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Research article

Impact of the phytochemicals cocktail "breast safeguard" in regulating the interplay between redox signalling and murine adenocarcinoma cell proliferation, survival and angiogenesis

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ABSTRACT

Phytochemicals are natural plant extracts with a potent antioxidant, anti-inflammatory and anticancer characteristics by acting as a cell signalling modulator. This study aims to evaluate the effect of a commercial cocktail of phytochemicals "Breast safeguard" (BSG) in upregulating the expression of antioxidant enzymes to counteract signalling pathways that promote Ehrlich cells progression. The potent antioxidant activity and total phenolics and flavonoids contents of BSG was chemically validated, BSG treated mice showed a significant reduction at the tumor size, along with significant reduction in the expression of prognostic markers CEA and TNFα and induction of cell cycle arrest at G1/S phase as well as downregulation of Ki67. BSG supplementation significantly diminished H_2O_2, NO, MDA levels and upregulated the expression of SOD, CAT, GPx and GSH antioxidant enzymes in plasma and tumor tissues. BSG treatment markedly activated P53/Bax/Bcl2/c-caspase 3 signalling for cell apoptosis and attenuated the expression of antiapoptotic survivin protein. Meanwhile, BSG significantly diminished the expression of VEGF as an indication of angiogenesis inhibition.

In conclusion, BSG exerted a significant upregulation of antioxidant enzymes which may be involved in upregulating P53/Bax/c-caspase 3 expression and attenuation of cell proliferation and angiogenesis.

1. Introduction

Breast cancer is one of the most dreaded diseases threatening female lives worldwide. Since 1970s the incident rate of breast cancer among women has dramatically increased by 30–40% [1, 2]. Ehrlich ascites carcinoma (Eh.) is one of the models that has been developed to study growth and proliferation of mammary cancerous cells. Due to its inability to adhere to a synthetic substrate; Eh. cells were passage from mouse to mouse via I.P transplantation [3]. Despite the research efforts directed to develop new chemotherapeutic drugs, this approach of treatment has two major limitations. First, the non-specific targeting of cancer cells; leading to off target effects on normal cells. Second, is the ability of cancer cells to develop a resistance against drugs with a high incidence of tumor recurrence [4]. These facts shed the light on the urgency to find alternative approaches to the currently used protocols. Phytochemicals are natural plant extracts known by their high biological activities. Last decade, several studies illustrated the anti-inflammatory, anti--proliferative and anti-cancer effects of phytochemicals [5, 6, 7].

Recent researches elucidated the effective prophylactic role of plant extracts in targeting several molecules and signalling pathways involved in cancer development and progression [8]. The blue green algae extract, C-Phycocyanin showed a potent anticancer characteristics via sensitizing the colon cancer cell lines for ionizing radiations by acting as an inhibitor for COX-2 signalling [9]. Indole-3-carbinol acts as an estrogen receptor antagonist to induce mammary cancer apoptosis and inhibition of cell proliferation using a xenograft model of canine inflammatory mammary cancer [10].

In carcinogenesis, Phytochemicals showed efficacy in inhibiting the onset of cancer by upregulating the expression of cytoprotective genes that drive the synthesis of anti-oxidant enzymes and carcinogen detoxifying enzymes. The effectiveness of phytochemicals as anti-oxidants comprehends their ability to antagonise the carcinogen dependent

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cellular stress of redox-imbalance and genotoxic damage [11]. Furthermore, the upcoming *in vivo* and *in vitro* data indicated a significant effect of phytochemicals, as a chemo-preventive agents, either independently or synergistically on induction of cell cycle arrest and cell apoptosis in treated cancers [12, 13].

The downregulation of antioxidant enzymes expression in cancer cells was correlated to nuclear localization of NFκB, which in turn stimulates the transcription of pro-inflammatory and anti-apoptotic proteins leading to cancer progression [14]. Thus, targeting of oxidative stress and its regulated signalling cascades in cancer cells by plant and fruit extracted phytochemicals may be considered as an imperative goal of researchers in this endeavour.

Ouhtit et al., illustrated the effect of a combination of six phytochemicals (resveratrol, quercetin, indole-3-carbinol, c-phycocyanin, curcumin and genistein) on halting breast cancer progression via targeting several signalling pathways of cell proliferation, apoptosis and metastasis [6].

Stemming from these data, a commercial product of these phytochemicals was developed as a nutritional supplement under the name of "breast safeguard" (BSG) for women breast health. Recently, BSG showed a profound effect in sensitizing Hepatocellular carcinoma for ionizing radiations by targeting P53-dependent apoptosis and inhibition of cell proliferation and migration [15].

This study aimed to evaluate the possible effects of BSG, as a commercial product of a phytochemicals cocktail, on targeting adenocarcinoma cell proliferation, apoptosis and angiogenesis by modulating oxidative stress signalling using Eh. cells as an *in vivo* model of murine adenocarcinoma.

2. Materials and methods

2.1. Chemicals

BSG which consisted of seven different phytochemicals "curcumin, indol-3-carbinol, resveratrol, quercetin, C-Phycocyanin, genistein and gallic acid" was kindly provided by Dr. Mahdwa Raj (Proteigene, USA), Phosphate buffer saline, Collagenase enzyme (Gibco, CA, USA).

2.2. Assessment of BSG antioxidant activity

2.2.1. DPPH assay

A working solution of BSG was prepared by reconstituting each capsule (480 mg) in 1ml PBS contains 10% DMSO. The mixture was vortexed for 2 min, then centrifuge for 20 min at 260gx. and the supernatant was harvested. The DPPH assay was conducted by mixing 0.1 mM DPPH solution prepared in methanol (V:V) with a serial dilutions of BSG supernatant conc. The percentage of antioxidant activity was calculated by measuring the ability of BSG different conc. to scavenge the generated radicals of stable 1, 1- diphenyl 2-picrylhyorazyl (DPPH) (0.135 mM). A serial dilution of L-Ascorbic acid (1–100 μg/mL) was used as a reference standard according to the method described by Kedare and Singh [16]. After 30 min incubation in dark, the change at absorbance of three separate experiments were measured at 517 nm using spectrophotometer (DU 800; Beckman Coulter, Fullerton, CA, USA). The percentage of inhibition at free radicals was estimated according to the following Eq. (1):

$$\text{Inhibition } \% = \text{Ac–As/Ac} \times 100 \quad (1)$$

Whereas, Ac is the control absorbance, As is the sample absorbance.

