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Research Article
Effect of Heavy Metal Contamination on the DNA Mutation on *Nepenthes* Plant from Abandoned Mine

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Abstract

Objective: Heavy metal contamination on water, soil, crops and including to the other living organisms, including their effects on DNA mutation in abandoned mine is highlighted in this study **Methodology:** In this study, 6 toxic metals, Zn, Pb, Fe, Cd, Cr and Mn in *Nepenthes* plants at an abandoned copper mine site were investigated. A total of 20 *Nepenthes* plants were collected from different sites of the abandoned mine. Metal concentration was determined using the Inductively Coupled Plasma/Optical Emission Spectrometry (ICP/OES) technique and electrophoresis gel and Inter Simple Sequence Repeat (ISSR) amplification processes for DNA mutation analysis. The total concentration trend of metal concentrations recorded with Cu (up to 88.797 mg kg^{-1})>Mn (39.018 mg kg^{-1})>Zn (30.260 mg kg^{-1})>Pb (8.206 mg kg^{-1})>Cd (0.168 mg kg^{-1}). **Results:** The results also showed that concentration of heavy metals in *Nepenthes* plants collected from the abandoned mine were much higher than in control plants. However, the concentrations were still under the tolerance limit of heavy metals in plants except for Cu concentration. Besides, the concentration of heavy metals generally is higher in the flowers of the plants. For DNA analysis, based on the image obtained the result showed the DNA bands were located at the same location with the control *Nepenthes* which indicated there is no mutation occurred for the *Nepenthes* collected from the abandoned mine. **Conclusion:** From the result it is suggested that pitcher plant such *Nepenthes* may act as a potential phytoextraction of heavy metal from contaminated soil or water compounds at the abandoned mine.

Key words: Heavy metal, *Nepenthes* plant, DNA mutation, abandoned mine

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Toxic heavy metals represents one of the most prominent environmental hazards due to contaminated and abandoned mine lands, which affecting many countries during the entire historic of mining industries[1]. As a direct impact of the open-pit mining operations, soil and water compounds are destroyed over a considerable amount and what is left of it is generally degraded and may continue causing environmental damage for longer time even after the mining period[2]. The disposal of mine wastes often produces more environmental problems than the mining operations themselves[3]. The pollutants may be transferred from tailings and waste rock dumps to nearby soil and water contact by acid mine drainage into atmospheric deposition of wind-blown-dust which depends on the climatic and hydrological conditions[4-10]. Heavy metal contaminations on soil, water, air, crops surrounding the abandoned mining area are a serious and critical challenge in environmental problem for scientific community, thus their impact on residents health risk is a persistent social issue especially the one living nearby the abandoned mine[11-12]. The concentrations of metals in plants often serve to indicate the metal contamination status of the site and also reveal the abilities of certain plant species that could take up and accumulate the metals from the mine site[13]. In this study, the *Nepenthes* plants were chosen because they are found be highly toxicity-tolerated after it seen to naturally grown successfully in the abandoned contaminated mine. This type of ability may link to *Nepenthes* potential as phytostabilization and phytoextraction that tolerate and able to hyperaccumulate heavy metals, at the same time to remove and decontaminate the toxic elements. But then there is no study has done for the hyperaccumulation ability effect on DNA mutation and damage of the accumulator and tolerant plants.

Heavy metals pollution is one of the environmental pollutions and known as the trace metals which damage the health of living organisms such as plants and it has at least five times of density heavier than water[14]. Heavy metals may enter the body of living organisms through some channels once it liberates into the environment, for example through inhalation, ingestion and skin absorption[4,14,15]. Heavy metals are also known as the persistent environmental contaminants as they cannot be degraded or destroyed and it can be easily discharged into ecosystems due to human activities[4,16]. There are various types of heavy metals that can be commonly found in environment and obtained from mining activity, such as lead (Pb), copper (Cu), zinc (Zn), cadmium (Cd), manganese (Mn), arsenic (As), iron (Fe) and mercury (Hg)[17]. They will become more harmful to organisms when the accumulation rate of heavy metals is higher than its discharge rate as heavy metals might accumulate in body of organisms and becoming toxic over a long time[14]. Heavy metals are considered as trace metals and toxicants to living organisms once the concentrations of heavy metals reached a certain level which cannot be afforded by the living organisms[18]. The heavy metals that exceed the allowable standard will bring the negative effects to human, fauna and also flora. For examples, heavy metals inhibit the physiological process of plants such as the photosynthesis process, respiration and roots elongation[19]. It is been reported by Di Salvatore *et al.*[20], Guala *et al.*[21] and Franco-Hernandez *et al.*[22] that roots of different plants getting shorter when the concentration of heavy metals getting higher and this showed that the heavy metals affected the roots elongation of plants. There are some reports showed that heavy metals might inhibit plants growth, prevent the uptake of nutrients and affect the metabolic process of the plants[23].

