Research article

Isolation, characterization and *in vitro* cytotoxicity studies of bioactive compounds from *Alseodaphne semecarpifolia* Nees

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ABSTRACT

Alseodaphne semecarpifolia (Lauraceae) is a traditional medicinal plant historically used in Indian Western Ghats to treat various human diseases. From the past few decades it has been traditionally recognized as an effective agent in cancer treatment. The phytochemical investigation of the stem bark and leaves of *A. semecarpifolia* led to the isolation of bioactive flavonoid compounds Icariin and Baicalein. Their structures were elucidated from obtained spectral data (^{1}H NMR, ^{13}C NMR, ^{1}H ^{1}H COSY, FTIR and MS). Cytotoxic activity of Icariin and Baicalein evaluated against MCF-7 cells revealed their potent activity with an IC_{50} of 42.15 ± 4.78 μg/ml and 44.37 ± 3.46 μg/ml, respectively, while very least effect was observed on normal cells (L6). Present study has suggested that both Icariin and Baicalein have potent cytotoxic activity against MCF-7 cells.

1. Introduction

Plants have been vast resources of traditional medicines over the years. The ancient practice of using traditional medicinal plant extracts to treat human diseases has greatly increased over the time [1]. The phytochemicals derived from medicinal plants continue to play a significant role in medicinal system [2]. Indigenous expertise is the predominant aspect involved in exploring herbal drugs from medicinal plants and this indigenous knowledge is the baseline for initiation of ethno pharmacological investigation [3]. It is appropriate to search for safe and potent herbal medicines which are used in traditional practices in order to strengthen their efficacy and safety levels through scientific validation.

Several clinically useful plant derived compounds have been developed and proved as inevitable resources used as substitute for modern synthetic drugs [4]. In Indian traditional medicinal system, many of the plants and their bioactive components are known to possess therapeutic properties and are being used as an alternative approach to minimise the irreversible effects of modern drugs on the immune system [5, 6].

Alseodaphne semecarpifolia Nees is an evergreen plant belongs to the family Lauraceae [7, 8, 9], and confined to Central Western Ghats region of Karnataka, India [10, 11]. It is also distributed in evergreen forests of peninsular India and Srilanka [12, 13]. The stem bark and leaves of this plant have been found to be used as an indigenous remedy to treat various human diseases [14, 15, 16].

The secondary metabolites of *A. semecarpifolia* are known for their potent pharmacological activities [13, 17]. Even though *A. semecarpifolia* has a traditional history of treating human diseases over the years, still it is lacking support from scientific validation. Also, there is no report on the isolation of bioactive compounds from this plant and evaluation of their bioactivity. In central Western Ghats region of Karnataka, India, it is traditionally employed in the treatment of human cancers. Hence, it was imperative to take up a thorough investigation of its bioactive components and their inhibitory effects on cancer cell proliferation. Herein, the isolation, structural elucidation and cytotoxic activity of bioactive compounds from stem bark and leaves of *A. semecarpifolia* are reported.

2. Experimental section

2.1. General experimental procedures

Thin-layer chromatography (TLC) was carried out on a pre-coated silica gel plates (Merck, Silica gel 60 F$_{254}$) and UV light was used to visualise the obtained products. The compounds were isolated by Column Chromatography (CC) using silica gel 60 (0.063–0.200mm; Merck,

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70–230 mesh ASTM). The ^{1}H NMR and ^{13}C NMR spectra were recorded by using (JNM-ECZ400S/L1; JEOL, USA) a spectrometer with 400MHz for ^{1}H NMR and 100MHz for ^{13}C NMR and a 2D NMR (^{1}H ^{1}H COSY) spectrum was obtained using 500MHz spectrometer (JNM-ECZ500R/S1; JEOL, USA) using DMSO-d6 solvent. The chemical shifts (δ) were recorded in parts per million (ppm), relative to internal reference standard tetramethylsilane (TMS). The infrared (IR) spectrum was recorded on FT/IR-4100 (JASCO, Germany) spectrometer, using triglycine sulphate (TGS) detector and KBr pellets. The mass spectrum was obtained using Waters, SQD-2 mass spectrometer with Single-quadruple detector. All the solvents used for extraction, fractionation and isolation were of analytical grade.

2.2. Plant material

The stem bark and leaves of *A. semecarpifolia* Nees were harvested from Karigudda, central Western Ghats region of Karnataka, India. The plant was identified and authenticated by Dr. Y. L. Krishnamurthy., Taxonomist, Kuvempu University, Karnataka, India. A voucher specimen (KUBPHS78) has been deposited in the herbarium of DBT-BUILDER project repository, Kuvempu University.

