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Restraining effect of *P. macrocarpa* methanol extract on ROS generation in SK-N-SH cell

Haider Abd Jabbar AL-AMMAR

Department of medical chemistry, College of Medicine, University of Al-Qadisiyah.

Abstract

Phaleria macrocarpa, is widely known as Mahkota dewa is a herb which is used in medical treatments and found in Malaysia & Indonesia. Extract of *P. Macrocarpa* contains a chemical composition which is useful in anti-cancerous, anti-fungus, anti-oxidation, anti-diabetic treatments. Reactive oxygen species (ROS) production in mammalian cell is responsible for many cell function, absence of detoxification for removal of this radical, affects cell functions and may produce abnormalities and diseases. Several nervous abnormalities were found be to the effect of ROS generation. Currently there are no effective medicinal options for its treatment. This study focuses on the effect of *P. Macrocarpa* leaf extract on the restraining reactive oxygen.

Methods: *P. Macrocarpa* leaf extract contains 80% methyl alcohol. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) is used for cytotoxicity analysis and restraining reactive oxygen is studied using 2′,7′-Dichlorofluorescin diacetate in SK-N-SH cell model.

Result: ROS Restraining performance of leaf extract is analysed between the group that treated only with crude extract, group treated with extract with Hydrogen peroxide and group treated only with Hydrogen peroxide. HPLC methods shows that Vitexin & iso-vitexin, this bioactive compound were present in the crude extract at retention time 20.98 & 22.94 for Vitexin & iso-vitexin respectively.

Conclusion: Anti-oxidation & low toxic characteristic of *P. Macrocarpa* extract concludes it to be beneficial for its usage as herbal supplement/medicine.

Keywords: Restraining effect of P. macrocarpa, methanol extract on ROS, generation in SK-N-SH cell

Background

Occurrence and Botanical description

With the advancement in the study of mammalian genes, interaction of genome and nutrient is also in focus [8]. Reactive oxygen species (ROS) is a cell molecules which are responsible transfer of signal. But the generation of Reactive oxygen species could also lead to abnormal functionality of biological or cellular processes. If the generation of ROS and defence doesn't balance then it may result into various diseases including cancer, neural damage, hypertension and asthma. This result is entirely depend on the molecular interaction [14]. Increased ROS generation and reduced level of endogenous antioxidant enzymes causes several causes for neural damage. Increasing the exogenous antioxidants will resist the neural cell damage [13]. There are some natural methods from herbs and plant which acts as a natural exogenous antioxidants, eventually reduces the risk of neural

damage [5].

Phaleria macrocarpa (*P. macrocarpa*) is commonly known as Mahkota dewa. In few region it is also called as God's crown and Pau. It is mostly observed in the tropical region of Indonesia specifically in Papua island and New Guinea up to 1,200 m above sea. It belongs to family Thymelaeaceae and Genus Phaleria. *P. macrocarpa* is a complete tree with stem, fruit, leave and flower. Figure 1 shows the botanical view of flower, leave, un-ripped fruits and fruits. It has been used in processed and unprocessed form, but a processed form is recommended to avoid any toxic effects [10]. Extract of *P. Macrocarpa* contains a chemical composition which is useful in anti-cancerous, anti-fungus, anti-oxidation, anti-diabetic treatments. *P. macrocarpa* leaves are useful in the treatment of blood pressure, various allergy reactions, heart issue and tumours [16].

Phytochemical Studies

P. Macrocarpa has high concentration of various bioactive chemical component. *P. macrocarpa* seeds may contribute to the presence of bioactivities such as anti-bacterial, an analgesic, an anti-fungal, an anti-inflammatory agent, and cytotoxicity. This bioactive components includes Lauric acid, hexadecanoic acid, phorboesters, fema 3490, sucrose [17]. Along with mahkoside A *P. macrocarpa* isolates Phalerin, Icaricide C, magniferin, gallic acid, palmitic acid, dodecanoic acid alkaloids and saponins [14]. Out of this components alkaloids, saponins, phenols, lignins tannins has anti-oxidant properties [4]

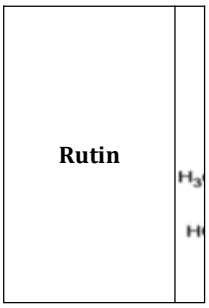
Figure 1. Botanical view of flower, leave, un-ripped fruits and fruits *P. Macrocarpa*

However there are few research which identified the toxic nature of P. Macrocarpa [1]. canker sores, Embryotoxicity, Cell injury in the region of convoluted tubule and feto-toxicity is also caused by . Macrocarpa leaf extract [1]. Enlargement of hepatocytes and increased concentration of Alanine transaminase enzyme in liver is observed in few research [11].

