

# Evaluating the cytotoxic effects of the water extracts of four anticancer herbs against human malignant melanoma cells

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**Abstract:** Malignant melanoma (MM) is the most dangerous type of skin cancer, killing more than 1,100 people each year in Canada. Prognosis for late stage and recurrent MM is extremely poor due to insensitivity to chemotherapy drugs, and thus many patients seek complementary and alternative medicines. In this study, we examined four commonly used anticancer herbs in traditional Chinese medicine, *Hedyotis diffusa*, *Scutellaria barbata*, *Lobelia chinensis*, and *Solanum nigrum*, for their in vitro antitumor effects toward human MM cell line A-375. The crude water extract of *S. nigrum* (1 g of dry herb in 100 mL water) and its 2-fold dilution caused 52.8%±13.0% and 17.3%±2.7% cytotoxicity in A-375 cells, respectively ( $P<0.01$ ). The crude water extract of *H. diffusa* caused 11.1%±12.4% cytotoxicity in A-375 cells with no statistical significance ( $P>0.05$ ). Higher concentrated formulation might be needed for *H. diffusa* to exert its cytotoxic effect against A-375 cells. No cytotoxicity was observed in A-375 cells treated with crude water extract of *S. barbata* and *L. chinensis*. Further high performance liquid chromatography-tandem mass spectroscopy analysis of the herbal extracts implicated that *S. nigrum* and *H. diffusa* might have adopted the same bioactive components for their cytotoxic effects in spite of belonging to two different plant families. We also showed that the crude water extract of *S. nigrum* reduced intracellular reactive oxygen species generation in A-375 cells, which may lead to a cytostatic effect. Furthermore, synergistic effect was achieved when crude water extract of *S. nigrum* was coadministered with temozolomide, a chemotherapy drug for skin cancer.

**Keywords:** herbal extract, malignant melanoma, cytotoxicity, HPLC-MS/MS, reactive oxygen species

## Introduction

Malignant melanoma (MM) is a less common but more dangerous type of skin cancer. It accounts for about 75% of skin cancer-related deaths.<sup>1</sup> In 2015, Canadian Cancer Statistics reported that the incidence rate of MM was increased at 2.3% per year in men and 2.9% per year in women, respectively, between 2001 and 2010.<sup>2</sup> It was estimated that there would be 6,800 new MM cases and 1,170 MM deaths in Canada in 2015.<sup>2</sup> Like other types of cancer, early diagnosis is the key prognostic factor for MM. The 5-year relative survival rate is higher than 90% for early diagnosed MM patients (stages IA and IB); however, prognosis is extremely poor for deeper (>4 cm) and metastatic MM partially due to resistance development toward chemotherapy drugs.<sup>3</sup> Thus, many advanced or recurrent MM patients seek complementary and alternative medicines, expecting to achieve better therapeutic efficacy, reduced chemotherapy-related side effects, and/or a boost to the immune system.<sup>4-6</sup>

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Oral and topical administrations of herbal extracts have been widely used to treat skin diseases including MM in traditional medicines for thousands of years even without much knowledge of the active ingredients.<sup>7,8</sup> Furthermore, these practices are rarely documented in English and usually lack sufficient quality control. In recent years, a significant amount of research effort has been put into identifying and isolating anticancer components from medicinal plants, such as paclitaxel that was isolated from the bark of the Pacific yew tree.<sup>9,10</sup> In fact, more than 70% of the anticancer drugs approved worldwide are natural products or their mimetic analogs.<sup>11</sup> However, this research effort did not reduce herbal usage in cancer patients.<sup>12</sup> Recently, the US Food and Drug Administration (FDA) has noticed increased herbal usage in cancer patients, updated its regulatory protocols and approved several herbal extracts, such as BZL101 and PHY906, for clinical trials.<sup>13–15</sup>

*Hedyotis diffusa*, *Scutellaria barbata*, *Lobelia chinensis*, and *Solanum nigrum* are four commonly used anticancer herbs, classified as nontoxic for both oral and topical administrations, in traditional Chinese medicine (TCM). *H. diffusa* is a major component in several TCM formulations. It has been used to treat liver, lung, colon, brain, and pancreatic cancers.<sup>16</sup> Our previous studies showed that the water extract of *H. diffusa* was highly cytotoxic toward human breast cancer MCF7 cells.<sup>17</sup> However, coadministration of the water extract of *H. diffusa* diminished the anticancer activity of three chemotherapy agents, doxorubicin, cyclophosphamide, and docetaxel.<sup>18</sup> Triterpenes and polysaccharides have been identified to be the active components in *H. diffusa*.<sup>16,19</sup> *S. barbata* is another widely used herb to treat lung, liver, breast, and gastric cancers in TCM.<sup>20</sup> Its water extract, BZL101, has been approved by FDA for clinical trials and has shown promising efficacy against metastatic breast cancer.<sup>13,14</sup> *L. chinensis* is traditionally used to treat snake bites and skin abscess. Recently, the water extract of *L. chinensis* was shown to possess antitumor activity against lung, colon, and liver cancers and several types of alkaloids have been identified as the active ingredients.<sup>21–24</sup> *S. nigrum* is traditionally used to treat liver disorders, chronic skin ailments, inflammations, and diarrhea. Both water and alcohol extracts of *S. nigrum* exhibited anticancer activities against liver, colorectal, breast, and cervical cancers.<sup>25–28</sup> The water extract of *S. nigrum* was also reported to inhibit the metastasis of mouse melanoma B16-F1 cells.<sup>29</sup> Various active components, including glycoalkaloids, polyphenols, polysaccharides, glycoproteins, and peptides, have been isolated from *S. nigrum*.<sup>30–34</sup>

