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In vitro Cytotoxicity and Antioxidant Study of *Rhodomyrtus tomentosa* (Aiton) Hassk. Ethanolic Leaf Extract on LPS-induced RAW 264.7 Macrophage Cells

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Authors' contributions

This work was carried out in collaboration among all authors. Author AAK conceived the study, concept, and design and conducted most of the laboratory experiments, analyzed and interpreted experiment results. Authors NAJ, EK, HH, RH and MB contributed to the supervision of the study, drafting, and critical revision of the manuscript of the article. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Ethanol extract of *Rhodomyrtus tomentosa* leaves found specifically on Malaysian soil was used to further investigate the antioxidant properties and cytotoxicity against RAW 264.7 macrophage cells in the search for a safer and effective natural antioxidant agent.
Study Design: Antioxidant potential of *R. tomentosa* were analyzed through series of spectrometric assays and cell-based bioassays model.

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Place and Duration of Study: This study was carried out at Faculty of Pharmacy, Universiti Teknologi MARA (UiTM), Puncak Alam Campus, 43200, Selangor, Malaysia from the year of 2019 to 2021.

Methodology: *R. tomentosa* leaves were subjected to extraction with 95% ethanol. The extracts were then denoted as ethanolic leaves extract of *R. tomentosa* or EtRT extract. EtRT extract were then screen for its antioxidant activity (AOA) and total antioxidant capacity (TAC) through DPPH radical scavenging assay and ferric reducing antioxidant power (FRAP) assay. After that, EtRT extract were brought to observe its toxicity against RAW 264.7 macrophage cells in MTT assay. Once their toxicity was obtained, EtRT extracts were finally tested for their ability to inhibit intracellular reactive oxygen species (ROS) and nitric oxide (NO) inhibition in RAW 264.7 macrophage cells to further analyze their antioxidant properties.

Results: In this study, EtRT extracts dose dependently showed the ability to scavenge DPPH radicals and reduce ferric ions during DPPH radicals scavenging activity assay and ferric reducing antioxidant power assay (FRAP), respectively. In DPPH radical scavenging activity assay, EtRT extracts showed EC_{50} value at 12.37 ± 1.73 µg/mL with ARP value of 0.08 almost as near as ascorbic acid's ARP value which is 0.09. Further into the study, EtRT extract were not cytotoxic to RAW 264.7 macrophage cells at concentrations 3.91 µg/mL and lower which showed more than 86.4% cell viability with IC_{50} value at 204.70 ± 5.30 µg/mL. EtRT extract possessed the ability to inhibit ROS production on LPS-induced RAW 264.7 macrophage cells at 7.813 µg/mL and lower, with the highest concentration can reduce up to 30.20% ± 1.01 out of the total ROS produced by the induced cells. Furthermore, EtRT extract also have evidenced that it is able to significantly inhibit NO production by the LPS-induced RAW 264.7 macrophage cells at 7 µg/mL and lower being the highest at 56.73% ± 0.11 inhibition of the highest concentration tested.

Conclusions: This study suggests that EtRT extracts have the potential to reduce LPS-induced oxidative stress due to the antioxidant activities of phenolic compounds in the extracts, and that at low doses, EtRT extracts had low to no cytotoxicity on RAW 264.7 macrophage cells. As a result, EtRT extract could be a promising natural medicinal agent for the treatment of oxidative stress.

Keywords: Rhodomyrtus tomentosa; antioxidant; oxidative stress; phenolics; Malaysia.

1. INTRODUCTION

Rhodomyrtus tomentosa (Aiton) Hassk. or commonly known as downey rose myrtle by the western, is a tropical medicinal plant that is extensively distributed throughout several nations in southern and south-eastern Asia, including India, China, Philippines, Malaysia, and Indonesia [1]. For a long time, the entire plant (leaves, roots, buds, and fruits) has been utilized in traditional Vietnamese, Chinese, and Malaysian medicine [2]. A plethora of phytochemical such as triterpenoids, flavonoids, polyphenols and meroterpenoids have been isolated from this plant, as described in a review article made by previous studies [3]. The extensive review also described that this plant exhibit variety of pharmacological properties ranging from antibacterial, antitumor, anti-inflammatory and antioxidant potencies.

Reactive oxygen species (ROS) are generated and removed from an organism in a balanced manner during normal metabolism, but following severe stimulation, a substantial increase in ROS production may occur, resulting in oxidative stress [4]. ROS includes superoxide anions (O_2^-), hydroxyl radicals (·OH), nitric oxideradicals (·NO), and hydrogen peroxide (H_2O_2) [5]. The interaction of nitric oxide radicals with other oxygen-derived free radicals can result in the formation of highly reactive species that can harm the host tissue by damaging important biological macromolecules like deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), as well as causing inflammation and cell death [6,7]. In addition, the presence of nitic oxide radicals willlead to nitrosative stress in which may alsocause damage to membrane fatty acids, DNA, and its repair mechanisms [8].

