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Antiproliferative and anticancer activities of *Neurada procumbens* L. against epithelial carcinoma and breast cancer cell lines

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Neurada procumbens L. commonly called as sand button, is used as a medicinal herb by Bedouin in the Arabia Peninsula for heart and respiratory functions. This little known plant has not been investigated in detail for its biological activities. Hence, we have made an attempt to investigate the plant for its anticancer activities. Methanol and chloroform extracts of *N. procumbens* were evaluated for antiproliferative and anticancer activities in HEp-2 and MCF-7 cancer cell lines. Human foreskin epithelial cells showed an IC_{50} value of 34 µg and 149 µg for chloroform and methanol extracts, respectively. The chloroform extract of *N. procumbens* @3 µg showed 50% reduction of HEp-2 cell population in 24 h, while 5.5 µg showed similar effects in MCF-7 cells. The results of the methanol extracts were little varied, where 49 µg showed slow inhibition of HEp-2 cells over a period of 72 h. In another similar observation, 76 µg of methanol extract inhibited 50% MCF-7 cell proliferation. The concentrations which could inhibit complete turnover of the experimental cancer cell lines at 24 h were >5 µg and 6 µg of chloroform extract and > 60 µg and 80 µg of methanol extract for HEp-2 and MCF-7, respectively. The micro therapeutic index of methanol extract in HEp-2 and MCF-7 were 3 and 1.9, while chloroform extract was 11.3 and 6.1. This is an initial observation of *N. procumbens* that shows antiproliferative activities against cancer cell lines.

Keywords: Quercetin, Rutin, Sand button

Cancers are a collection of diseases in which abnormal cells can divide and spread to nearby tissue. Cancers can arise in many parts of the body and spreads through the blood and lymph systems. The most commonly diagnosed cancer is the female breast cancer, followed by lung, colorectal and prostate cancer. The number of new cancer cases per year is expected to rise to 28.4 million by 2040[1]. The number of people affected by cancer is increasing around the world. Most of the anticancer agents are derived from natural products or their derivatives. Natural products or their derivatives are likely to provide many lead molecules that will be used as templates for building novel compounds with improved biological activities[2]. Some of the chemotherapeutic agents derived from natural products are etoposide, teniposide, paclitaxel, vinblastine, vincristine and camptothecin[3,4]. Hence, phytochemicals obtained from plants continue to offer opportunities as sources of new anticancer agents[5].

Neurada procumbens, L (Family-Rosaceae) is an annual herb used in Saudi Arabia to treat diarrhea and dysentery[6]. It is also used as a tonic to increase heart and respiration functions[7]. The dried powder of whole plant mixed with goat's fresh milk or water is given to the patient suffering from heat stroke during summer season[8]. The fruit is used as a nerve tonic[9]. The plant is a food of camels and it has been considered safe and edible. The plant shows woolly appearance due the dense hairs. The leaves are blue-green colour and oval shaped. The flowers are small with greenish or pinkish petals. The fruits are disc shaped. The bottom side of the fruits are smooth, but the upper side has spines. At maturity, the fruit becomes hard and woody. The plant is distributed from North Africa and the Mediterranean region and also found in Middle East, Afghanistan, Pakistan and India[10]. The plant has been reported to contain alkaloids, flavonoids, saponins, sterols, triterpenes, coumarins, volatile oil,

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and tannins[11]. The aqueous extract increases diastolic and systolic blood pressure in rats[9]. Anticancer activities of this plant, *Neurada procumbens* have not been studied. It is therefore, imperative to screen anticancer potential of the aerial parts of the plant against certain cancer cell lines.

Materials and Methods

Plant material and extraction

The plant was identified by Prof. Hassan Y Al Ayedh, King Abdulaziz city for Science and Technology, Riyadh. A voucher specimen was deposited in the department of Pharmacognosy, King Khalid University, Abha. The aerial parts were collected, washed with water, dried in shade and powdered. About 200 gm of powder was extracted with 1500 mL of methanol by maceration for 24 h. The content was stirred with a mechanical stirrer for about 3 h for effective extraction. The extract was filtered and the solvent was removed under vacuum by a rotary evaporator (percentage yield of extract - 2% w/w). Chloroform extract was also prepared by maceration (percentage yield of extract - 1.2% w/w). The dried extracts were used to study anticancer activities.

