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Centella asiatica aqueous extract inhibits motility of VGSCs-expressing MDA-MB-231 cells without affecting cell growth

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Abstract
Targeting nNav1.5, a potent metastasis marker in breast cancer using VGSCs blockers founded in a 'back-to-nature' strategy might improve treatment avenues with fewer or no undesirable side effects. The potential of *Centella asiatica* aqueous extract to inhibit cell motility was examined on the strongly metastatic breast cancer cell line MDA-MB-231 cells. Cells were treated with *Centella asiatica* aqueous extract ranging from 0.1-10µg/ml for 24h followed by total RNA extraction, cDNA synthesis and PCR. MTT and motility assays were conducted to measure the effect of the plant extract on growth and cell motility. Growth of MDA-MB-231 cells was not affected by any tested concentration (0.1- 10 µg/ml) of *Centella asiatica* aqueous extract but importantly, motility of the cells was suppressed significantly in a dose-dependent manner followed by down-regulation of the nNav1.5 gene expression was observed. Overall, *Centella asiatica* aqueous extract inhibit cell motility without affecting cell growth via nNav1.5 gene down-regulation.

Keywords: *Centella asiatica*, anti-metastatic, lateral motility, voltage-gated sodium channels, nNav1.5

1. Introduction
Among various recently identified metastasis-related genes, voltage-gated sodium channels (VGSCs) has consistently been highlighted. Although VGSCs is 'classically' abundant in excitable cells such as the neuron and muscle cells where its role is important in mediating cell regenerative membrane depolarization and conduction of electrical signaling (action potentials) [1]. However, recent discoveries have shown that VGSCs are also highly expressed in metastatic cancer cells.

Particularly in breast cancer, high VGSCs expression and activity correlate positively with its metastatic potential [2]. A whole-patch clamp recordings from a variety of human breast epithelial cells ranging from the normal to highly metastatic cell lines demonstrated that metastatic potential increases from the non-cancerous human breast epithelial cells, MCF-10A < weakly metastatic breast cancer cell line, MCF-7 < aggressive breast cancer cell line, MDA-MB-468 < strongly aggressive breast cancer cell line, MDA-MB-231. Only the strongly metastatic, MDA-MB-231 cells generate a fast inward current, representing high VGSCs activity [2]. Using PCR, high level of VGSCs mRNA expression particularly of the splice variant 'neonatal' Nav1.5 (nNav1.5) was detected in MDA-MB-231 cells. When these cells were treated with VGSCs specific blocker, tetrodotoxin (TTX), suppression of metastatic parameters was obtained [2]. Other research have reported that nNav1.5 can be blocked with other VGSCs modulators e.g local anesthetics and analgesics which altogether supports the potential of VGSCs as target in controlling breast cancer metastasis [3, 4, 5, 6, 7, 8].

Centella asiatica also known locally as 'pegaga' is a tropical plant commonly consumed by Malaysians as 'ulam'. In folk medicine, the plant has been claimed to have therapeutic potential to treat numerous conditions e.g wound healing, leprosy, psoriasis, lupus, eczema, varicose ulcers, diarrhoea, amenorrhea, diseases of the female genitourinary tract and also for relieving anxiety and improving cognition [9]. Importantly, *Centella asiatica* has been claimed to present central analgesic activity [10]. In one *in vivo* example, asiatic acid, a pentacyclic triterpene compound from *Centella asiatica* was demonstrated to have analgesic mechanisms due to its anti-inflammatory effects in the edema paw of a mice [11].

Subsequently, the main purpose of this study was to assess the anti-metastatic effect of *Centella asiatica* aqueous extract *in vitro*, on the lateral motility of strongly aggressive

MDA-MB-231 cells. We also analyzed the effects of the plant extract on nNav1.5 expression in order to link the mechanism of motility suppression by the plant extract.

2 Materials and Methods

2.1 Cell culture

Highly metastatic human breast cancer cell line, MDA-MB-231 was cultured in 10cm or 35mm Falcon plastic dishes (Becton Dickinson) and maintained in Dulbecco's minimum essential medium (DMEM) (Nacalai Tesque) supplemented with 4mM L-glutamine (Gibco) and 5% of fetal bovine serum (FBS) (Gibco). Cultured cells were kept in a humidified incubator at 37 ºC, 100% relative humidity and with 5% regulated CO_2 [2].

2.2 Identification and preparation of the plant extract

After appropriate identification, washed *Centella asiatica* were shade dried at room temperature for an hour and further dried in a hot-air oven (Memmert) at 50 ºC for another 48h. The dried samples were grind to produce fine powder and 50g was placed in a clean flask containing 200ml of aqueous. The flask was placed in a shaker incubator (Max^Q 4000, Barnstead) for 48h at 37 ºC and 150rpm. The aqueous extract of *Centella asiatica* was filtered using filter paper (Whatman No.1) and concentrated using rotary evaporator (Rotaver) at 80 ºC. The concentrated aqueous extract was further dried for an overnight in oven (Memmert). The extract was weighed and stored at -20 ºC until further use.