Apart from that, heavy metals might cause tumor and mutation to living organisms when they intake the heavy metals[24]. It is been reported that reported that toxic metal, Cd has caused the induction off number of mitotic abnormalities in *Capsicum annuum* as the abnormalities higher than control samples. The increases of concentration of Cd have caused the increasing chromosomal abnormalities, decrease of cell division frequency, shoot-root length, protein and pigment content reduction[25]. Plants that are highly exposed to heavy metals will undergo the reduction off photosynthesis, water uptake, nutrients uptake and show the visible symptoms of injuries reflected in term of chlorosis, growth inhibition, browning of root tips and even death[26]. Li *et al.*[27] studied earlier the induction of DNA damage in plants caused by heavy metals. It is reported that Zn, Cu and Cd induced the DNA damage in *Arabidopsis thaliana*. The increasing concentration of heavy metals increased the DNA damage in plants. The Cu^{2+} ion and Cd^{2+} ion caused much severe damage of DNA than Zn^{2+} at the same concentrations. There is study on *Boletus edulis* reported that Cd, Zn, Cu and Hg have caused structural damage to lipids and DNA in fruit bodies of the plants[28]. The DNA damage of potato plants also has been reported that associated with Cd toxic metal exposure that inhibited the plant growth and distorted yellowish leaves of the potato plants[29]. The mutation occurs during the damage of DNA which can cause by the hydrolysis, deamination and induced by chemicals that are known as mutagens, such as Cu, Cr, Hg and Fe[30,31]. The DNA may also damage by alkylation, oxidation and radiation[32]. There are few types of mutations that might occur in the cells of living organisms, which are the missense mutation, nonsense mutation, insertion, deletion, duplication, frameshift mutation and

repeat expansion[33]. The insertion means the number of DNA bases is changed by inserting a DNA bases which causes the protein cannot be functioned properly[34]. On the other hand, deletion of DNA bases refers to the number of DNA bases is changed by deleting a DNA which might be removing one or few base pairs or removing the entire gene or several neighboring genes[33]. Duplication means that a piece or a sequence of DNA bases copy for one or more times. All the mutations caused the protein and DNA cannot be functioned well, in the way alter the functions of protein or caused the protein cannot function[35]. Therefore, the aims of this study are: (1) To determine the concentrations of Cd, Cu, Mn, Pb and Zn in *Nepenthes* plants that grew in abandoned copper mine and (2) To investigate the effects of heavy metals contamination on the mutation of DNA in *Nepenthes* plants.

MATERIALS AND METHODS

Mamut copper mine was the largest and the only copper mine in Malaysia which located in Sabah, Malaysia, the Northern part of Borneo. The Environmental Ministry of Malaysia have done a lot of monitoring work at more than 40 sites of surrounding areas of the abandoned mine to observe the current state of the contaminated and abandoned mine. The monitoring work showed that a continuous state of water and soil contamination on most of the sites that even contained copper concentration at level up to 2 ppm, which is 10 times to the allowable standard level at 0.22 ppm[36]. Water at the main pit areas have low pH as low[37] 2.90-3.75 which is very acidic and this can cause dangerous contaminations of streams and rivers nearby the mine area. Few rivers such Bongkud river, Mamut river and Lohan river are the rivers that located surrounding the Mamut copper mine. In addition, this contaminated condition has caused a lot of negative side effects especially to residents nearby that lives surrounding the mine area. For example, the rain water washed the muds into surrounding river, Lohan river and then overflowed into some of the rice paddies, making the rice planting impossible[36]. Apart from that, the location of Mamut copper mine is located at the highest altitude of 1600 m that serves as one of the water catchment points, which receives 4000 m of rain every year based on its subtropical weather[38]. Dissolved metals effluent that flow from the mine to the streams and rivers will bring serious impacts to the domestic use and aquatic life[39]. Many residents nearby depending on the river as their main water resource for their daily use such as drinking purpose and for crops and vegetable irrigation. Moreover, there were many reports recorded for dead fish, livestock, crops decreases and skin rashes cases from the Mamut mine area especially residents living downstream that more expose to health hazards as heavy metals will enter their body through ingestion of foods and drinking water which are contaminated[14,40-42]. During the mining closure period have occurred several environmental incidents among which involved the pipeline leakage events in 1975 hence more than 800 ha of land were contaminated, 1977 heavy rains have washed parts of mine tailings in Lohan river and polluted farmland and along the river streams, in 2000 residents nearby complained of blown dust from the abandoned mine. Apart from that the *Nepenthes* plants which grew in Mamut copper mine were chosen as the sample plants for the analysis due to the physical characteristics of the *Nepenthes* plants from the abandoned mine that shows differences with other typical or regular *Nepenthes* plants from other place. The abandoned *Nepenthes* plants are seen to be shorter and very yellowish-brown in colour. Two parts of *Nepenthes* plant were used for this study, which are the stems and flowers. The samples were collected by using hand with gloves and quickly inserted into the plastic bags with label of time, date and location of sample collection. The samples were then kept inside the refrigerator with temperature of 4°C for preservation of sample plants.

DNA extraction: The DNA extraction was carried out for both *Nepenthes* plants from Mamut abandoned mine and control plants. There were two parts of both plants being analysed, which are stems and flowers. The DNAs obtained from both plants were compared by using gel electrophoresis.

Preparation of solutions: Solutions that are needed to prepare the TE buffer and DNA extraction buffer, which are 1 M tris-base solution at pH 8.0, 0.5 M ethylenediaminetetraacetic acid (EDTA) at pH value of 8.0, 5 M NaCl and 10% of Sodium Dedocyl Sulphate (SDS) solution. The 1.211 g of tris-base powder was measured by using an analytical balance and poured into a beaker. After that, 8 mL of distilled water was added into the beaker. The tris-base solution needed must achieve the pH value of 8.0, hence hydrochloric acid (HCl) is needed in order to adjust the pH value of tris-base solution. About 0.42 mL of HCl was measured by using a measuring cylinder and then added into the beaker. The solution was stirred and then it was adjusted to 10 mL. For the preparation of EDTA, first of all, 1.861 g of EDTA was measured and the powder was poured into a beaker. After that, 8 mL of distilled water was added into the beaker as well. Then, NaOH was needed for the adjustment of pH value of EDTA. Thus, 0.2 g of NaOH was measured and added into the beaker which contained EDTA powder and distilled water. The solution was then diluted to 10 mL. Preparation of 5 M NaCl needed 1.461 g of

NaCl in solid form and 5 mL of distilled water. The powder and distilled water were mixed and stirred by using a glass rod. Ten percent of SDS solution was prepared by mixing 1 g of SDS powder and 9 mL of distilled water. The solution was mixed and stirred by using a glass rod. After SDS powder was dissolved, the solution was then diluted to 10 mL.

