SCAVENGING ACTIVITIES AND PROTECTIVE EFFECTS OF *SYZYGIUM CUMINI* (L.) SKEELS ON H_2O_2 INDUCE OXIDATIVE STRESS IN NORMAL HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

Lanchakon Chanudom, Jitbanjong Tangpong*

School of Allied Health Sciences and Public Health, Walailak University, Nakhon Si Thammarat, 80160, Thailand

ABSTRACT:

Syzygium cumini (L.) Skeels is the most commonly used as medicinal plants on diabetes and anti-inflammation. Hence, effects of aqueous leaf extracts of *Syzygium cumini* (L.) Skeels (ASC) on scavenging activities and protective effects of H_2O_2- induced oxidative damage in human peripheral blood mononuclear cells (PBMC) were determined in this study. Total phenolic and flavonoid contents of ASC at 2,000 µg/ml were 694.44±3.64 µgGAE/ml and 8.27±0.10 µg quercetin/ml respectively which were correlated with the r^2 value at 0.93. IC_{50} values of ASC on free radical, hydroxyl and superoxide were 87.79±0.85, 766.62±28.61 and 1,299±25.13 µg/ml respectively while the nitric oxide scavenging activities were not higher than 50% at the concentration of 2,000 µg/ml of ASC. The protective effects of ASC in H_2O_2-induced PBMC cell damages were significantly increased to 83.13±3.57–112.32±4.47 % when treated with 0.25–1 µg/ml of ASC after oxidative induced by 5 mM of H_2O_2 for 3 h. Suggest that ASC is a potential source of natural antioxidant properties and protective defense system of PBMC.

Keywords: Protective effects, Scavenging capacities, *Syzygium cumini* (L.) Skeels

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INTRODUCTION

Syzygium cumini (L.) Skeels, Myrtaceae family, is a native tree of the tropics, originally from India and South-east Asia. It is widespread in north, northeast and south of Thailand and used as a popular treatment against various diseases. The bark, fruits, seeds and leaves were used for the treatment of diabetes and administered in various pharmaceutical preparations in Brazil [1]. Seeds have shown hypoglycemic and antioxidant activities. The bark is also used for dysentery and diarrhea. Moreover, *Syzygium cumini* (L.) Skeels has been shown to have sedative and anticonvalsant effects and a potent central nervous system depressant effect [2]. This plant is rich in compounds containing anthocyanins, glucoside, ellagic acid, isoquercetin, kaempferol and myricetin. The leaves are claimed to contain acylated flavonol glycosides, quercetin, myricetin, myricetin 3-O-4-acetyl-L-rhamnopyranoside, triterpenoids, esterase, galloyl caboxylase and tannin [3].

Reactive oxygen species (ROS) are associated with pathophysiology of aging and progression of a number of human diseases, such as cancer, rheumatoid arthritis, and atherosclerosis [4, 5]. The evidence has proven that the oxidative damages caused by ROS may be seriously deleterious and may be concomitant, and these oxidative damages occur continuously in the human body [6]. Among ROS, reactive oxygen intermediates such as hydrogen peroxide (H_2O_2) has been shown to alter the immune responses of lymphocytes in inflammatory systems. At sublethal concentrations, H_2O_2 inhibits the proliferation and effecter function of human T cells without cellular damage [7]. H_2O_2 and free radicals produce cell injury by oxidative modifications of membrane lipids and proteins [8]. The polyunsaturated fatty acid moieties of membrane phospholipids are particularly susceptible to oxidant injury because of the relative reactivity of the bis-allylic hydrogen atoms within the fatty acid