Phytochemical studies

The powdered material is used for screening the phytochemical constituents such as carbohydrates, proteins, amino acids, alkaloids, phenolic compounds including tannins, flavonoids, steroids, gums and mucilages[12]. Thin layer chromatographic (TLC) analysis was carried out detect rutin using silica gel as stationary phase with ethyl acetate-glacial acetic acid-formic acid-water (100:11:11:26) as mobile phase[13].

Estimation of total phenolic compounds

The total phenolic compounds were estimated by Folin-Ciocalteu method[14]. Various concentrations of standard and extract solutions were added with 1 mL of Folin-Ciocalteu reagent (1:10 diluted) and 2 mL of 20% sodium carbonate. The resulting solution was incubated at room temperature (20°C) and the absorbance was measured in a UV Vis spectrophotometer (Shimadzu, Japan) at 755 nm. The results were expressed as gallic acid equivalents (% w/w).

Estimation of total flavonoids

Total flavonoidal content was measured in a UV Vis spectrophotometer (Shimadzu, Japan) using aluminium chloride[15]. About 10 mg of rutin was dissolved in 10 mL of methanol to get 1.0 mg per mL stock solution. The stock solution was further diluted to get lower concentrations. Various aliquots of standard and extract solutions were added with 0.1 mL of 10% aluminium chloride solutions and incubated for 30 min at room temperature. The absorbance was measured at 432 nm with UV/Vis spectrophotometer. The results were expressed as rutin equivalents (% w/w).

Estimation of rutin and quercetin by high performance liquid chromatography (*HPLC*)

Rutin and quercetin in the methanol extract and chloroform extract was identified and estimated by HPLC[16]. The analysis was carried out using Shimadzu instrument with class VP software. Various concentrations of standard and test solutions (20 µL) were injected in to C18 column (150 mm × 4.6 µm). The compounds were eluted using methanol-water as mobile phase by gradient program starting from 10 to 80% within 30 min. The compounds were detected at 254 nm by a UV-Visible detector at ambient temperature. The retention time and peak area were noted for identification and estimation of rutin and quercetin in the extracts. The method validation parameters such as system suitability, linearity, limit of detection, limit of quantification, repeatability, precision and accuracy were performed[17].

Cell lines

Human epithelial type 2 (HEp-2) ATCC CCL23 representing human cervix carcinoma and human breast cancer MCF-7 cells (Kind gift from Dr. Serag, College of Science, KKU, Saudi Arabia) were used as cancer models. Normal foreskin fibroblast cells were used as controls owing to its epithelial origin. All the cell lines were cultured in (Dubeco minimum essential medium) DMEM medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% Eagle's nonessential amino acids and 1.0 mg/mL penicillin-streptomycin. The cells were exponentially passaged and after passage 9, used for the current study.

Preparation of 24-well Tissue culture plates for cytotoxicity and anticancer assays

Briefly, tissue culture bottles with complete monolayer of fibroblast or HEp-2 or MCF-7 cells were washed, trypsinized, harvested and seeded in 24 well tissue culture plates at a concentration of 10^5 cells/mL. The plates were incubated for 24 h at 37°C in 5% CO_2. The well with complete monolayer were selected for toxicity and anti-cancer assays.

Cytotoxicity assay

Cytotoxic concentration of the extracts in the normal control cells is the upper limit concentration,

which is non-toxic to the cell line. The concentration less than the upper limit will be used in subsequent anticancer and anti-proliferative studies[18]. Briefly, the growth medium from 24-well plate prepared with human fibroblast cells is removed and washed twice with phosphate buffer solution (PBS). Increasing concentration of extracts (from 1.0 µg to 500 µg/mL of DMEM W/O FCS) was added in tetrads. Cell control containing extract free medium, solvent control (0.25% DMSO) and neat extract controls were included. The plates were incubated at 37°C in 5% CO_2 environment. The changes in the morphology or cytotoxicity were microscopically examined at 24, 36, 48 and 72 h. The highest concentration of extracts showing no cytotoxic effect, 50% and 100% cell death were recorded. MTT assay were performed to confirm the cytotoxicity[19,20]. The concentration of the extract which was able to kill the control cells by 50% as evidenced by both microscopic scoring and MTT assay was recorded as inhibitory concentration 50 or IC_{50}.

Antiproliferative assay

Mode 1, Cell death assay

Briefly, the 10^5 HEp-2 or MCF-7 cells were seeded on 24 well tissue culture plates, incubated overnight 37°C in 5% CO_2 environment. At 70% confluence, the cell monolayer was washed once with 0.1% PBS and graded dose of the extracts in the growth media below the cytotoxic doses were added and incubated back at 37°C in 5% CO_2 environment. The cell death mode was graded microscopically and up to 72 h, while MTT assay were performed in the duplicate plates simultaneously. The concentration of the extract which was able to kill the cells by 50% as evidenced by both microscopic scoring and MTT assay was recorded as effective dose 50 or ED_{50}.