TE buffer: The TE buffer stands for tris-base EDTA buffer. There are 3 types of solutions needed in order to prepare the TE buffer, which are 1 M tris-base solution, 0.5 M ethylenediaminetetraacetic acid (EDTA) solution and purified water (ddH_2O). About 50 mL of TE buffer which consisted of 0.5 mL 1 M tris-base solution, 0.1 mL of EDTA and 49.4 mL of ddH_2O.

DNA extraction buffer: The DNA extraction buffer was prepared by mixing 2.5 mL of 1 M tris-base solution, 2.5 mL of 0.5 M EDTA, 3 mL of 5 M NaCl solution, 5 mL of 10% SDS solution and 37 mL of sterile ddH_2O. The final volume of the solution was 50 mL.

Extraction of DNA from samples: The sample plants were wiped and cleaned to remove the undesired particles that stick on the surface of samples. Then the plant tissue was cut into 0.5 cm long segments and placed in a crucible. Then, 400 µL of DNA extraction buffer was added into the crucible. The tissue was grinded with a glass rod until the colour of buffer turns dark green which indicates the releases of chlorophyll. After that, another 400 µL was added into the crucible. The solution was then mixed and transferred into a 1.5 mL microcentrifuge tube. About 400 µL was added into the microcentrifuge tube and mixed well. The microcentrifuge tube was then spin by using a microcentrifuge machine at full speed (13 rpm) for 30 sec. After the spin, the aqueous phase of the solution was transferred into another 1.5 mL microcentrifuge tube. About 800 µL of absolute ethanol was added into the microcentrifuge tube and the tube was spin again with microcentrifuge machine at full speed for 3 min. The supernatant was then discarded by removing it with micropipette without disturbing the lower layer of solution. The DNA pellets obtained was washed with 70% ethanol and the DNA pellets were allowed to dry. After that, the DNA pellets were resuspended in 50 µL of TE buffer. The DNA was stored in -20°C.

ISSR amplification: Inter Simple Sequence Repeat (ISSR) amplification was used to amplify the number of DNA with the use of PCR machine and ISSR primers. Before the ISSR amplification was carried out, the samples for ISSR amplification were prepared by using the DNA pellets obtained from the DNA extraction. The other materials needed for ISSR amplification were ddH_2O, ISSR buffer, magnesium chloride ($MgCl_2$), dNTPs which contained dATP, dTTP, dCTP and dGTP, Taq DNA polymerase buffer and the ISSR primers. A total volume of 12.85 µL of final product was produced which consisted of 5.15 µL of ddH_2O, 2 µL of ISSR buffer, 0.6 µL of dNTPs, 0.1 µL of Taq DNA polymerase buffer, 1 µL of template DNA and 1 µL of each ISSR primer. The ISSR amplification was carried out with a PCR machine. First, an initial denaturation period of 3 min at 95°C, followed by 30 cycles of 20 sec at 95°C, 30 cycles of 40 sec at 55°C, 30 cycles of 1 min at 72°C and then 10 min at 72°C for final extension. Then the final products of ISSR amplification were stored in -20°C before the gel electrophoresis carry out.

Gel electrophoresis: The gel electrophoresis was carried out in order to analyse and separates the DNA bands based on the sizes. The materials and apparatus needed for the gel electrophoresis are 1x TAE buffer solution, conical flask, gel casting tray, gel comb, ethidium bromide, agarose powder, bladder, loading dye and DNA samples after ISSR amplification. First of all, 1 L of 10x tris-acetic-EDTA solution was prepared by dissolving 48.5 g of tris-base powder into 800 mL of deionised water. Then, 11.4 mL of glacial acetic acid and 20 mL of 0.5 M EDTA with a pH value of 8.0 (preparation of 0.5 m EDTA at pH 8.0) were added into the tris-base solution. The solution was poured into a 1 L volumetric flask and diluted to 1 L. The 1x of TAE buffer solution needed in gel electrophoresis instead of 10x of TAE buffer solutions. Hence, the 10x TAE buffer solution was diluted to 1x TAE buffer solution by calculating the ratio, which means that 100 mL of 10x TAE buffer solution was diluted to 1 L by using deionised water. Two percent of agarose gel was prepared by using 2 g of agarose powder and 100 mL of 1x TAE buffer solution. The mixture was heated with an oven and allowed to cool at room temperature after heating. During the cooling down process, a little bit of ethidium bromide was added into the solution. The precaution should be taken during the adding of ethidium bromide because it is a mutagenic reagent which may cause cancer. When the solution reached around 70°C, it was poured into a gel casting tray with a gel comb and allowed it to solidify. Once the agarose gel solidified, the comb is removed and makes sure that not to rip the bottom of the wells. Then the agarose gel was placed into the electrophoresis chamber. The 1x TAE buffer was poured into the electrophoresis chamber and slightly covered the agarose gel. After that, 5 µL of 1 kb bladder was injected into the first well. For the DNA samples obtained after the ISSR amplification, 2 µL of loading dye mixed with 5 µL of DNA

pellet, pipette it in and out to make sure that the loading dye is well mixed with DNA pellet. Then the sample was injected into another well. The step for DNA pellets was repeated for all the DNA pellets. The gel electrophoresis was started at 70 V at 200 min. When the step of gel electrophoresis was completed, the gel was taken out carefully without damage the gel and DNA bands and then the gel was scanned by using a gel imager.

