Sesamol induces apoptosis in human platelets via reactive oxygen species-mediated mitochondrial damage

R.M. Thushara, M. Hemshekhar, K. Sunita, M.S. Kumar, S. Naveen, K. Kemparaju, K.S. Girish

Department of Studies in Biochemistry, University of Mysore, Manasagangothri, Mysore 570 006, Karnataka, India
Department of Biochemistry, Government College for Women, Mandya, Karnataka, India
Biochemistry and Nutrition Discipline, Defence Food Research Laboratory, Siddarthanagar, Mysore 570011, India

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Platelets play an indispensable role in human health and disease. Platelets are very sensitive to oxidative stress, as it leads to the damage of mitochondrial DNA, which is the initial step of a sequence of events culminating in the cell death through the intrinsic pathway of apoptosis. Owing to a lot of reports on secondary complications arising from oxidative stress caused by therapeutic drug overdose, the present study concentrated on the influence of sesamol on oxidative stress-induced platelet apoptosis. Sesamol, a phenolic derivative present in sesame seeds is an exceptionally promising drug with lots of reports on its protective functions, including its inhibitory effects on platelet aggregation at concentrations below 100 μM, and its anti-cancer effect at 1 mM. However, the present study explored the toxic effects of sesamol on human platelets. Sesamol at the concentration of 0.25 mM and above induced platelet apoptosis through endogenous generation of ROS, depletion of thiol pool, and Ca2+ mobilization. It also induced mitochondrial membrane potential depolarization, caspase activation, cytochrome c translocation and phosphatidylserine exposure, thus illustrating the pro-apoptotic effect of sesamol at higher concentration. However, even at high concentration of 2 mM sesamol effectively inhibited collagen/ADP/epinephrine-induced platelet aggregation. The study demonstrates that even though sesamol inhibits platelet aggregation, it has the tendency to elicit platelet apoptosis at higher concentrations. Sesamol has a potential as thrombolytic agent, nevertheless the current work highlights the significance of an appropriate dosage of sesamol when it is used as a therapeutic drug.

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1. Introduction

Platelets are the discoid-shaped blood cells originating from bone-marrow megakaryocytes, and have a lifespan of around a week. They have well recognized roles in hemostasis, thrombosis, wound healing and angiogenesis. A shortfall in platelet production and function may lead to unrestrained bleeding, while upregulated platelet activation may be the basis for arterial thrombosis. Altered platelet functions are also the root cause of the pathophysiology of multifactorial diseases including coronary heart disease and other cardiovascular diseases (CVDs) [1]. They also release a whole host of biologically active substances like growth factors, arbitrate inflammation and are a major source of pro-inflammatory molecules [2]. Although platelets are anuclear, they do undergo apoptosis, a process of programmed cell death. The events of platelet apoptosis via the intrinsic pathway include only the cytoplasmic events initiated by the increased rate of endogenous reactive oxygen species (ROS) principally hydrogen peroxide (H2O2) [3]. Apart from contributing towards the cell’s energy metabolism, the role of mitochondria as pivotal mediators of intrinsic pathway of apoptosis is well documented. The primary target of oxidative stress is mitochondrial DNA (mtDNA) and its damage leads to the down regulation of electron transport chain, eventually leading to an intensification of ROS generation and formation of mitochondrial permeability transition pore (MPTP) resulting in the inner transmembrane potential depolarization [4]. H2O2 induces changes in mitochondrial membrane integrity, followed by leakage of cytochrome c (cyt c) from the inner mitochondrial membrane into the cytoplasm. The cytoplasmic cyt c along with pro-apoptotic factors such as Apoptotic Protease Activating Factor-1 (Apaf-1) mediate the activation of caspase 9 (elicitor caspase), which in turn activates caspase 3 (executioner caspase). Ultimately there is externalization
of phosphatidylserine (PS), which is a signal from the apoptotic cells for phagocytosis [4,5]. During the process the cells also release phosphatidylserine (PS)-positive membrane fractions called microparticles (MPs). Activated platelets and MPs actively participate in the promulgation of atherosclerosis and related diseases, pathologies of the central nervous system and neoplasia [6].

