution also increases due to a large number of compression and rarefaction cycles, which results in a decrease in the peak area of ASA.[30] Therefore, an ultrasonication time of 1 min was selected in order to get an optimum extraction efficiency of DLLME for ASA.

Effect of pH and Ionic Strength

The pH of aqueous phase (sample solution) plays an important role in the extraction efficiency of DLLME, since at optimum pH, the analyte can be extracted in its non-ionized form. In order to investigate the pH effect, samples were prepared in the pH range of 2-12. As is evident from Figure 4b, the maximum peak area of ASA (pKa=3.5) was obtained at slightly acidic conditions wherein ASA behaves as a weak acid. Therefore, an extraction pH of 5 was chosen as the optimized value.

Following pH, the ionic strength of the aqueous phase was optimized by adding NaCl in the range of 0-10%. Generally, an increasing amount of salt in the aqueous phase decreases the solubility of the analyte in the sample solution and facilitates the mass transfer of the analyte in the extraction solvent. However, the recorded results revealed that the peak area of ASA decreases with an increasing amount of salt (data not shown). This can be explained by the fact that an increasing amount of salt enhances the viscosity and the density of the aqueous phase. This can lead to the generation of heat due to absorption of ultrasonic energy when the sample is subjected to ultrasonication.[31] Therefore; no salt was added in the sample solution for DLLME procedure.

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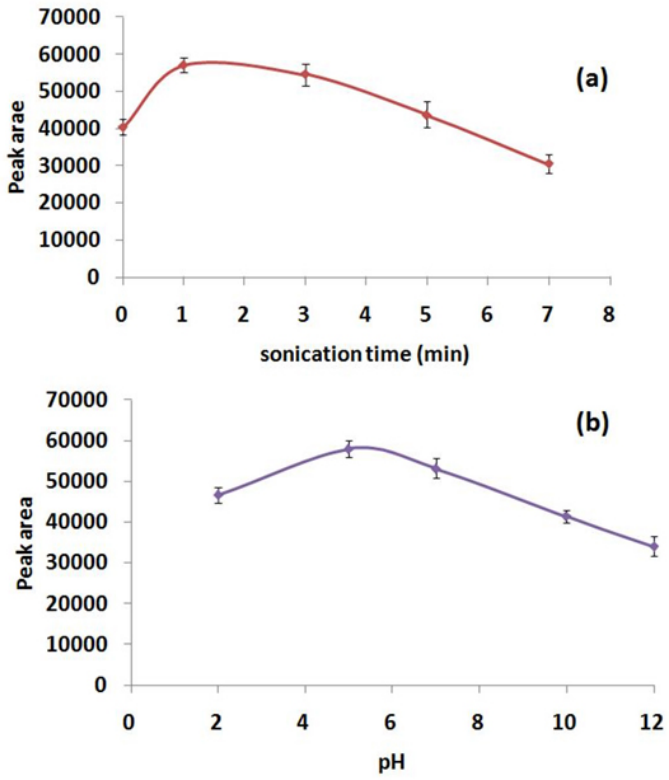
Figure 4: Effect of (a) Sonication Time and (b) pH on Extraction Efficiency of DLLME.



Method Validation Parameters

The developed method has been thoroughly validated for its linearity, precision, recovery, limit of detection (LOD) and limit of quantification (LOQ) in spiked water samples. Under optimized conditions, the method was found to be linear in the range of 1-250 µg/spot with a square of correlation coefficient (R^2) of 0.999. LOD and LOQ were found to be 0.1 and 0.35 µg/spot with a signal to noise ratio of 3 and 10, respectively. Intra-day and inter-day precision of the method were expressed as percent relative standard deviation (%RSD) and were studied at three different concentration levels of the calibration graph (i.e., 1, 25 and 250 µg/spot). Recovery assay of ASA in pharmaceutical formulations and urine were performed by the standard addition method at 1, 25 and 250 µg/spot and were found to be 90- 99% and 84-92%, respectively with an RSD in the range of 1.69-5.61%, respectively (Table 1). Intra and inter-day precisions were found to be in the range of 2.64-9.67% and 3.64-11.43% for pharmaceutical formulations and urine, respectively (Table 2). The ASA content of the tablet was quantified at three different concentration levels (n=5) of the calibration graph (i.e. 1, 25 and 250 µg/spot) and the results were shown in Table 3. UV-Visible spectrophotometric analysis of pharmaceutical tablets after US-DLLME was also performed in the concentration range corresponding to 1-250 µg/spot. However, the final extract obtained was diluted to bring the concentration within the working range of the UV-Visible spectrophotometer (Table 3).

CONCLUSION

TLC is still a widely used separation and identification technique in analytical laboratories. Modern image processing

Table 1: Recovery Assay of ASA in Pharmaceutical Formulation and Urine (µg/spot, n=3).

Total concentration	Amount taken		Amount added		Amount found		Recovery (% RSD)	
	Tablet	Urine	Tablet	Urine	Tablet	Urine	Tablet	Urine
1	0.5	-	0.5	1	0.9	0.84	90 (3.78)	84 (5.61)
25	12.5	-	12.5	25	23.5	22.2	94 (2.84)	89 (4.17)
250	125	-	125	250	247.5	230	99 (1.69)	92 (3.45)

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Table 2: Intra and Inter Day Precision of US-DLLME-TLC Image Processing Method for Analysis of ASA in Urine and Pharmaceutical Formulation (n=3).