2.3 Cell viability and proliferation assays

Proliferation was measured using an assay based upon colorimetric quantification of yellow 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) (Sigma) reduced to purple formazan in living cells [12].

2.4 Motility assay

Motility of cells was assessed using a monolayer 'wound-heal' assay (Fraser *et al*. 2003). Cells were plated in 35mm dishes at an initial density of 5×10^5 cells/dish and allowed to settle for 24h prior to wound creation and any pharmacological treatment (T_0). Wounds were created by scratching the monolayer cells using a p200 pipette tip at 90º angle. Wound widths were measured again after 24h (T_{24}). Motility was calculated according to $1 - (T_{24}/T_0)$ and percentage of lateral motility was normalized to Control.

2.5 Polymerase chain reaction (PCR)

Total RNA was extracted directly from a ~80% confluent dish of cells using the 'Trizol method' according to the protocol provided by the manufacturer (Invitrogen). Total RNA (1µg) was used to synthesis cDNA according to the QuantiTect Reverse Transcription Kit (Qiagen). PCR was performed using MyTaq HS (Bioline) and primers for nNav1.5: 5'-CTGCACGCGTTCACTTTCCT-3' (F) and 5'-GACAAATTGCCTAGTTTTATATTT-3' (R). β-actin: ATTGCCGACAGGATGCAGAAG-3' (F), 5'-TAGAAGCATTTGCGGTGGACG-3' (R). The intensity of PCR product was measured using ImageJ (free online software: http://rsbweb.nih.gov/ij/).

2.6 Statistical analysis

All values are expressed as the mean ± SEM. Student's *t*-test for unpaired results was used to evaluate differences between two groups. Differences were considered to be significant for values of $P<0.05$.

3 Results

3.1 Effect of *Centella asiatica* aqueous extract on cell viability and growth

Initial observations showed no apparent change in cellular morphology of MDA-MB-231 cells after 24h treatment with aqueous extract of *Centella asiatica* up to 10µg/ml. There was no effect on cell viability and the extract also did not affect cell number ('proliferation') after 24h treatment where, viability/cell number remaining between 98–100% of controls (Figure 1).

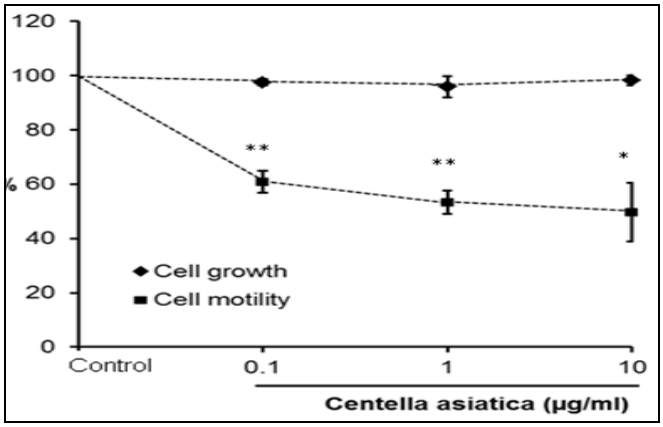

Fig 1: Effect of *Centella asiatica* aqueous extract on cell growth and motility. Cell growth was not affected after 24h treatment with aqueous extract of *Centella asiatica*. However, motility was reduced dose-dependently after 24h treatment with aqueous extract of *Centella asiatica*. Values are mean ± SEM ($n = 3$) with * and ** indicates significant differences $p<0.05$ and $p<0.01$, respectively.

3.2 Effect of *Centella asiatica* aqueous extract on motility

Motility was assessed according to the 'wound-heal' assay after 24h treatment with the plant extract (0.1 - 10µg/ml). The effect of *Centella asiatica* aqueous extract on motility was normalized to the control level; results showed that lateral motility was reduced by *Centella asiatica* aqueous extract dose-dependently with highest effect was seen at 10µg/ml which reduced the motility index by ~50% to 49.7 ± 10.8% (Figure 1).

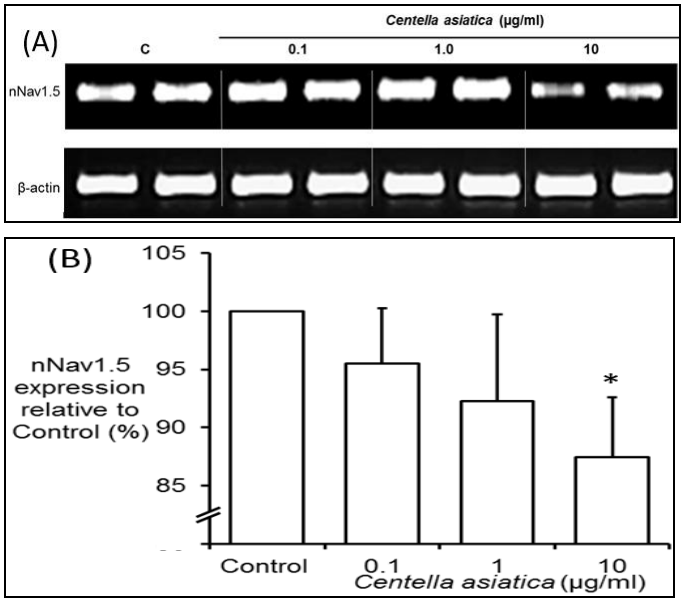

Fig 2: Effect of *Centella asiatica* aqueous extract on nNav1.5 gene expression. (A) PCR products (duplicate) of nNav1.5 and β-actin from of each samples; C – Control, 0.1–10µg/ml of *Centella asiatica* aqueous extract. (B) nNav 1.5 expression was reduced dose-dependently after 24h treatment with aqueous extract of *Centella asiatica*. Values are mean ± SEM ($n = 4$) with * indicates significant differences $p<0.05$