Analysis of heavy metals: The analyses of heavy metals were carried out by using the ICP-OES machine. The heavy metals that were involved in this research are Cd, Cu, Mn, Pb and Zn.

Preparation of samples: All the apparatus that used in the analysis were immersed in the solution of 10% nitric acid and left for a day. The apparatus were taken out and washed with distilled water. The plants were divided into 2 parts, which are the stem and flowers in order to justify which parts of *Nepenthes* plants accumulate more heavy metals. The different parts of plants were cut into small pieces by using a blender. After that, the samples were dried in the oven for 24 h at 65°C.

Digestion of samples: The samples were sieved and measured approximately 1 g by using the analytical balance. The sample was then poured into a 50 mL conical flask. After that, 10 mL of 65% nitric acid (HNO_3) was poured into the conical flask which contained the sample. The conical flask was then heated for 2 h at 120°C. Then, the solution was heated at 140°C until the clear solution was achieved and the solution remained around 5 mL. After cooling, the sample was filtered with Whatman nylon filter with the size of 45 µm. After that, distilled water was added to the filtered sample until the volume reach 50 mL. Then, the sample was poured into a 120 mL polyethylene bottle and kept into the refrigerator until the analysis of heavy metals by using the ICP-OES is carried out.

Determination of the concentration of heavy metals: The determination of concentration of heavy metals is carried out by using the ICP-OES Spectrometer Optima 5300 DV machine. The readings that obtained from the operation of ICP-OES are in unit of mg mL^{-1}. Hence, a calculation is needed to convert the unit. The equation is given as:

$$C = \frac{A \times Y}{W}$$

Where:

C = Concentration of heavy metals (mg kg^{-1})
A = Absorbance readings of samples from ICP-OES
Y = Volume of sample (mL)
W = Mass of sample (g)

RESULTS AND DISCUSSION

There are five types of heavy metals being studied, which are Cd, Cu, Mn, Pb and Zn. The *Nepenthes* sample plants were collected from the abandoned Mamut Copper Mine (MCM) by randomly pick and the sample plants were namely as MCM plant 1 from site sampling 1, MCM plant 2 site sampling 2, MCM plant 3 for site 3 and MCM plant 4 for site 4. In order to study the concentration of heavy metals in *Nepenthes* plants obtained from MCM, concentration of 4 regular *Nepenthes* plants which obtained from different locations of plant nursery and market that is away from MCM area were analysed and used as a benchmark and control sample plants. The control plants named as BM (benchmark) plant 1 market 1, BM plant 2 market 2, BM plant 3 market 3 and BM plant 4 market 4. Two parts of each plant were studied, which are the stem and the flower of *Nepenthes* plants to justify the part that accumulate more heavy metals.

Concentration of heavy metals in abandoned MCM plants: Based on Table 1, the concentration of Cu showed the highest concentration in MCM plants, which are 22.9265±0.2093 mg kg^{-1} in MCM flower 1, 23.0662±0.1080 mg kg^{-1} in MCM flower 2, 54.1379±0.7365 mg kg^{-1} for MCM flower 3, 88.7969±0.9709 mg kg^{-1} for MCM flower 4, 17.8221±0.0349 mg kg^{-1} for MCM stem 1, 32.2520±0.4035 mg kg^{-1} for MCM stem 2, 16.3554±0.2074 mg kg^{-1} for MCM stem 3 and 42.4164±0.6829 mg kg^{-1}. The concentrations of Mn and Zn in MCM plants are relatively higher than the other heavy metals but lower than the concentration of Cu. The concentrations of Mn in plants which obtained from MCM are range from 8.5941-39.0184 mg kg^{-1}, which are 8.5941±0.0505 mg kg^{-1} in MCM flower 1, 8.9185±0.09338 mg kg^{-1} in MCM flower 2, 32.7109±0.4361 mg kg^{-1} for MCM flower 3, 39.0184±0.3281 mg kg^{-1} for MCM flower 4, 2.7221±0.0095 mg kg^{-1} for MCM stem 1, 5.4022±0.0312 mg kg^{-1} for MCM stem 2, 11.7030±0.1398 mg kg^{-1} for MCM stem 3 and 37.1863±0.5854 mg kg^{-1} for MCM stem 4. On the other hand, the concentrations of Zn in flower of MCM plant are

Table 1: Concentration of heavy metals (mg kg^{-1}) in MCM plants

Heavy metals	Parts of MCM plants							
(mg kg^{-1})	MCM flower 1	MCM flower 2	MCM flower 3	MCM flower 4	MCM stem 1	MCM stem 2	MCM stem 3	MCM stem 4
Cd	0.0542±0.0182	0.0169±0.0015	0.1356±0.0157	0.1676±0.0153	0.0958±0.0151	0.1259±0.0054	0.1625±0.0115	0.0±0.0
Cu	22.9265±0.2093	23.0662±0.1080	54.1379±0.7365	88.7969±0.9709	17.8221±0.0349	32.2520±0.4035	16.3554±0.2074	42.4164±0.6829
Mn	8.5941±0.0505	8.9185±0.0934	32.7109±0.4361	39.0184±0.3281	2.7721±0.0095	5.4022±0.0312	11.7030±0.1398	31.1863±0.5854
Pb	0.9431±0.1496	0.9166±0.1783	6.7183±0.1526	8.2062±0.1146	1.1292±0.1233	1.7546±0.1490	1.4843±0.0898	3.5849±0.2952
Zn	14.6031±0.0132	15.4908±0.1416	30.1649±0.1784	30.2603±0.0422	12.2309±0.0634	13.0400±0.0435	18.3980±0.0665	26.7615±0.0972