* *Correspondence to: Jitbanjong Tangpong*
E-mail: njibjoy@yahoo.com

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double bond systems [9]. It has been shown that altering the fatty acid composition of membrane phospholipids by culturing cells in supplemented medium modulates the susceptibility of the cells to oxidative damage. H_2O_2 lower than 2 mM induces lipid peroxidation by increasing malondialdehyde (MDA) only 2-fold, whereas a larger increase (10-fold) could be observed in PBMC at the highest dose (5 mM) [10]. The induction of lipid peroxidation by H_2O_2 is generally thought to be due to its ability to stimulate OH° formation through the transition metal-catalyzed Haber–Weiss reaction [11]. Normally our cells have endogenous antioxidant defenses, but they are not enough to prevent the oxidative damage [12]. Therefore, extraneous antioxidants appear to be very important in the prevention of many diseases [13]. Because of toxicity and carcinogenic effects, synthetic antioxidants have begun to be restricted. Thus, interest in finding natural antioxidants without undesirable side effects, has greatly increased. At present, most of natural antioxidants such as traditional nutrients and polyphenols, are obtained from vegetables and fruits [14].

Among these significant pharmacologically actions of *Syzygium cumini* (L.) Skeels, the purpose for this study is to explore the scavenging capacity and the protective effects of *Syzygium cumini* (L.) Skeels on H_2O_2-induced oxidative damage to PBMC, and the data gathered is hoped to provide some baseline information which may highlight the potential of *Syzygium cumini* (L.) Skeels as a new source of natural antioxidants.

MATERIALS AND METHODS

Chemicals

Aluminum chloride, quercetin, methyl violet, pyrogallol, sodium nitroprusside, sulfanilamide, naphthylethylenediamine dihydrochloride, were purchased from Sigma Chemical Company.

For cell viability determination, RPMI-1640 media and 100 IU/mL of penicillin, 100 µg/mL of streptomycin were purchased from Flowlab (Australia), Ficoll-Paque Plus (Amersham Biosciences, USA), fetal bovine serum (FBS) (PAA, Austria) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, USA). All reagents are analytical grade.

Plants

Syzygium cumini (L.) Skeels was collected during March-May, 2012 from different natural habitats at Nakhon Si Thammarat province, Thailand. The fresh leaves were rinsed with distilled water. To prepare aqueous leaf extracts of *Syzygium cumini* (L.) Skeels (ASC), *Syzygium cumini* (L.) Skeels was chopped and homogenized into distilled water in a blender for 1 min. After that the suspension was filtered by Whatman No.1 and then lyophilized by freeze dryer at – 20 °C for 20 h. The powder was resuspended in RPMI-1640 media as an ASC stock solution of 1 mg/ml. The substock solutions of 1, 2, 10, 20 and 100 µg/ml. were prepared by diluting into RPMI-1640 media for cell viability test. The stock and substock solutions were both stored at 4 ° C until used.

Determination of total phenolic and flavonoid contents of ASC

The total phenolic of ASC was determined with the Folin-Ciocalteu reagent using the method of Lister and Wilson [15], 20 µl of sample in triplicate and 2 N Folin-Ciocalteau's reagent of 100 µl was added and incubated at room temperature for 5 min. Na_2CO_3 (25% w/v) of 300 µl was mixed and incubated at 45 °C for 30 min. The absorbance of ASC was read at 765 nm using UV–visible spectrophotometer. Results were expressed as milligram of gallic acid equivalent per gram of dry weight (mgGAE/ml).

Aluminum chloride colorimetric method was used for flavonoid determination [16]. 1 ml of ASC (100 µg/ml) was mixed with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water. Then it was incubated at room temperature for 30 min and the absorbance of the reaction mixture was measured at 415 nm with UV/Visible spectrophotometer. The calibration curve was prepared by preparing quercetin solutions at various concentrations in methanol. The flavonoid content was then calculated and expressed in milligram quercetin per milliliter of dry weight (µg quercetin/ml).