The percentage of remaining DPPH of each tested concentration (conc.) was estimated according to the following Eq. (2):

$$\%\ \text{DPPH remaining} = [\text{DPPH}]T/[\text{DPPH}]T0 \times 100 \quad (2)$$

Where (T) is the conc. at the end of experiment and (T0) is the initial conc.

The IC 50 of BSG extracts concs. (mg/ml) was calculated by plotting BSG extracts conc. vs the decrease at the initial DPPH conc.

2.2.2. Detection/quantification of BSG phenolic and flavonoids content

2.2.2.1. Total flavonoids content. Total Flavonoid's content of BSG extract was quantified using aluminum chloride colorimetric assay developed by Zhishen et al., [17]. 1 ml of BSG extract or standard catechin solution (20, 40, 60, 80, 100 and 200 μg catechin/ml) was added to 4 ml distilled water. 0.3 ml of 5 % sodium nitrite was added and left for five minutes, 0.3 ml of 10 % aluminum chloride was added and incubated for 6 min. Then, 2 ml of 1 M sodium hydroxide was added the total volume was completed to 10 ml by distilled water, the absorbance was finally measured at 510 nm. Total flavonoids content was assessed as mg catechin equivalent per gm of BSG with reference to standard curve ($y = 0.003x$, $R^2 = 0.99$).

2.2.2.2. Total phenolic compounds content. The total phenolics content of BSG was measured using the modified Folin Ciocalteu colorimetric assay developed by Wolfe et al., [18]. 1 ml of the BSG extract or standard solutions of gallic acid (10, 20, 40, 60, 80, 100 and 150 μg gallic acid/ml) was added to 5 ml of Folin Ciocaltu reagent (10%). After 3 min, 4 ml of $NaCO_3$ (7.5%) was added, then vortexed for 20 s and incubated at 40 °C for 30 min in a dark place. The absorbance was measured at 765nm. Total phenolics content was assessed as mg gallic acid equivalent per gm of BSG, with reference to the standard curve ($y = 0.006x$, $R^2 = 0.99$).

2.3. Experimental design and animals grouping

Female Swiss albino mice weighing 25–30 gm were purchased from the animal house of the Egyptian national vaccine institute (Giza, Egypt). The animals were grouped in a randomized manner and housed under ambient room temperature at day/night cycle. They were then assigned into four groups of 10 mice each. Group I: negative control group, Group II (BSG group): BSG was orally given daily by intragavage (1 g/kg b.w.) for 2 weeks, Group III (Eh. Group): mice were injected once subcutaneously by 3×10^6 Eh. cells to develop solid tumor, Group IV (Eh.+BSG group): mice injected subcutaneously by 3×10^6cells of Eh., after tumor appearance (two weeks of injection) a dose of BSG 1 g/kg b.w. was given daily by intragavage for extra 2 weeks. At the end of experiment, mice were sacrificed and samples were collected from all groups and stored at-20 °C until biochemical analyses. The animal experiments in this research were conducted according to the code of ethics of Mansoura University (DZ18002).

2.4. Biochemical analysis

2.4.1. Anti carcinogenic effect of BSG

The anti-tumor effect of BSG was assessed by evaluating the change at mean tumors volume of the treated group in comparison with untreated tumors [19]. In addition, the expression of breast cancer progression markers CEA and TNFα was tested using quantitative ELISA according to manufactures' protocol (Mybiosource, CA, USA).

2.4.2. Oxidative stress in plasma and tumors tissue

Expression of NO, H_2O_2 free radicals and antioxidant enzymes super oxide dismutase (SOD), catalase (CAT), Glutathione peroxidase (GPx) and reduced Glutathione (GSH) was estimated in plasma of all groups. The expression of the same parameters was estimated in tumor tissues of group III and IV using quantitative Elisa approach according to manufactures' protocols (Mybiosource, CA, USA). The level of oxidative stress was estimated by evaluating the expression of MDA, in both plasma and tumor tissues using quantitative elisa according to manufactures' protocol (Mybiosource, CA, USA).

2.5. Flow cytometry

2.5.1. Cell cycle analyses

Cell suspensions of (1×10^5 cell) were prepared in phosphate buffered saline (PBS) after solid tumor digestion by collagenase enzyme. Afterwards, cells were fixed overnight in 70% ethanol at 4 °C. Then, Cells were incubated with propidium iodide (PI) in dark for 1 h at room temperature (Sigma, CA, USA). To eliminate aggregates, cells were filtered through a 30mm nylon mesh filter of flow cytometry tubes (BD Bioscience, US). The cell cycle profile analysis was conducted using CellQuest analysis program (FACS can, Becton Dickinson, Germany).

2.5.2. Cell apoptosis (annexin V)

The fixed cells were incubated with 2.5% of FITC- conjugated annexin V for 30 min, then nuclear staining of dead cells was conducted via incubating the cells in dark with PI for extra 30 min. The incidence of cell apoptosis and necrosis was indicated using FACScan software (Becton Dickinson, Germany).

2.6. Enzyme-linked immunosorbent assay (ElISA)

To assess the effect of BSG on angiogenesis and cell apoptosis, the expression of vascular endothelial growth factor (VEGF), Bax and Bcl2 in tumors tissues was estimated using quantitative ELISA according to the manufactures' protocols (Mybiosource, CA, USA).