Human platelets reportedly undergo apoptosis upon stimulation with a suitable agonist or under stress and are very sensitive to nutritional and therapeutic components in the circulatory system [7]. There are numerous studies of therapeutic compounds influencing platelet functions. Thereby, the present study focuses on sesamol, a therapeutic compound reported to possess potent antiplatelet activity [8]. Sesamol (3,4-methylenedioxyphenol or 1,3-benzodioxol-5-ol), a phenolic derivative of sesame seed (Sesamum indicum L.) lignans, is reported to possess antioxidant potential. It has a plethora of therapeutic potentialities such as antiaging, hepatoprotective, neuroprotective, antiinflammatory, chemoprotective, chondroprotective, and anti-arthritis properties [9,10]. Besides, it is also used in the production of drugs for depression [11]. Recent studies have reported that sesamol in the concentration range 2.5–100 μM inhibits platelet aggregation by increasing the rate of cyclic AMP (cAMP) formation and attenuating the Nuclear Factor-kappa B (NF-κB) signaling events, thus depicting it to be an anti-thrombotic and cardioprotective compound [8,12]. However, the present study sought to demonstrate the effect of sesamol at higher concentrations on platelet functions, which is unknown hitherto. Moreover, to the best of our knowledge, there is no data regarding the effect of sesamol on platelet apoptosis. Whilst, there are reports of sesamol at the concentration of 1 mM exhibiting pro-apoptotic effects on mouse Leydig tumor cell line and endothelial cells [13,14].

2. Materials and methods

2.1. Chemicals/reagents

Rotenone, calcium ionophore A23187, benzamidine hydrochloride, glutaraldehyde, N-acetyl-Leu-Glu-Asp-Asp trifluoromethylcoumarin (AC-LEHD-FMC), acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AC-DEVD-AMC), sodium orthovanadate (Na3VO4), fluorescein isothiocyanate-labeled annexin V, 5-(and-6)-chloromethylfluorescein diacetate acetyl ester (CM-H2DCFDA), dithiothreitol (DTT), 5',5'-6',6'-tetrachloro-1',3',3'-tetraethyl benzimidazolylcarbocyanine iodide (JC-1), leupeptin hydrochloride, N-(2-hydroxyethyl) piperazine-N'-ethanesulfonic acid (HEPES), enhanced chemiluminescence detection reagents, and hyperfilm ECL were purchased from Sigma Chemicals, St. Louis, USA. Homovanillicacid (HVA) was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Sesamol (purity – 98%) was purchased from Acrros Organics, New Jersey, USA. Horseradish peroxidase-conjugated rabbit anti-rabbit IgG antibody and anti-cytocchrome c antibody were purchased from Epitomics, Inc., Burlingame, USA. Collagen was from Vitrogen 100; Cohesion, Palo Alto, CA USA. All other reagents were of analytical grade.

2.2. Preparation of sesamol solution

Sesamol was dissolved in 0.5% Dimethyl sulfoxide (DMSO) to obtain the solution of required concentration. DMSO (0.5%) was chosen as the solvent based on literature survey, and also preliminary studies with the solvent control neither showed any adverse effects on platelets nor did it influence the results. Further, the dosage of sesamol for the following assays was also fixed on the basis of preliminary studies.