Determination of scavenging activities of ASC

The free radical scavenging of ASC at various concentrations were determined by ABTs cation decolorization assay [17] and results were expressed as a percentage of the scavenging. The scavenging of ASC was considered more on hydroxyl, superoxide anion and nitric oxide radical. Hydroxyl radical assay according to the Fenton-type reaction [18] was measured. The reaction mixture contained 1 ml of 0.1 mM methyl violet, 0.5 ml of 5 mM $FeSO_4$, 0.5 ml of 1% H_2O_2, and 2 ml Tris buffer (pH 4.0). The reaction volume being 10 ml. 0.5 ml of ASC at a different concentration was added. The absorbance of the reaction mixture was measured at 565 nm by a spectrophotometer. The absorbance with ASC was measured as A_s, the absorbance

without ASC was A_0 and absorbance without $FeSO_4$ and H_2O_2 were determined as A. Scavenging activity (D) of ASC on hydroxyl radical was calculated according to the following formula of D = [(As-Ao)/(A-Ao)] X 100%.

The scavenging activity of the ASC on superoxide anion was calculated by comparing ΔA_1/min (without ASC) and ΔA_2/min (with ASC) of the pyrogallol system, according to the following formula [18]. 100 µl of 3 mM pyrogallol and 3 ml Tris buffer (pH 8.2) were mixed with 0.5 ml ASC at 10 mg/ml and the auto-oxidation rate of pyrogallol was measured by determining the changes in the absorbance at 325 nm in 4 min by UV–Vis spectrophotometer. The absorbance with ASC was A_2 and the absorbance without ASC was determined as A_1 following the formula of D = [(ΔA1 - ΔA2)/ΔA1] X 100 %. Ascorbic acid was used as natural antioxidant compound to compare the free radical, hydroxyl and superoxide radical scavenging action with ASC. Nitric oxide scavenging capacity assay was followed by Hoque et al. [19] and was measured at 550 nm. Gallic acid was used as a reference of natural antioxidant compound.

Cell viability determination

Isolation of PBMC

Blood (20–25 ml) was taken from same donor throughout this research by using the 25 ml syringe. The blood sample was diluted with the same volume of RPMI-1640 media. After that, the diluted blood sample was carefully layered on Ficoll-Paque Plus. The mixture was centrifuged under 400×*g* for 40 min at 4 ºC. The undisturbed lymphocyte layer was carefully transferred out. The lymphocyte was washed and pellet down with RPMI-1640 media twice and was resuspended with RPMI-1640 media with 100 IU/ml of penicillin, 100 µg/ml of streptomycin, 10%, v/v fetal bovine serum (FBS). Cell counting was performed to determine the PBMC cell number with equal volume of trypan blue [20].

The cytotoxic effect of the ASC on PBMC cells

The effect of the ASC on cell viability of PBMC was first determined by using a colorimetric technique, which is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 100 µl of PBMC (1 X 10^6 cell/ml.) in RPMI-1640 media with 100 IU/ml of penicillin, 100 µg/ml of streptomycin, 10%, v/v FBS was added into the 96-well plate. Then, 100 µl of each diluted ASC was added to get a final concentration of 0.5, 1, 5, 10 and 50 µg/ml. Then, 96-well plate was incubated in 37 ºC, 5% CO_2 and 90% humidity incubator for 24 h. After the corresponding period, 20 µl of MTT at 5 mg/ml was added into each well in the 96-well plate and incubated for 4 h in 37 ºC, 5% CO_2 and 90% humidity incubator. 100 µl of 10% SDS in 0.01 M HCl was added to each well to extract and solubilized the formazan crystal by incubating for 20 min in 37 ºC, 5% CO_2 incubator. Finally, the plate was read at 570 nm wavelength and percentage of cell viability was calculated.

The effect of H_2O_2 on PBMC cells

100 µl of PBMC (1 X 10^6 cells/ml) was mixed with 100 µl increasing concentrations of H_2O_2 at 5, 10, 15, and 20 mM (final concentration) and cells were incubated for 1, 2 and 3 h at 37 ºC, 5% CO_2 and 90% humidity incubator. After 20 µl of MTT at 5 mg/ml was added into each well in the 96-well plate and incubated for 4 h 100 µl of 10% SDS in 0.01 M HCl was added to each well and was incubated for 20 min in 37 ºC with shake. Finally, the plate was read at 570 nm wavelength and percentage of cell viability was calculated.

