

## Identification of Anticancer and Antioxidant phytoconstituents from chloroform fraction of *Solanum nigrum* L. berries using GC-MS/MS analysis

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*Solanum nigrum* is a traditional Indian plant acclaimed for its medicinal properties since antiquity. Among all plant parts fruit berries have shown to be most pharmacologically active part. In the present investigation, we tried to characterize the bioactive principles of chloroform fraction of *S. nigrum* (CFSn) fruit berries using GC-MS analysis. We could identify 29 compounds belonging to different chemical classes viz. alkaloids, flavonoids, carbohydrates, glycosides, phytosterols, proteins, phenolic compounds, and saponins. More specifically, we found two novel phenolic compounds, benzoisovanillin and syringic acid (4-hydroxy-3, 5-dimethoxybenzoic acid), which may be responsible for its pharmacological properties. Our phytochemical investigation of CFSn was well supported by its total phenolic content and antioxidant activity which we evaluated subsequently. Further, we investigated the anticancer activity against breast cancer cell lines (MDA-MB-231 and MCF-7) as well. Our *in vitro* results indicated that CFSn exhibited significant antiproliferative activity against both these cell lines and due induction of cancer cell death through apoptosis. Our study emphasizes the need for isolation and characterization of specific bioactive compounds of CFSn and determination of their mechanism of action responsible for its anticancer activity in breast cancer cells.

**Keywords:** Antiproliferative, Black nightshade, Breast cancer, Phenols, Tri-terpenoids

Cancer is one of the most life threatening diseases with more than 27 major types challenging the humankind with a striking fatality rate of 8.2 million people per year<sup>1,2</sup>. Worldwide, it registers 14.1 million fresh incidences every year<sup>2</sup>. It is no more a disease of the West. Less developed countries account for about 57% of cases and 65% of cancer deaths<sup>2</sup>. Breast cancer, being the most commonly diagnosed and highly prevalent malignancies among women and the leading cause of cancer death among them in less developed countries<sup>2</sup> (second leading in developed countries next to lung cancer<sup>3</sup>), still remains an important health issue of public concern. Globally, breast cancer constitutes to nearly a quarter (23%) of all cancers in women between the age group of 45-55<sup>4</sup>. In India, breast cancer leads at least in 19 registry areas with the highest rates observed in the Northeast and in major metropolitan cities such as Mumbai and Delhi<sup>5,6</sup>. The limited success of clinical therapies including radiation, chemotherapy,

immunomodulation and surgery in treating cancer, as evident by the high morbidity and mortality rates, indicates the need for relook in cancer management.

Natural phytochemicals derived from medicinal plants have gained significant recognition in the potential management of several human clinical conditions, including cancer<sup>7-14</sup>. Simultaneously, the synergistic effects of the cocktail of plant metabolites and the multiple points of intervention offer higher efficacy during chemoprevention regimens<sup>7</sup>. There are number of natural agents which are used in treatments against breast cancer such as curcumin, sauchinone, lycopene, denbinobin, genipin, capsaicin, and ursolic acid<sup>15</sup>. The Indian sub-continent has great botanical diversity and widespread use of traditional medicine practice known as ayurvedic medicine; however, only a relatively small number of these plants have been subjected to accepted scientific evaluation for their potential anticancer effects<sup>14,16,17</sup>.

*Solanum nigrum* L. (Solanaceae) commonly known as 'Black nightshade'; is a widely used medicinal plant in India and other parts of the world<sup>18</sup>. The folk medicine 'Gewai saag' of the tribals of central

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Himalayan region, Uttarakhand prepared from leaves and young stem of *S. nigrum* attenuates various body pain, joint pain and rheumatism<sup>19</sup>. Communities in Jeju Island, Korea are known to use its leaf paste to treat skin diseases<sup>20</sup>. The phytochemical studies have revealed that the whole plant contains glycoalkaloids, steroidal glycosides, steroidal saponins (diosgenin), steroidal genin (gitogenin), tannin and polyphenolic compounds with mature fruits having low alkaloid content<sup>21,22</sup>. Among many bioactive entities, solanine has been extensively studied and comprises of 95 percent of the total alkaloid<sup>23</sup>. Fruit berries of *S. nigrum* are considered to be pharmacologically most active part of the plant. The fruit of *S. nigrum* is reported to have antiulcer, antioxidant, anti-inflammatory, antituberculosis antidiuretics and antitumor activity<sup>24</sup>. Qureshi *et al.*<sup>25</sup> has also documented the use of *S. nigrum* leaf juice for cough and fever and fruit juice for stomach ailments including abdominal pain. The antiproliferative activities of the crude organic extract and isolated compounds were studied on tumor cell lines of liver, colon, breast, and cervical<sup>26-29</sup>.