Concentration (µg/spot)	Tablet		Urine	
	Intra-Day	Inter-day	Intra-day	Inter-day
1	4.75	9.67	5.46	11.43
25	3.81	8.28	4.39	8.19
250	2.64	6.78	3.64	6.46

Table 3: Analysis of ASA in Commercially Available Pharmaceutical Formulations of Different doses by US-DLLME-TLC Image Processing and US-DLLME-UV-Visible Spectrophotometric Method.

Tablet (Label Claim)	US-DLLME-TLC-Image Processing	US-DLLME-UV-Visible Spectrophotometry
Class 1 (350 mg)	349.0 (3.21)	349.2 (2.89)
Class 2 (75 mg)	74.2 (3.58)	74.6 (3.54)
Class 3 (75 mg)	74.5 (3.94)	74.4 (3.12)

*% RSD is mentioned in parenthesis.

techniques have helped in overcoming the drawback of TLC of being a qualitative analytical technique only. DLLME is an environmentally benign, rapid and cost-effective modern microextraction technique which consumes only microliters of the extraction solvent. For the first time, coupling of US-DLLME with TLC-image processing analysis have been proposed and successful application is demonstrated for the analysis of ASA in pharmaceutical formulations. The method can be used routinely to check the purity of the drug as well as in forensic toxicological laboratories for the analysis of ASA in tablets and capsules in drug overdose cases. The proposed method will pave the path for analytical chemists to develop cost-effective and eco-friendly analytical methods for a variety of analytes such as pesticides, drugs of abuse, pharmaceutical formulations etc. which will be useful for the resource limited laboratories. However, more efforts are needed to enhance the sensitivity of the TLC-image processing methodology for the trace level analysis of target analytes in biological matrices.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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TOXICOLOGY AND FORENSIC MEDICINE

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Research

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Cadmium Induced Oxidative Stress in Wistar Rats: Ameliorative Effect of Quercetin and *Embilica Officinalis* Plant Extracts

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ABSTRACT

Background: Cadmium is a naturally occurring metal that is widely distributed throughout the biosphere. The present investigation is aimed to assess the antioxidant potential of Quercitin and *Embilica officinalis* (amla) extracts, which are reported to have a wide range of pharmacological properties including the efficacy of these selected substances on antioxidants and lipid peroxidation status in the liver tissue of rats during cadmium intoxication.
Methods: After three weeks of cadmium oral administration, certain specific enzymes of the hepatic tissue of Wistar rats were assayed.
Results: After three weeks of cadmium oral administration, certain specific enzymes of the hepatic tissue including aspartate aminotransferase (AST), alanine aminotransferase (ALT), acid phosphatase (ACP), γ-glutamyl transferase (GGT), and lactate dehydrogenase (LDH) were significantly elevated, thus indicating cellular damage. The concentration of lipid peroxidation markers represented by thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides and protein carbonyl contents of the liver tissue were significantly elevated, whereas vitamin C and E levels were found to be significantly reduced. But the activity of the antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GPx) and glutathione S-transferase (GST) activities were found to be significantly inhibited.
Conclusion: The results obtained in the present investigation clearly demonstrates the antioxidant potential of Quercetin and *E. officinalis* extract, by ways of decreasing lipid peroxidation against cadmium induced oxidative stress in rats.

KEY WORDS: Quercetin; *Embilica officinalis;* Antioxidant enzymes; Oxidative stress.

ABBREVIATIONS: AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ACP: Acid phosphatase; GGT: γ-glutamyl transferase; TBARS: Thiobarbituric acid reactive substances; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidise; GST: Glutathione S-transferase.

INTRODUCTION

Heavy metals are the most dangerous group of anthropogenic environmental pollutants which are highly toxic and persistent in the environment. The major anthropogenic sources of environmental pollution include smelting operations, phosphate fertilizers, pigments, cigarette smoke, automobiles, etc., which constitutes almost 90% of cadmium levels in the environment leading to its bioaccumulation in humans and animals. Cadmium has been reported to exert deleterious effects in organisms at low levels of exposure,[1] which are nephrotoxic, cytotoxic, genotoxic and carcinogenic.[2-4]

The literature is replete with the reports on metal induced oxidative stress that has been recently implicated in the pathogenesis of metal toxicities. It is established that metals

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can generate reactive oxygen species (ROS), which in turn overwhelms the cell's innate antioxidant defences leading to oxidative stress. Studies have demonstrated that cadmium stimulates free radical production, resulting in oxidative damage to lipids, proteins and DNA, eventually resulting in membrane damage, protein dysfunction and DNA damage. These conditions can further culminate into pathological conditions both in humans and animals,[4,5] including diabetes, cardiovascular diseases, cancer, etc. Antioxidants are compounds that mop up the free radicals and prevent cellular damage.[6-11] Although, several chelating agents and antagonistic compounds are available which helps to reduce cadmium toxicity, some of them are burnt with undesirable side effects. Due to the intrinsic limitations and variability of the effectiveness of heavy metal chelating agents, research is being undertaken to apply cadmium intoxication therapy with the development of new therapeutic agents especially from flavonoids and other related phytometerials. In the recent years, phytometerials have been addressed as potent free radical scavengers and have attracted tremendous interest as possible therapeutics, against free radical material diseases.[12,13] The efficacy of a wide variety of phytocompounds has been reported to have a wide spectrum of pharmacological properties including anti-inflammatory,[14] anti-allergic,[9] anti-tumour[3] and anti-oxidant characteristics.[15-17] With this premise, the present investigation was designed to study the ameliorative effect of phytocompounds like Quercetin and *Embilica officinalis* (amla) extracts on Cadmium-induced oxidative stress in Wistar rats.