2.3. Crude extraction and fractionation

The shade dried and powdered stem bark and leaf samples of *A. semecarpifolia* were subjected to sequential Soxhlet extraction for 48 hours using petroleum ether, chloroform and methanol solvents. The individually obtained extracts were concentrated under reduced pressure using rotary evaporator and stored in a desiccator until further analysis.

The stem bark methanol extract and leaf methanol extracts were selected (based on extraction yield, presence of phytochemical constituents in crude extracts and their cytotoxic potential against certain cancer cells) and fractionated by using liquid-liquid partition chromatography. The compounds are separated under equilibrium by two immiscible liquid phases. 10g of the stem bark and leaf methanol extracts were dissolved separately in 100ml of chloroform and centrifuged at 10,000rpm for 10 minutes. The obtained residue and supernatant were collected separately and the supernatants were considered as chloroform liquid fractions. The obtained residue was further partitioned in 100ml of each methanol and hexane in separating funnel. The mixture was then shaken vigorously for the separation of compounds in two immiscible liquid phases of hexane and methanol. Further the two distinct layers of methanol and hexane were collected separately and considered as methanol and hexane liquid fractions. The resulted hexane, chloroform and methanol fractions were vacuum dried and stored in a desiccator.

2.4. Isolation and characterization of bioactive compounds

The stem bark and leaf methanol fractions obtained in liquid-liquid partition chromatography were selected for the isolation of bioactive compounds (based on fractionation yield, presence of phytochemical constituents in liquid fractions and their cytotoxic potential against MCF-7 cells). Thin-layer chromatography (TLC) was carried out for both stem bark and leaf methanol fractions and then they were subjected to isolation of bioactive compounds using column chromatography by preparing silica gel bed in chromatographic column in hexane employing wet column packing method. The elution of the compounds was initially started with 100% hexane and further continued by gradually increasing the solvent polarity. The elution was continued with hexane containing increased concentration of ethyl acetate as eluent (100:0–0:100 v/v); followed by ethyl acetate containing increased concentration of petroleum ether as eluent (100:0–0:100 v/v); subsequently petroleum ether containing increased concentration of chloroform as eluent (100:0–0:100 v/v) and chloroform containing increased concentration of methanol as eluent (100:0–0:100 v/v). The eluted sub fractions were monitored by TLC and then they were pooled based on their TLC profile and

pigmentation. Further the percentage purity of eluted sub fractions was evaluated by HPLC.

The methanol fractions of stem bark and leaf have resulted in the elution of 9 and 11 sub-fractions respectively. Among them, four major sub-fractions from each stem bark and leaf methanol fractions were considered for further analysis (based on their yield and percentage purity). From stem bark methanol fraction, sub-fraction B1 (101mg) was eluted as a brownish amorphous powder in the solvent ratio of 70:30 percentage of petroleum ether and chloroform; sub-fraction B2 (37mg) was separated as a brick red amorphous powder in the ratio of 45:55 percentage of petroleum ether and chloroform; sub-fraction B3 (134mg) was obtained as a colourless crystal by eluting with 10:90 percentage of petroleum ether and chloroform respectively and sub-fraction B4 (162mg) was purified as a faint yellow amorphous powder in the solvent ratio of 85:15 percentage of chloroform and methanol respectively. From leaf methanol fraction, Sub-fraction L1 (84mg) was eluted as a colourless amorphous powder in the solvent ratio of 80:20 percentage of petroleum ether and chloroform, sub-fraction L2 (146mg) was purified as brownish amorphous powder in 35:65 percentage ratio of petroleum ether and chloroform respectively, sub-fraction L3 (107mg) was separated as a white amorphous powder in 95:05 ratio of chloroform and methanol, and sub-fraction L4 (123mg) was purified as bright yellow amorphous powder in the solvent ratio of 25:75 chloroform and methanol respectively. Among all these sub-fractions, the sub-fraction B4 from stem bark methanol fraction and sub-fraction L4 from leaf methanol fraction were obtained with both high yield and percentage purity (Suppl. Figure 1 to Figure 8). Hence, they were considered for the structural elucidation and evaluation of their cytotoxic potential.

