Assessment of two different extraction methodology on the anticancer activity of a commercial cocktail of phytochemicals on proliferation and migration of liver cancer

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Assessment of two different extraction methodology on the anticancer activity of a commercial cocktail of phytochemicals on proliferation and migration of liver cancer

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Abstract

Phytochemicals are natural products which extracted from vegetables, fruits or plant roots by chemical methods. The interest in phytochemicals as anticancer agents were progressively developed in the last decades as they are safer than chemotherapy on human health. Breast safeguard (BSG), the drug under study, is a commercial product consisted of seven phytochemical compounds. This study aims to assess the impact of two different extraction approaches in increasing the efficacy of BSG in targeting HepG2 cells. The extraction of BSG was conducted by either 20% DMSO or 100% DMSO then diluted to 20% concentration using a complete media of cell culture. The phenolics and flavonoids contents and the antioxidant activity using DPPH assay was evaluated in both extracts. The effect of the two extracts on HepG2 cell proliferation and migration was tested using MTT assay and wound healing assay, respectively. The data revealed a significant effect of the second approach (100% DMSO) in increasing the antioxidant activity of BSG indicated by the significant increase at phenolic, flavonoid content confirmed by the marked decrease at IC50 compared to ascorbic acid as a reference standard. MTT assay indicated a significant effect of the second approach in inhibiting HepG2 cell proliferation in a dose dependent manner compared to the first approach (20% DMSO). Estimation of the wound size of the treated cells revealed a marked effect of second approach in inhibiting cell migration in comparison with the first approach. We concluded that the extraction of phytochemicals content using 100% DMSO not the 20% pre-diluted DMSO would provide a significant increase at the phenolic and flavonoid content which in turn lead to a significant increase at antioxidant activity and significant inhibition at HepG2 cell proliferation and migration pointing out the important role of the method of extraction plays for good assessment of BSG as a potent anticancer agent.

Keywords: Phytochemicals, Breast safeguard, Docetaxel, HepG2, liver cancer.

1. Introduction

Cancer is an abnormal cell growth in an uncontrolled manner with the ability for dissemination to another distant organs forming new tumor (Eble and Niland, 2019). Liver cancer is the fifth cause of death worldwide. The exact cause of liver cancer is not known yet, but researchers have identified Cirrhosis, a condition marked by scarring of the liver, as a major risk factor for liver cancer (Rashed et al., 2020). In early stages, symptoms of liver cancer could not be recognized but in late stages patient can suffer from jaundice, weight loss, loss of appetite and pain at the right side of the abdomen (Dayeh et al., 2014). In 2018, an estimated 18.1 million new cases of cancer occurred worldwide which are likely to increase to 23.6 million of new cases by 2030 (Choudhari et al., 2020). The World Health Organization reported in 2019 that cancer is considered as the first or second main cause of death in patients younger than 70 years old at 112 out of 183 countries worldwide (Sung et al., 2021). Hepatocellular carcinoma (HCC) ranked as the sixth most common cancer worldwide. In Egypt, it ranks the fourth common cancer (Rashed et al., 2020). HepG2 is an immortalized cell line of human liver carcinoma, derived from the liver tissue of a 15-year-old human who had a well differentiated hepatocellular carcinoma (Luckert et al., 2017). Treatment of liver cancer routinely carried out via adjuvant therapy which involves surgical operations, followed by chemo- and/or radiotherapeutic treatment (Wang et al., 2013). Several...
drawbacks were recorded for chemo- and radio-therapies in liver cancer including the high incidence of recurrence with a high mortality rate within 6 months of tumor appearance. Meanwhile, this approach of treatment showed several side effects on patients health due to their non-specific targeting of cancer cells (Abduljauwad et al., 2021). Hence, searching for an alternative approach instead of chemotherapy for liver cancer treatment was a target for several cancer researchers. Literature’s survey, pointed out phytochemicals as a potential target for cancer treatment due to their potent antioxidant, anti-inflammatory and anti-cancer characteristics (Ouhtit et al., 2014). Approximately, 50% of approved anticancer drugs from 1940 to 2014 originated from natural products or a direct derivative of their structure (Newman and Cragg, 2016).