MATERIALS AND METHODS

Quercetin ($C_{15}H_{14}O_9$; MW 338.27; 2-(3,4-dihydrophenyl)-3,5,7-trihydroxy-4H-chromen-4-one; a plant derivative of Rutin) and shade dried amla fruit were obtained from the local market. Ten grams of dried amla fruits were ground to powder form in an electric grinder and is dissolved in 50 ml of distilled water and was room evaporated for further use in experimentation.

Experimental Animals

Male albino rats weighing 100±5 g were used for the present investigation. The animals were fed with commercial standard pellet diet (Lipton India Ltd, Mumbai, MH, India) and had free access to water under well ventilated conditions of 12 h (day :night). The animals were acclimatized to laboratory conditions prior to the experiment under standard laboratory conditions (temperature 24±2 °C) for at least one week before the experiments, were performed, maintained on standard diet, and given free access to food and water. The animals were housed in specially designed plastic rodent cages in the animal house in Sri Venkateswara University, Tirupati, Andhra Pradesh. This study and all procedures were approved by the Animal Care and Bioethical Committee. Cadmium chloride was dissolved in distilled water and administered orally to rats. The animals were divided into six groups each comprising of six rats. The experimental period was three weeks (21 days) and the group were as follows:

Group 1: Control group (kept under standard laboratory conditions);
Group 2: Received cadmium chloride (5 mg/kg Body weight/day);
Group 3: Received cadmium chloride (5 mg/kg Body weight/day) along with Quercetin (100 mg/kg Body Weight/day orally) prior to the administration of $CdCl_2$;
Group 4: Received Quercetin (100 mg/kg Body weight/day orally) alone for 21 days;
Group 5: Received cadmium chloride (5 mg/kg Body weight/day) along with *E. officinalis* extract (150 mg/Kg Body weight/day);
Group 6: Received *E. officinalis* extract (150 mg/kg Body weight/day orally) alone for 21 days.

At the end of the experimental period, all the animals were starved overnight and then they were killed by cervical decapitation with mild ether anaesthesia. The liver tissue was dissected out, weighted and washed using chilled saline solution. For all the enzyme assays, the liver tissue was preserved at -80^{0}C till further analysis was performed.

Biochemical Analysis

Liver tissue homogenates from each experimental group were prepared in accordance with the standardized protocols. The methodologies adopted in the present investigation have been presented in the following tablulation:

1	Lipid peroxidation (Thiobarbituric acid reactive substances TBARS)	Fraga et al[18]
2	Lipid hydroperoxides	Jiang et al[19]
3	Protein carbonyl content	Levine et al[20]
4	Reduced Glutathione (GSH)	Ellman[21]
5	Vitamin C	Omaye et al[22]
6	Vitamin E	Desai[23]
7	Superoxide dismutase (SOD)	Kakkar et al[24]
8	Catalase (CAT)	Sinha[25]
9	Glutathione peroxidise (GPx)	Rotruck et al[26]
10	Glutathione S-transferase (GST)	Habig et al[27]
11	Aspartate amino transferase (AST)	Reitman & Frankel[28]
12	Alanine amino transferase (ALT)	Reitman & Frankel[28]
13	Reduced GSH assay	Moron et al[29]
14	Alkaline phosphatise (ALP)	Bodansky[30]
15	Lactate dehydrogenase (LDH)	Srikanthan & Krishnamoorthy[31]
16	γ-Glutamyl transferase (GGT)	Spectrophotometrically
17	MDA	Ohkawa et al[32]
18	Protein	Lowry et al[33]

All the chemicals and reagents used in the present study were analytical grade and were obtained from Sigma Chemical Company, Himedia Laboratories and local firms.

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Statistical Analysis

The results were expressed as the Mean±SD of six individual observations. The data was subjected to statistical analysis by ANOVA by using Statistical Packages (SPSS) Program.

RESULTS

The results presented in Table 1, indicate that the body weight of the wistar rats was increased during the three weeks of experimental period in both control and experimentally treated rats of all groups. The liver tissue weights were also shown to be increased, both in control and experimentally treated rats of all groups. Table 2, presents the changes in the level of lipid peroxidation products including thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides and protein carbonyl contents were significantly higher, whereas the levels of vitamin C and vitamin E were significantly lower in both control and experimentally treated rats (Figure 1). The administration of Quercetin and *E. officinalis* plant extracts significantly decreased the levels of lipid peroxidation products, lipid hydroperoxides and protein carbonyl contents in liver tissue of experimentally treated rats. Table 3, shows the levels of serum hepatic marker enzymes in control and experimentally treated rats of all groups. The oral administration of cadmium for 21 days induced several abnormalities in the levels of serum hepatic marker enzymes. The levels of serum hepatic marker enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline Phosphatase (ALP), lactate dehydrogenase (LDH), and γ-glutamyl transferase (GGT) were found to be significantly increased in cadmium treated rats. Consequently, the administration of quercetin and plant extract of *E. officinalis* significantly decreased the activity levels of serum hepatic marker enzymes compared to cadmium treated rats (Table 3). Table 4, presents changes in hepatic antioxidants including superoxide dismutase (SOD), catalase (CAT), glutathione peroxide (GPx), glutathione s-transferase (GST) and reduced glutathione (GSH) which were found to be increased significantly with the administration of Quercetin and *E. officinalis* plant extract to the cadmium-treated experimental rats (Figures 2 and 3).