2.3. Preparation of washed platelets

Washed platelets were prepared according to the method of Kumar et al. [15]. Briefly, venous blood was drawn from healthy drug-free human volunteers (non-smokers) with informed consent as per the guidelines of Institutional Human Ethical Committee (IHEC–UOM No. 72/Ph.D/2012-13), University of Mysore, Mysore. It was immediately mixed with acid citrate dextrose (ACD) anticoagulant (85 mM sodium citrate, 78 mM citric acid and 111 mM d-glucose) in the ratio 6:1 (blood:ACD v/v). It was then centrifuged at 90× g for 15 min and the supernatant thus obtained was the platelet-rich plasma (PRP). For the preparation of washed platelets, the PRP was centrifuged at 1700× g for 15 min at 37 °C. The platelet pellet thus obtained was washed after suspension and incubation in Tyrode’s albumin buffer (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 0.5 mM Na2HPO4, 1 mM MgCl2, 6 mM glucose, and 0.3% bovine serum albumin) pH 6.5 for 10 min at 37 °C. The platelets were again washed with Tyrode’s albumin buffer by repeating the previous step. Finally, the platelets were suspended in the Tyrode’s albumin buffer, pH 7.4. The platelet count was determined in both PRP and washed platelet suspension using Neubauer chamber. For all the following assays, the platelet count was adjusted to 5×107 cells/mL in the final suspension using Tyrode’s albumin buffer (pH 7.4).

2.4. Determination of endogenously generated reactive oxygen species (ROS)

An ROS-sensitive fluorescent probe CMH2DCFDA was used to detect endogenous ROS production as described by Lopez et al. [3], with slight modifications. Pre-treated (with 10 μg/mL calcium ionophore A23187 as standard agonist or with varying concentrations of sesamol as test group for 1 h at 37 °C) and control (untreated) PRP as well as washed platelet suspensions were made up to a final volume of 200 μL with HEPES-buffered saline (HBS) HEPES-buffered saline (HBS), pH 7.45, containing 145 mM NaCl, 10 mM HEPES, 10 mM d-glucose, 5 mM KCl, 1 mM MgSO4 and supplemented with 0.1% Bovine Serum Albumin (BSA), incubated with 10 μM CMH2DCFDA in a polystyrene 96-well microtiter plate for 30 min at 37 °C. Fluorescence was recorded with a Varioskan multimode plate reader (Thermo Scientific, USA) by exciting the samples at 488 nm and the resulting fluorescence was measured at 530 nm.

2.5. Determination of endogenously generated H2O2

According to the method of Barja [16], endogenously generated H2O2 in platelets was evaluated using HVA as a specific H2O2-sensitive fluorescent probe. Pretreated and control PRP as well as washed platelets (as described in Section 2.3) were incubated with HVA (100 μM) for 30 min at 37 °C, centrifuged and the pellets were suspended in HBS. Fluorescence was recorded using the multimode plate reader by exciting the samples at 312 nm and the resulting fluorescence was measured at 420 nm.

2.6. Estimation of total thiol, GSH and GSSG levels

The total thiol level in platelets was measured according to the method of Mokrasch and Teschke [17], with minor modifications. The assay mixture consisted of 20 μL treated (with 2 mM H2O2 as standard control) and varying concentrations of sesamol as test) and control PRP and washed platelet suspension were made up to 100 μL with buffered formaldehyde (1:4 V/V, 1 mL formaldehyde: 0.1 M Na2HPO4) kept at 37 °C for 5 min then 150 μL of 0.1 M phosphate buffer (pH 8.0 containing 5 mM EDTA) and 25 μL of Orthohaldehyde (1 mg/mL) were added incubated at 37 °C for 45 min.
and the fluorescence was recorded using fluorescence plate reader by exciting the sample at 340 nm and the resulting fluorescence was measured at 425 nm. For the measurement of oxidized glutathione (GSSG), equal volume of 0.04 M N-ethylmaleimide (NEM) is added to the treated platelet suspensions and incubated for 30 min to prevent GSH from undergoing further oxidation. The difference between total thiol and GSSG levels gives the GSH levels.