Phytochemicals are natural compounds extracted from organic nature sources such as seeds, grains, plant leaves, herbs, fruits and vegetables. They can be classified into three main classes according to their functional chemical group as follows; terbinoids, polyphenols and thiols (Kotecha et al., 2016). Flavonoids, tannins, phenols, and alkaloids are bioactive compounds. In medicinal plants have a vital role for keep the cell in the best case. These bioactive compounds have OH group which can reduce the odd electron present in free radical. Antioxidants inhibit the oxidative damage by protecting the cell from oxidative stress produced from the excess in reactive oxidative species (ROS). The Presence of free radicals in the cell will cause damage for macromolecules which will leads to health problems (Khan et al., 2019).

Breast safeguard (BSG), the drug under study, consisted of seven different phytochemicals “quercetin, genistein, ellagic acid, curcumin, resveratrol, indol-3-carbinol and c-phycocyanin” which are extracted from tea, soyabeans, nuts, curcuma roots, grape seeds, cruciferous plants and spirulina platensis respectively (Ouhtit et al., 2014).

2. Materials and Methods

2.1. Chemicals and reagents

BSG is a commercial product consisted of seven different phytochemicals” Indole-3-carbinol, Quercetin, Resveratrol, Gallic acid, Geneticin, Curcumin and C-phycocyanin” that was kindly provided by Prof. Madhwa Raj the founder of Proteigene company. The culture medium consists of Gibco Dulbecco’s Modified Eagle's Medium (DMEM) supplemented with 10% Gibco fetal bovine serum (FBS) which used as a nutrient supplement to basal growth medium in cell culture applications, 1% Gibco Penicillin-Streptomycin contains 5000 units/mL of penicillin and 5000 µg/mL of streptomycin. The DMSO solvent was purchased from (Serva, Germany)

2.2. Chemical extraction of BSG:

The first extraction approach was conducted by addition of 4ml of complete media to 1ml of the solvent Dimethyl sulfoxide (20% DMSO) then BSG powder 500mg was added in 15ml falcon tube. The second extract was prepared in 100% (DMSO) by dissolving the BSG powder 500mg in 1ml DMSO, with vortex for 5 min, then the extract was diluted using 4ml of complete media in 15ml falcon tube, put the mixture again on vortex for 30 min then centrifuge at 260 gx at room temperature. The supernatant of both extracts was used for assessing of BSG antioxidant and anticancer activity.

2.3. Assessment of BSG antioxidant activity

2.3.1. DPPH assay:

The DPPH radical scavenging activity of the BSG supernatant samples were determined according to the reported method by Blois (1958). DPPH radical (0.012%) was prepared in methanol and then 1 mL of the solution was mixed with 1 mL of a serial dilution of the BSG supernatant samples. After incubation in the dark for 30 minutes at room temperature, the absorbance of the serial dilution of BSG samples was measured against a blank sample at 517 nm. The radical scavenging activity (RSA)% of the DPPH radicals was calculated from (Eq. (1)):

\[
RSA(\%) = \frac{Blank \ Abs-Sample \ Abs}{Blank \ Abs} \times 100
\]

Where, (Abs) is the absorbance

The Half maximal Inhibitory Concentration (IC50) value is inversely proportional to the free radical scavenging activity/ antioxidant property of the sample. The IC50 of the sample was obtained by a linear regression analysis curve plotting the different concentrations of a sample against the percentage of remaining DPPH compared to ascorbic acid as a standard reference. The data of samples and standard were calculated from (Eq. (2)):

\[
% \ DPPH^\prime \ remaining = \frac{\left[DPPH^\prime\right]_T}{\left[DPPH^\prime\right]_{T=0}} \times 100
\]

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

2.3.2. Determination of total phenolics content of BSG:

The total phenolics of the prepared extracts of BSG were determined using the modified Folin Ciocalteu assay described by Wolfe et al. (2003). 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 minutes, 4 ml of NaCO3 (7.5%) was added, then vortexed for 20 seconds and incubated at 40°C for 30 minutes in a dark place. The absorbance was measured at 765nm. The phenolics were expressed as milligram gallic acid equivalent/ gram BSG powder, with reference to standard curve (y = 0.006x).