Hence, oxidative stress-associated diseases such as neurodegenerative disease, cardiovascular disease, diabetes and even cancer have attracted many researchers embarking in a journey to find its potential therapeutic agent from natural sources since the current synthetic drugs may cause detrimental side effects [9]. Previously, acetone extract of *R. tomentosa* leaves originated from Thailand have been explored and reported to have potential antioxidant properties [10]. To date, there is still

no study have been made on the cytotoxicity of ethanolic extracts of *R. tomentosa* leaves against RAW 264.7 macrophage cells. In this study however, ethanol extract of *R. tomentosa* leaves found specifically on Malaysian soil was used to further explore the antioxidant properties and its cytotoxicity against RAW 264.7 macrophage cells in search for a safer and effective natural antioxidant agent through a series of*in vitro* spectrometric assays and cell-based bioassay.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide), DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), DCFDA (2',7'-dichlorofluorescin diacetate), sodium nitrate ($NaNO_3$), N-(-1-naphthyl)-ethylenediamine dihydrochloride (NEDD), sulphanilamide ($C_6H_8N_2O_2S$), phosphoric acid (H_3PO_4), lipopolysaccharide (LPS), α-tocopherol ($C_{29}H_{50}O_2$), absolute methanol (CH_3OH), sodium acetate trihydrate ($NaC_2H_3O_2{\cdot}3H_2O$), glacial acetic acid ($C_2H_4O_2$), hydrochloric acid (HCl), ferric chloride hexahydrate ($FeCl_3.6H_2O$) and gallic acid (C_7H_6O5)were purchased from Merck (Germany) whereas anhydrous iron (III) chloride ($FeCl_3$) were purchased from R & M Chemicals (India). For the maintenance of cell cultures, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL) solution were all purchased from Nacalai Tesque (Japan). Trypsin-EDTA and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (Germany). Phosphate buffered saline (PBS) tablets were purchased from Oxoid (United Kingdom). RAW 264.7 macrophage cells were obtained from American Type Culture Collection (ATCC no. CRL-2266 and no. TIB-71™, Manassas, VA, USA).

2.2 Plant Materials

R. tomentosa leaves were collected in February 2019 at the peak of Bukit Batu Putih, Malacca, Malaysia at 2° 24' 40.572" N latitude and 101° 51' 1.4754" E longitude. Verification of specimen identification was made by a botanist from Forest Research Institute of Malaysia (FRIM) with an identification number of PID 050319-05.

2.3 Sample Preparation and Extraction

R. tomentosa leaves were dried in a 40°C oven for three days. Then, the leaves were grounded into a fine powder form with the aid of a mechanical blender. The powdered dried leaves (50g) of *R. tomentosa* were extracted with 500 mL of 95% ethanol and were placed on an orbital shaker with a speed of 100 rpm for 7 days. The solvent layers were collected and filtered using No. 1 Whatman filter paper. The filtered extracts were evaporated to dryness in a rotary evaporator at 40℃ by adjusting the pressure to less than 70 mbar to obtain the crude leaves extract. Finally, the crude leaves extract was freeze dried until crystalline residue was formed. The obtained standardized leaves extract was denoted as ethanolic leaves extract of *R. tomentosa* (EtRT). EtRT extract were kept at -20℃ until further used.

2.4 DPPH Radical Scavenging Assay

DPPH assay was carried out according to previous study with some modification [11]. In a 96-well microtiter plate, 100 µL of different concentrations of EtRT extracts ranging from 0.977 µg/mL to 62.5 µg/mL were mixed with 100 µL of 0.1 mM DPPH solution. The mixture was allowed to incubate in room temperature for 30 minutes in a dark surrounding. Then, the absorbance was measured at 517 nm. The control well was DPPH solution without sample. The antioxidant activity (AOA) in terms of percent radical scavenging activity of the samples were calculated according to equation 1. The optical density (OD) value obtained during the assay were used in the equation. L-ascorbic acid was used as positive control. The half maximal effective concentration (EC_{50}) value of EtRT extracts and L-ascorbic acids were obtained to find their antioxidant radical power (ARP) value (equation 2).These values were used to compare the ARP between the sample and the positive control. Higher ARP value showed higher radical power of the substances.All determination was performed in triplicates.