Nevertheless, neither the water extracts of these four commonly used herbs nor their respective active components have been examined for anticancer activity against human MM. In the current study, we evaluated whether the water extracts of the herbs possess any antitumor activity and exhibit synergistic/additive effects with temozolomide, an oral analog of dacarbazine (DTIC) used for stage IV melanoma, against human MM A-375 cells.

## Materials and methods

### Materials

Dried whole plants of *H. diffusa*, *S. barbata*, *L. chinensis*, and *S. nigrum* were purchased from a TCM store (Calgary, AB, Canada). All chemicals and fetal bovine serum used in the current study were purchased from Sigma-Aldrich (Oakville, ON, Canada). Human MM cell line A-375 was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cell culture medium, Dulbecco's Modified Eagle's Medium (DMEM), was purchased from Cedarlane Laboratories (Burlington, ON, Canada). Cytotoxicity assay kit, CytoTox96 non-radioactive cytotoxicity assay, which quantitatively measures lactate dehydrogenase (LDH), was purchased from Promega Corporation (Madison, WI, USA).

### Water extract preparation

For each herb, the crude water extract was prepared by boiling 1 g of the chopped herb in 100 mL deionized water for 1.5 hr. The water solution was allowed to cool down at room temperature (~23°C) for at least 2 hr before the supernatant was collected. The supernatant was then diluted serially up to 16-fold with deionized water. Both the supernatant and its serial dilutions were used for cell treatments within 24 hr.

### Cell culture

Human MM cell line A-375 was cultured in T-75 culture flasks under ATCC-recommended culture conditions (DMEM media with 10% fetal bovine serum and 1% penicillin/streptomycin) at 37°C under a humidified atmosphere (5% CO<sub>2</sub>) in a Forma™ series II water-jacketed CO<sub>2</sub> incubator purchased from ThermoFisher Scientific Inc. (Waltham, MA, USA). Cell culture media were changed every 2–3 days.

### Cytotoxicity assay

All experiments except the high performance liquid chromatography-tandem mass spectrometry analysis were carried out in triplicate in the current study. For the cytotoxicity assay, human MM A-375 cells were collected from the T-75 cell culture flasks, resuspended in the culture media,

and plated in 96-well culture plates with each well containing about 10,000 cells. The cells were allowed to attach and grow for 24 hr (reaching 70%–80% confluence) before being treated with the water extractions (1 g/100 mL) of *H. diffusa*, *S. barbata*, *L. chinensis*, and *S. nigrum*, as well as their respective serial-diluted solutions (2-, 4-, 8-, and 16-fold), for another 24 hr, which was the optimal treatment time predetermined from a pilot study. Cytotoxicity of the herbal water extracts was measured using the CytoTox 96® non-radioactive cytotoxicity assay. Cells treated with the culture media were used as negative control. The cytotoxicity was calculated using the following equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental} - \text{Control}}{\text{Maximum (Lysis)} - \text{Control}}$$

### High performance liquid chromatography-tandem mass spectroscopy (HPLC-MS/MS) analysis

HPLC-MS/MS analysis of the water extracts of *H. diffusa*, *S. barbata*, *L. chinensis*, and *S. nigrum* was performed in both positive and negative ionization modes with spectra acquired in the mass range of 100–1,500 *m/z* using an Agilent 1200 HPLC system (Mississauga, ON, Canada) interfaced to an AB Sciex 4000 QTRAP® hybrid triple quadrupole/linear ion trap mass spectrometer equipped with the TurbolonSpray™ interface (Concord, ON, Canada). Applied Biosystems/MDS Sciex Analyst Software (Version 1.6.0) was used for system control and analysis. The reverse-phase chromatography was performed using an Agilent ZORBAX Eclipse C18 column (5 µm, 4.6×150 mm<sup>2</sup>) at 40°C. For each herbal water extract, 5 µL sample was injected into the C18 column using the Agilent 1200 Autosampler (set to 4°C) and delivered with a gradient mobile phase. The column was equilibrated with 90% water acidified with 0.1% formic acid and 10% acetonitrile acidified with 0.1% formic acid at a flow rate of 0.2 mL/min. The gradient was run for 20 min to 10% water acidified with 0.1% formic acid and 90% acetonitrile acidified with 0.1% formic acid. Then, the column was returned back to 90% water acidified with 0.1% formic acid and 10% acetonitrile acidified with 0.1% formic acid over 2 min and held under the final condition for 1 min. Total run time was 23 min. For the tandem mass spectrometric analysis, the condition was set to source temperature at 500°C, ion spray voltage at 5,500 V in positive ionization mode and –4,500 V in negative ionization mode, curtain gas at 40 psi, nebulizer gas (GS1) at 40 psi, heater gas (GS2) at 40 psi, collision cell