2.7. Histopathology& immunohistochemistry

Tumors of group III and IV were collected and fixed in 10% formalin for histological studies. Paraffin embedded tissues were sectioned at 4 μm thickness and stained with Haematoxylin and Eosin (H&E) for histological examination. For immunohistochemical studies, antigen retrieval was performed by boiling the samples in 9 mmol/L citrate buffer (PH 6) (Invitrogen, CA, USA) for 25 min. Adjacent sections were examined for expression of VEGF, P53, Ki67 and c-caspase 3 (1:100 dilution; Genemed) and anti SVV polyclonal antibody (1:75 dilution; Santa Cruz, CA) HRP conjugated secondary antibody (1:500 dilution, Santa Cruz, CA). Staining was performed using the Vector lab detection kit according to manufactures' instructions (Vector Lab, CA, USA).

2.8. Statistical analyses

Differences between mean values were assessed for statistical significance using the two-tailed Student's *t*-test (GraphPad Prism 5.0 software, La Jolla, CA). *P* values <0.05 were considered statistically significant.

3. Results

3.1. Characterization of BSG antioxidant activity

The ability of different conc. of BSG extracts to scavenge DPPH generated free radicals was compared to ascorbic acid as a reference standard. The data revealed a marked effect of BSG in scavenging DPPH generated free radicals in a dose dependent manner. Meanwhile, the IC50 of BSG extract was calculated (0.00696 mg/mL) while the IC50 of the reference standard ascorbic acid was (0.023 mg/mL). The lower IC50 of BSG compared to ascorbic acid revealed its potency as an antioxidant (Table 1). Assessment of phenolics and flavonoids content of BSG extract, which was conducted compared to reference standards of gallic acid and catechins, respectively, indicated appreciable levels of phenolics (109.71 mg gallic acid equivalent/gm BSG); whereas flavonoids content was (18.935 mg catechin equivalent/gm BSG). The high phenolic and flavonoid content of BSG may elucidate its potent antioxidant activity.

3.2. Effect of BSG on tumor progression

In order to study the curative effect of BSG in diminishing the tumor growth, experimental animals were divided into four groups. The data showed a non-significant effect of BSG treatment on the mean of change in body weight nor mice survival (data not shown). At the end of experiment, effect of BSG on tumor growth was determined by assessing the mean change at tumors volume for groups III and IV which indicated a significant effect of BSG treatment (two weeks) on diminishing the tumor growth. Histopathological examination of solid tumors sections of group IV showed BSG dependent increase in multiple apoptotic areas with few islands of Eh. cells that have signs of disintegrating nuclei. Meanwhile, photomicrograph of untreated Eh. solid tumor showed sheets of higher chromatophilic tumor cells of variable shape signifying proliferating cells surrounding necrotic area at the core due to rapid tumor growth. The antitumorigenic effect of BSG treatment was further confirmed by evaluating the expression of tumor progression markers CEA and TNFα in plasma of the four studied groups using quantitative ELISA approach. The data indicated a significant increase in plasma levels of CEA and TNFα in untreated group of Eh. adenocarcinoma (group III) which was significantly disputed by BSG treatment in group IV (Figure 1).

3.3. BSG anti-oxidative effects

3.3.1. In blood plasma

The plasma of the four groups was collected and screened for the levels of free radicals and expression of the antioxidant enzymes SOD, CAT GPx and reduced glutathione (GSH) and total antioxidant capacity (TAC). The results showed a significant impact of cancer growth on upregulating the plasma levels of H_2O_2 and NO and diminishing the expression of the studied anti-oxidant enzymes in comparison to the negative control. Meanwhile, the BSG treated groups showed a significant effect in lessen the generation of H_2O_2 and NO and incrementing of the expression of SOD, CAT, GPx, and the levels of GSH and TAC (Figure 2). The effect of BSG on downregulating the incidence of oxidative stress in plasma of the four studied groups was revealed via the significant downregulation at the level of lipid peroxidation marker MDA compared to its levels in plasma of untreated mice (Figure 2).

3.3.2. In tumor tissue

Similarly, to what have been indicated in blood plasma, assessment of oxidative stress status in Eh. Solid tumors showed a significant effect of BSG treatment in deescalating the rate of H_2O_2 and NO production. Meanwhile, a significant upregulation at the expression of SOD, CAT, GPx and GSH generation was recorded in BSG treated tumors in comparison with untreated tumors. This upregulation at antioxidant enzymes expression in tumors of BSG treated mice led to a significant downregulation at the MDA cellular levels (Figure 3).

Table 1. Assessment of BSG antioxidant activity using DPPH assay. This approach estimate the ability of a serial dilution of BSG extract to scavenge the DPPH generated free radicals.

Stock soln.	Sample conc.(mg/mL)	% Remaining DPPH	% inhibition	IC_{50} mg/mL
Concentration 480 mg/mL BSG	0.12	29.56	70.44	0.00696
	0.06	57.06	42.92	
	0.03	77.79	22.21	
	0.015	90.44	9.56	