$$\text{AOA}(\%) = (OD\ DPPH\ without\ sample - OD\ DPPH\ with\ sample)/(OD\ DPPH\ with\ sample)\ x\ 100 \quad (1)$$

$$\text{ARP} = 1/EC50 \quad (2)$$

2.5 Ferric Reducing Antioxidant Power Assay

FRAP solution were freshly prepared prior to experiment. This solution made up of four different solution of 300 mM acetate buffer solution (pH 3.6), 10 mM TPTZ mixture in 40 mM

dilute hydrochloric acid, 20 mM ferric chloride solution and distilled water, mixed with a ratio of 10:1:1:1, respectively [12]. Then, in atransparent 96-well microtiter plate, 3 µL of different concentrations of EtRT extractsranging from 250 µg/mL to 1000 µg/mL were mixed with 100 µL of FRAP solution. The mixture was allowed to incubate in 37℃ water bath for 30 minutes in a dark surrounding. Then, the absorbance was measured at 593 nm. A standard curve of gallic acid ranging from 0.977 µM to 2000 µMwere also prepared by using the same steps. The total antioxidant capacity (TAC) of FRAP analysis were calculated using equation 3. The TAC value obtained were expressed as micromolar gallic acid equivalents (µM GAE). Ascorbic acid was used as positive control. All determination was performed in triplicates.

$$TAC=(OD\ of\ gallic\ acid\ from\ standard\ curve\ x\ dilution\ factor)/(Volume\ of\ samples\ (\mu L)) \quad (3)$$

2.6 Cell Culture Maintenance

Dulbecco's Modified Eagle's Medium (DMEM) complete medium containing 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin was used to sustain RAW 264.7 cells. The cells were held at 37°C in a humidified atmosphere with 5% CO_2 [13]. The culture medium was replaced every two days until the cells reached an 80 % to 90% confluent state. Then, with 0.25% Trypsin-EDTA solution, adherent cells were removed from the culture flask. For all cell culture analyses, the extracts were dissolved in dimethyl sulfoxides (DMSO) and diluted with DMEM complete medium to the intended concentrations. Note that the concentrations of DMSO must not exceed 1% of the final volume.

2.7 Cell Viability Assay

Cytotoxicity was assessed with MTT cell viability assay with slight modifications [14]. Cells were seeded in a 96-well microplate at a density of 30,000 cells/well with 100 µL/well final volume of DMEM complete medium. After 24 hours of incubation, the medium was discarded and replaced with different concentrations of EtRT extracts ranging from 0.976µg/mL to 250 µg/mL. The next day, treatment medium was discarded and replaced with 100 µL of 0.5 mg/mL MTT solution. This step was done in a dark surrounding. Then after four hours of incubation at 37℃ in a 5% CO_2 atmosphere, the MTT solution was discarded and replaced with 100 µL DMSO. The plate was agitated for 10 minutes in a dark surrounding to dissolve the purple formazan crystal salts within the cells. The absorbance of this purple solution was measured with a microplate reader at 570 nm. The percent cell viability was calculated using equation 4. All samples were performed in triplicates.

$$\text{Cell viability (\%)} = (OD\ sample - OD\ blank)/(OD\ control - OD\ blank)\ x\ 100 \quad (4)$$

2.8 Intracellular ROS Assay

ROS reduction was assessed with intracellular ROS assay protocol and are fluorescently detected with few modifications [15]. Cells were seeded in ablack 96-well microplate at 30,000 cells/well (100µL/well). After 24 hours incubation, the media was discarded and replaced with different concentrations of EtRT extracts ranging from 0.976 µg/mL to 7.813 µg/mL. These plates were incubated for 1 hour in an incubator with 5% CO_2 at 37℃. After an hour incubation, 4 µg/mL LPS was added to each well of the plateand were incubated for another 23 hours in the same incubator. The next day, treatment medium was discarded, and the wells are washed with PBS. Then the wells were treated with 20 µM 2',7'-dicholorofluorescin diacetate (DCFHDA) solution. The plate was incubated for 45 minutes in the same incubator. After incubation, the wells are again washed with PBS three times. The last wash was discarded, and all the wells were filled with 200 µL PBS. The absorbance was measured with a microplate reader in which the excitation wavelength was set at 485 nm and the emission wavelength was set at 520 nm. α-tocopherol (vitamin E)were used as the positive control and all determination was performed in triplicates.