exit potential at 10 V, and declustering potential at 40 V in positive ionization mode and –40 V in negative ionization mode. Nitrogen was used as the gas for all cases and the interface heater was on.

### Reactive oxygen species (ROS) measurement

The intracellular ROS level in human MM A-375 cells was measured using modified published protocol for the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay.<sup>35</sup> Briefly, A-375 cells harvested from the T-75 flasks were plated on a black flat-bottom 96-well plate at 10,000 cells per well and incubated at 37°C for 24 hr before being treated with the water extract of *S. nigrum* and its serial diluted solutions (2-, 4-, 8-, and 16-fold) for another 24 hr. Cells treated with cell culture media were used as negative control. At the end of treatment, 5 µL DCFH-DA working solution (concentration: 0.1 mM) was added to each well and allowed to react with the cells for 30 min before being aspirated out. The cells were then washed twice with 200 µL 1× phosphate buffered saline (PBS) buffer; and finally 100 µL 1× PBS buffer was added into each well and fluorescence was read at excitation of 485 nm and emission of 528 nm using an Agilent 8453E UV-visible spectroscopy system.

### Cytotoxicity of the water extract of *S. nigrum* and temozolomide combination

Human MM A-375 cells were plated in 96-well culture plates at 10,000 cells per well and allowed to attach and grow for 24 hr (reaching 70%–80% confluence) before being treated with temozolomide (final concentration: 200 µM) and its combination with the water extract of *S. nigrum* (1 g/100 mL) for another 24 hr. Treatment with dimethyl sulfoxide (DMSO), in which temozolomide solution was prepared, was used as negative control. Cytotoxicity of each treatment was measured using the CytoTox 96® non-radioactive cytotoxicity assay.

### Statistical analysis

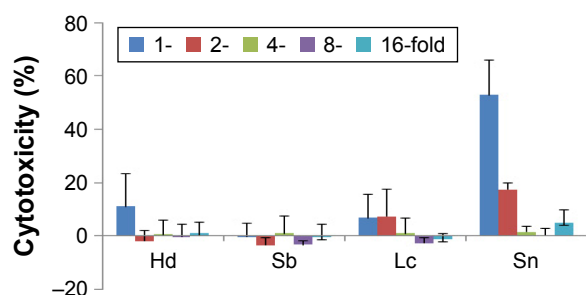
The experimental data were processed using Microsoft Excel 2013 and presented as mean ± standard deviation. Unpaired *t*-test was performed for statistical analyses using GraphPad QuickCalcs. A *P*-value <0.05 was considered to be statistically significant.

## Results and discussion

### Cytotoxicity of the herbal water extracts

*H. diffusa*, *S. barbata*, *L. chinensis*, and *S. nigrum* are four commonly used herbal plants to treat various types of cancer,

such as breast, liver, and colorectal cancers, in TCM. However, there is limited literature available regarding their applications against human MM. Only two previous studies reported that water extract of *S. nigrum* inhibited the metastasis of mouse melanoma B16-F1 cells and the ethanol extract of *H. diffusa* suppressed the growth of mouse melanoma B16-F10 cells.<sup>29,36</sup> To evaluate the applicability of these four herbs in treating MM, we measured the cytotoxicity of their water extracts toward MM cell line A-375. As shown in Figure 1, only *S. nigrum* exhibited a substantial and concentration-dependent cytotoxic effect against the A-375 cells. The water extract of *S. nigrum* and its 2-fold dilution increased the cytotoxicity by  $52.8\% \pm 13.0\%$  ( $P < 0.01$ ) and  $17.3\% \pm 2.7\%$  ( $P < 0.01$ ) compared to the control, respectively. As the water extract of *S. nigrum* was further diluted, no cytotoxicity was observed. The water extract of *H. diffusa* also increased the cytotoxicity by  $11.1\% \pm 12.4\%$  compared to the control; however this increase was not statistically significant ( $P = 0.23$ ). The clinical dose for *H. diffusa* is normally 2–5 times of that for *S. nigrum* in TCM; thus higher concentrated formulation of the water extract of *H. diffusa* may exhibit cytotoxic effect against A-375 cells. The water extracts of *S. barbata* and *L. chinensis* did not show any cytotoxic activity toward the A-375 cells. Higher concentrated formulation of the water extract of *S. barbata* or *L. chinensis* might still not be cytotoxic to the A-375 cells as their clinical doses are comparable to that for *S. nigrum*. Since ethanol extraction is also a common practice in TCM, further studies are warranted to investigate whether the ethanol extracts of these four herbs are cytotoxic toward human MM cells.