DISCUSSION

Cadmium is one of the main environmental and occupational pollutants in the industrialized countries. Exposure to cadmium is linked with serious health hazards and unlike other heavy metals, is unable to generate free radicals by itself; however, reports have indicated that the superoxide radical, hydroxyl radical and nitric oxide radicals could be generated indirectly.[34] A study by Watnabe et al[10] showed the generation of non-radical hydrogen

Table 1: Changes in Body Weight (g) and Liver Tissue (g) During Cadmium Induced Toxicity in Rats.

Groups	Body weight (g)		Liver weight (g)	
	Initial	Final	Initial	Final
Group-I	100.25±2.88 PDC	122.45±3.12 (+22) *p*<0.05	6.45±0.28 PDC	7.08±0.31 (+9.8) *p*<0.05
Group-II	100.08±2.84 PDC *p*>0.05	120.12±2.75 (+20)	6.45±0.24 PDC *p*>0.05	6.59±0.28 *p*<0.05
Group-III	100.49±2.72 PDC *p*>0.05	122.58±3.15 (+22) *p*>0.05	6.45±0.25 PDC *p*>0.05	6.89±0.29 (+6.8) *p*>0.05
Group-IV	100.54±2.59 PDC *p*>0.05	123.19±3.42 (+ 23) *p*>0.05	6.45±0.26 PDC *p*>0.05	7.13±0.24 (+10.5) *p*>0.05
Group-V	100.72±2.89 PDC p>0.05	120.13±2.82 (+19) *p*>0.05	6.45 ±0.27 PDC p>0.05	6.94±0.28 (+8) *p*>0.05
Group-VI	100.45±2.85 PDC *p*>0.05	123.34±3015 (+23) *p*>0.05	6.45±0.26 PDC *p*>0.05	7.15±0.26 (+11) *p*>0.05

Values are Mean±SD of six individual observations.
PDC: Percent Deviation over Control
Group-I : Control
Group-II : Received Cadmium Chloride 5 mg/Kg Body weight /day)
Group-III : Received Cadmium Chloride 5 mg/Kg Body weight/day along with Quercetin 100 mg/Kg Body weight/day
Group-IV : Received Quercetin (100 mg/Kg Body weight/day orally for 21 days.
Group-V : Received Cadmium Chloride 5 mg/Kg Body weight/day along with *Embilika officinalis* extract (150 mg/Kg Body weight/day)
Group-VI : Received *Embilika officinalis* extract alone for 21 days.(150 mg/Kg Body weight/day orally

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Table 2: Changes in the Lipid Peroxidation levels in the Tissue of Rats in Different Treatments.

Groups	TBARS[a]	Hydroperoxide[a]	Protein Carbonyls[b]	VitaminC[c]	VitamiE[c]
Group-I	2.88±0.12	114.42±6.13	204.18±10.12	2.48±0.12	2.13±0.14
Group-II	6.13±0.34 (+112) $p<0.001$	274.14±10.12 (+90) $p<0.001$	415.42±15.44 (+103) $p<0.001$	0.94±0.05 (+163) $p>0.05$	1.11±0.10 (+92) $p<0.001$
Group-III	3.72±0.25 (+30) $p>0.05$	175.13±8.12 (+54) $p<0.001$	315.49±10.75 (+54) $p<0.001$	2.19±0.15 (+13) $p>0.05$	1.45±0.12 (+47) $p<0.001$
Group-IV	3.13±0.22 (+9) $p>0.05$	134.18±7.42 (+17) $p<0.05$	249.45±10.13 (+22) $p<0.001$	2.77±0.18 (+12) $p>0.05$	1.94±0.11 (+10) $p<0.01$
Group-V	4.13±0.28 (+43) $p>0.05$	163. 42±6.77 (+42) $p<0.001$	285.77±8.85 (+40) $p<0.001$	2.11±0.12 (+18) $p>0.05$	1.58±0.12 (+26) $p<0.001$
Group-VI	3.05±0.22 (+6) $p>0.05$	128.42±6.75 (+12) $p>0.05$	238.41±7.77 (+17) $p<0.001$	2.68±0.12 (+8) $p>0.05$	1.96±0.12 (+8) $p<0.05$

Values are Mean±SD of six individual observations.
Values in parenthesis are Percent deviation over their respective Control
Group-I : Control
Group-II : Received Cadmium Chloride 5 mg/Kg Body weight /day)
Group-III : Received Cadmium Chloride 5 mg/Kg Body weight/day along with Quercetin 100 mg/Kg Body weight/day
Group-IV : Received Quercetin (100 mg/Kg Body weight/day orally for 21 days.
Group-V : Received Cadmium Chloride 5 mg/Kg Body weight/day along with *Embilika officinalis* extract (150 mg/Kg Body weight/day)
Group-VI : Received *Embilika officinalis* extract alone for 21 days. (150 mg/Kg Body weight/day orally)

[a]mM/100 g weight of tissue
[b]nmoles/mg protein
[c]µmoles/g weight of tissue

Figure 1: Changes in Percent Deviation Over Control Values of Selected Antioxidant Substances in Different Groups of rats Treated with Cadmium.