SK-N-SH is a Neuroblastoma cell line that shows epithelial morphology . It is derived from a human bone marrow metastasis. A primary mammalian neurons has limitation , that once it is mature, it stops propagating. Hence neuronal-like cell lines can be used for the research purpose [9]. It is used in cell model system for studying cell structures. SH-SY5Y is a sub cloned which is derived 3 time from the SK-N-SH neuroblastoma cell line. First to SH-SY, then to SH-SY5, and finally to SH-SY5Y. For the differentiation, this cell was treated with retinoic acid for 7 days [2]. Besides from retinoic acid other components like herb A, tetradecanoylphorbol acetate, Dibutyryl cyclic adenosine monophosphate can be used for differentiation purpose. Using this cell effect of ROS generation can be studied , hence in this research SK-N-SH cells were used to study the Restraining effect of P. macrocarpa methanol extract on ROS generation.

Fevicordin-A	
Myricetin	
Gallic acid	

Chemical structures *P. Macrocarpa* extracts:



MATERIAL & METHOD

Identification and collection

Collection of *P. Macrocarpa* doesn't involve any endangered species or location. *P. Macrocarpa* is collected from the University of Al-Qadisiyah, Al-Diwaniya, Iraq in year of collection. *P. Macrocarpa* was identified and authenticate from university botanist. Ethyl alcohol extract of *P. Macrocarpa* were obtained for analysis of its restraining effect on ROS generation.

Extraction

Cleaning and separation was carried out on the leaves of *P. Macrocarpa*. After that using Pruning Secateurs, leaves were cut into tiny pieces. Following this step for two week all the pieces of leave were dried at conditions near to room temperature of 25°C. This procedure was carried out in the Laboratory environment. After this the dried leaves are crushed into the semi-powered form. One litter solution was prepared with the 80% of methyl alcohol and around 250 gram of semi-crushed form leave were placed in the solution for 72 hours. Using vortex shaker, the solutions consist of methyl alcohol and semi-crushed leaves were shake on daily basis for 3 days at room temperature. This step produces an extract. Using the filter paper, this extract were filtered. The filtered extract was evaporated at 40°C and rotatory evaporator into the semi-solid form.

Sample Dilution

The obtained solution was diluted to the lower concentration. For this purpose the extract of crude was dissolved into one millilitre of 100% dimethyl sulfoxide. Obtained extract from the extraction step is not soluble into normal solvent hence dimethyl sulfoxide was used for dilution. After that the preparation were dissolved into the water at the concentration. Concentration of the solution were reduced by 2 fold that one half of the original concentration.

Cells Viability Assay

Cell viability test is useful to determine the active cells which further help in analysis of cell. cytotoxicity test is also equally important as cell viability test in an experiment. Cytotoxicity analysis, were carried out using Methylthiazolyldiphenyl-tetrazolium bromide (MTT) on the SK-N-SH crude extract. The cells were incubated in the Minimum Essential Media which consist of higher concentrations of essential nutrients of 150 μL at 38°C for one day. After the incubation cell were observed for another one day. After that the cells were treated with various concentration of crude and minimum essential media. This cells then incubated again for 3 days at 38°C and 5% of carbon di-oxide. Control well is used to analyse the cells, it consist of blank, which contain 100% and media with 0.1% of dimethyl sulfoxide. Methylthiazolyldiphenyl-tetrazolium bromide solution were obtained by 5 milligram of MTT re-agent and water-based salt solution containing disodium hydrogen phosphate and sodium chloride as a buffer. This concentration of MTT to buffer of 10% generates 10 µL Methylthiazolyldiphenyl-tetrazolium solution. This solution is then exposed to the cells at 36°C in microplate for three hour. Using Dimethyl sulfoxide, minimum essential media was removed which has extract of crude and cell and then incubation is carried out for half hour. The Formazan dyes which is artificial chromogenic products is dissolved and using the microplate photometers, the optical density (OD) that is quantity of light absorbed by a solution is measured. For obtaining the safe dose measurement for cytotoxicity analysis of hydrogen per oxide on the SK-N-SH cell, this same steps was carried out again.

