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Chemical investigation and cytotoxic activity of bee venoms collected by dissection and electric-shocked methods

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ABSTRACT

Introduction: Cancer is a large group of disorders characterized by uncontrolled cellular proliferation. It is one of the most devastating diseases all over the world. Recently, there is an increased interest in the clinical use of natural products as a safe, efficient, and economic therapeutic alternative. Honeybee products therapy, apitherapy, was used to control various diseases including cancer.

Objectives: The primary objective of this study was to screen the potential cytotoxic effects of bee venoms (BVs) against different cancer types and also to evaluate the reported bee venoms-caused hemolysis on human red blood cells.

Materials and Methods: Samples of venom were collected from different Egyptian localities with different techniques and tested by 3-(4,5-dimethyl-thiazolyl)-2,5-diphenyltetrazolium bromide cell-based assay against liver (Hep-G2), colorectal (Caco-2), and breast (MCF-7) cancer cell lines. Also, different concentrations of BVs were used to evaluate the hemolysis percentage caused by BVs if injected into the circulation.

Results: The results showed that Italian dissected method-BV was the most active among all the tested samples against Hep-G2 (93.92%), Caco-2 (93.92%), and MCF-7 (90.17%). In addition, all BVs at all concentrations caused high hemolysis percentage.

Conclusion: The results showed that BV is an interesting agent that has valuable activities against Hep-G2, Caco-2, and MCF-7. Being the most effective agent against cancer cell lines, BV might be incorporated in cancer remedy regimens after further studies but the inherent limitation that BVs cause severe hemolysis to blood cells at low concentrations would require more research to avoid the risk of using BV *in vivo*.

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Introduction

Cancer is a large group of disorders characterized by uncontrolled cellular proliferation. Cancer cells are also capable of metastasizing to other regions causing a number of devastating outcomes [1]. Nearly, all body organs are vulnerable to cancer with liver, colon, and breast being the most common ones. Hepatic cancers are the third leading cause of cancer-associated deaths worldwide, and currently, the frequent cause of deaths in cirrhotic patients [2]. Colon cancer has an estimated incidence of over 1 million new cases annually worldwide [3]. Almost one of the three patients with colon cancer dies from the disease. Colon cancer also more often affects people in well-developed countries in comparison to less developed countries [4]. Breast cancer is the leading cause of female mortality, more than half a million deaths were reported in 2012. It continues to represent the most frequently diagnosed cancer in females with more than 1.7 million new cases diagnosed in 2012; it represents 25% of all new cancer cases diagnosed in women [5].

Does this mean that we are helpless against cancer? The answer to this question was presented

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in 1994 in Cancer letters by Badria [6], whereas 62 Egyptian food and medicinal preparations were extensively examined for antimutagenic/anticarcinogenic activity using short-term and host-mediated assays. The antimutagenic activity of the substances examined was ranked as follows: 13 (strong), 7 (mild), and 5 (weak) after metabolic activation. Metabolic activation seems to be necessary for most antimutagenic substances in this study, e.g., radish inhibits 29% of mutagenicity produced in the direct antimutagenic assay and inhibits 89% of mutagenicity induced in the host-mediated assay. Hence, there is an urgent need for the discovery of a new regimen for hepatocellular carcinoma (HCC) treatment. Recently, many antitumor compounds with new structural features and mechanism of action have been isolated from natural products. Natural products serve as a good and affordable source for new drug entities. Different vaccines and biologics have been inspired from natural products structure. Such as betulinic acid and its analogous had an inhibitory activity against topoisomerase [7]. Oleogum resin of *Boswellia carterii* showed antiproliferative activity on T-lymphocyte culture [8]. Moreover, the combination of Boswellia serrata, licorice root (*Glycyrrhiza glabra*), and turmeric root (*Curcuma longa*) was used in the control of bronchial asthma because of their leukotriene inhibition, anti-inflammatory, and antioxidant activity, respectively [9]. These combinations of terpenoids were also used in the treatment of knee osteoarthritis and hepatitis C [10]. Cucurbitacin proved to have potent *in vitro* and *in vivo* activities toward HCC [11]. Recently, cucurbitacin B used as antitumor activity against ovarian cancer cell line (A2780) and as a chemosensitizer for cisplatin cytotoxicity in cisplatin-resistant ovarian cancer cell line (A2780CP) in 2-D and 3-D culture model [12]. Recently, many antitumor compounds with new structural features and mechanism of action have been isolated from natural products. Natural products serve as a good and affordable source for new drug entities [13–19].