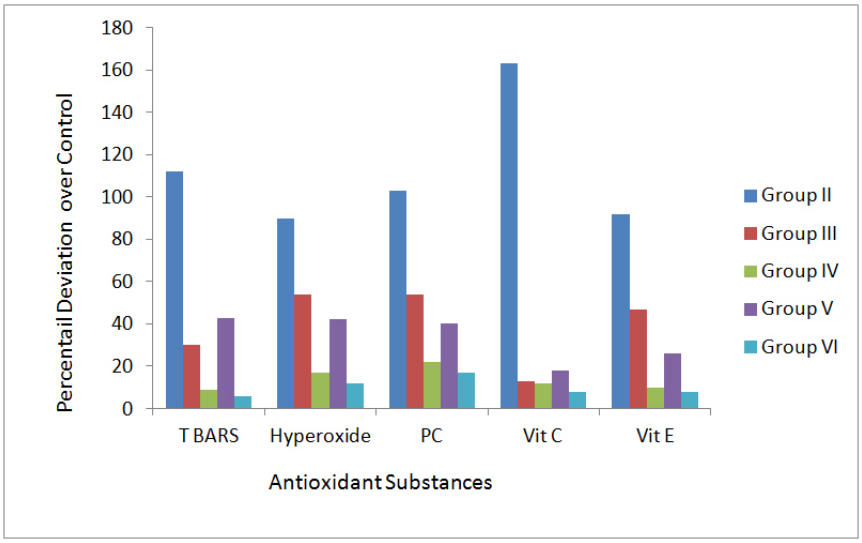

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Table 3: Changes in the Activities of Hepatic Marker Enzymes during Cadmium Induced Toxicity.

Groups	AST[a]	ALT[a]	ACP[b]	LDH[c]	GGT[d]
Group-I	65.72±2.45	39.42±1.94	102.45±3.78	145.44±6.42	102.41±6.75
Group-II	95.14±3.78 (+45) *p*<0.001	72.41±2.74 (+84) *p*<0.001	175.48±5.94 (+72) *p*<0.001	184.49±6.18 (+27) *p*<0.001	175.48±5.72 (+72) *p*<0.001
Group-III	175.48±5.72 (+72) *p*<0.001	46.43±1.78 (+18) *p*<0.001	159.42±5.12 (+56) *p*<0.001	174.58±5.13 (+21) *p*<0.001	168.41±5.15 (+65) *p*<0.001
Group-IV	68.49±2.75 (+5) *p*<0.001	42.49±2.05 (+8) *p*<0.001	128.49±4.75 (+26) *p*<0.001	155.45±4.88 (+7) *p*<0.001	142.41 ± 3.99 (+40) *p*<0.001
Group-V	76.74±3.49 (+17) *p*<0.001	42.74±2.05 (+9) *p*<0.001	165.48±5.15 (+62) *p*<0.001	165.48±5.15 (+62) *p*<0.001	172.43±5.42 (+69) *p*<0.001
Group-VI	70.43±3.45 (+8) *p*<0.001	40.11±1.42 (+2) *p*<0.05	125.44±5.12 (+23) *p*<0.001	150.42±4.88 (+4) *p*<0.001	138.74±4.88 (+36) *p*<0.001

Values are Mean±SD of six individual observations.
Values in parenthesis are Percent deviation over their respective Control
Group-I : Control
Group-II : Received Cadmium Chloride 5 mg/Kg Body weight /day)
Group-III : Received Cadmium Chloride 5 mg/Kg Body weight/day along with Quercetin 100 mg/Kg Body weight/day
Group-IV : Received Quercetin (100 mg/Kg Body weight/day orally for 21 days.
Group-V : Received Cadmium Chloride 5 mg/Kg Body weight/day along with *Embilika officinalis* extract (150 mg/Kg Body weight/day)
Group-VI : Received *Embilika officinalis* extract alone for 21 days. (150 mg/Kg Body weight/day orally)

[a]µmoles of Pyruvate liberated/mg protein/hr
[b]µmoles of Pi liberated/mg protein/hr
[c]µmoles of Formazon formed/mg protein/hr
[d]units/mg protein/hr

Table 4: Changes in the Antioxidant Levels in the Liver Tissue of Control and Experimental Rats.