Analysis of oxidation stress

SK-N-SH cell were treated with 10 µ M Retin-A for five day at room temperature in the laboratory. After the treatment, it was incubated again for one day in carbon di-oxide at 38 °C. After that the cells were treated with various concentration of crude and minimum essential media for one day. This cells then incubated again for 3 days at 38°C and 5% of carbon di-oxide. 150mM Hydrogen peroxide and the cell were incubated in the microplate for one day. Using Dimethyl sulfoxide, minimum essential media was removed which has extract of crude and cell and then it was placed in dark or less light conditions. Then dichlorofluores cin diacetate was used as an indicator for reactive oxygen species (ROS) in cells. It was diluted with the phosphate buffer solution and incubation was carried out for half hour. Using microplate photometers, measurement of generation of ROS was noted. In the well, because of oxidation dichlorofluores cin diacetate was generated which causes the increase in percentage fluorescence, this was used to measure the concentration of intracellular reactive oxygen species in assay.

Liquid Chromatography

To analyse the presence of bio-active compounds in the crude extract, High Performance Liquid Chromatography technique was used. The main aim of High Performance Liquid Chromatography is to identify, separate and quantify each component from the solution. It is depended on the pump which absorb the distinct compound and hence we can distinguish it. In this experiment High Performance Liquid Chromatography with 500 pumps as a absorbents and 3000 photo diodes of 300nm were used to detect the presence of vitexin and isovitexin. octadecylsilyl groups is used to separate it at 39°C. For separation gradient method was used. A gradient method is recommended for samples that cannot be easily separated by isocratic methods. It was carried out using methyl alcohol and Distilled, re-condensed water. This process was carried out for 1 hour, in the first 45 minutes the concentration of methyl alcohol was gradually increase from 10% to finally 90%. After this the solution is kept for 15 minutes at room temperature.

Statistical Analysis

The cytotoxicity test and H2O2 scavenge output was recorded by mean of standard deviation. Analysis of variance was first applied to the solution. It is a ground of statistical method and procedure to analyse the

difference between groups. After that honestly significant difference was applied to analyse whether the relationship between two sets of data is statistically significant.

RESULTS

Crude extract percent yield

Percent yield is the percent ratio of actual yield to the theoretical yield. To compound the percent yield, at 43°C using rotational evaporator herb was treated with 80% of methyl alcohol. Results shows that 16.9% w/w percent yield was obtained.

Cell Toxicity

1) Methyl Alcohol

Cytotoxicity analysis were carried out using Methylthiazolyldiphenyl-tetrazolium bromide (MTT) on the SK-N-SH crude extract. The result of SK-N-SH and crude extract at different concentration were recorded. above the concentration 125 µg/mL, observed higher cell death which was 78.32% at 125 µg/mL. At the concentration 300 µg/mL, cell viability quantity decreases to 47.2%. At 500 µg/mL, this count further deceased to 33.25%. When the concentration was kept at 1000 µg/mL cell viability was extremely reduced to 16.45%.

Table 1. Analysis of SK-N-SH and crude extract concentration effect on cell death

SK-N-SH and crude extract concentration	Cell viability percentage
125 µg/mL	78.32%
300 µg/mL	47.2%
500 µg/mL	33.25%
1000 µg/mL	16.45%

Table 1 shows the analysis of SK-N-SH and crude extract concentration effect on cell death. It was observed that, as the concentration is higher, cell death percentage increases. Which shows the invers relation of SK-N-SH and crude extract concentration and cell death.

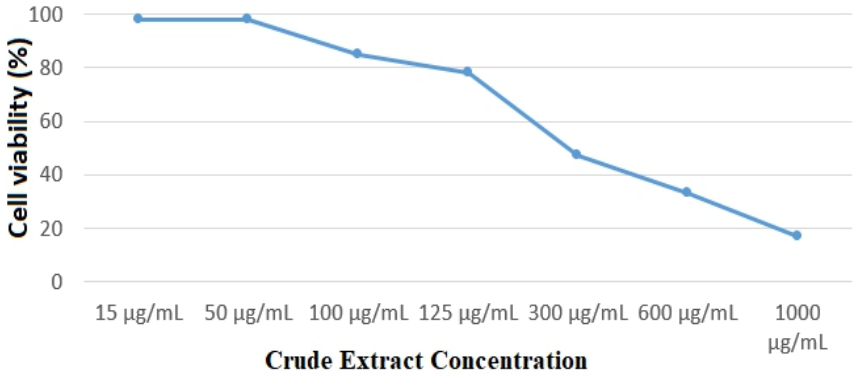


Figure 2. cell viability test of SK-N-SH cell with various concentration of crude extract

Figure 2 shows the cell viability test of SK-N-SH cell with various concentration of crude extract. It was observed that the higher concentration decreases the cell viability.