Apitherapy (*Apis* is a Latin word means bee) is the practice of using bee products such as honey, bee venom (BV), and propolis for disease prevention or treatment. It can be also described as the science (and art) of using honey-bee products, to maintain health and assist the individual in regaining health [20]. Researchers have concluded that BV, propolis, and honey are interesting agents that have valuable activities against human liver cancer, epithelial colorectal adenocarcinoma, and breast cancer cell lines [21]. BV has been used to treat diverse disorders.

BV has long been used to relieve pain symptoms and to treat inflammatory diseases, such as rheumatoid arthritis. The underlying mechanisms of the anti-inflammatory and analgesic actions of BV have been proved to some extent. Additionally, recent clinical and experimental studies have demonstrated that BV and BV-derived active components are applicable to a wide range of immunological and neurodegenerative diseases, including autoimmune diseases and Parkinson's disease. These effects of BV are known to be mediated by modulating immune cells in the periphery, and glial cells and neurons in the central nervous system [22]. Crude BV was found to have antibacterial effects against gram-positive bacteria strains. *Staphylococcus aureus*, Methicillin resistant *Staphylococcus aureus* (MRSA), and *Staphylococcus epidermidis* were found to be the most sensitive to crude BV with small minimum inhibitory concentrations (MIC) [23].

The main component of BV, which constitutes approximately 50% of its dry matter, is melittin (MEL) [24–27]. MEL is 26 amino acids long peptide. Modern pharmacological studies showed that MEL exerts various antitumor effects by inhibiting tumor cell growth [28,29], promoting tumor cell apoptosis [30,31], and inhibiting angiogenesis [32] and migration [33,34]. It is also reported to have strong hemolytic activity [35]. It has been used traditionally against chronic inflammation and cancer [36] and also used for the therapeutic agent of arthritis, rheumatism, and atherosclerosis [37,38]. The use of MEL in cancer therapy is limited due to the inherent issues accompany the use of BV that include toxicity, non-specificity, degradation, inefficient systemic delivery, limited bioavailability, and hemolysis [39].

Apitherapy could be used as a cancer therapeutic agent or to complement conventional cancer treatments.

Materials and Methods

Bee venoms

Powdered BV was obtained via lyophilization (freeze-drying) after collection from diverse honey bee strains by two different collection methods. Figure 1 shows BV from Craniolian (dissected and electric shock) and Italian (dissected and electric shock).

For hemolysis assay, different concentrations of BVs were prepared by dissolving the venoms in the

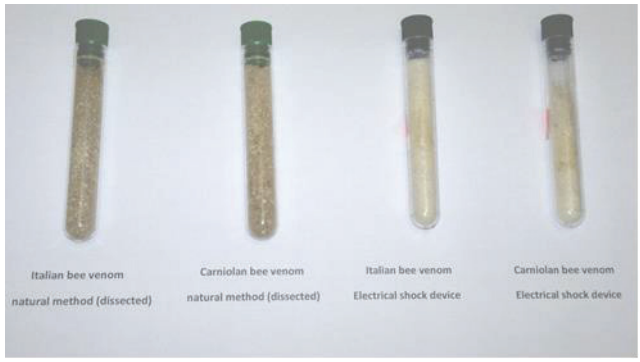

Figure 1. BV (Craniolian—dissected, Craniolian—electric shock, Italian—dissected, Italian—electric shock).

least amount of dimethyl sulfoxide (DMSO) and the required volume was completed with saline.