Mode 2, Cell turnover inhibition assay

Briefly**,** the 10^5 HEp-2 or MCF-7 cells were incubated at 37°C with graded doses of extracts in the growth medium. The treated cells were then seeded on 24-well tissue culture plates and incubated at 37°C in 5% CO_2 environment. Post 24 h of the incubation, the cells were enumerated for turnover and compared with cell control and solvent which were similarly treated. This assay was primarily done to check whether the extracts can inhibit the cell proliferation or metastasis of the newly developing cancer or tumor mass[21].

Micro Therapeutic Index

The micro therapeutic index for the cell line data was calculated form the formula

$$\text{Therapeutic Index (TI)} = \frac{\text{Inhibitory Concentration 50 } (IC_{50})}{\text{Effective Concentration 50 } (EC_{50})}$$

MTT assay

Briefly, the cells were washed once with 200 µL of PBS, and incubated with 100 µL of 500 mg/mL MTT/well in PBS at 37°C for 3 h. The insoluble formazan product was dissolved in 200 µL of DMSO. The plates were read at absorbance of 570 nm using a ELISA plate reader. The same protocol was repeated to anticancer activity protocols of MCF-7 and HEp-2 cell lines.

Statistical analysis

The cytotoxicity data are presented as mean percentages of control ± SD and linear regression analysis was used to calculate the IC_{50} values for the cytotoxicity assays. Other data were analyzed using analysis of variance (ANOVA) followed by Tukey-HSD test and the results were considered statistically significant if $P < 0.05$.

Results

The percentage yield of methanol and chloroform extracts were found to be 2 and 1.2%, respectively. The phytochemical studies revealed the presence of carbohydrates, proteins, amino acids, alkaloids, phenolic compounds, tannins, flavonoids and steroids. TLC analysis confirmed the presence of rutin in methanol extract and chloroform extract with Rf value of 0.46. The TLC plates were developed with the mobile phase, ethyl acetate-glacial acetic acid-formic acid-water (100:11:11:26) was found to be suitable for the separation of rutin. The rutin was visualized in iodine chamber as yellow brown spot. Total phenolic compounds in the methanol extract and chloroform were found to be 9.2 and 3.4%, respectively. Methanol extract showed higher quantity of total phenolic compounds than chloroform extract. Methanol extract showed 2.65% of total flavonoids by aluminium chloride method whereas, chloroform extract showed 2.28% of total flavonoids. Both the extracts were found to have almost equal amount of total flavonoids. Rutin and quercetin were identified in methanol and chloroform extract by HPLC. The retention time of rutin and quercetin were found to be 15 min and 16.05 min respectively (Fig. 1 A-C). The mobile phase, methanol and water with gradient program separated rutin and quercetin from other compounds, showing well resolved sharp peaks. A calibration curve was constructed for rutin and

quercetin with the concentration ranging from 50 to 600 µg/mL. About 0.667% of rutin was found in methanol extract, while chloroform extract showed 0.561%. However, quercetin content in methanol extract was found to be 0.496%, while chloroform extract showed 0.507%. The developed HPLC method was simple, accurate, precise and reproducible.

The results of the cytotoxicity assay of the methanol and chloroform extracts of *N. procumbens* over normal human foreskin epithelial cells were varied. The cumulative results of cytotoxicity and concentrations that are antiproliferative of cancer cell lines are depicted in Table 1. The median concentrations obtained from 3rd day readings of extracts are well below 28.6 µg and 135.7 µg, revealing no cytotoxicity (Fig. 2 A-D). The IC_{50} values of methanol and chloroform extracts over human foreskin epithelial cells were 149 µg and 34 µg respectively (Fig. 2 A & B).