Table 2: Concentration of heavy metals (mg kg^{-1}) in BM plants

Heavy metals	Parts control plants							
(mg kg^{-1})	BM flower 1	BM flower 2	BM flower 3	BM flower 4	BM stem 1	BM stem 2	BM stem 3	BM stem 4
Cd	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Cu	0.0617±0.0109	0.3918±0.0099	0.0793±0.0094	0.1242±0.0018	0.2009±0.0091	0.2161±0.0121	0.1068±0.0178	0.0488±0.0080
Mn	14.1605±0.1586	12.2657±0.0720	8.2367±0.0657	7.8980±0.0475	7.2673±0.0605	7.3286±0.0398	6.9454±0.0453	9.8334±0.0558
Pb	0.3403±0.0739	0.3577±0.1151	0.4905±0.0390	0.4164±0.0264	0.2722±0.0732	0.3178±0.0028	0.3766±0.1064	0.2808±0.0926
Zn	3.5920±0.0464	3.4349±0.0578	5.4665±0.0439	5.1800±0.0313	3.0378±0.0214	3.0292±0.0049	4.1837±0.0405	2.7770±0.0185

range from 14.6031-30.2603 mg kg^{-1}, which are 14.6031±0.0132 mg kg^{-1} for MCM flower 1, 15.4908±0.1416 mg kg^{-1} for MCM flower 2, 30.1649±0.1784 mg kg^{-1} for MCM flower 3 and 30.2603±0.0422 mg kg^{-1} for MCM flower 4.

The concentrations of Pb are relatively lower than the other heavy metals but higher than the concentration of Cd, which showed the lowest concentration in MCM plants. The concentrations of Pb for MCM flower 1, MCM flower 2, MCM flower 3 and MCM flower 4 are 0.9431±0.1496, 0.9166±0.1783, 6.7181±0.1526 and 8.2062±0.1146 mg kg^{-1}, respectively and 1.1292±0.1233 mg kg^{-1} for MCM stem 1, 1.7546±0.1490 mg kg^{-1} for MCM stem 2, 1.4843±0.08985 mg kg^{-1} for MCM stem 3 and 3.5849±0.2952 mg kg^{-1}. The concentrations Pb in most of the sample plants were low, which range from 0.9166-8.2062 mg kg^{-1}. The concentrations of Cd for MCM flower 1, MCM flower 2, MCM flower 3 and MCM flower 4 are 0.0542±0.0182, 0.01689±0.00158, 0.1356±0.0157 and 0.1676±0.0153 mg kg^{-1}, respectively; 0.0957±0.0151 mg kg^{-1} for MCM stem 1, 0.1259±0.0054 mg kg^{-1} for MCM stem 2, 0.1625±0.0115 mg kg^{-1} for MCM stem 3 and the concentration of Cd for MCM stem 4 was below the limit detection. Generally, concentrations of Cu are higher in flower parts. Reilly[43] and Guleryuz *et al.*[44] done the researches and reported that the concentrations of Cu are higher in flowers and leaves compared to the concentrations of Cu in stems. This is related to the function and basic metabolic activities in flowers[44]. Translocation rate is higher in flower part compared to the stem part[45].

Concentration of heavy metals in control plants: The concentration of heavy metals in control plants followed the trend of Mn>Zn>Pb>Cu>Cd, which means that the highest concentration is Mn, then followed by Zn, Pb, Cu and the lowest concentration is Cd as shown in Table 2. Highest concentration is found in Mn which range from 6.9454-14.1605 mg kg^{-1}, followed Zn which range from 2.7770-5.4465 mg kg^{-1}. The concentrations of Cu and Pb are more or less the same, which are range from 0.0488-0.4905 mg kg^{-1}. The concentrations of Cd are negative value due to lower detection limit. Based on 2, generally the concentrations of heavy metals in BM flowers are higher than the concentration of heavy metals in BM stems. This is because the concentrations of heavy metals are dispersed more to the roots and flowers tissues instead of stem part[45,46]. The concentrations of Mn and Zn are higher than the other heavy metals in both flowers and stems part of BM plants. This is due to both Mn and Zn is the essential nutrients for plants to grow and increase the efficiency of enzymes to work and co-exist already in the plants cells[47]. Thus, the concentrations of these heavy metals will be higher compared to the other heavy metals in plants.

Comparison of concentration of heavy metals in BM plants and MCM plants: The concentrations of each heavy metal in BM plants and MCM plants were compared and the concentrations were compared to the toxicity range of each heavy metal in order to determine whether the plants are polluted by heavy metals due to the abandoned mine.

Based on Fig. 1, the concentrations of Cd are higher in the plants that have been collected from MCM, which range from 0.01687-0.1676 mg kg^{-1}. However, the concentrations of Cd in MCM stem 4 and in all the BM plants showed negative readings. This is because the concentrations of Cd in these sample plants are lower than the detection limit of ICP-OES. According to Opaluwa *et al.*[48], the normal range of Cd in plants is below 2.4 mg kg^{-1} while toxicity of Cd occurs when the concentration of Cd exceeds 2.4 mg kg^{-1}.