Materials used in the biological assays

Cell lines; Hep-G2, Caco-2, and MCF-7 (Holding Company for Biological Products and Vaccines, VACSERA, Agouza, Giza, Egypt), Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), an antibiotic/antimycotic solution containing 1,000 U/ml penicillin, 1,000 µg/ml streptomycin and 25 µg/ml fungizone, phosphate buffer saline, DMSO and 3-(4,5-dimethylthiazolyl)-2,5-diphen-yltetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO), 96-well plates, tissue culture treated polystyrene (#3512, Corning Inc., NY), and DMSO (Cornell Lab, UK).

Blood samples used in hemolysis assay

The blood was obtained from healthy male adult humans and immediately collected and defibrinated as described by Norred et al. [40] before use in the hemolysis assay. The blood was defibrinated by swirling gently with five to seven glass beads for about 5 minutes, then the defibrinated blood was decanted in a clean container without touching the beads to be employed in the hemolysis assay.

The crude BVs were collected and prepared for chemical and biological studies during 2013–2015. Two honeybee hybrid strains venom were collected (Carniolan—Italian) by dissected method (manual) and BV electric shock device (automated) obtained from the Department of Bee Research Qalyubia—Agricultural Research Center—Egypt.

Dissected method (manual way method) according to the method of Pence [41] with one exception that the heat was replaced by cold. Five hundred random worker bees from every strain in the month middle of each month throughout the duration of the study, which extends to 12 months collected, then placed in the refrigerator for drugging the worker, removed the stinging with venom sac and placed in a 10 ml distilled water in a Petri dish. So placed in a centrifuge tubes device undercooling at a temperature of −15°C for 15 minutes, the number of laps was 5,000 laps/minutes. Then, we grinding output to bring out all the venom, the amount of liquid with venom takes, and put it in the drying device (lyophilizer) under cooling for a period of 8 hours, after the drying scraping venom by scalpel and then weighed.

So, venom compared to the amount and component of venom obtained from the two strains, compared the different seasons (Autumn–Winter–Spring–Summer), and amount and component of venom sample obtained by a way of electric shock and manual way. Stages of honey BV extract are shown in Figure 2.

Cytotoxicity assay using MTT

MTT assay is a colorimetric assay for assessing cell metabolic activity by reducing the tetrazolium dye MTT 3-(4,5-dimethyl-thiazolyl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color, through reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes. This, under defined conditions, can reflect the number of viable cells present [42]. Activity as cytotoxic agents against hepatic, colorectal, and breast cancers was tested in a cell-based assay using Hep-G2, Caco-2, and MCF-7 cell lines, respectively, by MTT assay. Cell lines were cultured in complete growth DMEM media containing 10% FBS, 1% penicillin/streptomycin, and incubated at 37°C with 5% carbon dioxide (CO_2) and 90% relative humidity. All cell passages used were between passages 30 and 40. 5-fluorouracil (5-FU) or cis-platin (cis-pt) was used as the standard cytotoxic agent. The compounds were dissolved in DMSO-free media (water-soluble components) or DMSO/Media vehicle so that the DMSO limit does not exceed 0.05%. The viability of cells was measured spectrophotometrically using a MTT assay which indicates mitochondrial metabolic activity. This assay depends on measuring the activity of mitochondrial NADPH oxidase-dependent reductase that converts the yellow tetrazolium salt, MTT, to a purple formazan product that is water-insoluble. After solubilization of formazan crystals, the purple solution is easily measured quantitatively using enzymelinked immunosorbent assay (ELISA) plate reader at a wavelength of 540 nm [43,44].