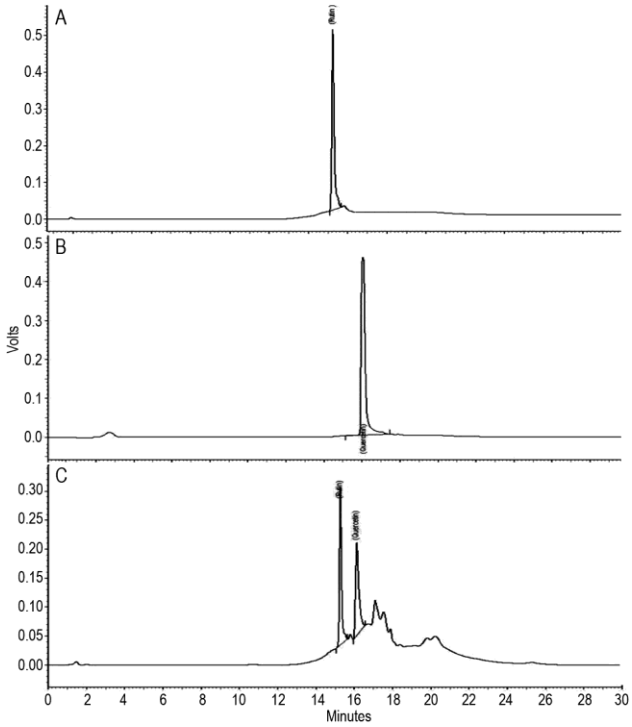

Fig. 1 — HPLC Chromatogram of (A) rutin; (B) quercetin; and (C) *Neurada procumbens* extract

Antiproliferative activity of the extracts by mode 1 (Cell death assay) showed 3 µg of chloroform extract inhibited 50% of HEp-2 cells, while similar effect was observed with 5.5 µg over MCF-7 cells (Fig. 3 A & B). The complete killing of the both the cell types (HEp-2 and MCF-7) were observed over a concentration of >5 µg and 6 µg of chloroform extract. On the other hand, the results of the methanol extracts were varied with a slow inhibition compared to the chloroform extract. About 49 µg of methanol extract inhibited 50% of the HEp-2 cells over a period of 72 h, while 76 µg inhibited 50% of the MCF-7 cells (Fig. 3 C & D). Complete inhibition of the methanol extract of both HEp-2 and MCF-7 cells were >60 µg and 80 µg, respectively. The effect of the extracts on the log phase cells (growing cells) were checked. The cells were incubated or pretreated with varying concentrations of the extracts, showed the complete killing of the cells at half the concentration of what was observed in the antiproliferative assay. The exact mechanism of action is not known, but eventually the normal hFFEC cells were killed in half of the concentration of the recorded IC_{50} (Fig. 4 A & B).

Since, the results showed a wide difference between the cytotoxicity to the normal cells and inhibiting anticancer proliferation, the micro therapeutic index of the chloroform extract was 11.3 for HEp-2 and 6.1 for MCF-7. However, the methanol extract showed the micro therapeutic index of 3 for HEp-2 and 1.9 for MCF-7, respectively (Table 1). The therapeutic window was wide over >2, except, the methanol extract, which showed 1.9 over MCF-7 cells.

Owing to the high therapeutic window, the extracts were tested for the inhibition of the turnover at the growth phase, which will mimic the control of metastasis, an inbound character of the cancer cells. The results showed that both the extracts at much lower concentration of EC_{50} could completely inhibit the turnover of the cells at a seeding concentration of 10^5 cells/well. Overnight growth in controls resulted in doubling of the cells in both HEp-2 and MCF-7, while human foreskin epithelial cells showed 52% turnover. Interestingly, the chloroform extract was

Table 1 — Cumulative cytotoxicity and anti-proliferative assays of the extracts over human Foreskin Fibroblast Epithelial cells, HEp-2 and MCF-7 cell lines

Extract	hFFEC		HEp-2			MCF-7		
	IC_{50} (µg)	No cytotoxicity (µg)	EC_{50} (µg)	Complete cytotoxicity (µg)	TI	EC_{50} (µg)	Complete cytotoxicity (µg)	TI
Chloroform Extract	34	28.6	3	> 5	11.3	5.5	> 6	6.1
Methanol Extract	149	135.7	49	> 60	3	76	> 80	1.9