2.7. Estimation of intracellular calcium

Intracellular Ca\(^{2+}\) concentration was measured in PRP and washed platelets as described previously [18]. Treated and control PRP and washed platelets [as described in Section 2.3] were taken in 96-well polystyrene microtiter plates and the final volume made up to 200 μL with modified Tyrode's solution (pH 7.4) containing NaCl (150 mM), KCl (2.7 mM), KH\(_2\)PO\(_4\) (1.2 mM), MgSO\(_4\) (1.2 mM), CaCl\(_2\) (1.0 mM), and HEPES (10 mM) with 1% BSA and incubated for 1 h at 37 °C to induce the release of Ca\(^{2+}\) from the intracellular Ca\(^{2+}\) stores. The platelets were then incubated for 45 min at room temperature with 2 μM fura-2/AM, a fluorescence Ca\(^{2+}\) indicator. The cells were subsequently washed twice with the modified Tyrode's solution to remove the dye from the extracellular fluid and finally the platelet pellet suspended in modified Tyrode's solution. The fura-2/AM absorption was determined by exciting the cells at 340 and 380 nm and the resulting fluorescence was measured at 500 nm. Data were presented as absorption ratios (340/380 nm).

2.8. Determination of changes in mitochondrial membrane potential (ΔΨm)

Changes in the ΔΨm were detected using JC-1, a cationic dye. JC-1 aggregates in mitochondria at high membrane potentials and emits red fluorescence, whereas, at low membrane potential it is converted to a green fluorescent monomeric form. Treated and control PRP as well as washed platelet suspensions (as described in Section 2.5) were loaded withJC-1 (10 μg/mL) and incubated at 37 °C for 10 min. The cells were then excited at 488 nm and emission was detected at 585 nm for JC-1 aggregates and 516 nm for JC-1 monomers using multimode plate reader and the emission ratios (585/516) were calculated. Changes in ΔΨm following the treatment with the agonist were considered as the integral of the decrease in JC-1 fluorescence ratio [19].

2.9. Preparation of platelet lysate

Platelet lysate was prepared by immediately lysing the treated and control platelets from PRP and washed platelet suspension with an equal volume of 2× Triton buffer (2% TritonX-100, 2 mM EGTA, 100 mM Tris/HCl – pH 7.2, 100 μg/mL leupeptin, 2 mM PMSF, 10 mM benzamidine, 2 mM Na\(_3\)VO\(_4\)) at 4 °C for 30 min. The lysate was centrifuged at 16,000 × g for 5 min. The pellet thus obtained is the cytoskeleton-rich (Triton-insoluble) fraction, which was subjected to caspase activity and Western blotting [20].

2.10. Detection of cytochrome c release

Cytochrome c (cyt c) release was measured according to the method of Lopez et al. [21]. The cytosolic fractions from the treated and control platelets (as described in Section 2.5) were subjected to immunoblotting. Cytosolic proteins were separated by 10% SDS-PAGE and electrophoretically transferred on to a nitrocellulose membrane for 1 h at 20 V using a wet blotter. Blots were then incubated overnight with 10% BSA in tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein binding sites followed by incubation with anti-cytochrome c antibody (1:1000) in TBST for 2 h. Finally, they were incubated with horseradish-peroxidase (HRP)-conjugated anti IgG antibody (1:10,000) in TBST, exposed to enhanced chemiluminescence for 3 min and then exposed to photographic films. β-Actin was used as loading control.

2.11. Assay of caspase activity

Caspase activity was determined by the method of Amor et al. [22]. The cell lysate (from treated and control platelets) is incubated in a 96-well polystyrene microtiter plate with substrate solution (20 mM HEPES – pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT and 8.25 μM caspase substrate AC-DEVD-AMC for caspase 3 or AC-LEHD-AFC for caspase 9) for 2 h at 37 °C. Substrate cleavage was measured with a multimode plate reader (excitation wavelength 360 nm and emission at 460 nm).

2.12. Determination of PS externalization

PS scrambling was determined by the method of Rosado et al. [23]. Treated and control PRP as well as washed platelet suspension samples were transferred to equal volumes of ice-cold 1% (w/v) glutaraldehyde in PBS for 10 min, and then incubated in 0.6 μg/mL buffered aqueous solution of annexin V fluorescein isothiocyanate for 10 min. This was followed by centrifugation for 60 s at 3000 × g and the cells were collected by suspending the pellet in PBS. Cell staining was analyzed in a multimode plate reader by exciting the samples at 496 nm and emission was recorded at 560 nm.