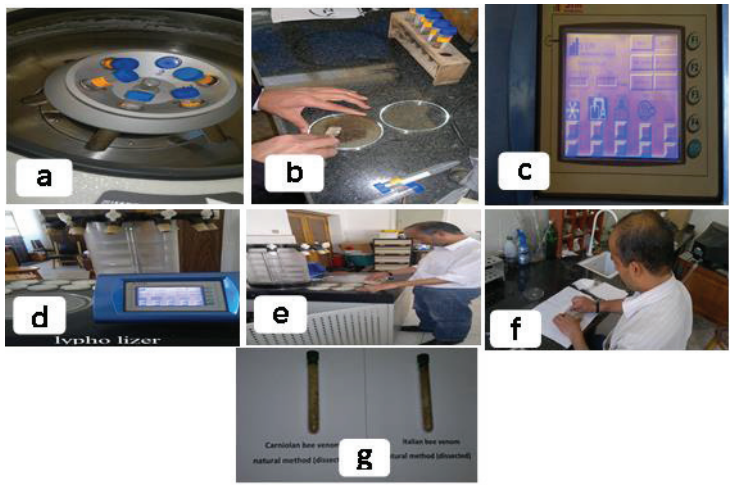

Figure 2. Stage of honey BV extract [(a) centrifuging samples, (b) homogenate venom sac, (c) lyophilizer, (d) samples drying, (e) dried samples, (f) venom's collect, and (g) dried venom].

Cell lines were cultured in 96-well plates (1 × 105 cells/ml). After incubation, the medium was removed and the wells were treated with 100 µl of 5 mg/ml MTT and incubated for 4 hours at 37°C. Then, 100 µl of solubilizing solution, DMSO, was added to each well and the produced purple solution was quantified spectrophotometrically at 540 nm using ELISA BioTek Lx800 microplate reader (BioTek, Bedfordshire, UK).

The cytotoxic activity of the test compounds was indicated by the ratio of tested well to negative control and half maximal inhibitory concentration (IC_{50}) was calculated. Cytotoxic activity was calculated from the following formula:

$$\text{Percentage of cytotoxic activity} = \frac{OD_{\text{negative control}} - OD_{\text{test}}}{OD_{\text{negative control}}} \times 100$$

Whereas OD indicates optical density.

Quantitative determination of percent of hemolysis caused by bee venoms

The colorimetric method described by Ansel and Cabre [45] was used to determine the percentage hemolysis inflected by BVs with some modifications. Briefly, 20 µl defibrinated blood was added to 2 ml of different concentrations of BVs and vortexed for 2–3 seconds. The tubes were then incubated for 45 minutes at about 30°C then centrifugated at 2,000 rounds per minute for 4 minutes to precipitated non-hemolyzed red blood cells (RBCs). The supernatant was measured spectrophotometrically at 540 nm. Saline and distilled water were used instead of samples to determine 0% and 100% hemolysis, respectively. 0% hemolysis was used as a blank. The percentage of hemolysis was obtained by dividing the absorbance of the sample by the absorbance of 100% hemolysis.

Table 1. Percentage of cytotoxicity of different BVs, with respect to their collection methods, in HEP-G2, Caco-2, and MCF-7 cell lines using 5-FU and cis-platin (cis-pt) as positive controls.

Honeybee products*	Percentage of inhibition activity**		
	HEP-G2	Caco-2	MCF-7
Control			
5-FU	12.46 ± 0.65	68.97 ± 1.36	0.75 ± 0.35
Cis-platin	85.65 ± 0.49	90.62 ± 0.54	86.62 ± 0.54
Bee venom			
Craniolian dissected	92.96 ± 1.36	93.25 ± 1.06	87.43 ± 2.02
Craniolian electric shock	91.93 ± 0.11	92.38 ± 0.88	84.14 ± 3.03
Italian dissected	91.96 ± 2.77	94.12 ± 1.25	90.67 ± 0.71
Italian electric shock	91.75 ± 2.47	92.86 ± 0.20	91.61 ± 1.97

*All compounds were used at a concentration of 100 µg/ml.
**Data are represented as mean ± SD.