3.3 Effect of *Centella asiatica* aqueous extract on nNav1.5 gene expression level

PCRs were conducted to measure nNav1.5 expression levels after 24h treatment with *Centella asiatica* aqueous extract. Figure 2(A) shows the products of nNav1.5 and β-actin of each sample. In comparison to control (untreated cells), nNav1.5 expression was reduced dose-dependently by *Centella asiatica* aqueous extract (0.1 to 10µg/ml). nNav 1.5 gene levels was reduced to ~95 ± 4.8, 92 ± 7.4 and 87 ± 5.2%, respectively (Figure 2(B)).

4. Discussions

The preferred future cancer therapy would be to use drugs that is non-toxic but could control cancer metastasis which has been the main cause for 90% of cancer mortality. Therefore, in this study, the cell viability and proliferation assays were primarily aimed to choose concentrations of *Centella asiatica* aqueous extract that would not give effect on growth. This is essential to achieve as to exclude any limitation on the analysis of anti-metastasis, where the effect should be exclusively *via* the suppression of metastatic parameter and not due to suppression of cells proliferation. Indeed, after 24h treatment with *Centella asiatica* aqueous extract ranging from 0.1 to 10µg/ml, cell proliferation of MDA-MB-231 cells were not affected (Figure 1). Therefore, tested concentration range remained throughout later experiments. Although the concentration range used in this study was not killing the cells, the cytotoxicity potential of *Centella asiatica* aqueous extract has been shown in other report where similarly, it was tested on MDA-MB-231 cells and the obtained IC_{50} was at 648µg/ml [13].

During metastasis, cell motility enables cancer cell to disseminate away from the primary tumour. Motility or 'wound-heal' assay has been used widely in research investigating or measuring cancer motility potential. After 24h treatment with *Centella asiatica* aqueous extract (0.1 to 10µg/ml), lateral motility of MDA-MB-231 cells was suppressed dose-dependently (Figure 1). This result supported our hypothesis; *Centella asiatica* aqueous extract exclusively inhibit the motility of MDA-MB-231 cells at concentrations not affecting the cell growth. Similarly, motility of aggressive prostate cancer cell line expressing Nav1.7, MAT-LyLu was suppressed without effect on cells growth by TTX (1 µM) [14], highlighting the role of VGSCs in controlling cancer motility and importantly the potential of TTX as anti-metastatic. However, since TTX is very toxic, using 'back-to-nature'-medicinal plants might be a safer alternative to target VGSCs in breast cancer. Indeed, our results indicate that *Centella asiatica* aqueous extract might have the right component to do so with the exact phytochemical component need to be determined.

After 24h treatment with *Centella asiatica* aqueous extract, down-regulation of nNav1.5 gene expression levels was observed (Figure 2(A) & Figure 2(B)), suggesting that suppression of motility by *Centella asiatica* aqueous extract may associate with down-regulation of nNav1.5 gene. Previously, down-regulation of nNav1.5 mRNA expression level by TTX has been well linked with suppression of metastasis parameters such as migration and invasion of MDA-MB-231 cells [7, 15]. Similarly, in prostate cancer expressing VGSCs isoforms namely, Nav1.7, down-regulation of the gene by TTX was followed by suppression of metastasis parameters (migration and invasion) [7]. Interestingly, comparable 'back-to-nature' examples have been reported by docosahexaenoic acids (DHA), a natural omega-3 polyunsaturated fatty (PUFA) abundant in fish oil; DHA reduced nNav1.5 mRNA and protein expression at plasma membrane hence suppressing migration of metastatic breast cancer cells [16]. Another omega-3 acid PUFA eicosapentaenoic (EPA) have also been reported to have similar observation; down-regulation of VGSC mRNA expression was obtained by EPA followed by inhibition of metastatic potential of prostate cancer cells [17].

5. Conclusions

The adverse effects of chemotherapy can be highly invasive and some breast cancer patients can have low tolerance to treatment. In this regard, targeting VGSCs in breast cancer using VGSC blockers founded in a 'back-to-nature' strategy might improve treatment avenues with fewer or no undesirable side effects. *Centella asiatica* aqueous extract might contain the right component to suppress nNav1.5 expression at the same time non-toxically control breast cancer aggressiveness. However, the exact phytochemical component responsible for its action on nNav1.5 needs to be determined. The effect of the responsible component should also be validated using a standard method to measure ion channel activity e.g. patch-clamps.

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