**Figure 1** Percentage increase (mean  $\pm$  standard deviation) in cytotoxicity of the crude water extracts of Hd, Sb, Lc, and Sn (extraction condition: 1 g herb in 100 mL water) and their respective serial dilutions (2-, 4-, 8-, and 16-fold) against human malignant melanoma cell line A-375.

**Notes:** The cytotoxicity was measured 24 hr after exposure using the CytoTox 96® non-radioactive cytotoxicity assay. Cells treated with the culture media were used as negative control. The increase of cytotoxicity was statistically significant only for the crude water extract of Sn ( $52.8\% \pm 13.0\%$ ,  $P < 0.01$ ) and its 2-fold dilution ( $17.3\% \pm 2.7\%$ ,  $P < 0.01$ ) compared to the control.

**Abbreviations:** Hd, *Hedyotis diffusa*; Sb, *Scutellaria barbata*; Lc, *Lobelia chinensis*; Sn, *Solanum nigrum*.

## HPLC-MS/MS analysis of the herbal water extracts

A major challenge in TCM is identification and quality control of biologically active ingredients in medicinal herbs, as the herbs are usually formulated in Fufang (combination of two or more herbs) with each herb containing hundreds of components. Recently, HPLC-MS, which allows fast separation, quantification, and even identification of compounds in a mixture, has been widely applied in analyzing herbal components.<sup>37</sup> Several classes of biologically active compounds including alkaloids, glycosides, flavonoids, polyphenols terpenoids, and saponins have been identified and characterized.<sup>37</sup> To identify why only *S. nigrum* was active against the MM A-375 cells, we analyzed the components of the four anticancer herbs using HPLC-MS/MS in both positive and negative ionization modes. In the positive ionization mode, the total ion current (TIC) chromatogram showed that a peak region with retention time between 1.8 to 5.5 min was only present in the water extracts of *S. nigrum* and *H. diffusa* (Figure 2A). We subsequently examined the ions in this peak region (Figure 2B and C). Surprisingly, we discovered that most of the abundant ions ( $m/z$  at 102.6, 279.6, 295.7, 352.9, 369.8, 761.3, 773.2, 775.2, 785.3, 801.3, 803.3, 809.3, and 817.3) were shared by both herbs, suggesting that *S. nigrum* and *H. diffusa*, which belong to different plant families (*Solanaceae* versus *Rubiaceae*), might use the same bioactive components for their cytotoxic effects against MM A-375 cells. The major difference in ion composition was that *S. nigrum* contained ions with  $m/z$  of 702.2, 747.2, 821.3, and 833.3, whereas *H. diffusa* contained ions with  $m/z$  at 704.3, 759.2, 839.2, and 855.5.  $\alpha$ -Solanin and solanidine, two major bioactive alkaloids identified in *S. nigrum*, were confirmed not to present or with minimal quantity in the fraction eluted between 1.8 and 5.5 min upon comparing with their fragmentation pattern reported previously.<sup>38</sup> We hypothesized that components eluted from the C18 column within this retention time range were responsible for the cytotoxicity of *S. nigrum* and more concentrated water extract of *H. diffusa* would be cytotoxic toward A-375 cells. Studies have already been initiated to prove our hypothesis and confirm the identities of the components. Furthermore, the purpose of coadministration of *S. nigrum* and *H. diffusa* in TCM might be to increase the concentration of bioactive components. As for the negative ionization mode, we observed a unique peak region with retention time between 5.5 and 6.8 min for *S. nigrum* from the TIC chromatogram (Figure 3A). The ions in this peak region were smaller in size compared to those in the positive ionization mode (Figure 3B), with the abundant



ions having  $m/z$  of 128.3, 165.3, 175.3, 197.4, 204.4, 227.4, 279.4, 281.5, 311.6, 317.5, 331.4, 347.4, 395.7, and 451.5. Further studies are warranted to elucidate whether components eluted from the C18 column at retention time between 5.5 and 6.8 min also contribute to the cytotoxic effect of *S. nigrum*.