Determination of cell viability of ASC on H_2O_2 induce oxidative stress

PBMC (1 X 10^6 cells/ml) were mixed with optimum concentration of ASC for 30 min at 37 °C, 5% CO_2 and 90% humidity in RPMI media in 24-well plate. At the end of the preincubation period, the optimum concentrations and optimum incubating time of H_2O_2 were added and incubated at 37 ºC, 5% CO_2 and 90% humidity incubator. After MTT assay was used to determine percentage of cell viability.

Statistical analysis

In this study, all analyses were run in triplicate. Data obtained were expressed as mean ± standard error of mean (SEM). Analysis of variance (ANOVA) was used to test any difference in ASC and concentration of H_2O_2 on cell viability percentage from these methods. Duncan's new multiple range test was used to determine significant differences. Differences at $p<0.05$ were considered significant.

RESULTS

Total phenolic and flavonoid contents

The amount of total phenolic content of ASC was determined by Folin-Ciocalteu reagent and gallic acid was used as a standard. Total phenolic content of ASC was found to be increased from 134.44±2.22–694.44±3.64 µgGAE/ml at the concentration of 62.5–2000 µg/ml of ASC respectively depended on dose dependent manner. Flavonoid content was calculated from the regression equation of the quercetin calibration curve (y = 0.023X+0.118). The total flavonoid

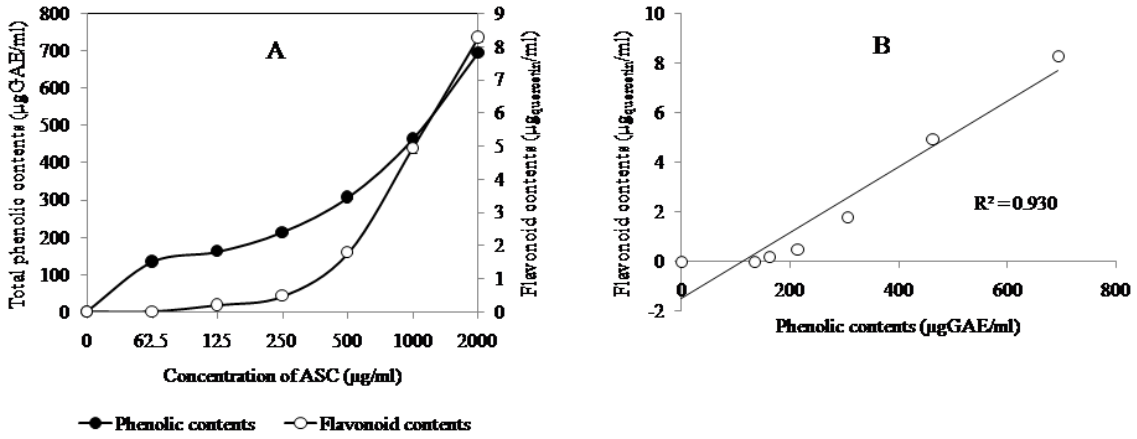

Figure 1 Total phenolic and flavonoid contents of ASC at various concentrations (A) and the correlation of phenolic and flavonoid contents of ASC (B)

Table 1 Scavenging activities at 50% (IC_{50}) of ASC and ascorbic acid. Data was expresses as mean±S.E.M of triplicates

Scavenging activities	IC_{50} (µg/ml)	
	ASC	Ascorbic acid
Free radical	87.79±0.85	123.44±0.42
Hydroxyl radical	766.62±28.61	553.23±7.29
Superoxide radical	1,299±25.13	905.61±24.01
Nitric oxide radical	> 2,000	>2,000

content was increased from 0.21±0.04–8.27±0.10 µg quercetin/ml at the concentration of 125–2000 µg/ml of ASC respectively. The result of total phenolic and flavonoid contents were summarized in Figure 1A and the correlation between total phenolic and flavonoid contents were almost related and depended on the concentration of ASC at 0.93 of r^2 value (Figure 1B).