2) H_2O_2

Cell viability test on H_2O_2 was carried out by following the same step as methyl alcohol. The result of SK-N-SH and H_2O_2 at different concentration were recorded. Above 125 µg/mL concentration shows higher cell death which was 74.32% at 125 µg/mL. At the concentration 250 µg/mL, cell viability quantity decreases to 48.2%. At 400 µg/mL, this count further deceased to 34.25%. When the concentration was kept at 650 µg/mL cell viability was extremely reduced to 17.4%.

Table 2. Analysis of SK-N-SH and H_2O_2 effect on cell death

SK-N-SH and H_2O_2 concentration	Cell viability percentage
125 µg/mL	74.32%
250 µg/mL	48.2%
400 µg/mL	34.25%
650 µg/mL	17.4%

Table 2 shows the analysis of SK-N-SH and H_2O_2 concentration effect on cell death. It was observed that, as the concentration is higher, cell death percentage increases. Which shows the invers relation of SK-N-SH and H_2O_2 and cell death.

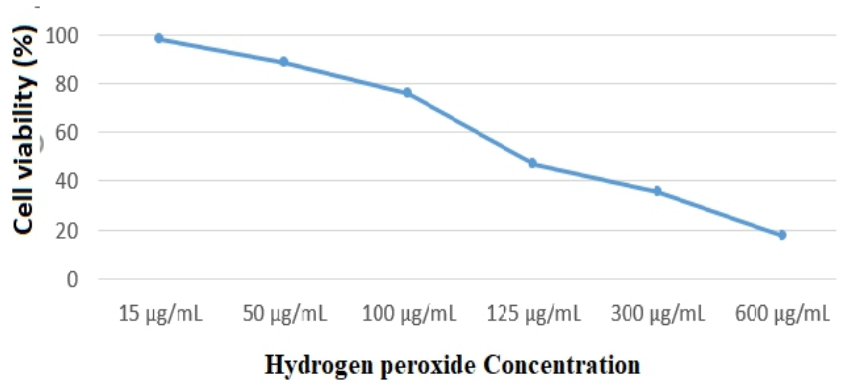


Figure 2. cell viability test of SK-N-SH cell with various concentration of crude extract

Figure 2 shows the cell viability test of SK-N-SH cell with various concentration of H_2O_2. It was observed that the higher concentration decreases the cell viability.

3) Oxidation stress

Dichlorofluores diacetate was used as an indicator for reactive oxygen species (ROS) in cells. It was observed that SK-N-SH cells has very less ROS inhibition when it was exposed to H_2O_2. Untreated SK-N-SH cells shows the highest ROS inhibition percentage. The result of SK-N-SH and crude extract at different concentration were recorded. At 1000 µg/mL concentration the ROS inhibitor percentage was at 24.23%. At 500 µg/mL concentration the ROS inhibitor percentage was at 30.15%. At 125 µg/mL concentration the ROS inhibitor percentage was rises to 61.78%. At 10 µg/mL concentration the ROS inhibitor percentage was rises to 83%.

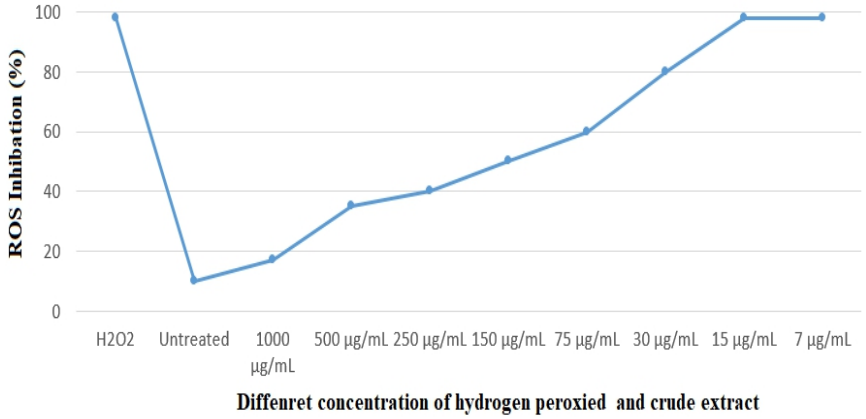


Figure 3. oxidation stress of SK-N-SH cell with various concentration of H_2O_2. Oxidation stress with untreated, exposed to H_2O_2 and Crude extract.