2.9 Nitric Oxide Assay

Nitric oxide (NO) inhibition was assessed with Griess assay with some optimization [16]. Cells were seeded in a transparent 12-wells microplate at 100,000 cells/well (500µL/well). After 24 hours of incubation, the media was discarded and replaced with different concentrations of EtRT extracts ranging from 0.875 µg/mLto 7 µg/mL. The plate was incubated for one hour in 37℃ incubator with 5% CO_2 atmosphere before the addition of 500 µL of 4µg/mL LPS. Then, the plates were incubated for another 23 hours in the same incubator. The next day, 100 µL of the supernatants were taken out and transferred into a transparent 96-wells microplate that has already being labelled with their respective

concentrations accordingly. The plate was then treated with equal amount of Griess reagent and were gently swirled. The nitrite was measured at 540 nm and the concentration was quantified using sodium nitrate standard curve. In this assay, dexamethasone was used as positive control and all determination were done in triplicates.

2.10 Statistical Analysis

Data are tabulated as the mean ± standard deviation (SD) and statistical analyses were performed using Graph Pad Prism software (Version 9). Results were compared to control and treated groups by one-way analysis of variance (ANOVA).Dunnett's post-hoc test was used for comparison between treatment groups and a single control.Differences were considered as statistically significant at **P≤ 0.05* versus the control group.

3. RESULTS AND DISCUSSION

3.1 Antioxidant Activity (AOA) during DPPH Radical Scavenging Analysis

The radical scavenging activity of a plant extract can be evaluated using a variety of *invitro* assay models. In this study, DPPH radical scavenging activity assay was employed on EtRT extract. The ability for the extract to stabilize DPPH radical were observed as antioxidant screening purpose.Fig. 1 showed the antioxidant activity (AOA) in terms of percent (%) DPPH radical scavenging activity of different concentration of EtRT extracts ranging from 0.97 µg/mL to 62.5 µg/mL. Fig. 1 Also demonstrated that the scavenging activity of EtRT extracts was dose dependent to the concentration of the extracts. The trend is that the higher the concentration of the extract added into the DPPH solutions, the higher the radical scavenging activity.It also showed that ascorbic acid is still the best and potent radical scavenger, but EtRT extracts also showed a promising result. Fig 2. showed the percent inhibition of different concentrations of EtRT extracts and L-ascorbic acids against DPPH radical. From these curves, the half maximal effective concentration (EC_{50}) and the antioxidant radical power (ARP) was obtained and recorded in Table 1. From the data, it showed that L-ascorbic acid and EtRT extract exhibit its EC_{50} at 10.41 ± 0.62 µg/mL, and 12.37 ± 1.73 µg/mL, respectively. The highest ARP is at 0.09 for positive control followed by EtRT extract at 0.08. This data showed that the ARP value of EtRT extracts is as near as ascorbic acid's ARP value at EC_{50}concentration that are not far from each other.

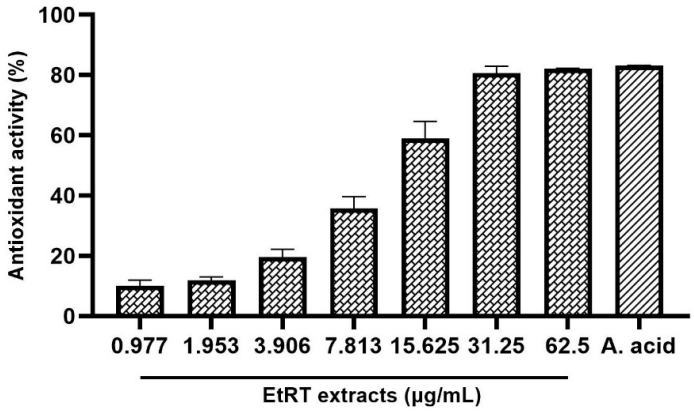

Fig. 1. Antioxidant activity (AOA) EtRT extracts
A. acid = ascorbic acid (22 µg/mL). Results were expressed as the mean ± SD in triplicates

Table 1. EC_{50} and ARP values of EtRT extracts as compared to L-ascorbic acids

Sample	EC_{50}, µg/mL	ARP
L-Ascorbic Acids	10.41 ± 0.62	0.09
EtRT Extracts	12.37 ± 1.73	0.08

**Values are expressed in mean ± SD where n = 3*

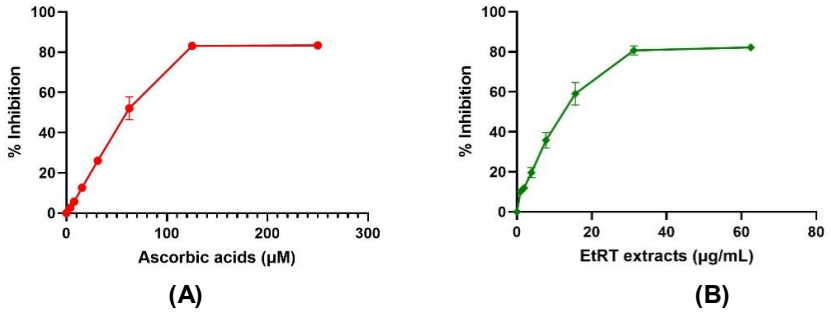

Fig. 2. Percent inhibition of (A) L-ascorbic acids and(B) EtRT extracts

Results were expressed as the mean ± SD in triplicates.