Scavenging activities of ASC

As shown in Table 1, IC_{50} values of ASC on free radical, hydroxyl and superoxide were 87.79±0.85, 766.62±28.61 and 1,299±25.13 µg/ml respectively. The data indicated that increasing of ASC concentration were related to the increased of radical scavenging activities, especially for free radical scavenging activity of ASC were comparable active than ascorbic acid (Figure 2A). The scavenging activities of ASC and ascorbic acid at the concentration of 62.5-2,000 µg/ml on hydroxyl radical were increased from 6.81±1.00-52.09±0.75 and 18.29±2.14-69.04±0.97 % respectively (Figure 2B) and superoxide radical scavenging activities of ASC and ascorbic acid at the concentration of 125-2,000 µg/ml were concentration dependent increased from 2.21±0.07-66.36±1.82 and 19.42±1.51-84.50±4.58 % respectively (Figure 2C). Nitric oxide scavenging activities of ASC was measured and compared to gallic acid (Figure 2D). The scavenging activities of ASC and ascorbic acid at the concentration of 62.5-2,000 µg/ml on nitric oxide radical were increased from 4.15±0.92-13.05±0.96 and 7.39±0.97-32.89±0.81 % respectively. The nitric oxide scavenging activities at 50% (IC_{50}) of ASC and gallic acid were not calculated because the scavenge action did not reach to 50 % even the concentration of ASC and gallic acid were increased up to 8,000 µg/ml (data not shown).

The effect of the ASC on PBMC cells viability

To study the effect of ASC activated PBMC in various dosages, MTT assay was used to determine cell viability. The increase of cell viability shows that the ASC is not toxic to the immune cell, only the viable and metabolically active cell can cleave the MTT to produce formazan [20]. The ASC did not display strong cytotoxic effect on PBMC with viability of 120.87±10.69–104.56±7.41 % at 0.25–2.5 µg/ml and IC_{50} was obtained at 7.58 µg/ml. The toxicity of ASC was significantly shown at 5–500 µg/ml by dropping the cell viability down to 60.37±3.05–6.74±1.28 % respectively. The result of percent on PBMC cell viability was shown in Figure 3.

The effect of H_2O_2 on PBMC cells viability

The PBMC were incubated with 5, 10, 15 and 20 mM for 1, 2 and 3 h of H_2O_2 and the MTT assay was used to measure the cytotoxicity of H_2O_2 on PBMC. After incubated for 2 h, the percentage of cell viability were decreased significantly about