### Water extract of *S. nigrum* reduced intracellular ROS level in A-375 cells

Excessive ultraviolet (UV) exposure has been identified as a major risk factor for MM.<sup>39</sup> UVA (320–400 nm) radiation can raise intracellular ROS generation and cause melanin-dependent oxidative DNA damages in melanocytes.<sup>40,41</sup> In fact, MM cells maintain a much higher level of intracellular ROS than most other types of cancer.<sup>42–44</sup> Furthermore, ROS are generally believed to promote MM invasion and metastasis.<sup>45,46</sup> Therefore, we investigated whether the water extract of *S. nigrum* has any effects on ROS generation. Cultured MM A-375 cells were treated with the water extract of *S. nigrum* and its serial dilutions for 24 hr and the intracellular ROS level was measured using the DCFH-DA assay. As shown in Figure 4, the ROS level in A-375 cells was reduced to  $24.7\% \pm 5.0\%$  ( $P < 0.01$ ) upon treatment with the water extract of *S. nigrum* compared to

the control. The 2- and 16-fold dilutions of the *S. nigrum* water extract also reduced the ROS level in A-375 cells to  $79.1\% \pm 36.3\%$  ( $P = 0.11$ ) and  $83.9\% \pm 35.9\%$  ( $P = 0.21$ ) compared to the control, respectively, however these ROS reductions were not statistically significant. Interestingly, the intracellular ROS level in A-375 cells was increased to  $122.1\% \pm 17.2\%$  (statistically significant,  $P = 0.03$ ) and  $110.9\% \pm 32.5\%$  ( $P = 0.37$ ) upon treatment with the 4- and 8-fold dilutions of the *S. nigrum* water extract, respectively. Thus, we concluded that the water extract of *S. nigrum* possessed cytostatic effect via reducing intracellular ROS generation in addition to its cytotoxic effect toward human MM A-375 cells. Further studies, including the expression of key ROS-related enzymes such as catalase, glutathione peroxidase, and superoxide dismutase, are warranted to fully understand how the water extract of *S. nigrum* reduces the intracellular ROS generation.

### Synergistic effect between the water extract of *S. nigrum* and temozolomide

Advanced MM generally does not respond to chemotherapy and DTIC is the only commonly used chemotherapy drug with a response rate of about 15%.<sup>47</sup> Temozolomide, an oral analog of DTIC, has been extensively evaluated in treating