3.2 Total Antioxidant Power (TAC) during FRAP Analysis

Another scavenging ability analysis for screening purpose can also be assessed by retrieving the total antioxidant capacity (TAC) from ferric reducing antioxidant power (FRAP) assay. Fig. 3 showed a linear graph of gallic acid standard curve. A satisfactory linear regression equation of gallic acid is displayed between the optical density (OD) value versus the concentrations of gallic acid based on the coefficient of determination and regression equation, R^2 = 0.9979 and y = 0.0008x + 0.1139, respectively. This standard curve was used to obtain the micromolar gallic acid equivalent (µM GAE) values for subsequent determinations of total antioxidant capacity (TAC) of EtRT extracts. From optimization, 44 µg/mL of ascorbic acid were used as comparison against EtRT extract for its TAC determination during this assay. Fig. 4 showed the TAC as expressed in µM GAEof EtRT extracts of various concentrations ranging from 62.5 µg/mL to 1000 µg/mL. A dose dependent trend was also observed in which the TAC increases as the concentrations of the extract increases.Compared to its AOA value in DPPH radical scavenging activity analysis which begins at a lower concentration, the TAC value however, begins at a very high concentration of the plant extract. Again, ascorbic acids showed greater TAC value at lower concentration as it is known as a potent radical scavenger, and this also can be seen in EtRT extracts but at higher concentrations.