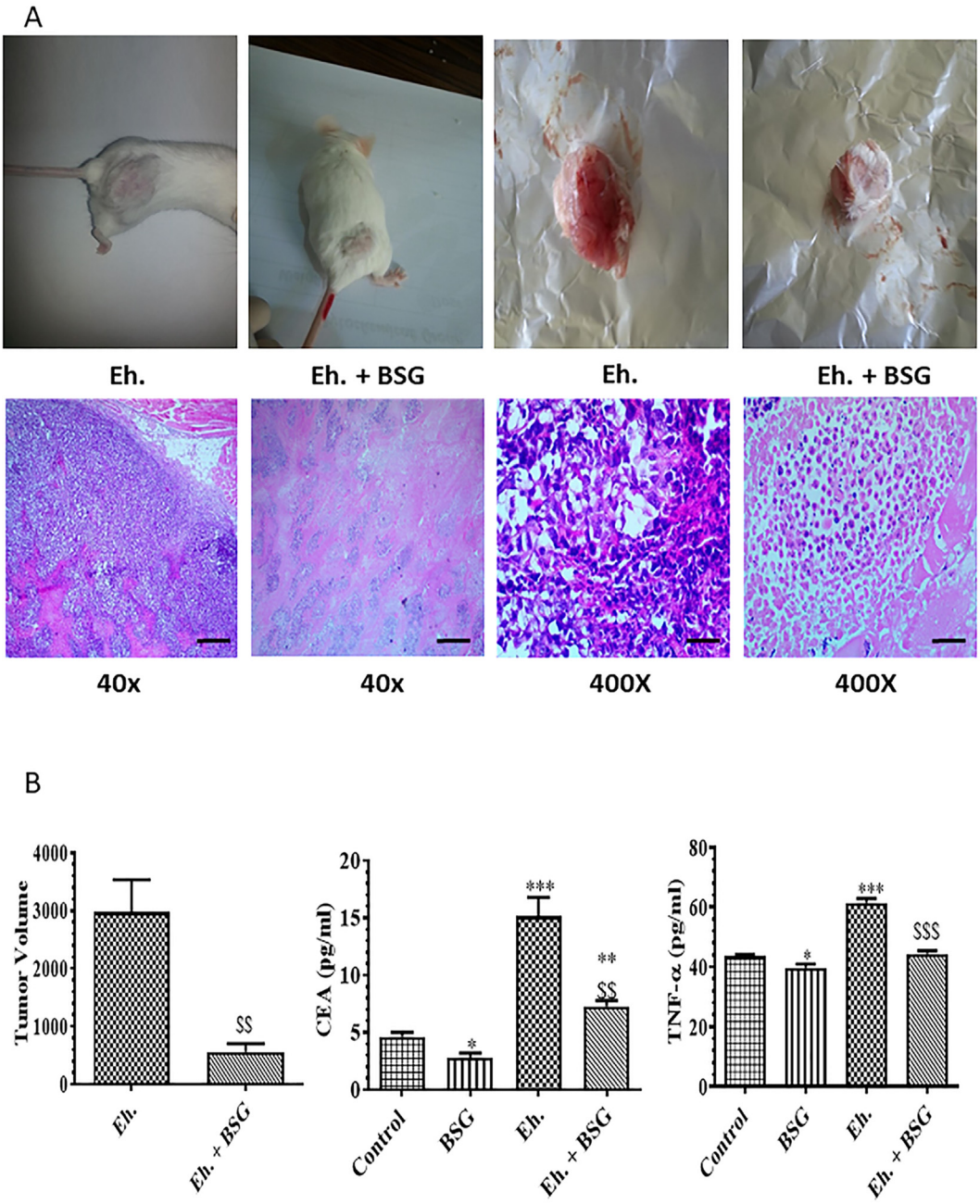

Figure 1. Effect of BSG on tumor growth. (A) Photography showing the decrease at tumor volume upon BSG treatment. Histopathological examination of tumor tissues indicated the BSG marked induction of cell apoptosis (disintegrating nuclei) with few spots of Eh. cells. (B) Statistical analyses of the expression of tumor progression markers CEA and TNFα indicated significant effect of BSG in downregulating their expression. Data were presented as mean of five tumors ±SEM. Significance was denoted as *<0.05, **<0.001, ***<0.0001 as compared to Eh. group. Significance was denoted as $<0.05, $$<0.001, $$$<0.0001 as compared to control.

3.4. BSG inhibits angiogenesis and cell proliferation

In order to assess the effect of BSG on cancer cell proliferation, flowcytometric cell cycle analyses were carried out. The data determined a significant effect of BSG in induction of cell cycle arrest at G1/S phase in BSG treated tumors compared to untreated tumors. In addition, the up-regulation of tumor cell populations at sub G1 phase in the BSG treated mice indicated a profound effect of BSG in induction of cell death. These data were further confirmed by immunolocalization of cell proliferation marker Ki67 in nuclei of Eh. solid tumor tissues. The nuclei of untreated Eh. tumors showed a marked expression of Ki67 which was demolished in BSG treated group (Figure 4a).

In order to assess the effect of BSG treatment on angiogenesis, tissues of Eh. solid tumor were evaluated for the expression levels of VEGF using quantitative ELISA approach. The resulting data revealed a significant effect of BSG on demolishing the levels of VEGF in BSG treated tumors compared to the untreated tumors of Eh. adenocarcinoma. These data were further confirmed via immunolocalization of VEGF in sections of Eh. solid tumor using immunohistochemistry approach (Figure 4b).

3.5. BSG significantly induced Eh. tumor cell apoptosis

The notable induction of cell death that has been indicated at sub G1 phase of cell cycle profile of BSG treated tumors was confirmed by double