2.13. Platelet aggregation

Platelet aggregation was determined by turbidimetric method with a dual channel Chrono-log model 700-2 aggregometer (Havertown, USA) [15]. Briefly, 240 μL of PRP was incubated at 37 °C in a siliconized glass cuvette and pre-incubated with different concentrations of sesamol (0–200 μg/mL) for 3 min, and the aggregation was initiated by the addition of collagen (10 μg/mL)/ADP (10 μM)/epinephrine (10 μM). To test whether high concentration sesamol itself causes platelet aggregation, platelets were treated with sesamol alone i.e., without the addition of any standard platelet agonists. The aggregation was then followed with constant stirring at 900 rpm for 6 min. Aggregation induced by collagen/ADP/epinephrine alone was considered 100% aggregation.

2.14. Statistical analysis

Results are expressed as mean ± SEM of five independent experiments. Statistical significance among groups was determined by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. Here, data were shown as mean ± SEM (n = 5), p < 0.05 (a*) was considered to be significant.

3. Results

3.1. Effect of on sesamol on endogenous generation of ROS and H\(_2\)O\(_2\), thiol levels and Ca\(^{2+}\) in platelets

Treatment of platelets with positive control A23187 (positive control) induced 2.84 and 2.65 folds increase of ROS in PRP (Fig. 1A) and washed platelets (Fig. 1B) respectively. While, it induced an increase of 1.9 fold in the generation of H\(_2\)O\(_2\) both in PRP (Fig. 1C) and washed platelets (Fig. 1D). Treatment of PRP with sesamol at increasing concentrations induced a dose-dependent increase in ROS and H\(_2\)O\(_2\) levels the platelet samples. At a concentration of 1 mM sesamol induced maximum of 4.32 and 6.3 folds increase of ROS in PRP and washed platelets respectively. While, it induced a
maximum of 2.27 and 2.1 folds increase of H$_2$O$_2$ in PRP and washed platelets respectively.

Sesamol (1 mM) brought down the GSH:GSSG ratio to a maximum of 65% in PRP as compared to 97% in control platelets. In washed platelets, the GSH:GSSG ratio was reduced to a maximum of 57% as compared to 93% in control platelets (Fig. 2A). Sesamol was also found to induce a dose-dependent decrease in the total thiol levels by a maximum of 35% and 71% respectively in PRP and washed platelets (Fig. 2B and C).

Considering the increase in intracellular Ca$^{2+}$ induced by A23187 (positive control) to be 100%, sesamol at the concentration of 1 mM induced mobilization of intracellular Ca$^{2+}$ by 77% and 93% in PRP (Fig. 3A) and washed platelets (Fig. 3B) respectively.

### 3.2. Effect of sesamol on caspase activity

Treatment of both PRP and washed platelets with 2 mM H$_2$O$_2$ (positive control), at 37°C for 2 h, induced the activation of caspase 3 by 1.4 and 1.58 folds respectively. Activation of caspase 9 by H$_2$O$_2$ reached 1.82 and 2.7 folds increase in PRP and washed platelets respectively. Stimulation of platelets with sesamol in the concentration range 0.25–1 mM induced the activation of caspases in a
concentration-dependent manner. At the concentration of 1 mM sesamol induced a maximum of 1.93 and 2.38 folds increase of caspase 3 and 9 activities respectively in PRP (Fig. 4A). In washed platelets, 1 mM sesamol could evoke a maximum activity of 3.8 and 4.4 folds respectively for caspases 3 and 9 (Fig. 4B).

3.3. Sesamol triggers $\Delta \Psi m$ depolarization and PS scrambling

Rotenone (positive control) treatment (10 $\mu$M) for 1 h at 37 °C evoked maximal decrease in JC-1 fluorescence and was considered as 100% $\Delta \Psi m$ dissipation. Treatment of platelets with sesamol at a concentration range 0.25—1 mM evoked a dose-dependent increase in the percentage of $\Delta \Psi m$ dissipation. At the concentration of 1 mM, sesamol was able to cause 105% and 126% $\Delta \Psi m$ dissipation in PRP and washed platelets respectively (Fig. 5A and B).