Later, the sub-fraction B4 and L4 were characterized by using a combination of standard analytical techniques such as ^{1}H NMR, ^{13}C NMR and 2D NMR (^{1}H ^{1}H COSY), FTIR and MS. The structures of the isolated compounds B4 and L4 were elucidated and confirmed by the obtained spectral data (Suppl. Figure 9 to Figure 18).

Compound B4: faint yellow amorphous powder; LC-MS (ESI, Positive): m/z: 677.32 $(M+H)^+$ and (ESI, Negative): m/z: 675.23 $(M-H)^-$. FTIR (KBr, cm^{-1}): 3648, 3388 & 3374 (OH str), 2971 (ArH str), 2923 & 2865 (CH str), 1653 (CO str), 1073 (C–O str). ^{1}H NMR (DMSO-d$_6$, 400MHz, δ_H ppm): 12.56 (s, 1H, Ar-OH), 7.90–7.88 (d, 2H, Ar–H), 7.14–7.11 (d, 2H, Ar–H), 6.63 (s, 1H, Ar–H), 5.36–5.34 (d, 1H, CH), 5.284–5.281 (d, 1H, OH), 5.18–5.13 (m, 2H, CH & OH), 5.06–5.05 (d, 1H, CH), 4.99–4.98 (m, 2H, CH$_2$), 4.73–4.72 (d, 1H, CH), 4.66–4.60 (m, 2H, CH & OH), 4.00–3.98 (q, 1H, CH), 3.73–3.69 (m, 1H, CH), 3.59–3.41 (m, 5H, CH), 3.30 (s, 2H, CH$_2$), 3.19–3.05 (m, 3H, CH), 3.85 (s, 3H, OCH$_3$), 1.68 (s, 3H, CH$_3$), 1.60 (s, 3H, CH$_3$), 0.79–0.77 (d, 3H, CH$_3$). ^{13}C NMR (DMSO-d$_6$, 100MHz, δ_C ppm): 178.28 (CO), 161.39, 160.49, 159.07, 157.31, 153.00, 134.62, 131.10, 130.56, 122.24, 122.11, 114.07, 108.28, 105.57, 101.97, 100.51, 98.10, 77.17, 76.58, 73.34, 71.08, 70.69, 70.06, 69.63, 60.60 (CH$_2$–OH), 55.49 (CH$_2$=CH), 25.45, 21.40, 17.85, 17.44 (CH$_3$).

Compound L4: bright yellow amorphous powder; LC-MS (ESI, Positive): m/z: 271.09 $(M+H)^+$ and (ESI, Negative): m/z: 269.01 $(M-H)^-$. FTIR (KBr, cm^{-1}): 3412, 3257 & 3235 (OH str), 3092 (ArH str), 1617 (CO str), 1084 (C–O str). ^{1}H NMR (DMSO-d$_6$, 400MHz, δ_H ppm): 12.65 (s, 1H, Ar-OH), 10.54 (s, 1H, Ar-OH), 8.90 (s, 1H, Ar-OH), 8.08–8.05 (m, 2H, Ar–H), 7.63–7.55 (m, 3H, Ar–H), 6.94 (s, 1H, Ar–H), 6.63 (s, 1H, ArH). ^{13}C NMR (DMSO-d$_6$, 100MHz, δ_C ppm): 182.11 (CO), 162.88, 153.63, 149.81, 146.93, 131.83, 130.95, 129.30, 126.30, 104.46, 104.25, 93.99.

2.5. Cell lines and culture condition

The human breast cancer (MCF-7) cells and normal rat myoblast (L6) cells were procured from ATCC (American Type Culture Collection). The DMEM (Dulbecco's Modified Eagle Medium) was used to culture the procured stock cells by supplementing with streptomycin, 10% inactivated Fetal Bovine Serum (FBS) and penicillin in a humidified atmosphere of 5% CO$_2$ and 95% air at 37 °C until the cells were confluent.

2.6. Cell seeding

The cells were dissociated by using cell dissociating solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The viability of the cells was checked, and centrifuged followed by seeding 50,000 cells/well in a 96 well plate and allowed to form a monolayer under regular growth conditions and these cells were harvested for cytotoxicity studies.