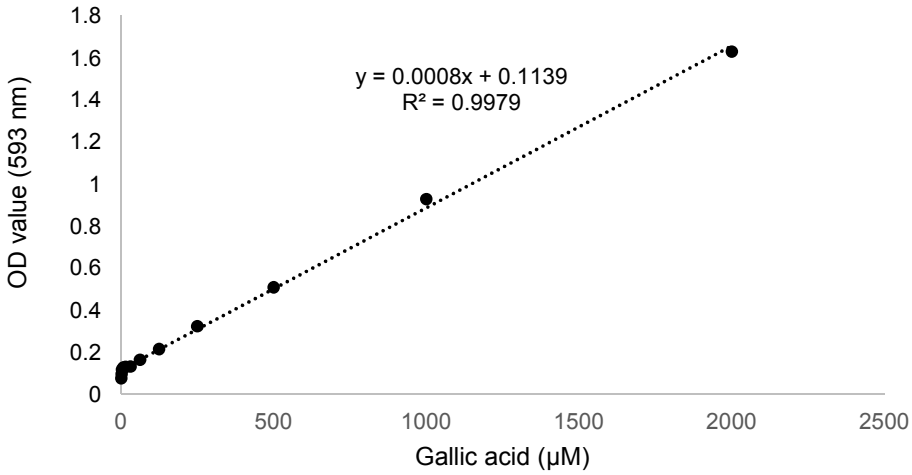

Fig. 3. Gallic acid standard curve

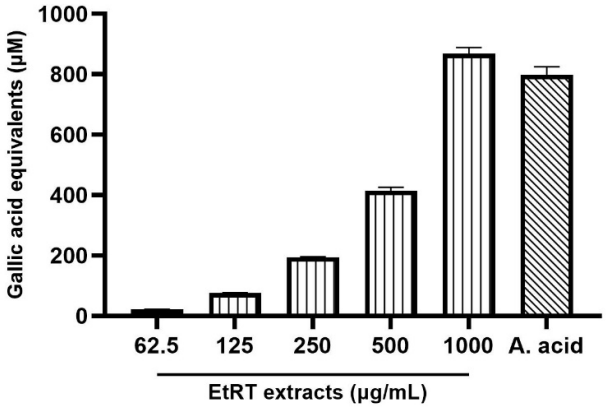

Fig. 4.Total antioxidant capacity (TAC) of EtRT extracts

A. acid = ascorbic acid (44 µg/mL). Results were expressed as the mean ± SD in triplicates.

3.3 Cytotoxicity Assessment

The cytotoxic effect of various concentrations of EtRT extracts was detected with MTT assay on RAW 264.7 macrophage cells. Fig. 5 showed the percent cell viability of RAW 264.7 macrophage cells treated with EtRT extracts ranging from 0.976 µg/mL to 250 µg/mL. The percent cell viability of the cells decreases along with increasing extract concentrations, meaning that higher concentrations of the extract is toxic to the cells resulting in lower percentage of living cells compared when treating it with lower concentrations of extract. According to Fig. 5, 125 µg/mL and 250 µg/mL of EtRT extracts are cytotoxic where they showed moderate viability with 58.84% ± 1.58 and 43.27% ± 0.76 cell viability, respectively. This result was compared to the morphology of the cells, where there are a lot of spindled-shaped macrophages in higher concentrations of EtRT extract treatment wells. However, EtRT extracts at lower concentrations such as 1.95 µg/mL and 3.91µg/mL are not cytotoxic in which they showed 98.30% ± 6.79 and 86.41% ± 0.10 cells viability, respectively as compared to control with 100% viability.

Furthermore, the half maximal inhibitory concentration (IC_{50}) was calculated to determine the concentration of EtRT extracts required for 50% cells inhibition *invitro*. It also indicates the concentrations of the inhibitor which in this context is the plant extract different concentrations, needed to inhibit a given biological or biochemical function by half. Any concentration higher than the IC_{50} will give out more than 50% to total cell death. Fig. 6 showed the percent inhibition of RAW 264.7 macrophage cells against EtRT extracts and it was used to determine the IC_{50}. The IC_{50} of RAW 264.7 macrophage cells were obtained to be 204.70 ± 5.30 µg/mL when treated with EtRT extracts at the specified concentrations range after 24 hours incubation, which is quite moderate. Therefore, higher concentrations of extracts could have toxic effect for RAW 264.7 macrophage cells viability. By carefully observing the percent viability against the concentrations of the plant extracts, hence, the safest concentration that can be used in the subsequent experiments that gives very low to no cytotoxicity to RAW 264.7 macrophage cells are 7.813µg/mL and below. Since the cytotoxicity of ethanol extracts of *R. tomentosa* leaves against RAW 264.7 macrophage cells have not yet being establish, hence this result would be a great benefit for future research.

3.4 Intracellular ROS Reduction Analysis

Fig. 7 showed the production of ROS in relative fluorescence units (RFU) of different concentrations of EtRT extracts ranging from 0.977µg/mL to 7.813 µg/mL on RAW 264.7 macrophage cells. α-tocopherol (vitamin E)with concentration of 40 µg/mL were taken as comparison, and it gave out lower amount of ROS production than EtRT extracts with concentration of 7.813 µg/mL and lower. From the results in Table 2, EtRT extracts with concentrations of 7.813 µg/mL and 3.906 µg/mL significantly ($P \leq 0.05$) reduce the amount of ROS produced by the cells upon 4 µg/mL LPS stimulation by 30.20% ± 1.01and 28.01% ±

2.03ROS inhibition, respectively. However, the ROS production is higher than the positive control which showed the highest activity with 52.24% ± 2.01ROS inhibition, but at 40 µg/mL concentration. This means that if the concentrations of α-tocopherol were to reduce a little, the percent ROS inhibition of the positive control would nearly be similar as EtRT extracts. The antioxidant properties of vitamin E are attributed to the hydroxyl group of the aromatic ring, which gives up one hydrogen atom to the free radicals or any reactive species around [17]. Because previous study has stated that *R. tomentosa* is a good source of phenolics, hence the mechanism of the antioxidant activity is quite similar to that of vitamin E in which they scavenged free radicals and/or by chelating metal ions as demonstrated during DPPH radical scavenging activity assay and FRAP assay analysis [18]. Epidemiological studies significantly indicate the significance of phenolic compounds in the protection of oxidative stress and chronic inflammation-related disorders such as cardiovascular disease, cancer, osteoporosis, diabetes mellitus, arthritis, and neurological disease [19, 20].

3.5 Nitric Oxide Reduction Analysis

Fig. 8 showed a linear nitrite standard curve. A satisfactory linear regression equation of nitrite is displayed between the OD value versus the concentrations of nitrite based on the coefficient of determination and regression equation, R^2 = 0.9993 and y = 0.0141x + 0.0572, respectively. This standard curve was used to obtain the nitrite valuein micromolar (µM) for subsequent nitrite concentrations determination on EtRT extracts.