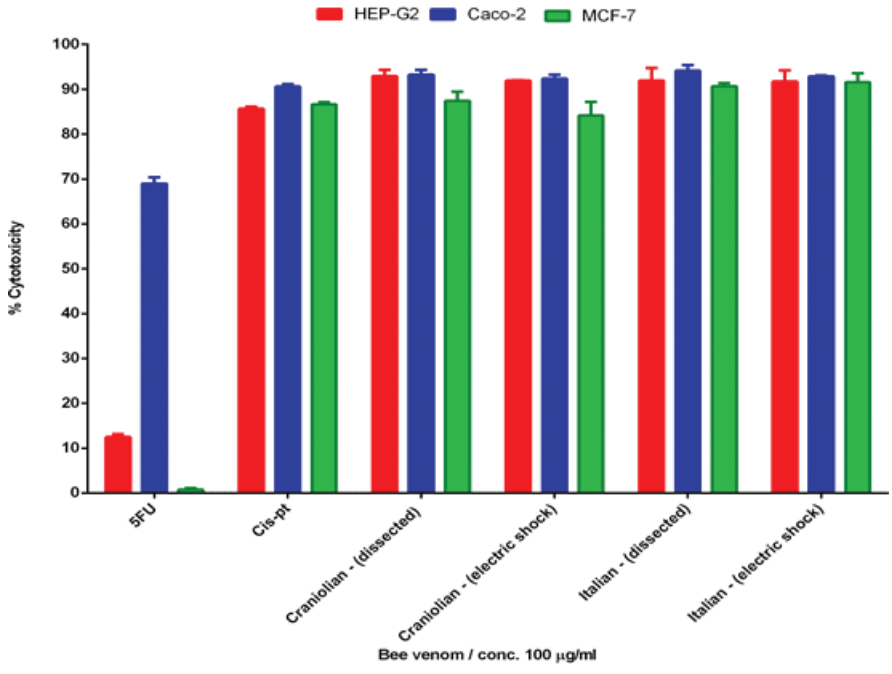

Figure 3. Percentage of cytotoxicity of different BVs at 100 µg/ml against Hep-G2, Caco-2, and MCF-7 cell lines, using 5-FU and cis-platin (cis-pt) as positive controls.

Statistical analysis

Data were represented as the mean ± standard deviation (SD). All assays were performed three times (n = 3). The significance between controls and BVs cytotoxicity was examined using one-way Analysis of variance (ANOVA) followed by Tukey's post-hoc test with p = 0.05. All statistical analysis was performed using Graph Pad Prism version 6.0 software.

Results

Craniolian and Italian BV (by dissected method) recorded the highest activity against Hep-G2, with 93.92% inhibition, followed by Italian venom (electric shock device) with 93.49%. In Caco-2 cell lines, the dissected Italian venom recorded the highest effect, 93.23%, followed by shock device Italian venom, 92.72%. On MCF-7, the dissected Italian venom recorded the highest effect, 90.17% followed by shock device Italian 90.21%. Results of cytotoxic activity of BVs are summarized in Table 1 and Figure 3. All BVs activities were highly significant in comparison to 5-FU as a control.

All BVs at 50, 100, and 200 µg/ml caused high hemolysis percentage ranging from 72.89% to 96.48% for electric shock and dissected craniolian BVs (100 µg/ml), respectively. Results of hemolysis caused by BVs are represented in Table 2 and Figure 4.

Table 2. Percentage of hemolysis caused by different BVs at 50, 100, and 200 µg/ml on human RBCs.

Bee venom	Concentration (µg/ml)	Percentage of hemolysis**
Craniolian, electric shock	50	77.10 ± 8.57
	100	72.89 ± 4.06
	200	92.68 ± 13.82
Craniolian, dissected	50	73.88 ± 7.45
	100	96.48 ± 8.57
	200	85.72 ± 4.26
Italian, electric shock	50	90.11 ± 11.42
	100	92.45 ± 4.19
	200	92.73 ± 5.84
Italian, dissected	50	93.15 ± 6.50
	100	92.31 ± 8.67
	200	90.28 ± 4.51

**Data are represented as mean ± SD.

Discussion

The BVs are an important weapon in protection of the colony of stinging bees. A single sting might kill other insects instantaneously but only will cause temporary local inflammatory symptoms characterized, in humans, by swelling, redness, and pain. Also, a bee sting is painful and it causes erythema, itching, inflammation, and edema [46]. Venom has been utilized as a pain reliever and as a treatment against inflammatory diseases since earliest history. In these aspects, the effect of venom against liver, breast, and colorectal cancer was explored.