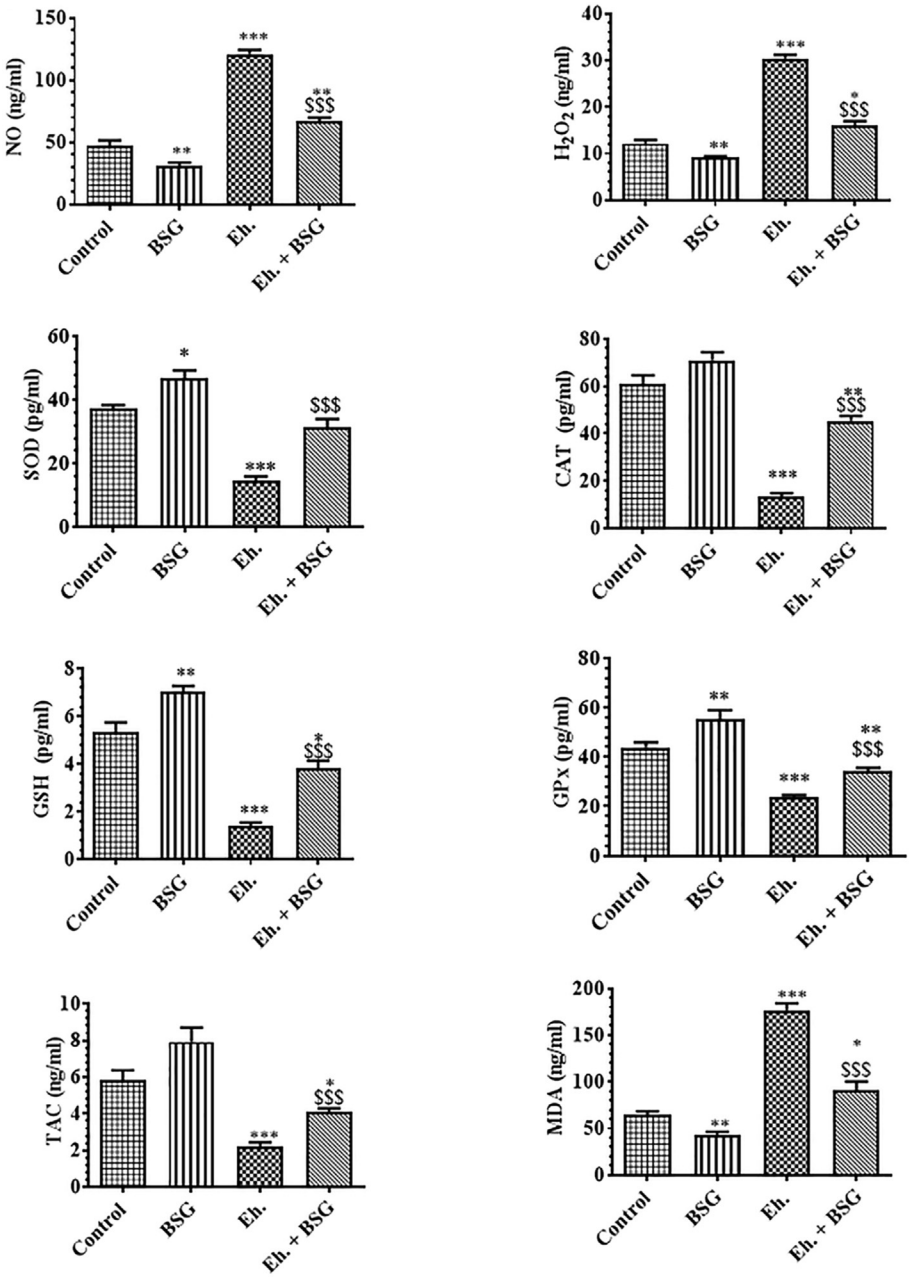

Figure 2. Effect of BSG on plasma oxidative stress. Quantitative Elisa approach was applied to determine a significant effect of BSG in reducing plasma levels of H_2O_2, NO free radicals' generation via upregulating the expression of SOD, CAT, GPx and GSH antioxidant enzymes. Data were presented as mean of five samples ±SEM. Significance was denoted as *<0.05, **<0.001, ***<0.0001 as compared to Eh. group. Significance was denoted as $^{\$}$<0.05, $^{\$\$}$<0.001, $^{\$\$\$}$<0.0001 as compared to control.

labelling of the solid tumors harvested cells with PI and annexin V. The data revealed a significant reduction in cell viability of BSG treated Eh., which was correlated with a significant upregulation at percentage of apoptotic and necrotic cells.

For further illustration of our results, the pro-apoptotic effect of BSG was confirmed initially by immunohistochemical localization of Pro-apoptotic P53 in tumor tissues which indicated a marked retrieval at the expression of P53 in BSG treated group in comparison with untreated tumors which have minimal signs of P53 expression. Meanwhile, immunoassay of the ratio of Bax/Bcl2 expression in solid tumors of both BSG treated and untreated mice using quantitative ELISA, showed a significant BSG dependent upregulation of pro-apoptotic Bax and downregulation of anti-apoptotic Bcl2 expression in comparison with untreated Eh. tumors (Figure 5). The positive labelling of the active form of caspase 3 (C-caspase 3), as a downstream target of Bax signalling for apoptosis, in sections of BSG treated tumors, indicated a significant effect of BSG in upregulating c-caspase 3 expression (Figure 5).

In the same context, the nuclear expression of anti-apoptotic protein SVV (IAP family member) was markedly recognized in sections of untreated tumors, which was markedly vanished in nuclei of the tumors of BSG treated mice upon BSG treatment (Figure 5).

4. Discussion

The low incidence of colon cancer in Asian countries, which are known by food meals rich with phytochemicals, such as soy beans products, shed the light on the significant implication of phytochemicals to either prevent the onset and/or diminishing cancer progression [20, 21, 22]. A recent study by our group was directed to use a cocktail of phytochemicals to target several signalling pathways to attenuate the progression and survival of both primary and metastatic human breast cancer cell lines MCF7 and MDA-MB 231 [6]. Relying on this work we developed a commercial product of this cocktail of phytochemicals named "Breast safeguard" for breast health.