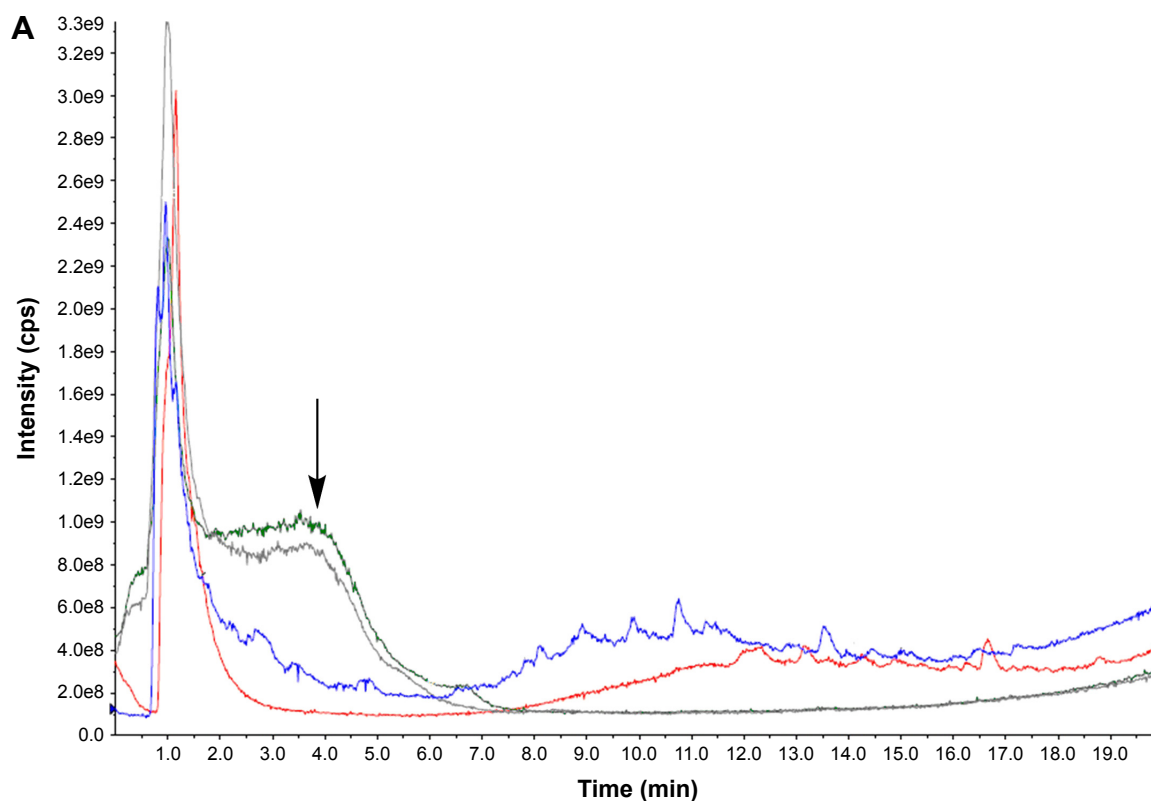
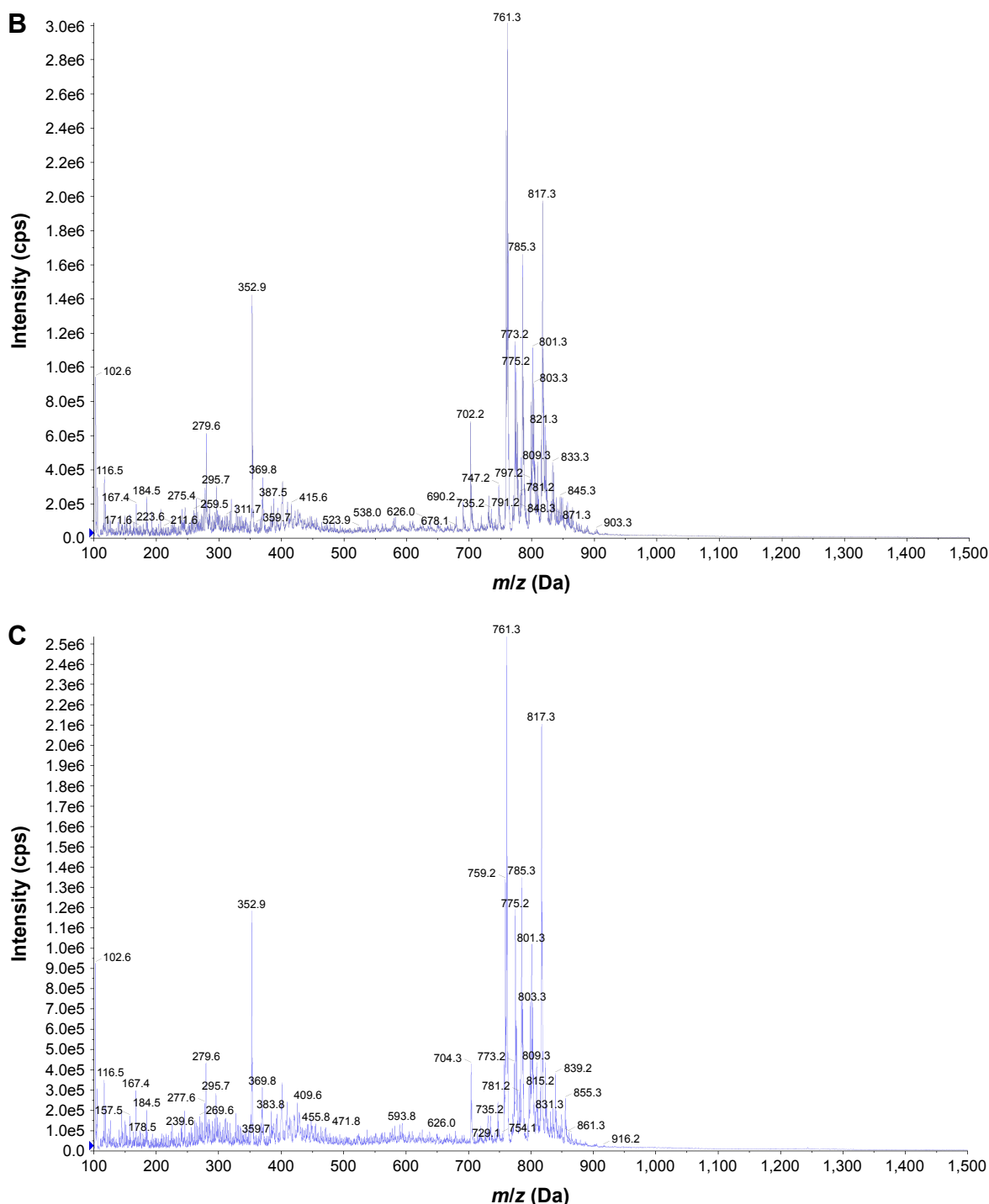


Figure 2 (Continued)