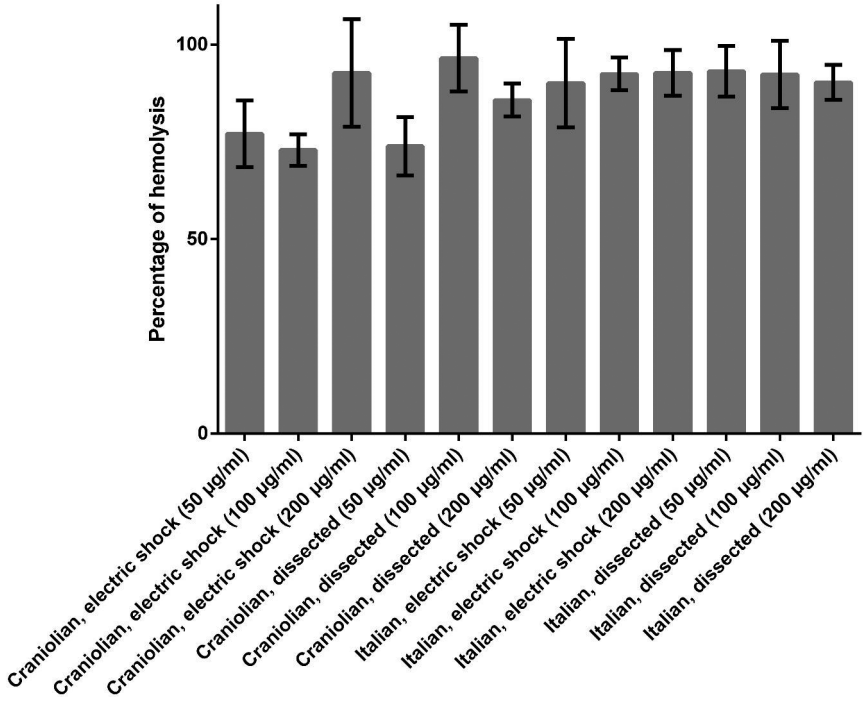

Figure 4. Percentage of hemolysis inflected by different BVs at 50, 100, and 200 µg/ml on human RBCs.

All venom treatments were found to have effect against liver, breast, and colorectal cancer as represented in Figure 3. The cytotoxic effect was ranged between the lowest inhibition activity 86.28% in Craniolian—electric shock on MCF-7 and the highest inhibition activity 93.92% in Craniolian, Italian—dissected on Hep-G2. Craniolian—electric shock, respectively.

These findings indicate that it could be suggested that BVs collected by (dissected method) showed the highest cytotoxic activity against cancer cell lines. It could be concluded that dissected methods are considered the best in the extraction of BV because this method preserves all BV components and unstable substances of biological significance. Unlike the BV which is traced in the electric shock collection method, this method causes the loss of many significant biological components and cannot get all the amount of venom.