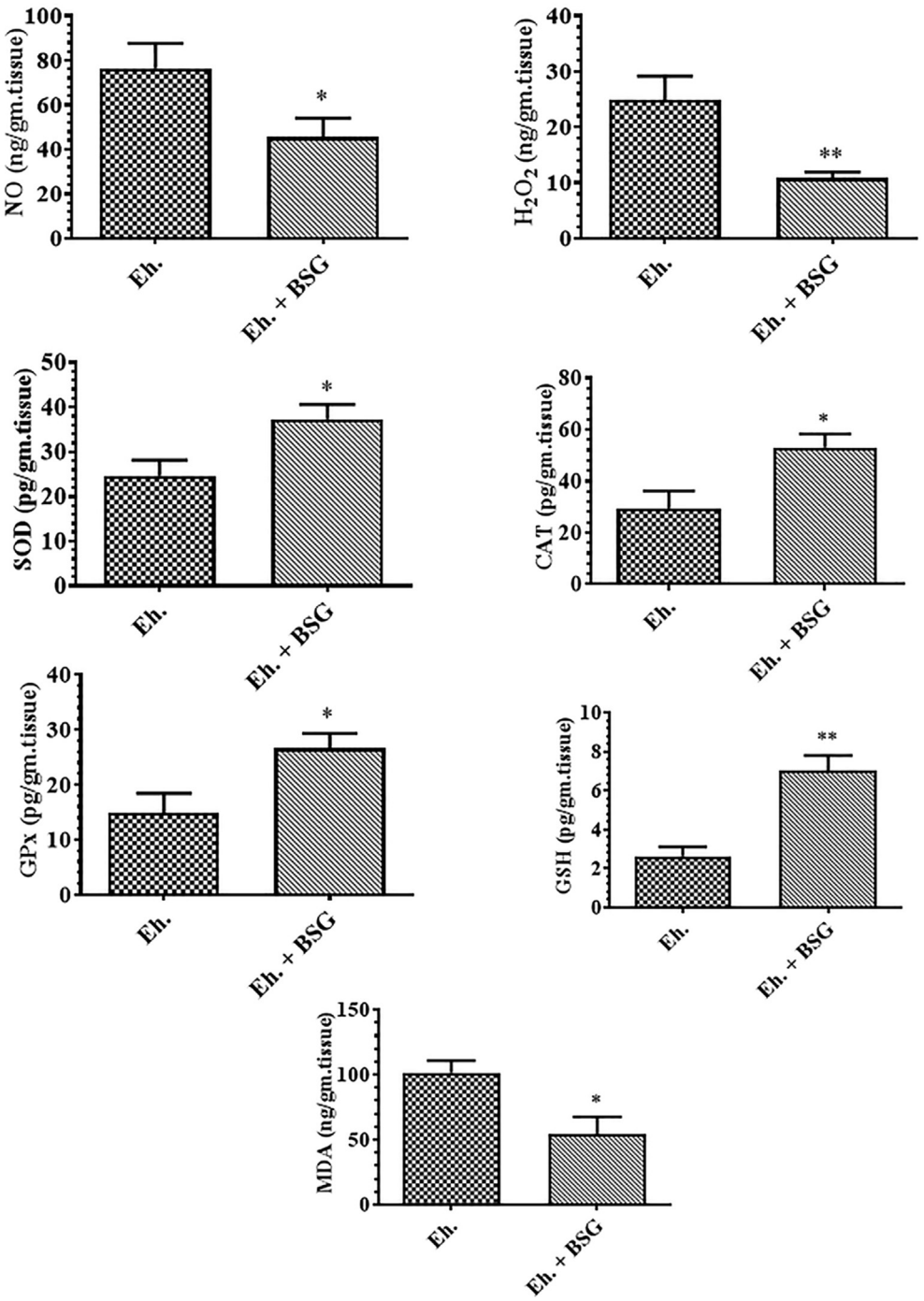

Figure 3. Effect of BSG on tumor tissue oxidative stress. Tumor tissues were processed for quantitative Elisa for determination of free radicals levels and antioxidant enzymes expression. Significant effect of BSG in reducing tumor tissue levels of H_2O_2, NO free radicals generation via upregulating the expression of SOD, CAT, GPx and GSH antioxidant enzymes. Data were presented as mean of five samples ±SEM. Significance was denoted as *<0.05, **<0.001, ***<0.0001 as compared to Eh. group.

The potent antioxidant activity of BSG was chemically validated by elucidating the significant effect of BSG in eradicating the free radicals generated by DPPH in a dose dependent manner, which indicated by the lower IC_{50} of BSG compared to ascorbic acid as a reference standard (Table 1). This potent antioxidant activity of BSG was further illustrated via its recorded high content of both polyphenols and flavonoids. Several literatures attributed the antioxidant activity of phytochemicals to both the total phenolics and flavonoids contents due to their high scavenging or chelating capabilities [23, 24, 25, 26].

The inhibitory effect of BSG on tumor growth was first illustrated by the decrease at the tumors mean volume and the histological presence of multiple apoptotic bodies in treated group. These findings were confirmed by the decrease at plasma levels of the tumor prognosis markers CEA and TNFα in treated tumors (Figure 1). The role of CEA and TNFα as prognostic markers of cancer progression was elucidated by several literatures [27, 28, 29]. Berberoglu *et al.*, correlates the down-regulation at serum levels of TNFα and the responsiveness of breast cancer patients' to neoadjuvant chemotherapy [30]. Literature survey illustrated the correlation between TNFα expression and tumor progression indicating its possible use as prognostic marker for chemotherapy [31, 32].

The rapid growth of cancer cells has a direct impact on increasing the level of ROS and/or RNS due to the cancer cells high metabolic rate and relative hypoxia [33]. This elevation at oxidative stress would stimulate several signalling pathways such as TNFα/NFκB and FOXOs which act as transcriptional factors activating the expression of proteins responsible