Groups	**SOD[a]**	**CAT[a]**	**GPx[a]**	**GST[b]**	**GSH[c]**	**MDA[d]**	**LPO[d]**
Group-I	16.18±0.72	105.42±5.38	18.45±0.38	42.45±1.38	75.18±3.42	6.78±0.25	12.14±0.35
Group-II	10.13±0.62 (+38) *p*<0.001	60.38±2.49 (+43) *p*>0.05	13.12±0.29 (+29) *p*<0.001	28.34±1.04 (+34) *p*<0.001	53.44±2.42 (+29) *p*>0.05	18.14±0.78 (+167) *p*>0.05	26.72±1.28 (+120) *p*<0.001
Group-III	12.42±0.58 (+24) *p*<0.001	70.13±2.84 (+34) *p*>0.05	14.15±0.32 (+24) *p*<0.001	32.41±1.04 (+24) *p*<0.001	60.13±2.41 (+20) *p*>0.05	13.19±0.68 (+94) *p*>0.05	16.42±0.72 (+36) *p*<0.001
Group-IV	14.18±0.59 (+13) *p*<0.001	88.42±3.49 (+17) *p*>0.05	16.89±0.34 (+9) *p*<0.05	40.78±1.19 (+4) *p*<0.001	74.79±2.55 (+05) *p*>0.05	10.45±0.66 (+55) *p*>0.05	13.77±0.58 (+14) *p*<0.01
Group-V	13.38±0.59 (+18) *p*<0.001	95.14±3.25 (+10) *p*>0.05	15.42±0.34 (+17) *p*<0.001	34.84±1.42 (+18) *p*<0.001	62.44±2.38 (+17) *p*>0.05	16.43±0.72 (+42) *p*>0.05	17.49±0.75 (+44) *p*<0.001
Group-VI	16.42±0.59 (+2) *p*>0.05	112.14±4.98 (+7) *p*>0.05	19.05±0.42 (+4) *p*>0.05	43.49±1.13 (+3) *p*<0.01	77.49±2.59 (+4) *p*>0.05	8.79±0.68 (+30) *p*>0.05	14.15±0.32 (+17) *p*<0.001

Values are Mean±SD of six individual observations.
Values in parenthesis are Percent deviation over their respective Control
Group-I : Control
Group-II : Received Cadmium Chloride 5 mg/Kg Body weight /day)
Group-III : Received Cadmium Chloride 5 mg/Kg Body weight/day along with Quercetin 100 mg/Kg Body weight/day
Group-IV : Received Quercetin (100 mg/Kg Body weight/day orally for 21 days.
Group-V : Received Cadmium Chloride 5 mg/Kg Body weight/day along with *Embilika officinalis* extract (150 mg/Kg Body weight/day)
Group-VI : Received *Embilika officinalis* extract alone for 21 days. (150 mg/Kg Body weight/day orally)

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Figure 2: Changes in Percent Deviation Over Control Values of Selected Enzymes in Different Groups of Rats Treated with Cadmium.

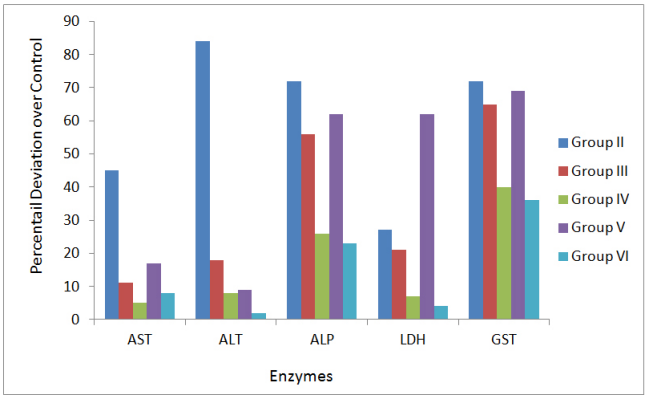
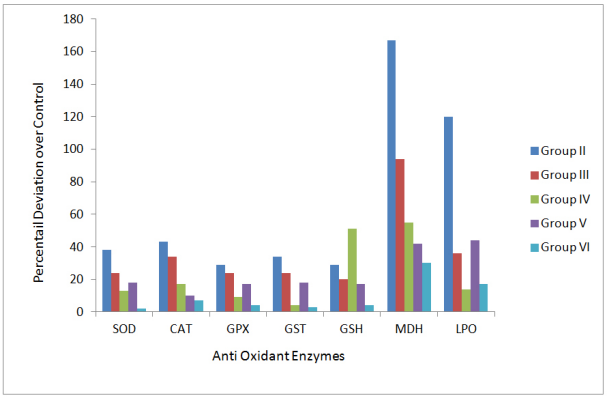


Figure 3: Changes in Percent Deviation Over Control Values of Selected Antioxidant Enzymes in Different Groups of Rats Treated with Cadmium.



peroxide, which by itself became a significant source of free radicals *via* the Fenton reaction. The replacement of iron and copper by cadmium from a number of cytoplasmic and membrane proteins like ferritin, in turn would release and increase the concentration of unbound iron or copper ions. These free ions participate in causing oxidative stress *via* the Fenton reactions.[35] The generation of reactive oxygen species (ROS) has been attributed to cadmium induced pathotoxicity that leads to a disruption in the pro-oxidant/anti-oxidant balance, a condition coined as oxidative stress. A quest for safe phytoconstituents as antioxidants has been the main stay of recent research in phytotherapy to alleviate the pathologies that are associated with oxidative cellular damage. Studies have indicated that cadmium is an inducer of cell oxidative stress, either in a variety of cell culture systems[36] or *in vivo* models through all routes of exposure.[37] Lipid peroxidation is one of the consequences of oxidative damage and is found to play an important role in the toxicity of cadmium.[38-39] Cadmium-induced oxidative stress is caused due to the production of hydroxyl radicals, superoxide anions, nitric oxide and hydrogen peroxide.[40-42]