2.3.3. Determination of total flavonoids content BSG:

Flavonoids content was determined using AlCl3 assay described by Zhishen et al. (1999). 1ml of the
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% aluminium chloride and at 6 minutes, 2 ml of 1 M sodium hydroxide was added then the total volume was completed to 10 ml by distilled water. The absorbance was measured at 510 nm. Total flavonoids were expressed as milligram catechin equivalent/gram BSG powder, with reference to standard curve (γ = 0.003x).

2.3.4. Determination of total Tannins content of BSG:
Tannins content was estimated by Vanillin hydrochloride assay (Gandhi et al., 2017). 5ml of vanillin hydrochloride reagent (8% HCl in methanol and 4% vanillin in methanol 1:1 v/v) was added to 1ml of the plant extract, then the change at the colour absorbance was measured at 500nm after 20 minutes of incubation. The tannins content was calculated as milligram gallic acid equivalent/gram BSG powder, with reference to standard curve (γ = 0.0009x).

2.4. The effect of BSG on cell proliferation and cytotoxicity:
HepG2 cells were cultured DMEM in Medium supplemented with 10% FBS, 1% Penicillin/Streptomycin. HepG2 cells were incubated at standard conditions of 37 °C and 5% CO₂. Cells were regularly subcultured using Trypsin (0.25%), during routine cell culture passaging. For detecting EC50 of the two extraction techniques, cells were cultured in 96 well plate (3000 cell/well) in 100μl of complete media. In second day, different concentrations of BSG (5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.06, 19.53 and 9.76 μg/ml) were added and untreated cells receives DMSO as a vehicle control. After 2 days of treatment, the EC50 of BSG and the inhibition at cell proliferation was measured at 570 nm (BIOTEK ELX 800, US) (Wilsher et al., 2017). The inhibition percentage in cell proliferation was calculated from (Eq. (3)):

\[
\text{Inhibition} = \frac{(\text{Control (OD)} - \text{Sample (OD)})}{\text{Control (OD)}} \times 100 \% \quad \text{Eq. (3)}
\]

Where, (O.D) is the optical density.

2.4.2. Wound healing assay
Cells were seeded in a six-well plate and let to grow to confluence. The monolayer cells were mechanically scratched using a 200-pipette tip to create a vertical wound, and cells were washed twice with PBS to remove floating cell debris. Cells were treated with a serial dilution of the calculated EC50 of both extraction approaches. For the first technique: the used concentrations of BSG were calculated to be 1066 μg/ml (EC50), 533 μg/ml and 266.5 μg/ml. For the second technique: the used concentrations of BSG were 263 μg/ml (EC50), 131 μg/ml and 65.7 μg/ml. The wounds are observed using phase contrast inverted microscopy over 33 hr. till wound closure (Martinotti and Ranzato, 2020).

2.5. Statistical analysis
Data were expressed as mean ± SEM. Student’s t-test was used to compare the mean differences between samples using Graphpad software version 6.01 (GraphPad Software, CA, USA). For all analyses, P < 0.05 was considered statistically significant.

3. Results
3.1. Assessment of BSG antioxidant activity
3.1.1. The ability of BSG to scavenge free radicals
The data revealed that the 2nd extraction approach showed a potent effect in scavenging the DPPH generated free radicals in a dose dependent manner compared to the 1st extraction approach, where The IC50 of BSG by 1st technique was found to be 1.2066 mg/ml, while the one of the 2nd extraction was 0.12356 mg/ml (fig. 1).

![Fig. (1): exponential curve calculating IC50 of BSG scavenging activity. DPPH assay data (Left panel) revealed a marked increase at the BSG conc. required to eradiate DPPH free radicals by 1st technique. (Right panel) The IC50 of the 2nd technique of extraction indicated a lower conc. of BSG required to scaveng free radicals compared to 1 st extraction approach which indicated its potent antioxidant activity.](image)

3.1.2. Assessment of total phenolics content
The phenolics content of BSG powder were assessed in both extracts. The data pointed out the high phenolic content of the 2nd extract of BSG (109.365 mg gallic acid equivalent/gm BSG) while the 1st extract of BSG showed a phenolic content (22.191 mg gallic acid equivalent/gm BSG). The gallic acid was used as a reference standard for phenolics content (Table 1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phenolics content mg gallic acid equivalent / g BSG</th>
<th>Flavonoids content mg Catechin equivalent /g BSG</th>
<th>Tannins content mg gallic acid equivalent / g BSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st technique</td>
<td>22.191 ±0.153</td>
<td>1.333 ±0.148</td>
<td>10.1 ±0.698</td>
</tr>
<tr>
<td>2nd technique</td>
<td>109.365 ±0.274</td>
<td>18.1025 ±0.137</td>
<td>30.944 ±0.012</td>
</tr>
</tbody>
</table>