The development of cancer in humans is a complex process including cellular and molecular changes mediated by diverse endogenous and exogenous stimuli. It is well established that free radical induced oxidative DNA damage, chromosomal defects and oncogene activation is responsible for cancer initiation and development<sup>30</sup>. A quantitative correlation between the antioxidant activity and the content of polyphenols has been seen in many studies, signifying that the phenolic compounds present in the plants contribute to their antioxidant activity<sup>31,32</sup>. On this note, large numbers of studies are conducted on medicinal plants, for their free radical scavenging and natural antioxidants activity which stabilizes their therapeutic use in treatment of various diseases such as cardiovascular diseases, diabetes, neurodegeneration, inflammation, and cancer<sup>9,13,17,32,33</sup>. Presence of carotenoids, phenolics, flavonoids and tannins contents in *S. nigrum* is suggestive of its immense antioxidant potential, and thereby explain its pharmacological properties *in vitro* as well as *in vivo*<sup>29,34</sup>.

However, as per the currently available literature, only a few studies have correlated the phytochemicals contents and medicinal properties of chloroform fraction of *S. nigrum* (CFSn) fruit berries. Consequently, the present study focuses on the

assessment of the entire phytochemical profile of CFSn. We also evaluated the antiproliferative activity of CFSn against the human breast cancer cells (MDA-MB-231 and MCF-7) in order to prove its medicinal property as an anticancer agent.

## Materials and Methods

### Collection of plant berries

The fresh berries of *S. nigrum* were collected from the local market of Lucknow in June 2014. The taxonomic identification of the plants was done from the Department of Botany, Shia PG College, Lucknow. All the plant berries were cleaned and shade dried.

### Preparation of methanolic extract

The shade dried berries of selected plants were pulverized. About 500 g of powdered sample was extracted with the 1000 mL of methanol by using a Soxhlet extractor for 24 h. The obtained extracts were concentrated using a rotary flash evaporator. The extracts were well preserved in airtight containers for further analysis.

### Fractionation of Chloroform

This crude methanolic extract was further utilized for the preparation of chloroform fraction by cold maceration process (Fig. 1). Vacuum evaporator was used to concentrate chloroform fraction, and then it was kept in a vacuum desiccator for complete removal of solvent leaving behind the weighed chloroform fraction.

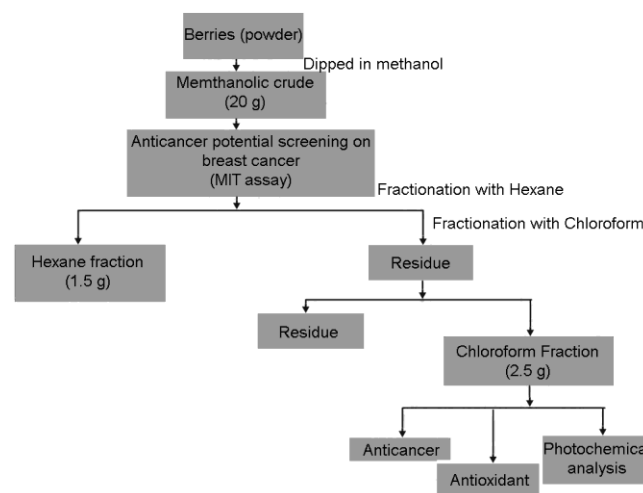


Fig. 1—Flow chart depicting processing of *Solanum nigrum* to produce chloroform fraction.

**GC-MS/MS analysis**

The GC-MS analysis was carried out using a Thermo TSQ Quantum XLS with Triplus autosampler equipped with triple quadrupole with an elite-1 (100% dimethyl poly siloxane), 30 m × 0.25 mm (inner diameter) × 0.25 μM of capillary column. Helium gas (99.99%) was used as carrier gas at a constant flow rate of 1.1 mL/min with splitless mode, and sample (1 μL) was first derivatized with 80 μL of Methoxy amine hydro-chloride and 100 μL of N, O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) at 65°C for 1 h and injected into the system. The instrument was set to an initial temperature of 250°C and maintained at this temperature for 2 min. At the end of the period, the oven temperature was rose up to 290°C at the rate of an increase of 6°C/min ending with a 20 min hold. Injection port temperature was maintained at 250°C. The ionization voltage was 70 eV and the solvent delay was 6.5 min and the total GC-MS running time was 55 min with full scan mode with injector port silylation in which derivatizing agent was used BSTFA (1.5 μL). Using computer searches on a National Institute of Standards and Technology ver.2.1 MS data library and comparing the spectrum obtained through GC-MS compounds present in the plants sample were identified.