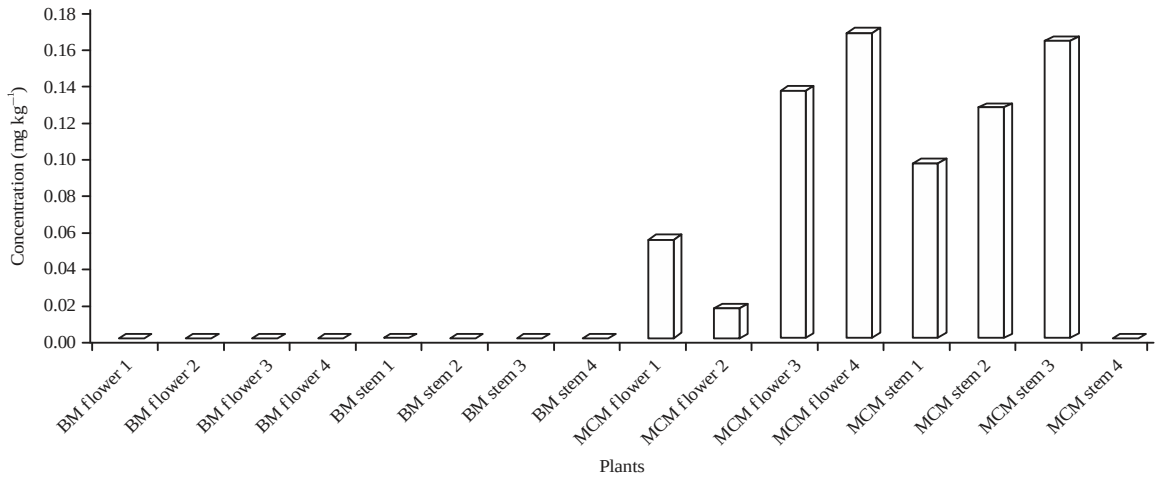

Fig. 1: Comparison of concentration of Cd in both BM plants and MCM plants

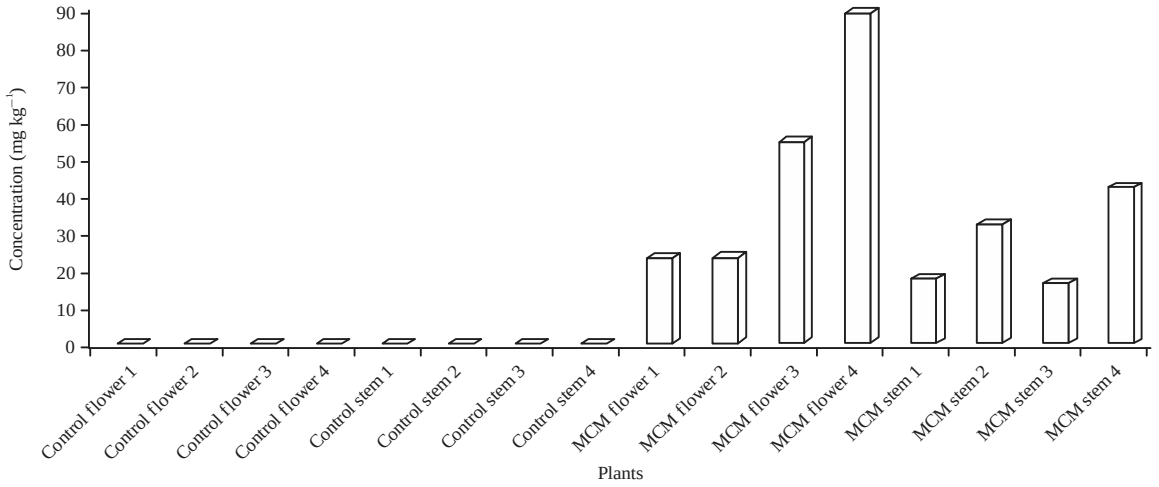

Fig. 2: Comparison of concentration of Cu in both control plants and MCM plants

The concentrations of Cu as shown in Fig. 2 are in range from 16.3554-88.7969 mg kg^{-1} for MCM plants while the range of concentrations of Cu is from 0.0488-0.3918 mg kg^{-1} for control plants. Dharani *et al.*[49] reported that the normal range of concentration of Cu in dried plants is between 2.0-25 mg kg^{-1}. The plants with concentration of Cu higher than 30 mg kg^{-1} are considered as phytotoxic, while the plants with concentration of Cu that less than 2.0 mg kg^{-1} are considered as deficiency of Cu. The concentration of Cu for MCM flower 3, MCM flower 4, MCM stem 2 and MCM stem 4 are more than 30 mg kg^{-1}, which indicates that these parts of plants are undergoing phytotoxic.

Based on Fig. 3, the concentration of Mn in BM plants and MCM plants are constant, however, the concentrations of MCM flower 3, MCM flower 4 and MCM stem 4 are generally higher than the others part of plants, which are 32.7109, 39.0184 and 37.1863 mg kg^{-1}, respectively, while the concentrations of Mn for the other parts are range from 2.7721-14.1605 mg kg^{-1}. According to WHO[5], the toxic concentrations of Mn in plants tissues are vary, with the critical values which range from 100-5000 mg kg^{-1}. The value indicates that the plant samples collected from MCM were not polluted by Mn as the concentrations were lower than the tolerance limit (Fig. 4).