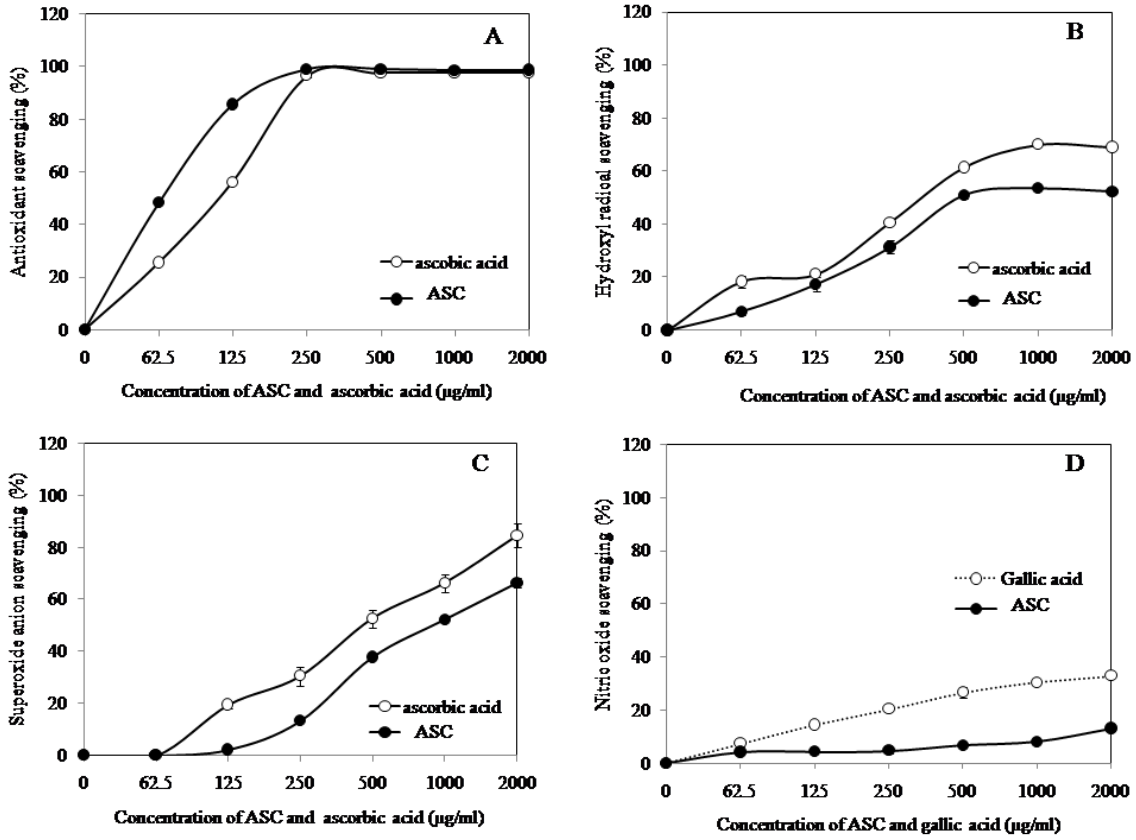

Figure 2 Scavenging activities of ASC, ascorbic acid and gallic acid on free radical (A), hydroxyl radical (B), superoxide radical (C) and nitric oxide radical (D)

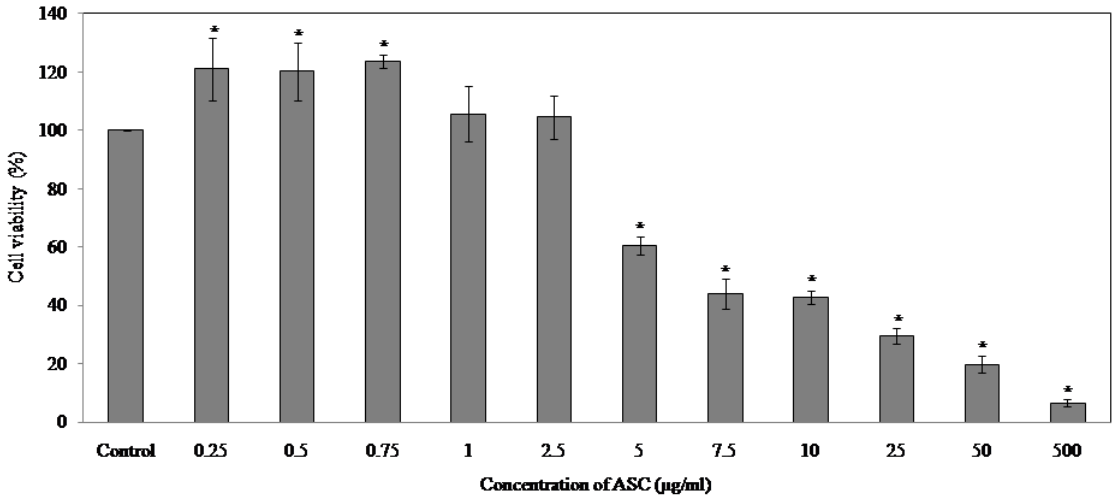

Figure 3 The percentage of PBMC cell viability on various dosage of ASC treated cells. Data are means±S.E.M. of three measurements and *indicates a significant effect of ASC to PBMC cell viable ($p<0.05$).

70% at the concentration of 5–20 mM and decreased to 50–60% after 3 h of treatment. From the toxicity of H_2O_2 on PBMC on Figure 4, it had been shown that H_2O_2 can induce cell injury. Thus, the optimum concentration of H_2O_2 at 5 mM was optimized to induce cell injury for 3 h.