**Determination of the total phenolic content**

Total phenol contents were determined by Folin-Ciocalteu reagent method<sup>35</sup> with slight modifications using Gallic acid as a standard phenolic curve. In brief, 0.1 mL of CFSn (1 mg/mL) was added to 1 mL 10% Folin-Ciocalteu reagent and incubated for 5 min. After 5 min, 0.8 mL solution of Na<sub>2</sub>CO<sub>3</sub> (10% w/v in distilled water) was added and allowed to stand for 15 min at 25°C with alternating shaking. The sample was vortexed again and the absorbance of resultant solutions was measured at 725 nm using Thermo-Fisher spectrophotometer after incubation at 25°C for 1 h. The total phenolic contents were expressed as mg of Gallic acid equivalent (GAE)/g plant dried wt.

**Free radical-scavenging activity**

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was evaluated based on method<sup>36</sup>. A stock solution of 0.052 mg/mL of DPPH was prepared in methanol. The sample extract (0.2 mL) was diluted with methanol and 2 mL of DPPH solution (0.5 mM) was added. After 30 min, the absorbance was measured at 517 nm. The percentage of the DPPH radical scavenging was calculated from

the measured absorbance data. Ascorbic acid was used as a standard antioxidant in this assay method. DPPH radical scavenging activity was calculated as follows:

$$\% \text{ inhibition} = \frac{[\text{Abs sample} - \text{Abs control}]}{(\text{Abs control})} \times 100$$

Control = 1.0 mL methanol + 1.0 mL DPPH

**Cell line and culture**

Human breast cancer (MDA-MB231 and MCF-7) cell line was obtained from Cell repository, National Center for Cell Science (NCCS), Pune, India. MDA-MB-231 and MCF-7 were cultured with medium RPMI supplemented with 5 and 10% FBS, respectively with 1% penicillin/streptomycin solution and grown at 37°C, 5% CO<sub>2</sub> in a humidified chamber.

**Cell Viability assay**

Approximately, breast cancer cells (5 × 10<sup>3</sup>) were seeded in 100 μL RPMI media in each well of a 96-well culture plate for 24 h at 37°C and humidity of 5% CO<sub>2</sub>. Cells were treated with different concentrations (6.25-400 μg/mL) of CFSn for 24, 48 and 72 h. There after, a 10 μL of MTT (5 mg/mL) dye was added to each well, and incubated for at 37°C until the development of formazan blue crystals. Formazons were dissolved 100 μL of DMSO 10 min at 37°C. The absorbance was recorded at 540 nm by a microplate reader (BIORAD-680). The percentage viability was calculated by using the formula:

$$\% \text{ Growth inhibition} = 100 - \% \text{ Cell viability}^*$$

$$\text{where } \% \text{ Cell viability} = \frac{[\text{OD of treated}]}{(\text{OD of control})} \times 100$$

**Colony formation assay**

Cells were seeded in 6-well plates at a density of 1000 cells per well and treated with different concentrations (6.25-100 μg/mL) of CFSn for 24 h. After 24 h of treatment, media was removed with fresh complete growth media. Media was changed every 3 days to sustain the viability of cells and the propagation to form visible colonies. Colonies formed after 7 days were fixed with methanol, stained with 0.05% Coomassie blue and counted under an inverted microscope.

The following formula was used to calculate the colony inhibition rate

$$\text{Colony inhibition rate (\%)} = \frac{(\text{Colony no of control group} - \text{Colony no of treated group})}{\text{Colony no of control group}} \times 100$$

**Hoechst 33342 staining**

MDA-MB-231 cells and MCF-7 (200 cells per well) were grown on a cover slip in 6-well plate and treated with different concentrations (6.25-100 µg/mL) of CFSn for 24 h, 0.1% DMSO in complete growth media was used as a control. At the end of incubation, cells were collected and fixed in 4% para-formaldehyde for 15 min at 4°C. Cells were then washed with ice-cold PBS thrice and stained with Hoechst 33342 dye (1 µg/mL in PBS) and incubated for 2 min in the dark, at 37°C. Fixed cells were again washed thrice with ice-cold PBS and observed under a Phase-Contrast and Fluorescence Inverted Microscope (ECLIPSE Ti-U, Nikon, Japan) with standard excitation filters.

**Results****Chemical composition of extracts by GC-MS analysis**

The chloroform fraction of *S. nigrum* was processed for GC-MS analysis to identify its phytochemical content. A high resolution qualitative GC-MS analysis of CFSn showed the presence of 29 chemical constituents eluted between 6.36 and

42.05 min (Fig. 2 and Table 1). In our GC-MS analysis, major bioactive components of CFSn were phenolic compounds among which we identified two novel phenolic compounds, isovanillic acid and syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), which were not previously reported in CFSn. Isovanillin is a phenolic aldehyde and isomer of vanillin well-known as anticancer agent<sup>37,38</sup>. Syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid) is a naturally occurring major phenolic compound found in many plants and food compounds and reported for its anticancer activity<sup>39</sup>.