Treatment of PRP and washed platelet suspensions with 2 mM H$_2$O$_2$ (positive control) for 1 h at 37 °C induced 1.55 and 1.97 folds increase in PS exposure respectively. Treatment of platelets with sesamol in the concentrations range 0.25—1.0 mM significantly induced PS externalization in a concentration-dependent manner. Sesamol at 1 mM induced a maximum of 1.84 and 2.16 folds increase in PS exposure in PRP and washed platelets respectively (Fig. 6).

3.4. Effect of sesamol on cytochrome c release

Treatment with 2 mM H$_2$O$_2$ (positive control) for 1 h at 37 °C induced cyt c release into the cytosol, which was detected by Western blotting technique. Treatment with sesamol at concentrations of 0.5, 0.75 and 1.0 mM triggered the cyt c release by the
platelets in a concentration-dependent fashion (Fig. 7 i). Fig. 7 ii represents the immunoblot of β-actin, which was used as the loading control.

3.5. Effect of sesamol on platelet aggregation

Treatment of PRP with sesamol alone at different concentrations (up to 2 mM) did not induce aggregation (data not shown). Conversely pre-treatment with sesamol at different concentrations followed by agonist treatment resulted in a dose-dependent inhibition of platelet aggregation. Sesamol (1 mM) was able to inhibit collagen-induced aggregation by 99% (Fig. 8A), at 0.5 mM it was able to inhibit ADP-induced aggregation by 75% (Fig. 8B) and at 5 μM it was able to inhibit epinephrine-induced aggregation by 96% (Fig. 8C). Fig. 8D depicts the graphical representation of the above data showing the percentage platelet aggregation.

4. Discussion

Sesame seeds and the oil have been a part of human diet in different parts of the world from time immemorial. Sesame has been traditionally considered as a food with nutritional and healing properties in the Indian subcontinent, which has been scientifically validated by several studies. Sesamol is the major constituent of sesame seed oil. It being a phenolic antioxidant renders sesame oil less vulnerable to oxidative deterioration than other oils. Besides, it is an effective free radical scavenger [24]. Numerous reports attribute oxidative stress to the development of several diseases such as cancer, CVDs, atherosclerosis, and Alzheimer’s disease [14,25–27]. Moreover, platelets have a role to play in these diseases. Therefore, the effect of oxidative stress and sesamol on platelets entailed a detailed investigation. Earlier reports are suggestive of the cytotoxic effect of sesamol (1 mM) on cancer cells. Therefore, the concentration range 0.25–1 mM was chosen to assess the effect of sesamol on platelets. With the results achieved in the current study, an interesting finding was made. Although sesamol inhibits aggregation in platelets, it was found to stimulate apoptosis at higher concentrations (≥0.25 mM). Focus was tended to the mitochondrial pathway as it is this pathway that is mainly activated by factors contributing to oxidative stress in the cell including activation of tumor suppressor genes, UV radiation, chemotherapeutic and dietary constituents, which leads to changes in mitochondrial membrane integrity. Sesamol, a dietary component, in its purified form is reported to possess chemotherapeutic property by virtue of its capacity to set off apoptosis in neoplastic cells. The present study asserts that sesamol has the potentiality to stimulate apoptosis even in platelets through the mitochondrial pathway.

In the first set of experiments, oxidative stress markers such as ROS, H2O2 and GSH were measured in platelets. Sesamol treatment induced a dose-dependent increase in ROS generation and it was found to be more potent than A23187 (positive control) at higher concentrations. Reports indicate that H2O2 is the predominant ROS which stimulates the apoptotic events in platelets via the intrinsic pathway by altering the Δψm [3]. Hence the endogenous generation of H2O2 was also measured. Here again sesamol-treated platelets showed dose-dependent increase in H2O2 generation. Fujimoto et al. [14] have earlier reported that the oxidation products of sesamol namely, trimer and tetramer exhibit cytotoxic effects on rat thymocytes and human leukemia K562 cells, but there are no reports of sesamol itself inducing free radical generation.