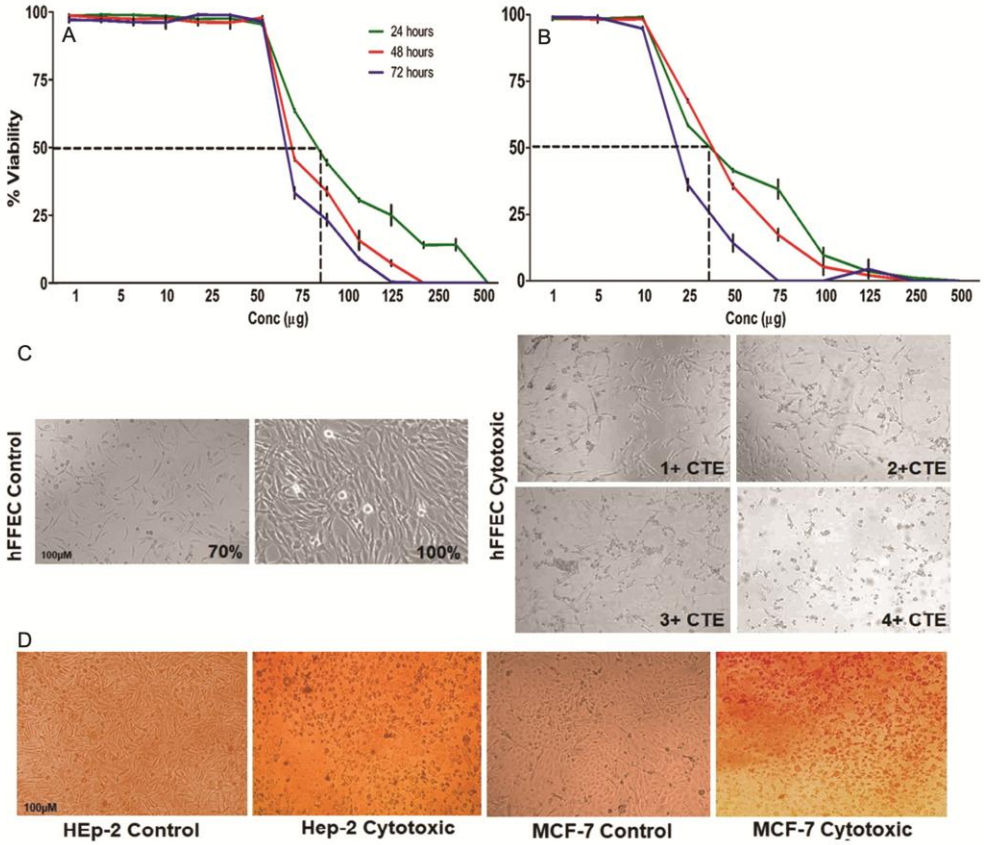

Fig. 2 — Cytotoxicity of methanol and chloroform extracts on normal human fibroblast cells. The minimal and maximum cytotoxicity is shown along with IC_{50} value of (A) methanol; and (B) chloroform extract on hFFEC cells; Representative cell line pictures showing (C) 70 and 100% cell growth and microscopic grading of the cytotoxicity as 1+ (~25% cell death), 2+ (~50% cell death), 3+ (~75% cell death) and 4+ (100% or complete cell death); and (D) HEp-2 and MCF-7 cell lines, normal and cytotoxicity

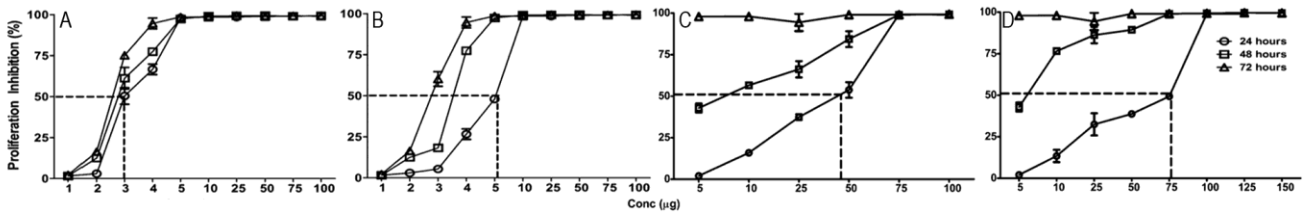

Fig. 3 — Anti-proliferative activity of chloroform and methanol extracts on HEp-2 and MCF-7 cell lines. The IC_{50} values of (A & B) chloroform; and (C& D) methanol extract inhibiting cells at various concentrations (non-toxic, minimal and maximum) c. HEp-2 and d. MCF-7, respectively

significantly toxic to the log phase cells than methanol extract indicating the extracts might be toxic to actively dividing cells.