2.7. Cytotoxicity assay

The *in vitro* cytotoxic effect of the isolated compounds B4 and L4 on MCF-7 and L6 cells was assessed by using MTT assay [18, 19]. The monolayer cell culture was trypsinized and the cell count was adjusted to 1×10^5 cells/ml using DMEM containing 10% FBS. 100μl (50,000 cells/well) of the diluted cell suspension was loaded onto the respective wells in 96 well plate and incubated for 24 hours in CO_2 incubator at 37 °C with 5% CO_2, 95% air and 100% relative humidity. After incubation, the supernatant was carefully flicked off when the partial monolayer was observed, followed by washing with DMEM. Later the cells were treated with different concentrations of test compounds (10, 20, 40, 80, 160 and 320 μg/ml) and incubated for 24 hours in CO_2 incubator with similar incubation conditions. After incubation, the test solutions in the wells were exchanged with 100μl (0.5 mg/ml) of MTT solution and again incubated for 4 hours in CO_2 incubator. The supernatant was removed and 100μl of DMSO was added and the plate was gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader (SpectraMax i3) at a wavelength of 590nm. The IC_{50} values were obtained from the dose-response curves and the percentage growth inhibition was calculated.

2.8. Statistical analysis

The statistical analysis was performed using GraphPad Prism Software v 5.01 (GraphPad Software Inc., San Diego, CA). The data of MTT assay is presented as mean $\pm$ SEM of three replicates and it is statistically analyzed using two way analysis of variance (ANOVA) followed by Bonferroni post-test. The differences were considered statistically significant when [ns]$p > 0.05$, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

3. Results and discussion

3.1. Structural elucidation of the isolated compounds

The "compound B4" was isolated as a faint yellow amorphous powder. The molecular formula ($C_{33}H_{40}O_{15}$) was assigned based on the positive ion peak in the mass spectrum of compound B4 (Suppl. Figure 9) at m/z 677.32 [M+1] along with its ^{1}H NMR and ^{13}C NMR spectral data. The IR
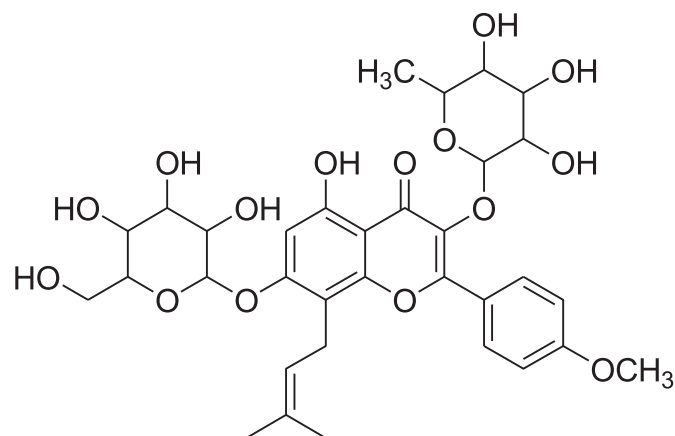
spectrum of compound B4 (Suppl. Figure 10) showed the presence of three hydroxyl groups (3648, 3388 and 3374 cm^{-1}) and carbonyl (1653 cm^{-1}) functional moieties. The ^{1}H NMR spectrum of compound B4 (Suppl. Figure 11) displayed the presence of aromatic hydroxyl proton signal in the downfield region at δ_H 12.56 ppm, five aromatic ring protons in the range of δ_H 7.90 to 6.63 ppm, methoxy protons at δ_H 3.85 and two allylic methyl protons at δ_H 1.68 and 1.60 ppm. Further the presence of vinyl proton at δ_H 5.36–5.34 ppm, allylic methylene protons adjacent to hydroxyl and alkene moiety at δ_H 3.30 ppm and δ_H 4.99–4.98 ppm respectively. The ^{13}C NMR spectrum of compound B4 (Suppl. Figure 12) showed lactone ring carbonyl carbon at δ_C 178.28 ppm, methylene carbons at δ_C 60.60 and 55.49 ppm attached to hydroxyl group and double bond respectively and presence of four methyl carbons at δ_C 25.45, 21.40, 17.85 and 17.44 ppm. Further, in 2D NMR ^{1}H ^{1}H COSY spectrum of compound B4 (Suppl. Figures. 13 & 14) showed the coupling interactions of protons. These data and observations suggested that the structure of the isolated compound B4 is similar to the previously identified compound Icariin (Figure 1).

Icariin is a natural bioactive compound and a major flavonol glycoside initially isolated from dried leaf and stem of *Epimedium brevicornum*, belonging to Berberidaceae family [20, 21]. Epimedium is a plant commonly known as Horny Goat Weed or Yin Yang Huo, used as a tonic agent in Chinese Traditional Medicinal system [22]. Icariin has been reported to possess several pharmacological and biological properties such as antidepressant, apoptotic, immunomodulating and preventing osteoporosis [23, 24]. Extensive literature indicated that Icariin also acts as a potent anticancer agent that can inhibit tumor cell growth and proliferation [25, 26, 27, 28]. Several previous studies also reported that, Icariin exerts antitumor effects on various tumor cells, including gall bladder cancer [29], ovarian cancer [27], colorectal cancer [30] and oesophageal cancer [31].