The concentrations of Pb in BM plants are range from 0.2722-0.4905 mg kg^{-1} while the concentrations of Pb in MCM plants are range from 0.9166-8.2062 mg kg^{-1}. Hence, the uptakes of Pb by plants in MCM are higher than control plants because of the copper mining activities that has been carried out in MCM. Davila *et al.*[50] reported that the range of toxic concentration of Pb in plants is range from 30-300 mg kg^{-1}. Mendoza *et al.*[51] stated that the permissible limit in plants recommended by WHO is 2 mg kg^{-1}. This means that the MCM flower 3, MCM flower 4 and MCM stem 4 having the

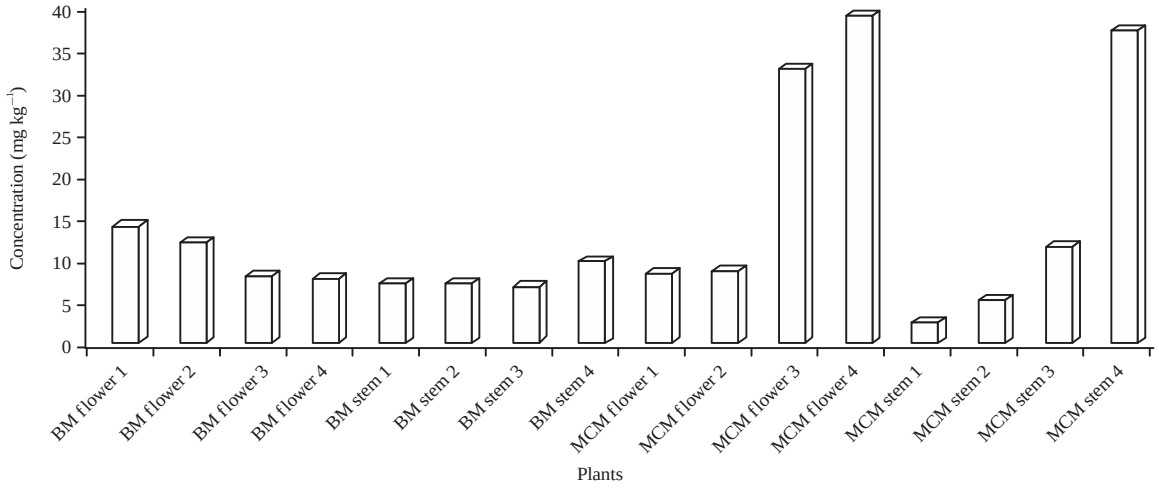

Fig. 3: Comparison of concentration of Mn in both BM plants and MCM plants

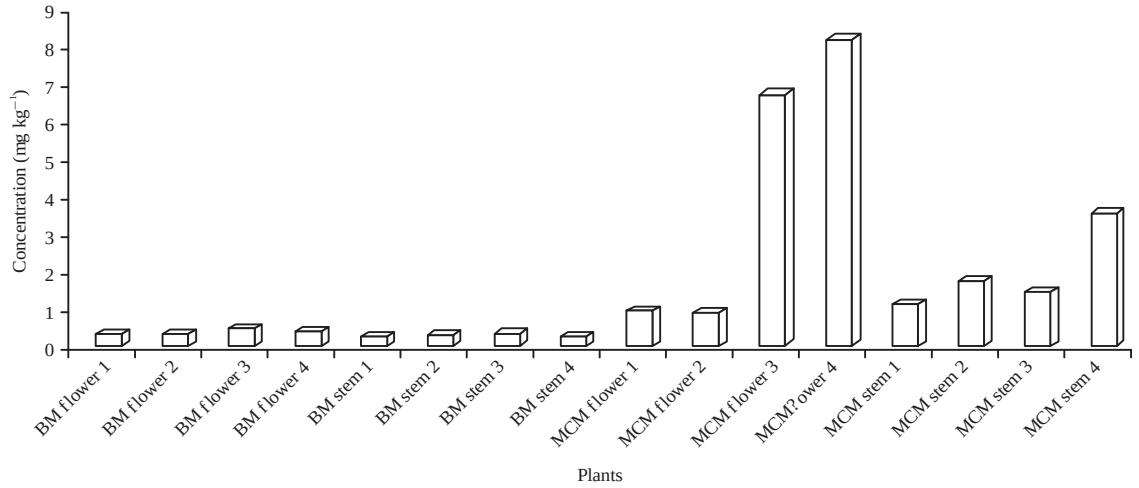

Fig. 4: Comparison of concentration of Pb in both Control plants and MCM plants

Table 3: Correlation coefficients between concentrations of heavy metals in both flower and stem part of MCM plant

Metals	R^2	R
Cd	-0.4192	-0.6475
Cu	0.3149	0.5612
Mn	0.4761	0.6900
Pb	0.3149	0.5612
Zn	0.7429	0.8619

R: Correlation coefficient value

concentration of Pb higher than the permissible limit. However, it is still under the acceptable concentration of Pb as the toxic concentration of Pb range from 30-300 mg kg^{-1}. Based on Fig. 5, the range of concentration of Zn for BM plants is from 2.7770-5.4665 mg kg^{-1} while it is range from 12.2309-30.2603 mg kg^{-1} for MCM plants. Davila *et al.*[50] has reported that the toxic concentration of Zn is range from 150-400 mg kg^{-1}. On the other hand[52], concluded that there is no effect to the plants when the concentration of Zn is between 27-150 mg kg^{-1}. In other words, the concentration of Zn in MCM plants and BM plants do not exceed the tolerance limit of plants.

Correlation between the flowers and stems in MCM plants: Table 3 shows the significant correlations for concentrations of heavy metals between flower and stem of MCM plants as all the R-values were greater than 0.5 (more than 50% strength relationship) with $p<0.05$ which shows a significant value on the correlations. However, the concentration of Cd in flower and stem of MCM plants showed a negative correlation while the other heavy metals showed the positive correlations. Negative correlation is due to decreased in concentration of Cd in stems when the concentration of Cd increased in flower[53,54]. This might due to the lower pH value in soils as soil pH affected the uptake of Cd in plants which soluble Cd increased with decreasing of pH value[55].