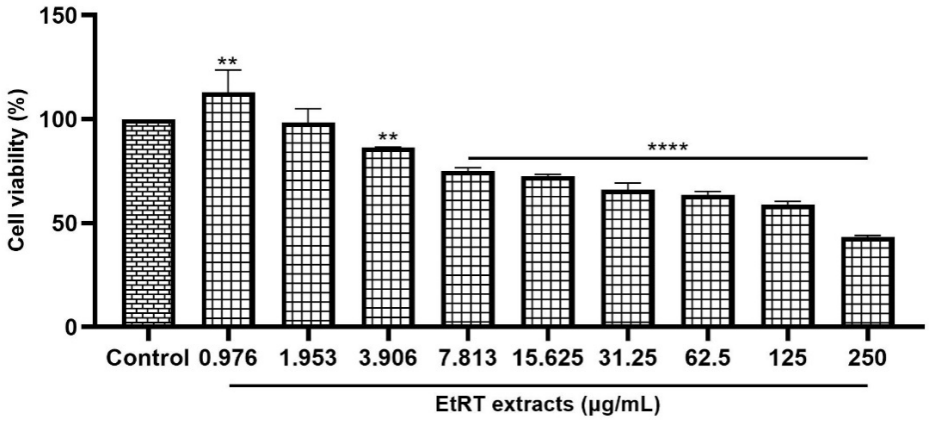

Fig. 5. Cell viability (%) of RAW 264.7 macrophage cells against EtRT extract
*Control = untreated cells. Result was expressed as the mean ± SD in triplicates. **P ≤ 0.01, ***P ≤ 0.001 and **** P ≤ 0.0001 compared to control (100%)*

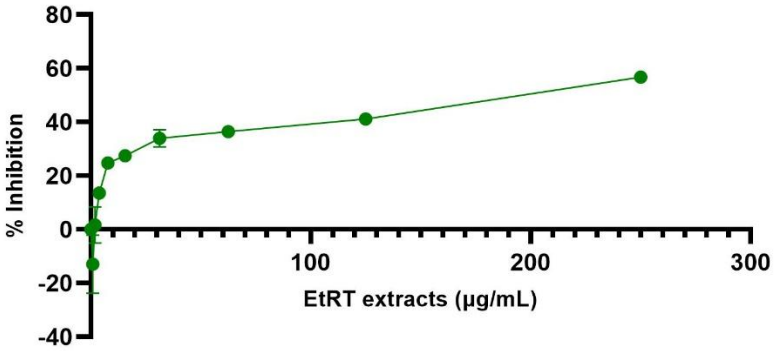

Fig. 6. IC_{50} of RAW 264.7 macrophage cells against EtRT extracts
Results were expressed as the mean ± SD in triplicate

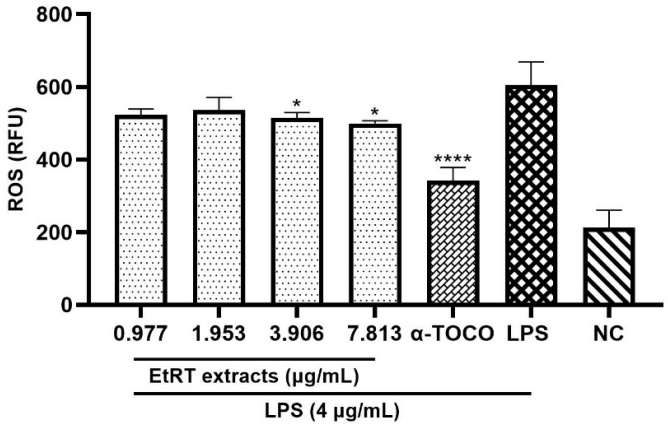

Fig. 7. Production of ROS on RAW 264.7 macrophage cells against EtRT extracts

*NC = negative control / untreated cells. α-TOCO = α-tocopherol (40 µg/mL). Result was expressed as the mean ± SD in triplicates. *P ≤ 0.05 and **** P ≤ 0.0001 compared to LPS (4 µg/mL)*

Table 2. Production of intracellular ROS on RAW 264.7 macrophage cells against ETRT extracts treatment

Sample	Concentrations, µg/mL	ROS Inhibition, %
EtRT extracts	0.977	26.93 ± 2.38
	1.953	25.02 ± 4.76
	3.906	28.01 ± 2.03
	7.813	30.20 ± 1.01
α-TOCO	40	52.24 ± 2.01
LPS	4	0
NC	-	-