3.1.3. Assessment of total flavonoids content
The 2nd extract of BSG showed a high flavonoids content (18.1025 mg Catechins equivalent/gm BSG) while the flavonoids content of the 1st extract of BSG was (1.333 mg Catechins equivalent/gm BSG). The catechin was used as a reference standard for flavonoids content (Table 1).

3.1.4. Assessment of total tannins content
Similarly, the total tannins contentment was assessed. The data showed that the tannins content of BSG by the 1st technique was 10.1 mg gallic acid...
equivalent/gm BSG while that by the 2nd technique was 30,944 mg gallic acid equivalent/gm BSG. This indicated a marked effect of the 2nd approach of extraction in upraising BSG antioxidant capacity (Table 1).

3.2. The effect of BSG on HepG2 cell toxicity and proliferation

Treatment of HepG2 cells with different concentrations of both extracts showed a significant dose dependent effect in inhibiting the ability of cells to proliferate. Furthermore, the effect of 2nd extract in inhibiting cell proliferation was compared to their relevant concentrations at the 1st extract. Interestingly, the data of the 1st extract revealed the significant effect of low doses (1250, 620, 313 and 156 μg/ml) in promoting HepG2 cell growth compared to its relevant doses of the 2nd extract. Meanwhile, the EC50 of each extract was calculated by plotting the BSG conc. Vs log of absorbance and the data revealed a lower EC50 of the 2nd extract (261.9 μg/ml) compared to EC50 of 1st extract (1066 μg/ml) Which means a potent antioxidant activity of 2nd extract compared to 1st extract (Fig. 2).

Fig. (2): Assessment of BSG extracts effects on HepG2 cell proliferation and toxicity. (a.) MTT assay revealed a significant dose dependent effect of the 2nd extraction approach (5000-156 μg/ml) on inhibiting HepG2 cell proliferation in comparison with the 1st extraction approach. Lower doses of 1st extract (1250-156 μg/ml) showed a dose dependent promotion of cell growth. (b.) Data presented at the curve of the calculated EC50 of 1st and 2nd extraction approach of BSG powder, indicated lower EC50 of the 2nd extract revealing its potent antioxidant activity. Data were presented as mean of three separate experiments SD. Significance was denoted as *p<0.05, **p<0.001, for each 2nd extracts dose as compared to 1st extract representative dose.

3.3. Effect of BSG on HepG2 cell migration

To evaluate the ability of both extracts to inhibit HepG2 cell migration, the size of the induced wound was monitored over 33 hr, till closure of the induced wound in control cells. The data illustrated a no marked effect in groups treated with BSG extracted with 1st technique except for cells treated with EC50 of BSG (0.261 μg/ml). Contradictory, the data showed a marked effect of BSG extracted by 2nd technique in inhibiting cell motility in a dose dependent manner (Fig.3).

4. Discussion

The one of the biggest disadvantages of implication of phytochemicals in clinical study is their low bioavailable levels. Therefore, our group hypothesized to use a cocktail of phytochemicals to target several signaling pathways to diminish breast cancer cell proliferation, metastasis and survival (Ouhtit et al., 2014). Relying on these data, a commercial product named “breast safeguard” was developed as a nutrient supplement to support breast health. Recently, BSG showed a profound effect in sensitizing liver cancer cell line HepG2 to x-ray radiations (Abdraboh et al., 2020). This work was directed to assess the best chemical extraction approach that shows potent antioxidant activity and significant inhibitory effect on HepG2 cell proliferation and migration.