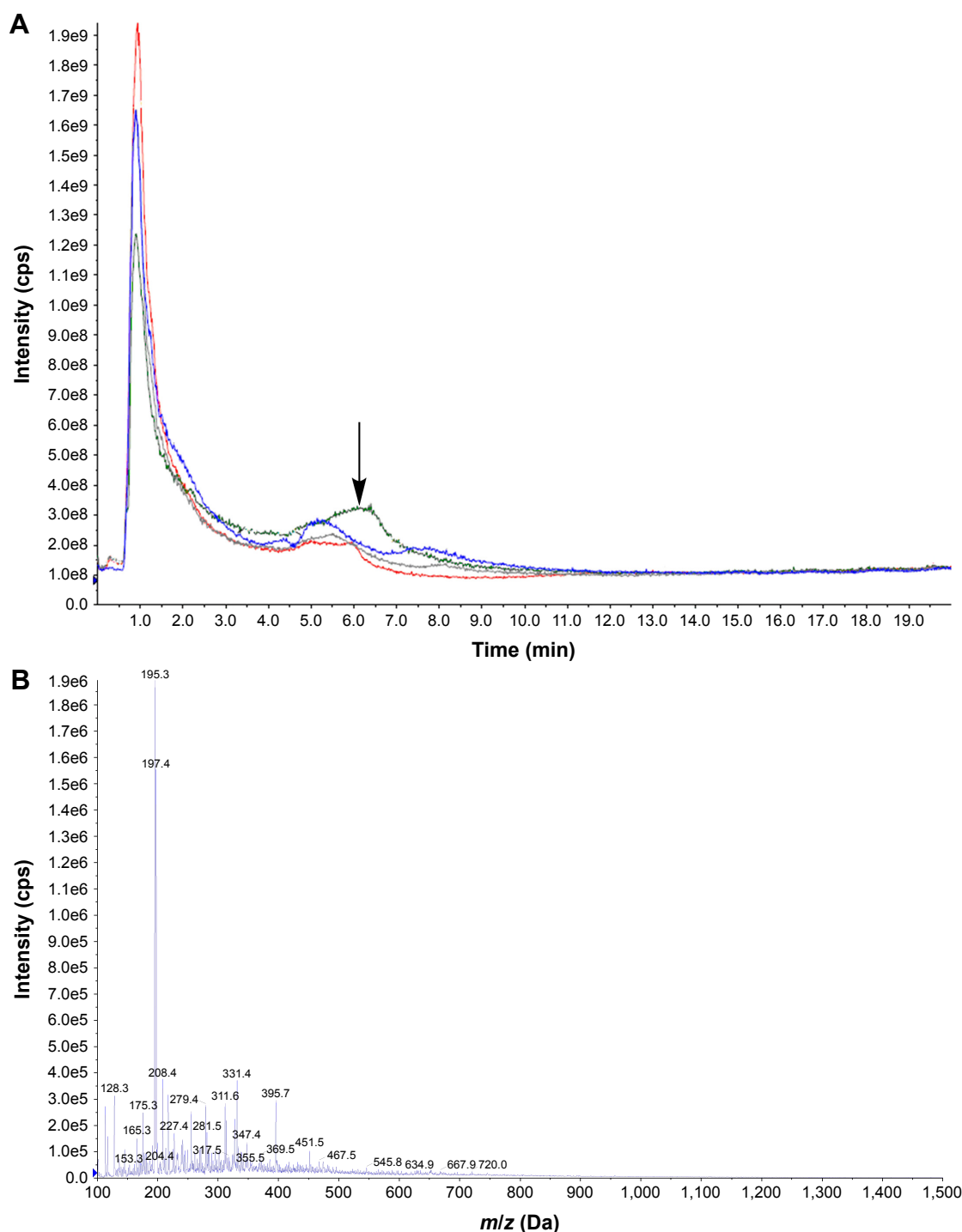
**Figure 2** HPLC-MS/MS analysis of the crude water extracts of *Hedyotis diffusa*, *Scutellaria barbata*, *Lobelia chinensis*, and *Solanum nigrum* (extraction condition: 1 g herb in 100 mL water) in positive ionization mode.

**Notes:** The total ion current (TIC) chromatograms were presented in panel (A) with *H. diffusa*, *S. barbata*, *L. chinensis*, and *S. nigrum* shown in gray, red, blue, and green, respectively. A peak region with retention time between 1.8 and 5.5 min, which was highlighted by an arrow, was observed only for the water extracts of *H. diffusa* and *S. nigrum*. Abundance of the ions within this peak region was shown in panel (B) for *S. nigrum* and panel (C) for *H. diffusa*.

**Abbreviations:** HPLC-MS/MS, high performance liquid chromatography-tandem mass spectroscopy; cps, counts per second.

advanced MM.<sup>48–52</sup> However, its new drug application (NDA 21-051) for melanoma was denied by FDA due to a lack of evidence showing therapeutic advantage over DTIC. A randomized phase III trial showed that coadministration of temozolomide and interferon  $\alpha$ 2b (INF- $\alpha$ 2b) increased

the response rate from 13% to 24% and overall survival time from 8.4 to 9.7 months.<sup>53</sup> Since various herbal water extracts have been reported to potentiate the therapeutic efficacy of chemotherapy drugs,<sup>25,26,54–56</sup> we evaluated whether the water extract of *S. nigrum* exhibits synergistic/additive effect



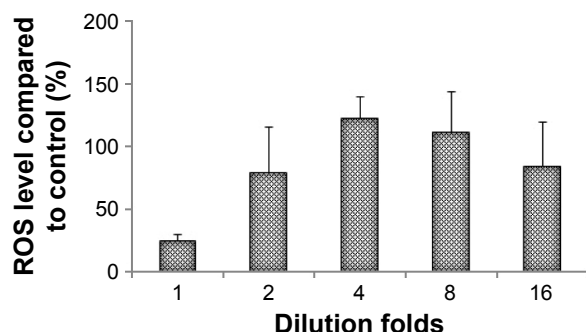
**Figure 3** HPLC-MS/MS analysis of the crude water extracts of *Hedyotis diffusa*, *Scutellaria barbata*, *Lobelia chinensis*, and *Solanum nigrum* (extraction condition: 1 g herb in 100 mL water) in negative ionization mode.