The hepatic TBARS content was shown to be significantly increased in the cadmium-treated rats which may be attributed to excessive formation of free radicals which subsequently led to changes in the biological macromolecules.[4,5,37] Several authors reported that lipid peroxidation is a sensitive maker of cadmium hepatic toxicity in vertebrate species.[4,5] In the present study, cadmium treatment resulted in an excessive production of free radicals such as hydroxyl radical, superoxide radical, peroxyl radical and hydrogen peroxide. All the above mentioned radicals have a great potential to react rapidly with lipids, which in turn leads to lipid peroxidation.[4] Decomposition products of lipid hydroperoxide such as malanoldehyde and 4-hydroxynonenal, can cause chaotic cross-linkage with proteins and nucleic acids, which plays an important role in the process of carcinogenesis. In the present investigation, hepatic lipid peroxidation (LPO) activities show significant increase due to cadmium intoxication. Furthermore, extensive damage to tissues in a free radical mediated LPO results in membrane damage and subsequently decrease the membrane fluid content. Consequently, the treatment of cadmium exposed rats to both Quercetin and plant extract of *E. officinalis* resulted in a significant decrease in the levels of TBARS, hydroperoxides and protein carbonyl contents compared with cadmium treated rats. This observation clearly demonstrates that the free radical scavenging efficiency of both Quercetin and *E. Officinalis* extract may be associated with the presence of two hydroxyl groups in the β-ring of its molecule.[41] The presence of polyunsaturated substitution on the β-ring of Quercetin together with 2,3 double bond, a free 3-hydroxyl substitution and a 4-ketogroup confer potent anti-peroxidative properties of Quercetin.[42] Several authors also reported that LPO was either blocked or prevented due to administration of Quercetin.[43] Thus, Quercetin effectively quenches free radicals, inhibits LPO and protects the hepatic tissue from cadmium-induced oxidative damage. Literature has reported that the antioxidative potential of amla fruit has been attributed to its high vitamin C content. However, recent studies report the presence of bioactive tannoid principles in the fruit, comprising of emblicanin A, emblicanin B, punigluconin and pedunculagin, which have been shown to exhibit antioxidant activity *in vitro* and *in vivo* and are responsible for preventing the oxidation of ascorbic acid.[44]

The results obtained in the present investigation showed a marked increase in lipid peroxidation in the hepatic tissue of rats after cadmium treatment, which is in consensus with the previous reports on cadmium-induced oxidative stress in rats,[45,46] in poultry[47] and in fish.[48] In addition to cellular lipids, studies have shown that cellular proteins may also be affected by radical accumulation. It is well established that cadmium does not directly generate free radicals like often heavy metals, but it is capable of generating non-radical hydrogen peroxide that eventually acts as a source of free radicals through the Fenton chemistry. The formation of carbonyl derivatives of proteins is suggested to be a useful measure of oxidative damage to proteins. The carbonyl derivatives of proteins may result from oxidative modification of amino acid side chains and reactive oxygen-mediated rapid cleavage.[49] Stolis and Bagchi[37] have reported that the primary target of the oxygen radical attack,

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promoted by cadmium, is represented on cellular proteins. The results obtained in the present investigation also showed an increased protein carbonyl content in the cadmium treated rat liver tissue. When cadmium-exposed rats were subjected to treatment with both Quercetin and *E.officinalis* extracts, protein carbonyl contents were significantly decreased, which may be attributed to the antioxidant properties of the selected compounds in the present study. Both the compounds selected in the present study, by its free radical scavenging action, prevents the attack of free radicals on amino acids and this reduces the production of the protein carbonyl groups thus driving cadmium intoxication of rats.

GSH, is a tripeptide (L-αglutamylcysteinol glycine), an antioxidant and a powerful nucleotide, critical for cellular protection such as detoxification of ROS, conjugation with xenobiotics, excretion of toxic molecules and control of inflammatory cytokine cascade.[50] General depletion of GSH in the tissues of animals leads to the impairment of cellular defence against ROS and may result in peroxidative tissue injury. In the present investigation, a significant reduction in the hepatic reduced glutathione (GSH) levels in the cadmium-treated rats and reduced levels of Vitamin C and Vitamin E in the liver tissue, portrays the implications of cadmium-induced stress condition in rats.

Vitamin C is the most potent water soluble antioxidant that scavenges a wide variety of ROS and nitrogen, including superoxide radical.[51] Vitamin E, is a major chain breaking antioxidant, found in the lipid phase of membrane, and acts as a powerful terminator of LPO.[52] In the present investigation, the non-enzymatic antioxidants levels were found to be significantly depleted in cadmium-intoxicated rats, signifying that increased levels of free radical generation by cadmium which is effectively managed by both Vitamin C and E, are considered as the most effective free radical scavengers. Several authors also reported reduced production of hepatic antioxidants including GSH, Vitamin C and Vitamin E during cadmium intoxication in rat.[53]

The results obtained in the present study demonstrated that cadmium exposure showed an increased activity level of markers such as ALT, AST, ALP in the liver tissue of rats. The increase in these enzymatic activities may be due to cellular necrosis of hepatocytes, which causes increase in the permeability of the cell. Lactate dehydrogenase (LDH) is an index of cell damage including hepatotoxicity and the endothelial disruption in blood vessel. In the present study, increased level of LDH was substantially detected in all cadmium-treated rats, compared with the control group. This result is suggestive of the beginning of cytolysis, which is a possible indication of membrane damage including the endothelial membranes of blood vessels. This disruption of endothelial membrane, directly or indirectly, as reported earlier, includes the generation of reactive oxygen species in endothelial cells.