**CFSn contains high amounts of polyphenols**

We intended to determine the total phenolic content (TPC) of CFSn using the Folin-Ciocalteu method. The total phenolic content (TPC) of CFSn was calculated using Folin-Ciocalteu reagent, expressed in terms of Gallic acid equivalent (the standard curve equation:  $y = 7.026x - 0.0191$ ,  $r^2 = 0.999$ ). The calculated TPC of CFSn is expressed as mg of GA/g of dried weight. The content of TPC in the CFSn was  $19 \pm 0.6$  mg/g GAE.

Table 1—Summary of compounds found in chloroform fraction of *Solanum nigrum*

Compounds (NIST)	R.T.	M.W.	Relative peak area (%)	Molecular formula
$\alpha$ -hydroxylsobutyric acid [tms]	6.34	248	0.19	C <sub>10</sub> H <sub>24</sub> O <sub>3</sub> Si <sub>2</sub>
D-lactic acid-ditms	8.05	234	0.13	C <sub>9</sub> H <sub>22</sub> O <sub>3</sub> Si <sub>2</sub>
Hexonic acid, tert-butyl dimethylsilyl ester	8.36	230	0.17	C <sub>12</sub> H <sub>26</sub> O <sub>2</sub> Si
Ethanedioic acid, bis(trimethylsilyl) ester	9.57	234	0.08	C <sub>8</sub> H <sub>18</sub> O <sub>4</sub> Si <sub>2</sub>
2-Deoxy ribose O,O',O''-tris(trimethylsilyl)-	10.60	350	0.46	C <sub>14</sub> H <sub>34</sub> O <sub>4</sub> Si <sub>3</sub>
1-O-pentadecylglycerol, bis(trimethylsilyl) ether	12.67	446	1.21	C <sub>24</sub> H <sub>54</sub> O <sub>3</sub> Si <sub>2</sub>
DL-Malic acid, O-trimethylsilyl-,bis(trimethylsilyl) ester	17.27	350	0.34	C <sub>13</sub> H <sub>30</sub> O <sub>5</sub> Si <sub>3</sub>
Pyroglutamic acid, bis(trimethylsilyl)	17.87	273	0.22	C <sub>11</sub> H <sub>23</sub> NO <sub>3</sub> Si <sub>2</sub>
$\alpha$ -D-Mannopyranoside, methyl cyclic-2,3,4,6-bis(butylboronate)-	18.86	326	0.17	C <sub>15</sub> H <sub>28</sub> B <sub>2</sub> O <sub>6</sub>
Bis trimethylsilyl fumaric acid	19.48	260	0.32	C <sub>10</sub> H <sub>20</sub> O <sub>4</sub> Si <sub>2</sub>
$\beta$ -D-galactopyranoside, methyl-2,3-bis-O-(trimethylsilyl)-, cyclic methylboronate	21.45	362	0.77	C <sub>14</sub> H <sub>31</sub> O <sub>6</sub> Si <sub>2</sub>
Isovanillic acid	22.47	312	0.86	C <sub>14</sub> H <sub>24</sub> O <sub>4</sub> Si <sub>2</sub>
Azelaic acid, bis(trimethylsilyl) ester	23.10	332	2.78	C <sub>15</sub> H <sub>32</sub> O <sub>4</sub> Si <sub>2</sub>
Tartaric acid, bis-O-(trimethylsilyl)-, bis(trimethylsilyl) ester	23.25	438	1.35	C <sub>16</sub> H <sub>38</sub> O <sub>6</sub> Si <sub>4</sub>
Isocitric acid, tms	23.45	480	0.69	C <sub>18</sub> H <sub>40</sub> O <sub>7</sub> Si <sub>4</sub>
L-(+)-tartaric acid, bis(trimethylsilyl) ether, bis(trimethylsilyl) ester	23.95	438	1.54	C <sub>16</sub> H <sub>38</sub> O <sub>6</sub> Si <sub>4</sub>
5-(4-Hydroxyphenyl)-3-methyl-5-phenylhydantoinic acid	24.59	688	0.28	C <sub>32</sub> H <sub>48</sub> N <sub>2</sub> O <sub>9</sub> Si <sub>3</sub>
Syringic acid d-tms	24.76	342	0.67	C <sub>15</sub> H <sub>26</sub> O <sub>5</sub> Si <sub>2</sub>
Hexadecanoic acid, trimethylsilyl ester	27.29	328	5.05	C <sub>19</sub> H <sub>40</sub> O <sub>2</sub> Si
Stearic acid, trimethylsilyl ester	30.18	356	3.2	C <sub>21</sub> H <sub>44</sub> O <sub>2</sub> Si
Cholesterol trimethylsilyl ester	31.93	458	4.97	C <sub>30</sub> H <sub>54</sub> O <sub>2</sub> Si
Octadecadienoic acid, [(trimethylsilyl)oxy]-, trimethylsilyl ester	32.16	440	4.18	C <sub>24</sub> H <sub>48</sub> O <sub>3</sub> Si <sub>2</sub>
1H-indole-3-carboxylic acid,2-ethoxy-1-(trimethylsilyl)-5-[(trimethylsilyl)-oxy]-,	33.56	393	4.51	C <sub>19</sub> H <sub>31</sub> NO <sub>4</sub> Si <sub>2</sub>
2-Oleoyl-bis trimethylsilyl glycerol	34.90	500	2.7	C <sub>27</sub> H <sub>56</sub> O <sub>4</sub> Si <sub>2</sub>
1-Monolinoleoylglycerol tms ether	35.17	498	3.76	C <sub>27</sub> H <sub>54</sub> O <sub>4</sub> Si <sub>2</sub>
Cholesterol trimethylsilyl ether	39.10	458	1.67	C <sub>30</sub> H <sub>54</sub> O <sub>2</sub> Si
Stigmasterol trimethylsilyl ether	40.97	484	0.32	C <sub>32</sub> H <sub>56</sub> O <sub>2</sub> Si
Sarsapogenin	41.76	416	0.62	C <sub>27</sub> H <sub>44</sub> O <sub>3</sub>
$\beta$ -sitosterol trimethylsilyl ether	42.05	486	1.38	C <sub>32</sub> H <sub>58</sub> O <sub>2</sub> Si