**Notes:** The TIC chromatograms were presented in panel (A) with *H. diffusa*, *S. barbata*, *L. chinensis*, and *S. nigrum* shown in gray, red, blue, and green, respectively. A peak region with retention time between 5.5 and 6.8 min, which was highlighted by an arrow, was observed only for the water extracts *S. nigrum*. Abundance of the ions within this peak region for *S. nigrum* was shown in panel (B).

**Abbreviations:** HPLC-MS/MS, high performance liquid chromatography-tandem mass spectroscopy; TIC, total ion current; cps, counts per second.

with temozolomide against human MM A-375 cells. As illustrated in Figure 5, the cytotoxicity of the coadministered temozolomide and water extract of *S. nigrum* was increased statistically significantly to  $97.0\% \pm 3.6\%$  from  $31.0\% \pm 7.5\%$  for temozolomide ( $P < 0.01$ ) and  $55.0\% \pm 17.6\%$  for the water

extract of *S. nigrum* ( $P = 0.03$ ), implicating synergistic effect was achieved between temozolomide and the water extract of *S. nigrum* against human MM A-375 cells. Our current study suggests that oral administration of temozolomide and the water extract of *S. nigrum* or oral administration of



**Figure 4** Effects of the crude water extract of *Solanum nigrum* and its serial dilutions (2-, 4-, 8-, and 16-fold) on intracellular ROS generation (mean  $\pm$  standard deviation) in human malignant melanoma A-375 cells.

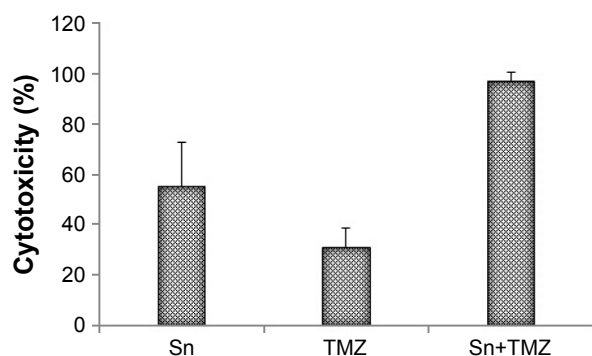
**Notes:** Treatment with cell culture media was used as negative control. Inhibition of ROS generation by the crude water extract of *S. nigrum* was statistically significant ( $P < 0.01$ ).

**Abbreviation:** ROS, reactive oxygen species.

temozolomide in combination with topical wash with the water extract of *S. nigrum* is worthy of further investigation using a patient-derived xenograft or a mouse xenograft melanoma model.

## Conclusion

In this study, we evaluated the cytotoxicity of the water extracts of *H. diffusa*, *S. barbata*, *L. chinensis*, and *S. nigrum* toward human MM A-375 cells. *S. nigrum* was the only cytotoxic herb at extraction condition of 1 g herb in 100 mL water. Our HPLC-MS/MS analysis showed that *S. nigrum* and *H. diffusa* might adopt the same bioactive components for their cytotoxic functions and higher concentrated formulation of the water extract of *H. diffusa* would exhibit cytotoxic effect against A-375 cells. The water extract of *S. nigrum*



**Figure 5** Percentage increase (mean  $\pm$  standard deviation) in cytotoxicity of the crude water extract of Sn (1 g herb in 100 mL water), TMZ (200  $\mu$ M), and their combination (Sn+TMZ) against human malignant melanoma cell line A-375.

**Notes:** The cytotoxicity was measured 24 hr after exposure using the CytoTox 96® non-radioactive cytotoxicity assay. Cells treated with DMSO, in which TMZ solution was prepared, were used as negative control. The cytotoxicity of Sn+TMZ was statistically significantly increased to  $97.0 \pm 3.6\%$  from  $31.0 \pm 7.5\%$  for TMZ ( $P < 0.01$ ) and  $55.0 \pm 17.6\%$  for Sn ( $P = 0.03$ ).

**Abbreviations:** DMSO, dimethyl sulfoxide; Sn, *S. nigrum*; TMZ, temozolomide.

was also shown to possess cytostatic activity toward MM A-375 cells through decreasing intracellular ROS generation. Furthermore, synergistic effect was observed between the water extract of *S. nigrum* and temozolomide. Based on the current results, we hypothesized that coadministration of the water extract of *S. nigrum* could improve the therapeutic efficacy of temozolomide, as well as DTIC, against human MM. Further studies are warranted to prove our hypothesis using a patient-derived xenograft or a mouse xenograft melanoma model.

## Acknowledgment

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## Disclosure

The authors report no conflicts of interest in this work.

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