**Values are expressed in mean ± SD where n = 3.*

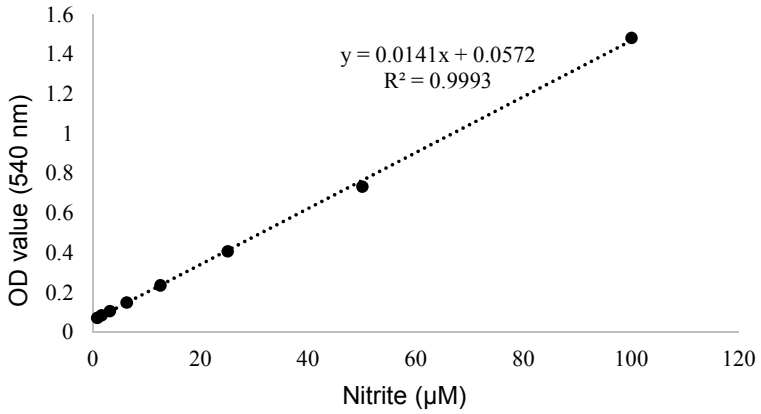

Fig. 8. Nitrite standard curve

The nitric oxide (NO)productions in LPS-stimulated RAW 264.7 macrophage cells to further evaluate the antioxidant activity of various concentrations of EtRT extracts ranging from 0.875 µg/mL to 7 µg/mL were illustrated in Fig. 9.Nitrite productions reflect the amount ofNO produced by each concentration of the samples when compared to LPS-stimulated

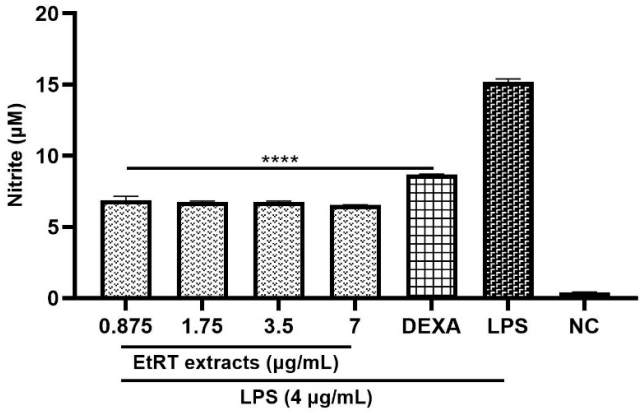

Fig. 9. Nitrite production from RAW 264.7 macrophage cells against EtRT extracts

*NC = negative control / untreated cells. DEXA = dexamethasone (7.8 µg/mL). Results were expressed as the mean ± SD in triplicates. **** P ≤ 0.0001 compared to LPS (4 µg/mL)*

Table 3. Nitrite production and percent inhibition (%) from RAW 264.7 macrophage cells against different concentrations of EtRT extracts

Sample	Concentrations, µg/mL	Nitrite Inhibition, %
EtRT extracts	0.875	54.45 ± 1.72
	1.75	55.32 ± 0.44
	3.5	55.45 ± 0.57
	7	56.73 ± 0.05
DEXA	7.8	42.49 ± 0.11
LPS	4	0
NC	-	-

**Values are expressed in mean ± standard error mean where n = 3*

only wells as shown in Table 3. Based on these data EtRT extracts significantly inhibited NO production (*P ≤ 0.0001*) being the highest concentration at 7 µg/mL with nitrite inhibition of 56.73% ± 0.05and the percentage slightly decreases as the concentration decreases. However, the positive control; dexamethasone, at 7.8 µg/mL showed 42.49% ± 0.11nitrite inhibition. This showed that EtRT extracts can inhibit more nitrite production compared to positive control at a slightly lower concentration with no cytotoxicity. Due to the fact that involvement of NO radicals with other oxide radicals can further deteriorate host tissue and cells through nitrosative stress, hence it is very much practical to prevent it [8]. In this context, EtRT extracts seem to be a suitable candidate in the prevention of nitrosative stress as it possessed the ability to inhibit NO production by LPS-stimulated RAW 264.7 macrophage cells.

4. CONCLUSIONS

This study suggest that EtRT extracts has the potential to prevent LPS-induced oxidative stress due to the antioxidant activities of phenolic compounds in the extracts and it showed low to no cytotoxicity to RAW 264.7 macrophage cells at low concentrations. Therefore, EtRT extracts may be a good candidate as a natural therapeutic agent for treating oxidative stress. However, further *invivo* treatment will be needed as it is pre-clinical requirement before it can be consume or use as a topical cream for human administration.

DISCLAIMER

The products employed in this study are routinely and often used in our field of study and country. There is no conflict of interest between the writers and makers of the products because we do not plan to use them as a means of pursuing legal action, but rather to further knowledge. Also, the research was not funded by the producing company rather it was funded by the institution.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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