These findings support the results of the previous study by Hamedani et al. [47] in which proposed the antitumorigenic activity of BV in the treatment process of cancer which was paralleled with findings of Magnan et al. [9,30]. It was concluded that honey BV affects matrix metalloproteinase-2 activity, cell proliferation, and interferon beta production in a time and dose-dependent manner. MEL is the main component found in BV [48]. Recent researchers have found that MEL exerts various antitumor effects by obstructing tumor cell growth [28,29], stimulating tumor cell apoptosis [27,30], and inhibiting tumor angiogenesis [26] and migration [34,37]. MEL have various effects on cellular functions of cancerous cells such as angiogenesis, proliferation, metastasis, apoptosis and cell cycle, and the anticancer processes involve various signal molecules and regulatory pathways, BV and its main component can inhibit the proliferation of cancer cells through stimulating of apoptosis, inhibition of tumor metastasis, and invasion by regulating the expression of caspases, calcium ion concentration, death receptors (DRs), extracellular matrix degradation enzymes, angiogenesis factors, and a variety of signaling pathways [49]. BV and its main constituent MEL have been found to influence numerous features of the tumor such as preventing cancer cell growth and/or proliferations, inducing apoptosis or suppressing tumor metastasis, signifying that it could be an exceptional replacement for the management of cancer [50]. The induction of apoptotic cell death through several cancer cell death mechanisms, including the activation of caspase and matrix metalloproteinases, is important for the MEL induced anticancer effects. The conjugation of cell lytic peptide (MEL) with hormone receptors and gene therapy carrying MEL can be useful as a novel targeted treatment for some forms of cancer, such as breast and prostate cancer [32]. There are

18 active components at least in the venom which have some pharmaceutical properties [51]. These compounds include, MEL, a small peptide containing about 26 amino acids, is the main effective toxin of BV [52], which forms about 50% of BV [53]. Also, several studies have confirmed that BV and/or MEL have anticancer effects on liver [54,55] and breast [56,57]. Genistein improved antitumor effects are due to a better decrease in the DNA-binding activity of nuclear factor kappalight-chain-enhancer of activated B cells (NF-κB) [58]. Natural toxin BV could be beneficial as an anticancer drug through the overexpression of DR3 and inactivation of NF-κB in the remedy of lung cancer cells and drug-resistant cancer cells [59]. It was proposed that the effects of BV on breast can-cer (MCF-7) may be suppressing cell motility and metastasis by inhibiting PI3K/Akt/mTOR signaling pathway [60]. As for liver cancer (Hep-G2), BV prevents cell metastasis through inhibition of the Rac1-dependent pathway [31] and for colorectal cancer (Caco-2), through inhibition of vascular endothelial growth factor (VEGF)-induced neovascularization [61], Blocking VEGF-R2, and the cyclooxygenase-2 mediated mitogen-activated protein kinase signaling pathway (tumor-promoting inflammation [62], blocking of epidermal growth factor—induced signaling (sustaining proliferative) [63] and immune activation (signaling) [64].

From the previous results, it could be summarized that BV under treatments exhibits a potential cytotoxic effect on Hep-G2, Caco-2, and MCF-7 but it is known that BVs have high risks when used *in vivo*. Immune response to BVs can range from skin reaction to life-threatening conditions [65]. As the results indicated, low concentrations of different BVs obtained by different methods caused a high percentage of hemolysis which limits the potential use of them as therapeutic agents. The result is in accordance with the findings in other studies [66,67]. In a study that investigated the effect of DMSO on hemolytic activity of antimicrobial preservatives, it was found that, at specific concentrations, DMSO can interfere with the hemolytic activities through a direct action on the RBCs or by changing their permeability barriers rather than by chemical interaction with the hemolytic agents [45]. This suggests that the hemolytic activity of BVs can be manipulated through various mechanisms. Another example is the usage of BV in producing a novel protein infusion of MEL and mutant human interleukin 2 which was found to be more effective against human ovarian cancer SKOV3 cells than interleukin 2 [39,68]. Also, the isolation of active components that have cytotoxic potentials against cancer cells would limit the risk factors associated with using BV in *in vivo* applications.

Conclusions

The results showed that BV is an interesting agent that has valuable activities against Hep-G2, Caco-2, and MCF-7. Being the most effective agent against cancer cell lines, BV might be incorporated in cancer remedy regimens after further studies to elevate the risks associated with using BVs in *in vivo* therapeutic applications and also to isolate and identify the specific BV components that are responsible for cytotoxic effects against cancer cells.

Conflict of interest

There is no conflict of interest to disclose.

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