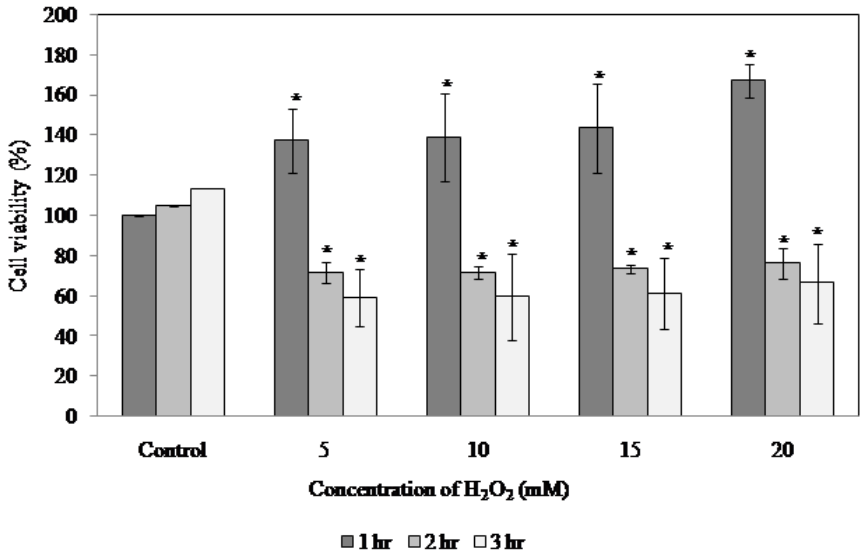

Figure 4 The toxicity of H_2O_2 to PBMC cell viability at various time and concentration. Data are means±S.E.M. of three measurements and *indicates a significant effect of H_2O_2 to PBMC cell viable compares with control group ($p<0.05$)

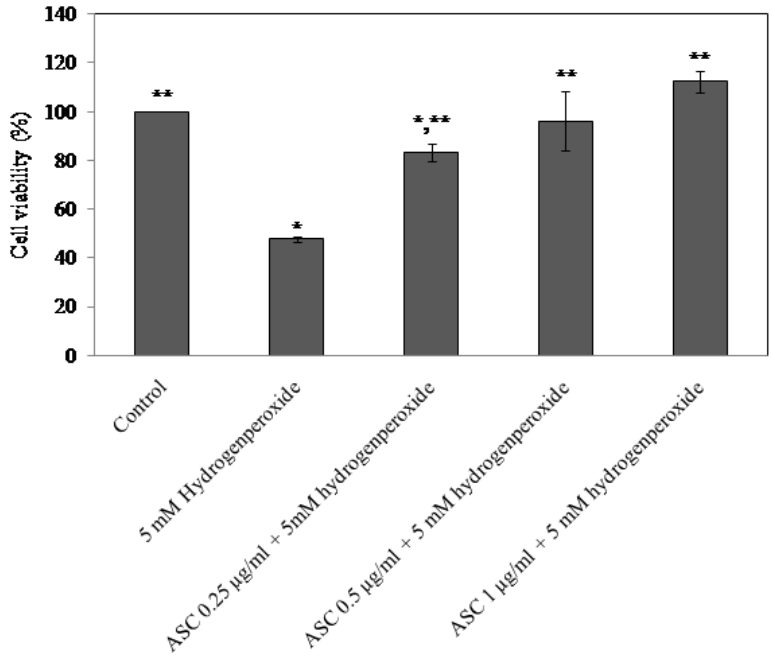

Figure 5 Protective effect of ASC on PBMC induced oxidative damage by H_2O_2. Data are means±S.E.M. of three measurements, *is significant when compared between control and H_2O_2 treated group and **is significant when compared with H_2O_2 treated alone treated groups at $p<0.05$