The chemical assessment of BSG extracts antioxidant activity was first conducted using DPPH assay and the data revealed a significant effect of the second extraction approach (100%DMSO) in scavenging DPPH generated free radicals in comparison with first extract. This potency may be referred to the significant increase at the flavonoid, polyphenols and tannin content of the second extract (18.1025 mg Catechin equivalent/gm BSG, 109.365 mg gallic acid equivalent/gm BSG and 30.944 mg gallic acid equivalent/gm BSG) compared to their content at the first extract (1.333 mg gallic acid equivalent/gm BSG, 22.191 mg gallic acid equivalent/gm BSG and 10.1 mg gallic acid equivalent/gm BSG), respectively.

The contents of polyphenols, flavonoids and tannins levels in phytochemical extracts determines their antioxidant capacity (Sivananth et al., 2017). The presence of phenolic groups in the chemical structure of resveratrol, indole-3 carbinol and gallic acid, potentiate their antioxidant capacity (González-Vallinas et al., 2013). C-phycocyanin free radicals scavenging activity may be related to its ability to chelating iron ions and inhibition of deoxyribose enzyme degradation (Bermejo et al., 2008). In addition, C-phycocyanin exploited its antioxidant activity, by showing a nephroprotective effect against cisplatin, via halting the H2O2 and ROS dependent impairment of mitochondrial membrane and the subsequent release of cytochrome C (Fernández-Rojas et al., 2014). The anticancer activity of C-phycocyanin against HepG2 cells line was elucidated by its significant effects in induction of cell apoptosis (Roy et al., 2007). The data of MTT assay revealed a significant effect of BSG both extracts on diminishing the ability of HepG2 cells to proliferate in a dose dependent manner, except for low doses of the 1st extract (1250-156μg/ml). The significance of the second extraction approach in inhibiting cell proliferation.
Fig. (3): Assessment of BSG extracts effects on HepG2 cell motility. (a.) Wound healing assay of the 1<sup>st</sup> extraction approach of BSG pointed out its unmarked effect at cell migration except for the higher dose (0.261mg/ml). (b.) The wound healing assay for the 2<sup>nd</sup> extraction approach showed a marked dose dependent effect in diminishing the ability of HepG2 on cell migration.

Quercetin showed significant activity in downregulating cancer cell proliferation via induction of cell cycle arrest and downregulation of cancer growth corelated inflammatory actions (Rather and Bhagat, 2020). Several literatures support the anti-cancer characteristics of indol-3-carbinol and its role in targeting cancer cell proliferation via induction of cell cycle arrest at G1 phase (Popolo et al., 2017; Chamovitz et al., 2018; Munakarmi et al., 2020). Furthermore, a recent in vitro study illustrated the effect of c-phycocyanin on induction of cell cycle arrest at G0/G1 phase and activation of breast cancer cell apoptosis (Jiang et al., 2018).

Resveratrol showed a profound effect on reducing the tumor volume of pancreatic, breast and colon Cancer in a new approach of neoadjuvant therapy. Moreover, resveratrol can increase cancer cell sensitivity for chemotherapeutic drugs such as, capsaicin, docetaxel and doxorubicin (Jiang et al., 2017). Clinical trials conducted by Giordano and Tommonaro proved that curcumin can act as antioxidant gent in low doses, but it can not damage cell DNA, which would be possible in case of high doses applications. In addition, curcumin acts on growth factors genes and cytokines levels to induce cancer cell apoptosis (Giordano and Tommonaro, 2019).

On the other hand, the marked effect of BSG second extract in demolishing the ability of HepG2 cell to migrate into the induced wound compared to the first extract support the anti-metastatic abilities of BSG. C-phycocyanin showed a profound effect in inhibiting the migration of the breast cancer cell line MDA-MB-231 by downregulating the expression of cyclooxygenase-2 (Jiang et al., 2018). A recent study illustrated the role of gallic acid nanoparticles in downregulating the ability of HepG2 and MCF7 cell lines to migrate using wound healing assay approach (Hassani et al., 2020). Resveratrol significantly inhibited cell metastasis at several cancers including liver, breast, lung and skin cancers, this effect was achieved by its targeting to signaling pathways of epithelial-mesenchymal transition, migration and metastasis (Kim et al., 2016).

In conclusion, method of extraction of phytochemicals plays an important role in good estimation of its anticancer activity. whereas, the extraction of BSG phytochemicals combination with 100% DMSO revealed a better content of antioxidant phenolics, flavonoids and tannins than 20% of DMSO.

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