Figure 4 shows the oxidation stress of SK-N-SH cell with various concentration of H_2O_2. Oxidation stress with untreated, exposed to H_2O_2, and various percentage of crude extract.

4) Chromatography

To analyse the presence of bio-active compounds in the crude extract, HPLC technique was used. retention time indicates the time taken for a compound to elute from the column. vitexin and isovitexin were used to determine the presence of bioactive components in the *P. macrocarpa.* In the HPLC test, the presence of bioactive compounds were observed at the retention time of 22.023 and 23.780.

DISCUSSION

To extract the bioactive components propanone, methyl alcohol and ethyl alcohol are mostly used. From previous research it was analysed that for the fruit extracts, the yield of molecule with phenol using propanone solvent was much higher than methyl alcohol. Also it was analysed that for leave extracts, the phenol molecules yield is higher in the methyl alcohol solvent as compare to propanone [7]. This is caused by the presence of different components at various part of the plant. This polar and non-polar components caused this. methyl alcohol and ethyl alcohol are also considered as good solvent to extract the molecules with phenol. Because of the ability of polar solvent to extract the high quantity of phenol components, it is mostly used in the extraction process of herbs in the experimental studies. 43°C using rotational evaporator herb was treated with 80% of methyl alcohol. Results shows that 16.9% w/w percent yield was obtained. This result is with alignment of previous extraction result on *P. macrocarpa* plant extract with 80% methyl alcohol.

When crude extract concentration test was carried out it was observed to be having a toxic. Cytotoxicity analysis, were carried out using Methylthiazolyldiphenyl-tetrazolium bromide (MTT) on the SK-N-SH crude extract. It was observed that, as the concentration is higher, cell death percentage increases. Which shows the invers relation of SK-N-SH and crude extract concentration and cell death. Presence of cytotoxic phytochemical

compounds in the extract is one of the major reason for the cell viability. It was also observed that the presence of phytohormones substances which are responsible for cell division or cytokinesis also increases the chances of cytotoxicity growth, which eventually leads to cell death [18]. Cell viability test on H_2O_2 was carried out by following the same step as methyl alcohol. It was observed that the cytotoxicity increases with the concentration of hydrogen peroxide On SK-N-SH cells. From the experimental results it was clearly observed that the cytotoxicity of hydrogen peroxide on SK-N-SH cells increases above 125 µg/mL concentration. Because of the increased cytotoxicity, the viability percentage drops gradually above 125 µg/mL. Previous researches stated that 250 µg/mL of hydrogen peroxide is a safe , but in this research we observed ROS generation above 125 µg/mL [15]. However this difference could have been caused because of variation in the chemical composition of experiments.

Dichlorofluores diacetate was used as an indicator for reactive oxygen species (ROS) in cells. It was observed that SK-N-SH cells has very less. ROS inhibition when it was exposed to H_2O_2. However there is no previous research to compare the result of this research on ROS inhibition effect of *P. Macrocarpa* on the SK-N-SH cells. Bioactive components present in the extract causes the reduction in light absorbance.

Vitexin and isovitexin were used to determine the presence of bioactive components in the *P. macrocarpa*. In the HPLC test, the presence of bioactive compounds were observed at the retention time of 22.023 and 23.780. In few research the herbal properties of *P. macrocarpa* were identified as the result from presence of vitexin and or isovitexin. Many researches has reported the medical properties of *P. macrocarpa* which includes anti-oxidant[12] , anti-cancer [19], anti-microbial activity [6]. This medicinal properties of *P. macrocarpa* were caused by various other compounds along with vitexin and isovitexin. Saponins, phenyl and alkaloids were also observed in the *P. macrocarpa* which shows medicinal property [3]. The concentration of vitexin and Isovitexin is very less but he additional herbal compounds in the *P. macrocarpa* increases its herbal effects.

CONCLUSION

It was observed that the vitexin and isovitexin is present in the crude extract which increases the ROS generation inhibitory effect of SK-N-SH cells. Presence of this compounds justify the use of *P. macrocarpa* as a herbal plant on Restraining ROS generation. Hence this research recommended the use of *P. macrocarpa* crude extract on mammalian animals for experimental purpose. Anti-oxidation effect of vitexin and isovitexin and its isolation is also recommended.

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