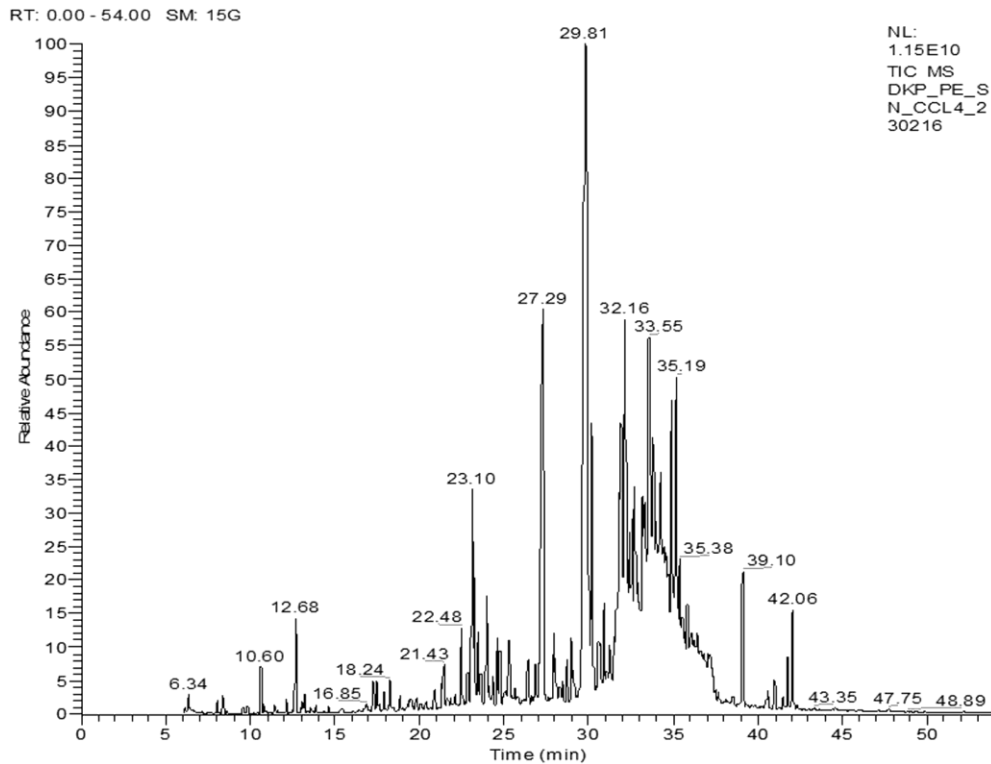


Fig. 2—GC-MS/MS chromatogram of Chloroform fraction of *Solanum nigrum*

**CFSn exhibits potent free radical scavenging activity**

The antioxidant activities of CFSn were determined according to DPPH free radical scavenging method in dose-dependent manner (Fig. 3) using ascorbic acid as standard compound. The IC<sub>50</sub> (amount needed to scavenge 50% of DPPH) of standard ascorbic acid was found to be 14±0.5 µg/mL. CFSn with concentration range (6.25-400 µg/mL) exhibited potent scavenging effects against DPPH free radical. The median effective concentration (IC<sub>50</sub>) of CFSn needed to scavenge 50% of DPPH radical was found as 39.5±0.3 µg/mL. These data indicate that CFSn possesses very potent antioxidant activity.