Further, sesamol induced a concentration-dependent reduction in the GSH:GSSG ratio as well as, total thiol levels. Considering the fact that GSH is a ubiquitous master antioxidant in all cells, this effect seems to be an obvious response to the increased ROS levels in the platelets following sesamol treatment. The normal GSH:GSSG ratio of 98% is very important to maintain the cellular...
health. Reduction in this ratio indicates a disease condition and it means that increasing amount of GSH (reduced glutathione) is being converted to GSSG (oxidized glutathione) by the pro-oxidants such as H2O2 in the cell. In addition, the depletion in the thiol pool further aggravates the stress condition.

Experiments were carried out to assess the influence of sesamol on platelet mitochondria. The current results demonstrate that sesamol at concentrations higher than 0.5 mM is more powerful than 10 μg/mL rotenone in evoking depolarization of ΔVm. It is suggestive of sesamol’s toxic effects on mitochondria, thereby paving way for the subsequent events of the apoptotic pathway. To further establish the potentiality of sesamol in inducing ΔVm depolarization, its effect on intracellular Ca2+ was investigated. The presence of high concentration of Ca2+ in the cytosol is one of the factors responsible for the changes in ΔVm and formation of MPTP [28]. Sesamol was found to trigger the increase in intracellular Ca2+ concentration.

Increased permeability of inner and outer membranes of mitochondria resulting from the depolarization, leads to the release of the pro-apoptotic proteins from mitochondria to cytosol, which is regarded as a reliable parameter to identify an apoptotic cell. The immunoblot of cytosolic cyt c in sesamol-treated platelets clearly points out the increase in protein concentration with corresponding increase in sesamol dosage. Once cyt c is released into the cytosol, it combines with Apaf 1 and procaspase 9 resulting in the formation of apoptosome. Subsequently, the prosapase 9 self activates to caspase 9, which leads to the activation of procaspase 3 to caspase 3. Caspase 3 being the executioner caspase provokes the disintegration of cytoskeletal proteins, which leads to morphological changes in the platelets [29]. Sesamol was capable of evoking the activation of both the caspases 9 and 3 in a concentration-dependent manner. These findings further confirm the pro-apoptotic effects of sesamol in platelets. Furthermore, it was shown that sesamol was also able to induce PS scrambling, a prominent biochemical feature of an apoptotic cell, which is a distinctive signal for phagocytic cells, finally leading the demise of the cell.

To investigate whether sesamol at high concentrations can induce platelet aggregation as well, aggregation studies were carried out. Sesamol alone, even at high concentrations (up to 1 mM) did not induce aggregation; on the other hand it was not only able to inhibit collagen-induced aggregation as reported in previous study, but also inhibited other agonist-induced (ADP and epinephrine) aggregations as evident by the present study. In an earlier study, Chang et al. [8] have reported that sesamol inhibits collagen-induced platelet activation via Ca2+ mobilization, thromboxane A2 (TXA2) formation, and phospholipase C γ2 (PLC γ2), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) phosphorylation. It also increases cAMP and cGMP levels, expression of endothelial nitric oxide synthase (eNOS) and NO release. In yet another study, Chang et al. [12] have further advanced the investigation wherein they found that sesamol (2.5–25 μM) stimulates cAMP-PKA signaling, subsequently inhibits NF-
kB-PLC-PKC cascade, thereby leading to attenuation of Ca$^{2+}$ mobilization and inhibition of platelet aggregation. ADP-induced platelet aggregation occurs via the coordinated activation of two G protein–coupled receptors, P2Y1 and P2Y12. P2Y1 is coupled to PLCβ and therefore binding of ADP to P2Y1 leads to the activation of PLCβ resulting in the granular secretion, TxA2 production and platelet shape change, finally culminating in platelet aggregation. On the other hand, binding of ADP to P2Y12 leads to the inhibition of adenylate cyclase and activation of fibrinogen receptor αIIbβ3 finally resulting in platelet aggregation. Like ADP, epinephrine is known to inhibit adenylate cyclase activity mediated by α2a-adrenergic receptor. However, there are no reports on the mechanism of inhibition of ADP- and epinephrine-induced platelet aggregation by sesamol. Thus, it would be interesting to investigate the effect of sesamol on these pathways. On the whole, from the current study and the previous studies it can be said that sesamol at concentrations below 0.1 mM inhibits platelet aggregation via the attenuation of the classical pathways, without inducing apoptosis. In contrast, sesamol at concentrations above 0.25 mM elicits apoptosis in platelets and also inhibits aggregation (Fig. 9).