The "compound L4" was isolated as a bright yellow amorphous powder. The molecular formula ($C_{15}H_{10}O_5$) was assigned based on the positive ion peak in the mass spectrum of compound L4 (Suppl. Figure 15) at m/z 271.09.32 [M+1] along with its ^{1}H NMR and ^{13}C NMR spectral data. The IR spectrum of compound L4 (Suppl. Figure 16) showed the presence of three hydroxyl groups (3412, 3257 and 3235 cm^{-1}) and lactone ring carbonyl (1617 cm^{-1}) functional moieties. The ^{1}H NMR spectrum of compound L4 (Suppl. Figure 17) showed the presence of three aromatic hydroxyl groups δ_H 12.65, 10.54 and 8.90 ppm, five aromatic ring protons in the range of δ_H 8.08–8.05 to 7.63–7.55 ppm, singlet signals at δ_H 6.94 ppm attributed to aromatic proton fused to pyran ring and one more singlet at δ_H 6.63 ppm due to vinylic proton of pyran ring. Further, ^{13}C NMR spectrum of compound L4 (Suppl. Figure 18) displayed lactone ring carbonyl carbon signal at δ_C 182.11 ppm, fused ring carbon signals in the range of δ_C 162.88 to 129.30 ppm and phenyl ring carbons in the range of δ_C 126.30 to 93.99 ppm. These spectroscopic observations suggested that the structure of the isolated

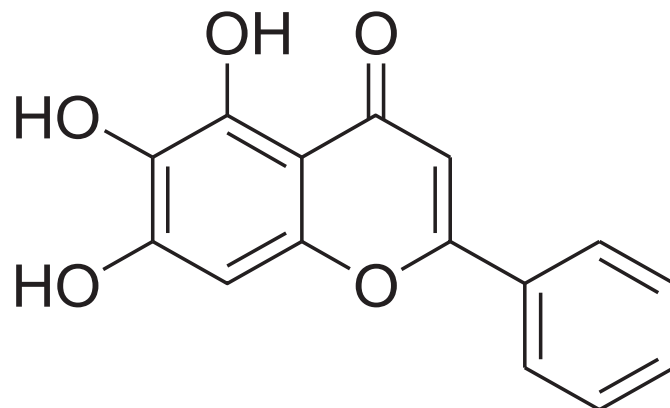**Figure 1.** Chemical structure of Icariin.
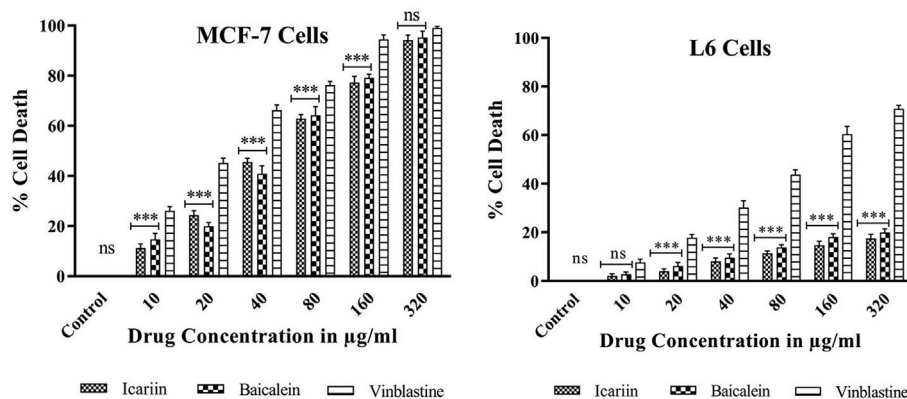

Figure 2. Chemical structure of Baicalein.

Figure 3. Cytotoxic effect of Icariin and Baicalein on MCF-7 and L6 cells. Values are presented as mean ± SEM of three replicates. ns: non-significant; *: significant; ***: highly significant.

compound L4 is similar to that of previously identified compound Baicalein (Figure 2).