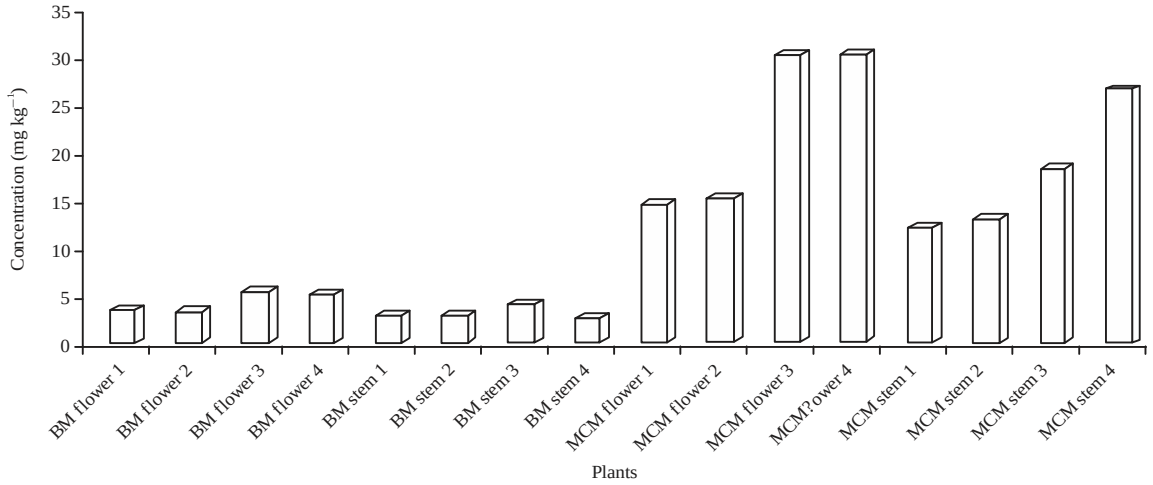

Fig. 5: Comparison of concentration of Zn in both BM plants and MCM plants

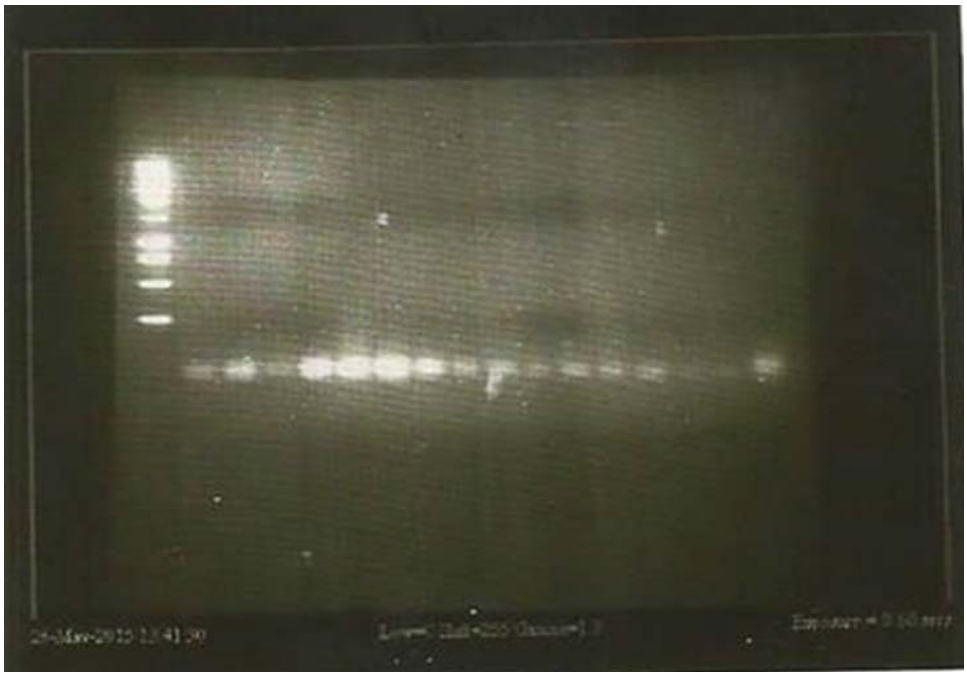

Fig. 6: DNA bands of samples for both BM plants and MCM plants in gel electrophoresis

DNA analysis of *Nepenthes* plants: The DNA template of MCM plants and BM plants were extracted using DNA extraction method. Once the DNAs of each of the samples were obtained, it was sent for the ISSR amplification of DNA and lastly gel electrophoresis has been carried out for running the DNA templates to compare the DNA bands for both MCM plants and BM plants. Figure 6 shows, the DNA bands showing no bands shift or extra band indicates the absence of mutation[56]. The DNA bands detected for all 16 samples were located in the same location which indicates there was no mutation occurred between the MCM plants and control plants. The high concentrations of heavy metals do not altered the DNA of *Nepenthes* plants which collected from MCM. Glutathione (GSH) has been detected in all compartments of cells such as cytosol, chloroplast and endoplasmic reticulum and it makes an ideal biochemical to protect plants against stress, for examples oxidative stress, heavy metals, exogenous and endogenous organic chemicals[26]. Hence, with the help of the protection of GSH, the mutation does not occur in *Nepenthes* plants from MCM even though with higher concentration of heavy metals.

CONCLUSION

In conclusion, the concentrations of heavy metals in *Nepenthes* plants that collected from abandoned Mamut Mine were much higher than the control plants. However, the concentrations of heavy metals in MCM plants are still under the tolerance limits for plants except for Cu. Apart from that, overall the concentrations of heavy metals in flower were found compared to stems. Correlation between flowers and stems of MCM plants showed significant correlation coefficient greater than 0.5, which indicates stronger correlation between flowers and stems. For DNA analysis, the result showed that the DNA for both plants indicates no mutation affect to the plants from heavy metals. However, for future recommendations, it is best to use PCR amplification for better result and accuracy.

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