**In vitro anticancerous activity of CFSn against MDA-MB-231 and MCF-7**

The preliminary screening of antiproliferation activity of CFSn was performed by MTT assay against human breast cancer cell lines (MDA-MB-231 and MCF-7). The cancer cells were exposed to increasing dose of CFSn for 24, 48 and 72 h. From bar diagram (Fig. 4), the antiproliferative activity revealed that CFSn had dose and time dependent cytotoxic effect. However, 50% killing (IC<sub>50</sub>) of MDA-MB-231 cells was observed at 42.5±1.87 and 55.5±0.3 µg/mL for MCF-7 after 24 h of exposure. The IC<sub>50</sub> as compared to that of untreated cells was

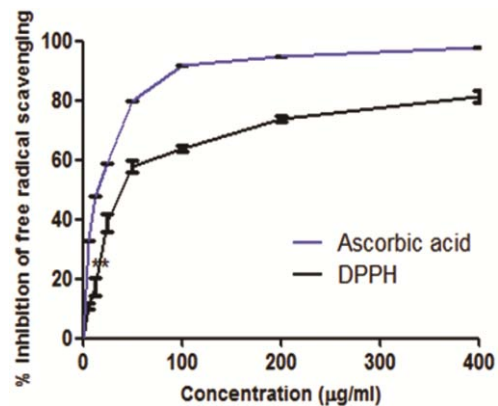


Fig. 3—Free radical scavenging effect of CFSn in DPPH assay.

Table 2—IC<sub>50</sub> value obtained for CFSn against MDA-MB-231 and MCF-7 cells after exposure of 24, 48 and 72 h

Time (h)	MDA-MB-231 IC <sub>50</sub> (µg/mL)	MCF-7 IC <sub>50</sub> (µg/mL)
24	42.5±1.87	55.5±0.3
48	35.5±1.5	37.5±2.5
72	17.5±4	27.5±0.5

Values are expressed as mean ± S.D. (n = 3).

determined in (Table 2). The CFSn demonstrated a potent anti-proliferative activity against MDA-MB-231 cells and MCF-7 cells in a dose and time dependent manner.

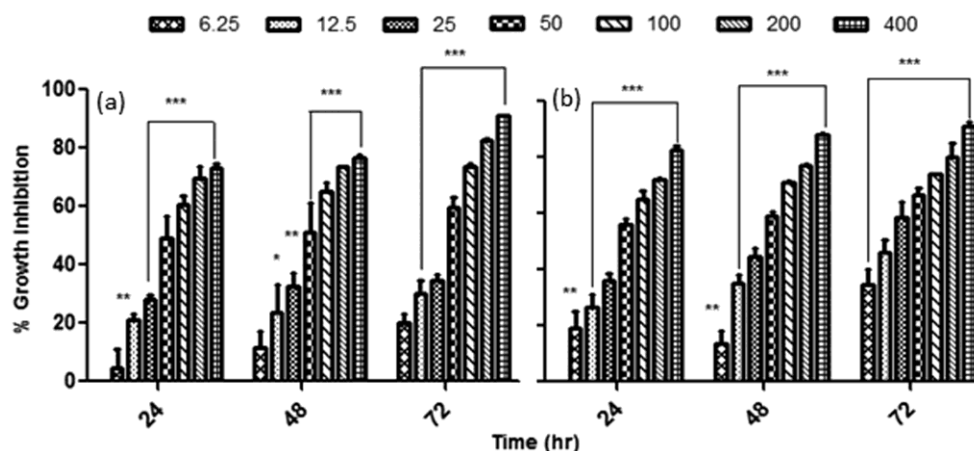


Fig. 4—Anti-proliferative effect of CFSn against human breast cancer cells. Human breast cancer (a) MDA-MB-231; and (b) MCF 7 cells were exposed for concentrations range (6.25–400  $\mu\text{g/mL}$ ) for 24, 48 and 72 h, and the viability of the cells was determined by MTT assay. [Cell viabilities are shown in percentages with respect to untreated control. Data represent the means  $\pm$ SD of three experiments conducted with  $*P \leq 0.05$ ]