The bioactive compound Baicalein isolated from *A. semecarpifolia* is a naturally occurring flavonoid compound and it was initially isolated from *Scutellaria baicalensis* belongs to Lamiaceae family [32, 33, 34]. It is a perennial herb used in Traditional Chinese Medicine for anticancer therapy [35]. Baicalein acts as a potent anticancer compound, reported to inhibit proliferation of lung cancer [35], prostate cancer [36], thyroid cancer [37, 38] and breast cancer cells [39, 40].

3.2. Icariin and Baicalein induced cytotoxicity on MCF-7 cells

Cytotoxic efficacy of Icariin and Baicalein on human breast cancer (MCF-7) cells was evaluated by comparison with a plant alkaloid Vinblastine used as a reference standard. Normal rat myoblast (L6) cells were used to evaluate the cytotoxic effects of Icariin and Baicalein on non-cancerous cells. Upon treatment with Icariin and Baicalein, cell viability was gradually decreased as the concentration of the compounds increased. Both Icariin and Baicalein showed potential cytotoxic activity on MCF-7 cells with an IC_{50} of 42.15 ± 4.78 μg/ml and 44.37 ± 3.46 μg/ml respectively, whereas the IC_{50} of Vinblastine on MCF-7 cells was 24.03 ± 2.12 μg/ml (Figure 3). The isolated compounds Icariin and Baicalein were found to have very least toxicity on L6 cells and it is lesser than that of Vinblastine (88.52 ± 3.56 μg/ml). The IC_{50} values of Icariin and Baicalein on L6 cells were unable to determine due to their lesser toxicity. The viability of the L6 cells was not much affected even when treated with the higher concentrations of Icariin and Baicalein. To be a potent anticancer drug it should be selectively toxic on cancer cells without affecting the viability of normal cells. From this investigation it is evident that both Icariin and Baicalein are selectively toxic on human breast cancer cells with very least and negligible toxicity on normal cells. Although Vinblastine is an efficient anticancer drug it induced toxicity on normal cells, and therefore Icariin and Baicalein are safer compared to Vinblastine.

The choices of modern drugs for the cancer therapies are limited and most of them are accompanied with dose-related toxicities. The development of an effective cytotoxic agent is crucial and new strategies need to be put forth which can help for the identification of natural compounds with superior clinical efficacy and lesser toxicity. Many anticancer drugs also influence toxicity on healthy cells, which in turn leads for the unbearable side effects on patients. For this reason, identification and development of novel anticancer compounds with selective toxicity on cancer cells is more of a concern [41]. Even though several natural active compounds have unique anticancer properties, they are not used in clinical practices due to limited bioavailability or due to their adverse toxicity [42, 43]. The present study has revealed the selective toxicity of Icariin and Baicalein on MCF-7 cells with no adverse toxicity on normal cells.

Both Icariin and Baicalein suppressed multiplication of MCF-7 cells in a dose dependent manner. This might be due to several cell toxic mechanisms that include arresting tumor cell cycle progression, induction of DNA damage and cell specific apoptosis by the compounds. The earlier investigations on anticancer properties of Icariin and Baicalein also revealed that they have the potential to suppress proliferation of breast cancer cells by inducing apoptosis and cell cycle arrest [40, 44]. In agreement with earlier studies [40, 44], the present investigation also reported the dose dependent inhibition of cell proliferation in breast cancer cells by Icariin and Baicalein.

4. Conclusion

The secondary metabolites derived from natural sources are the potent maneuver for drug discovery and development. Present study has suggested that *A. semecarpifolia* is a potential source of bioactive compounds which is evident by the phytochemical analysis. It is the first ever report on the isolation and characterization of bioactive compounds from *A. semecarpifolia*. Also, findings of the present investigation confirm the cytotoxic property of Icariin and Baicalein which might provide a basis for the development of Icariin and Baicalein as potential agents for breast cancer and authenticate the traditional medicinal claims of the plant as a potent anticancer agent. Further investigations on the anticancer properties of Icariin and Baicalein are in progress to validate their anticancer efficacy, by assessing their effect on induction of cell cycle arrest, apoptosis, DNA damage and expression of tumor suppressor genes in human breast cancer cells.

Declarations

Author contribution statement

Ganadhal Puttaramaiah Chethankumara: Conceived and designed experiments, Performed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools or data; wrote the paper.

Kakanahalli Nagaraj: Conceived and designed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools or data.

Venkatarangaiah Krishna: Conceived and designed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools or data.

Gurunathan Krishnaswamy: Analyzed and interpreted the data; contributed analysis tools or data.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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