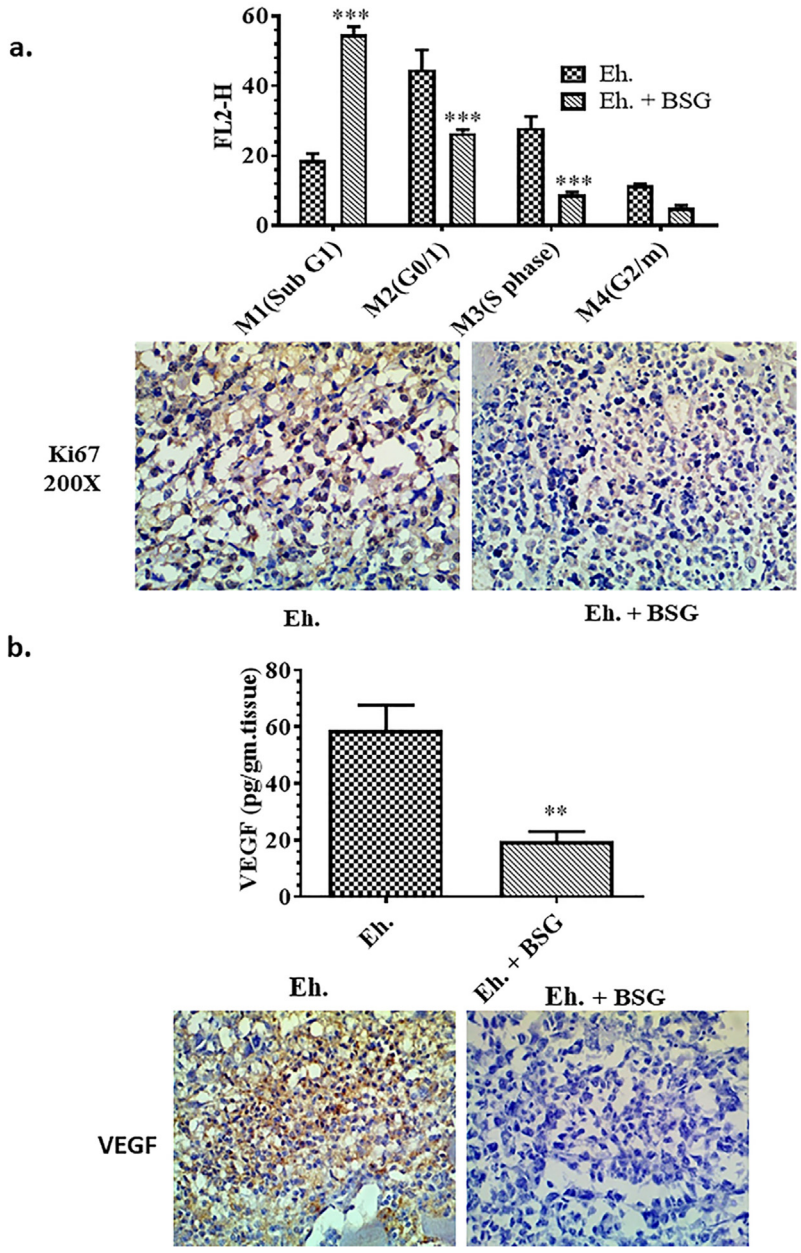

Figure 4. Effect of BSG on tumor dependent angiogenesis and cell proliferation. (a.) The expression of VEGF was assessed in tumor tissue using quantitative Elisa and Immunohistochemistry approaches. Data revealed a significant effect of BSG in downregulating VEGF expression to inhibit neo-angiogenesis process. (b.) The effect of BSG on cell proliferation were determined via cell cycle analysis and Ki67 expression. Data revealed a significant effect of BSG in ceasing cell cycle at G1/S phase and significant downregulation of Ki67 expression. Data were presented as mean of three replicates ±SEM. Significance was denoted as *<0.05, **<0.001, ***<0.0001 as compared to Eh. group.

for cancer cell proliferation and survival in a positive feedback mechanism [34, 35].

The potent anti-oxidant effects of BSG were illustrated by the significant effect of BSG in downregulating the level of H_2O_2 and NO and upregulating the expression of anti-oxidant enzymes SOD, CAT, GPx and GSH generation upraising which leads to significant reduction at the rate of MDA generation in tumor tissues and blood plasma (Figures 2 and 3). These data came in the same contest with what have been established of the antioxidant potentiality of BSG phytochemicals constituents [36, 37]. The tumor level of oxidative stress has a direct relation with the activation of signalling pathways involved in tumor cell proliferation, survival and metastasis [33, 38, 39].

In the same contest, the effect of BSG on downregulating the level of free radicals and antioxidant enzymes led to direct induction of cell cycle arrest at G1/S phase and a marked downregulation of the cell proliferation marker Ki67 expression illustrating the inhibitory effect of BSG on proliferation of murine adenocarcinoma (Figure 4a). The usage of ki67 as prognostic marker for cancer cell responsiveness to chemotherapy was recorded [40, 41].

The BSG dependent reduction of tumor oxidative stress has a direct impact on its molecular signalling for tumor angiogenesis which was indicated by the reduction at expression of VEGF in BSG treated tumors (Figure 4b). Several literatures illustrated the contribution of ROS upraise in signalling for VEGF upregulation and promotion of angiogenesis [42, 43, 44]. The role of VEGF in signalling for angiogenesis which is critical step for cancer cell growth, survival and metastasis is a fact that been established by several literatures [20, 45].

The significant effect of BSG on induction of late apoptosis in treated solid tumors was initially recorded by cells double labelling with PI/annexin V. Furthermore, the molecular mechanisms via which the BSG attenuation of oxidative stress was involved in induction of tumor cell apoptosis was assessed. BSG treatment markedly upregulated the expression of P53 in tumor tissues which significantly upregulated the expression of its signalling downstream target proapoptotic Bax and

downregulated Bcl2 expressions to induce tumor cell apoptosis. The marked elevation at the expression of cleaved caspase-3, as an end target of Bax activation of mitochondrial dependent apoptosis was recorded as well (Figure 5). This effect could be claimed to the profound effect of BSG in demolishing the redox-imbalance which has a direct effect on inactivation of P53. The P53 dependent apoptosis signalling cascade includes the stimulation of Bax expression which in turn leads to caspases activation via activation of cytochrome C release [46].

The implication of free radicals and anti-oxidant enzymes balance in regulation of cell apoptosis in cancer was illustrated by several literatures [47, 48]. The proliferation dependent induction of oxidative stress in cancer cells has a direct impact on inactivation of various tumor suppressor proteins such as P53 and Rb [38, 49]. The levels of ROS and/or RNS were considered as one of the main factors implicated in P53 activation and inactivation. Low and moderate RS will lead to activation of P53 which in turn activate mitochondrial dependent apoptosis. Meanwhile, high level of RS will lead to P53 inactivation by oxidating the cysteine residues of P53 [50,33].