#### Assessment of CFSn on colony formation of breast cancer cells

The clonogenic assay is considered as the gold standard to determine the anticancer activity of drugs. CFSn significantly ( $P \leq 0.05$ ) reduced the colony formation ability of both breast cancer cells (MDA-MB-231 and MCF-7) over a period of 10 days, thereby signifying the long-term anti-proliferative consequence of CFSn (Fig. 5). A significant decrease in colony formation was noted in both the treated cells with a decrease of 10-95% and 20-85% in the concentration range of 12.5-200  $\mu\text{g/mL}$  for MDA-MB-231 and MCF-7, respectively, compared to control after exposure with CFSn.

#### Assessment of CFSn induced apoptosis by Hoechst 33342 staining

Alteration in nuclear morphology is assessed for preliminary determination of apoptotic cancer cell death. Hence to confirm the nuclear and morphological changes in MDA-MB-231 and MCF-7 cells Hoechst 33342 staining were performed. After treatment with CFSn for 24 h, the treated cells revealed distinctive apoptotic features such as cell contraction, nuclear strengthening, and formation of plump apoptotic bodies which give the impression of round spherical globules (Fig. 6). Our results suggested that CFSn induced cell death is mediated by apoptotic induction in MDA-MB-231 and MCF-7 cells. The data also suggested that the phytochemicals present in CFSn might have activated the phenomena of programmed cell death of cancer cells.

#### Discussion

In the present study, CFSn showed cytotoxicity and cell proliferation inhibition in MDA-MB-231 and

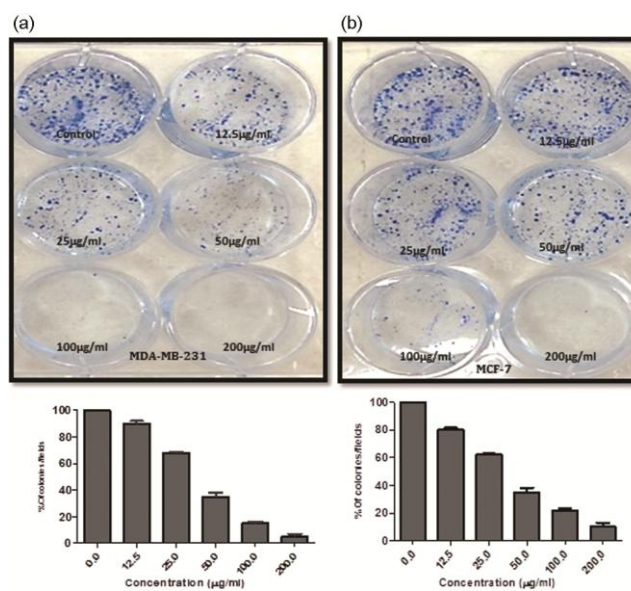


Fig. 5—Effect of CFSn on growth assay estimated by colony formation. Human breast cancer (a) MDA-MB-231; and (b) MCF-7 cells were grown in media along with specified concentration of the CFSn. [The number of colonies was recorded after 10 days of treatment. Data represents the mean  $\pm$  SD of three different assays with  $*P \leq 0.05$ ]

MCF-7 in dose and time dependent manner without affecting the normal cells with  $\text{IC}_{50} 42.5 \pm 1.87 \mu\text{g/mL}$  for MDA-MB-231 and  $55.5 \pm 0.3 \mu\text{g/mL}$  for MCF-7 cells after 24 h of exposure. We also found significant decrease in the number of colonies formed after CFSn treatment which suggested its antiproliferative activity. The preventive mechanisms of tumor promotion by natural phytochemicals range from the inhibition of genotoxic effects, increased antioxidants and anti-inflammatory activity, inhibition of proteases