MTT assay for cell viability of ASC on H_2O_2 induce oxidative stress

PBMC were incubated with ASC for 30 min and then treated with 5 mM of H_2O_2 for 3 h, and then MTT assay was used. The increase of cell viability shows that the ASC extract is not toxic to the immune cell and it potentially modulates the cellular immune response [21]. After treated with H_2O_2 alone the viability of PBMC cell was significantly decreased to 50% and cell viability was significantly increased to 83.13±3.57–112.32±4.47 % when 0.25–1 µg/ml of ASC was treated as protective agent when compared to H_2O_2 treated alone and the highest protective effect was shown on 1 µg/ml ASC treated and was not significant to control group (Figure 5).

DISCUSSION

Syzygium cumini (L.) Skeels leaves contain various medicinal compounds and were used for treatment in various pharmaceutical preparations. It is well-known that natural phenolic compounds contribute to quality and nutritional value in terms of modifying color, taste, aroma and flavor and also in providing health benefit effects. The natural phenolic compounds also serve in plant as defense mechanisms to counteract reactive oxygen species (ROS) in order to survive and prevent molecular damage and damage by microorganisms, insect, and herbivores [22]. Total phenolic compounds, including tannins and flavonoids have been reported to have multiple biological properties to possess general antimicrobial and antioxidant activity [23, 24]. The strong antioxidant activity of the ASC may be attributed to the phenolic compound and flavonoid content which were correlated with r^2 value at 0.93.

Methyl violet reacted as a trap with the hydroxyl radical produced in the Fenton system was decolorized and was monitored at 565 nm [18]. The pyrogallol auto-oxidation method is widely used for superoxide anion measurement. Superoxide anion was rapidly produced by the auto-oxidation of pyrogallol in alkaline solution and its amount was measured by determining the rate of absorbance's change in the solution at 325 nm. The compound sodium nitroprusside is known to decompose in aqueous solution at physiological pH 7.2 producing $NO^{\bullet}$ under aerobic condition, $NO^{\bullet}$ reacts with oxygen to produce stable products (nitrate and nitrite), which can be determined using Griess reagent. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride immediately read at 550 nm [19]. In this study, ASC scavenge hydroxyl radical, superoxide anion and nitric oxide in vitro indicated that the ASC may help to arrest the chain of reactions initiated by excess generation of $OH^{\bullet}$, $O_2^{\bullet}$ and $NO^{\bullet}$ that are effective to human health [25].

Reactive oxygen species such as H_2O_2 have been implicated in certain disease processes and aging. H_2O_2 was given to PBMC cell at 5 mM for 3 h in this experiment as optimum condition. It is known that, when H_2O_2 was added directly to cell culture medium, H_2O_2 concentration was quickly decreased by cellular catalase and glutathioneperooxidase. Bechoua et al. [10] were found that treated with H_2O_2 for 10 min with concentration of 3 mM did not alter cell viability. Thus, to increase more concentration and keep on incubated cell with H_2O_2 for longer time can drop PBMC cell viability. No cytotoxic effect of ASC on PBMC at range of concentration from 0.25 – 2.5 µg/ml but at the concentration of 5 – 500 µg/ml was dropped the cell viability down to 60%. This finding suggested that the high concentration of ASC may contain high capacity of antioxidant which can affect the PBMC immune cell. The protective effect of ASC was significantly increased PBMC cell viability by H_2O_2 induced oxidative damage at 1 µg/ml which is due to the strong potential on free radical scavenging capacity. However, the mechanism of the cytotoxic effect on ASC activated PBMC remains unclear but the action of ASC on scavenging activities can be marked as a potential natural antioxidant.

CONCLUSION

The results obtained in this study have considerable value with respect to the scavenging activities of aqueous extract of *Syzygium cumini* (L.) Skeels. There is a strong positive correlation between phonolic and flavonoid contents and showing its promising to be exploited as primary antioxidant. Moreover to apply the ASC in to cytotoxic test shown excellent protection on ROS induced oxidative stress which can be claimed as a good source of natural antioxidant against ROS caused disease.

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