Generally under normal conditions, the animals tend to maintain a balance between generation and neutralization of ROS in the tissues. But, when the organisms are subjected to Xenobiotic stress, the rate of production of ROS including O_2^-, H_2O_2, OH^-, ROO^{--}, exceeds their scavenging capacities. All the organisms have their own cellular antioxidant defence system composed of both enzymatic and non-enzymatic components. Enzymatic antioxidant pathway consists of SOD, CAT and GPx. Superoxide anion O_2^- is dismutated by SOD to H_2O_2, which is reduced to water and molecular oxygen by CAT or is neutralized by GPx, that catalyses the reduction of H_2O_2 to water and organic peroxide to alcohols using GSH as a source of reducing equivalent. GR regenerates GSH from oxidized glutathione (GSSG), which is a scavenger of ROS as well as a substrate for other enzymes. GST conjugates xenobiotics with GSH for exclusion. One non-enzymatic pathway components consists of small organic molecules such as β-carotene, GHS, Vitamin C and Vitamin E.[54] Some of these parameters could serve as stress indicators in animals when exposed to environmental pollutants. Superoxide dismutase is considered to be its first line of defence against the deleterious effects of oxygen radicals in the cells and it scavenges ROS by catalysing the dismutation of superoxide to H_2O_2. Several authors also reported that SOD activities was considerably reduced during cadmium intoxication.[55] The inhibition of SOD activity may be associated with an increased flux of superoxide into the cellular compartments which may be the reason for decreased lipid peroxide indices in the present investigation. Catalase acts as a preventive antioxidant and plays an important role in protection against the deleterious effects of LPO, GPx and GST. Several authors have also reported that hepatic antioxidant enzyme activities were significantly reduced during cadmium intoxication, which may be due to excessive production of ROS. Glutathione peroxidase is also a first line of defence against oxidative damage due to H_2O_2 or lipid hydroperoxides, thus protecting the membrane from oxidative damage. GST is considered to be the second line of defence against xenobiotics on account of its direct conjugation with the expense of GST, both being associated with glutathione dependent activities. In the present investigation, both GST and GPx activities were significantly reduced during cadmium intoxication due to excessive production of H_2O_2 by Cadmium. Thus, the analysis of antioxidant status in the present study, indicates that the levels of both non-enzymatic and enzymatic antioxidants were significantly reduced due to cadmium reduced oxidative stress. Administration of both Quercetin and *E.officinalis*, extract significantly modulates the antioxidant status in the liver tissue of rats, suggesting the enhancing effect of the above substances on cellular antioxidant defences. The antioxidant role of the above selected substances may include the following interventions- scavenging of O_2^- OH^- peroxyl radical and peroxynitrite.[4] Several authors also reported that the above substances were known to prevent DNA damage during cadmium intoxication, enhanced the GSH dependent protection and prevented the depletion of thiols during oxidative stress.[5] Thus, it was indicated that both the substances selected might have played a role in quenching the free radicals, inhibiting LPO and ultimately reducing the build up of antioxidants during Cadmium intoxication.

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CONCLUSION

The results of the present study clearly indicate that heavy metal cadmium causes oxidative stress in rats and concluded that, Quercetin and phytonutrient rich plant extract from *E.officinalis* possess antioxidant property, which may be attributed to its protective action on lipid peroxidation and to the enhancing effect on cellular antioxidant defences which might be further implicated in its protective role against the damage due to cadmium toxicity in rats. The plant extracts selected in the present investigation were known to contain rich phytocompounds that bring about free radical quenching effect. Thus, the use of Quercetin and phytonutrients to counter oxidative damage serves as a therapeutic approach to restore normal body function. The current results also contribute towards improving our knowledge on the possible development of oxidative stress induced by cadmium treatment in rats indicating a possible role of antioxidant systems in the prevention of induced damage in rats.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Letter to the Editor

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Health-Based Occupational Exposure Limits in Pharmaceutical or Chemical Manufacturing

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To the Editor,

Unexpected exposure to pharmaceuticals or chemicals at the site of manufacture can cause significant health hazards to the concerned personnel. Therefore, it is essential to protect the workplace from serious adverse effects by limiting the exposure to such chemicals to acceptable levels. Qualitative methods such as 'a fraction of therapeutic dose' or occupational exposure banding (OEB) are in practice and may be useful during the early periods of development, when insufficient toxicological and pharmacological data is available. However, this information is arbitrary and scientifically not defensible. With the implementation of risk-based manufacturing practices (Risk-MaP), the pharmaceutical or chemical industries are adopting a quantitative health-based approach to set an occupational exposure limit (OEL). This scientific approach utilizes all aspects of pharmacology, *in vitro* and *in vivo* toxicology, clinical therapeutics, and adverse effects profile, which will be put to perspective by qualified toxicologists to arrive at a maximum safety exposure limit. Among all, the no observed effect level (NOEL) for the most critical effect is the best starting point to derive the OEL limit. The limit at which the OEL is established related to the NOEL should be based on the presence or absence of the threshold value, the slope of the dose-response curve, the nature of the lead effect and the quality, relevance and extent of data available. The health-based OEL utilizes all the available data related to toxicology and applies scientifically acceptable daily exposure (ADE). Processes involved in OEL derivation including hazard identification, dose-response assessment, uncertainty factors analysis (e.g., interspecies, intraspecies, LOAEL to NOAEL, severity and bioavailability), measures to minimize subjectivity and the need for documentation to improve regulatory acceptance are described. Therefore, it is crucial to set or derive the OEL value for each pharmaceutical or chemical which does not pose a risk for the development of any health hazard to healthy workers in the pharmaceutical industry.