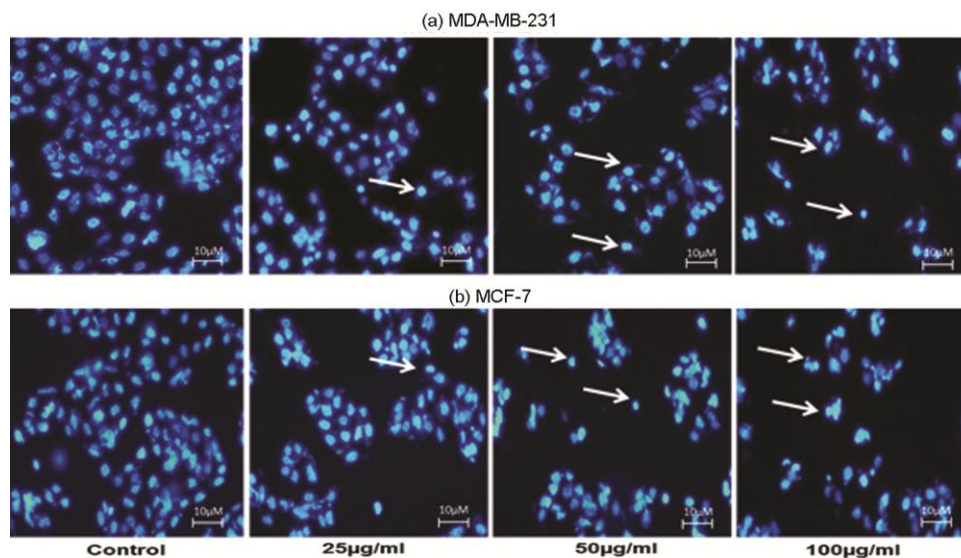


Fig. 6—Induction of nuclear fragmentation by CFSn in (a) MDA-MB-231; and (b) MCF-7 cells:  $2 \times 10^4$  cells/well were seeded in 12-well culture plate and allowed to grow for 24 h and then treated with different concentrations of CFSn for 24 h and stained with Hoechst 33242 stain following standard protocol. [Images were captured through inverted fluorescence microscope at 20X magnifications. Images are representative of three independent experiments]

and cell proliferation, protection of intracellular communications to modulate apoptosis and signal transduction pathways<sup>40</sup>. Among all these effects apoptosis induction is the principal mechanism to kill cancer cells. Apoptosis is programmed cell death which is characterized by morphological changes such as membrane bebling cell shrinkage, chromatin condensation and DNA fragmentation. Our results from Hoechst staining also showed features of apoptotic cell death in both the cancer cells. It can be presumed that apoptotic induction by CFSn may be responsible for its antiproliferative activity in MDA-MB-231 and MCF-7 cells. Previous studies on aqueous extract of *S. nigrum* have shown to exert inhibitory effects on cell growth and colony formation on different cancer types (prostate, breast, colorectal and cervical cancer)<sup>41,42</sup>.

Phytochemicals constitute one of the most numerous and ubiquitous group of plant metabolites such as alkaloids, phenols, fatty acids. It was found that in addition to their primary antioxidant activity, these compounds display a wide variety of biological functions which are mainly related to regulation of carcinogenesis<sup>43</sup>. In our GC-MS analysis, major bioactive components of CFSn were phenolic compounds along with identified two novel phenolic compounds Iovanillic acid and syringic acid, CFSn also comprises of many known phytosterol such as  $\beta$ -sitosterol, cholesterol and Stigmasterol which are reported in medicinal plants and have shown

antioxidant properties<sup>44</sup>. The berries of *S. nigrum* have been found to contain a saturated steroidal genin, which has been identified as tigogenin by mixed melting point and IR spectroscopy<sup>45</sup>. Sarsasapogenin (SAR) is a steroidal sapogenin that is used as starting material for the industrial synthesis of steroids. It has various pharmacological benefits, such as antitumor and antidepressant activities<sup>46</sup>. We also found indole alkaloids to be present in CFSn. Alkaloids are among the most important active components in *S. nigrum*, and some of these compounds have already been successfully developed into chemotherapeutic drugs, such as solasodine and solanidine<sup>47</sup>.

The free radical scavenging or antioxidant activity of phenolic compounds is mainly due to their reduced properties which allow them to act as metal chelators, absorber and neutralizes free radicals<sup>48</sup>. Therefore, based on the total phenolic contents of CFSn its antioxidant potential was also investigated by DPPH method. The redox properties of the phenolic compounds is main cause of its antioxidant activity, which can play an important role in absorbing and neutralizing free radicals ( $IC_{50}$   $39.5 \pm 0.3$   $\mu\text{g/mL}$ ), quenching singlet and triplet oxygen or decomposing peroxides and have inhibitory effects on mutagenesis and carcinogenesis in humans<sup>49</sup>. This result showed that greater rate of DPPH scavenging activity of CFSn may be due to the presence of high phenolic or flavonoid compounds as identified in our GC-MS analysis.

Conclusively, our study provided experimental evidence of antioxidant and antiproliferative activity of CFSn. The presence of high amounts of polyphenols, alkaloids and novel bioactive principles like isovanillic acid and syringic acid suggest that this fraction has a reservoir of pharmacologically active compounds, and it is apparent from our study that effective compounds produced from CFSn tend to support the traditional medicinal use of this plant in the treatment of cancer. Our results, therefore, promote isolation of these bioactive compounds from CFSn and assessment of their antiproliferative and pro-apoptotic activity.

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