In addition, the reduction at SVV expression in treated tumors elucidated the marked role of BSG in targeting several signalling pathways in order to induce apoptosis (Figure 5). IAP family member, SVV is a known anti-apoptotic protein that expressed in drug resistant tumor cells to inhibit caspases activation [51]. The expression of SVV is regulated by different cell receptors such as CD44 and TNFα which stimulated the nuclear localization and binding of transcription factors E2F1 and NFκB to SVV promoter, respectively [52, 53, 54, 55].

This *in vivo* study illustrated the role of BSG as a cocktail of phytochemicals in demolishing cancer progression via targeting signalling pathways that regulates cell proliferation, survival and angiogenesis. These effects were accomplished as a result of BSG dependent oxidative stress downregulation.

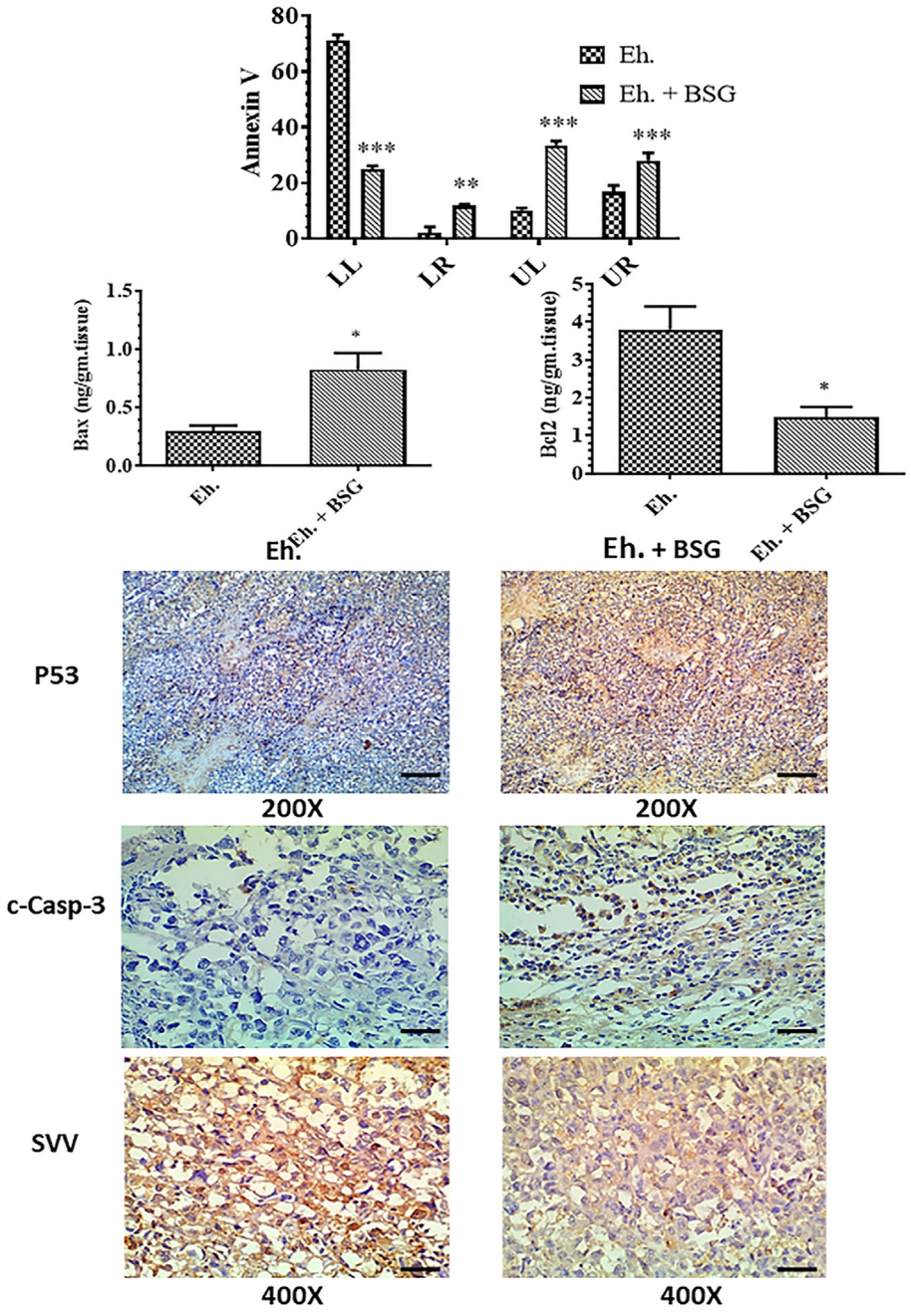

Figure 5. BSG significantly induced apoptosis in treated tumors. (a.) Double labeling of BSG treated Eh. cells with AnnexinV/PI indicated a significant induction of cell apoptosis. Quantitative Elisa results showed a significant BSG dependent upregulation of pro-apoptotic Bax expression and correlated downregulation of Bcl2 expression. (b.) Immunohistochemical localization indicated a marked increase of pro-apoptotic P53 and c-caspase 3 expression and downregulation of anti-apoptotic SVV nuclear expression in BSG treated tumors (two weeks). Data were presented as mean of three replicates ±SEM. Significance was denoted as *<0.05, **<0.001, ***<0.0001 as compared to Eh. group.

Declarations

Author contribution statement

Mohamed E. Abdraboh: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Daw Saad Daw: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Ali M. AbouEl-ezz: Performed the experiments; Wrote the paper.

Wafaa M. El-Kholy: Conceived and designed the experiments; Analyzed and interpreted the data.

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Data availability statement

The authors do not have permission to share data.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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