Apart from sesamol, resveratrol and thymoquinone are hitherto known phytochemicals that induce platelet apoptosis. Similar to sesamol, resveratrol (5–25 μM) inhibits platelet aggregation stimulated by collagen, and induces platelet apoptosis. Thymoquinone (≤5 μM) was shown to elicit platelet apoptosis in a PI3K-dependent manner, through a GPCR family receptor [30]. Results from the current study suggest that even though sesamol can be deemed as a thrombolytic and cardio-protective agent by virtue of anti-platelet activity, at higher concentrations (>0.25 mM) it has a propensity to induce apoptosis in platelets. Thus, the study strongly emphasizes on the critical evaluation of the toxicological aspects of phytochemicals before depicting them to be therapeutic drugs. Further, it also highlights that the extreme use of sesame seeds in day to day life may have adverse consequences due to loss of platelets. For instance, there is a report indicating the occurrence of thrombocytopenia and concomitant petechiae due to the consumption of tahini (pulped sesame seeds) [31,32]. The amount of sesamol in sesame seed oil is 0.26–0.32 mg/100 g, whereas the sesamol in tahini can be as high as 10.98–12.33 mg/100 g. The sesamol in halva (sweet meat made from sesame seeds) samples is reported to be 8.24–9.12 mg/100 g [33]. All said and done, there are few questions regarding the physiological relevance of high sesamol concentrations that still need to be answered. How much sesamol intake is needed to reach these intracellular concentrations? And can these concentrations of sesamol ever be reached in the plasma? Thus, further research in animal model is required to answer the above questions.

5. Conclusion

Previously, studies have explored the pro-apoptotic effects of therapeutic compounds such as resveratrol and thymoquinone [7,34]. The current piece of work stresses upon the pro-apoptotic effects of yet another therapeutic compound sesamol, at concentrations higher than 0.25 mM on human platelets. Apoptotic platelets reportedly release MPs, which cause fibrin deposition by making available effective surface area for the assembly of coagulation factors and thus aggravate thrombus formation. Circulating MPs are also regarded as pro-inflammatory particles that advance coagulation and influence vascular functions. These events augment the development of thrombotic disorders and CVDs. Thereby, MPs are considered to have a significant role in the disease etiology of CVDs [35]. Sesamol inhibits platelet aggregation at doses less than 100 μM as reported in earlier studies, and even at higher concentrations as observed in the present study, thus depicting it to be anti-thrombotic and cardioprotective. However, the study proves that it may turn toxic to platelets at higher doses (>0.25 mM). Since doses more than 0.25 mM up to 1 mM have been used to induce apoptosis in tumor cells, the current study highlights the importance of the dosage of sesamol when used as a therapeutic drug. There exists a

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**Fig. 9.** A schematic representation depicting the proposed mechanism of the differential action of sesamol on platelets, at different concentrations.
fine line between its beneficial effects at lower concentrations and detrimental effects at higher concentrations. Hence, it is absolutely necessary for a patient undergoing sesamol treatment, to be kept under constant vigilance. Besides, by virtue of its wide range of therapeutic properties, sesamol is used as one of ingredients in dietary supplements. However, reports of it turning into cytotoxic derivatives under oxidative conditions prompt us to keep in mind the storage environment for sesamol and sesamol-containing products so as to prevent oxidation.

Conflict of interest

The authors declare that there are no conflicts of interest.

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