Discussion

It was evident from the results that the presence of the secondary metabolites in the methanol and chloroform extracts were in agreement with other published sources especially from the family of Neuradaceae[22]. Especially, the detection of the rutin and quercetin in both extracts well coincided with the potent medicinal use of these compounds to treat various ailments[23,24]. These compounds may also be considered as markers of the plant. In our study, the plant showed the presence of rutin and quercetin which were identified and quantified by HPLC. Phytochemical studies along with quantification of marker (rutin and quercetin) may be helpful in assessing the quality of the plant. The plant extracts showed the inhibition of the proliferating cancer cells, may be well correlated to the presence of flavonoids especially, rutin and quercetin. Though, the methanol and chloroform extracts of the aerial parts of the plant contain equal concentration of flavonoids, our results

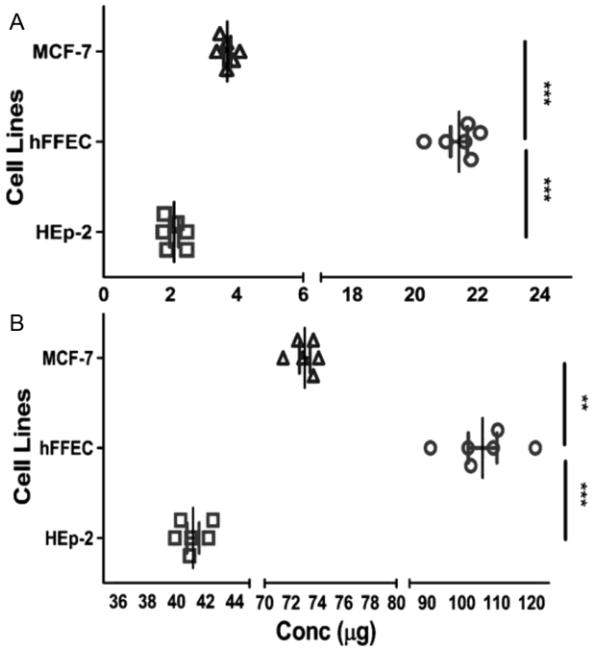

Fig. 4 — Cell turnover inhibition by pre-incubation of the extracts with log phase cells. (A) Chloroform; and (B) methanol extracts showing inhibition of the cells post pretreatment with lower concentrations

showed, profound activity with chloroform extract, while methanol extract showed comparatively milder effect. The other secondary metabolites like steroids or phenolic compounds might be responsible for the antiproliferative activity. Rutin and quercetin might possibly act as the protectant to cellular damage, modulating the harsh chemical constituents of the both methanol and chloroform extracts.

The current study is a preliminary investigation indicating anti-proliferative and anticancer activity leading to cell death[25]. The rationale behind screening of the crude extract was to determine whether the active lead molecules from plant origin show synergistic activity due to many structurally similar types of secondary metabolites. Therefore, conducting downstream studies aiming in determination of mechanism of the anti-cancer/tumor activity are indispensable. From our results, it was evident that both methanol and chloroform extracts showed varying degree of the cytotoxicity to each cell lines (HEp-2 and MCF-7) over normal cells. This was in agreement with several studies using methanol and chloroform for extraction of active components[26]. Large therapeutic window is more convenient during screening of the crude extracts, as the anticancer activity of phytochemical fractions are mostly synergistic.

However, the anticancer activity of the extracts with large therapeutic window alone could not be an exemplary solution in the screening of the new compounds. Extracts which extends antagonistic effect to the killing of the cancer cells by offering protection to the adjoining normal cells could be investigated for an extended drug use as observed in the cases of chronic and remission cancers. It was observed that the anticancer activity shown by the extracts over two different cancer cell lines were remarkably different. Though, such observations are well in agreement with other studies with many of the plant extracts[27] and pure small molecules[28], we do speculate such wider differences in the activity which may give us the clue in elucidating the anticancer mechanisms. The growth phase inhibition of the cancer cell lines for both the extracts showed higher toxicity level. This could be due to lower concentrations of extracts which is toxic to early stages of cellular cancer manifestation or to a large therapeutic window[29]. However, the extreme cytotoxic nature of the chloroform extract to even normal cells at low density needs to be further investigated as what implies the protection to the higher densities of the normal cells.

Conclusion

Neurada procumbens has not been much investigated or documented for bioactivity. In this study, the IC_{50} values of methanol extract was found to be higher (149 µg) than and chloroform extract (34 µg) over human foreskin epithelial cells. The methanol and chloroform extracts showed antiproliferative activity against epithelial carcinoma and breast cancer lines with high micro therapeutic index. The presence of the rutin and quercetin among the secondary metabolites and its proven use as cell protectant also favours the use of this plant in extending the screening in animal models.

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Conflict of interest

Authors declare no competing interests.

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