Capsaicin and dihydrocapsaicin extracted from *Capsicum chinenses* decrease cell viability of neuroblastoma SH-SY5Y cells *in vitro*

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Abstract: Neuroblastoma is an extra-cranial solid cancer that primarily affects children. Aggressive neuroblastoma tumors typically demonstrate resistance to conventional chemotherapeutic and radiotherapeutic regimens. Interestingly, the use of dietary supplements in the control of cancers has gained ascendance in recent scientific investigations.   Capsaicin and dihydrocapsaicin are bioactive components of *Capsicum chinenses* fruit. Qutenza (a high-dose capsaicin patch) is used in the management of neuropathic pain from postherpetic neuralgia and HIV-associated neuropathy. Research on the potency of capsaicin as an anticancer agent has been demonstrated on several cancer cell lines and in *in vivo* models. The possibility of conventional cancer therapies having long-term developmental and other side effects on pediatric patients invokes the need to search for other less toxic agents against neuroblastoma. In this study, we tested if *Capsicum chinenses* fruit extract has therapeutic potential against neuroblastoma. To carry out this study, capsaicin and dihydrocapsaicin extract were made from *Capsicum chinenses* red fruits via hexane extraction method. Then, a range of concentrations (1pg/mL–100 mg/mL) of the extract was administered to cultured SH-SY5Y neuroblastoma cells and their viabilities assessed. The potency of capsaicin in destroying neuroblastoma cells indicated that it might act via multiple routes, hence we screen for possible receptors in and on neuroblastoma cells that might interact with capsaicin using molecular docking techniques. Our findings showed that capsaicin and dihydrocapsaicin extracted from *Capsicum chinenses* reduced neuroblastoma cell viability in a concentration-dependent manner with an IC$_{50}$ of 69.75 μg/mL. Our *in-silico* analysis determined that capsaicin might potentially bind to other receptors on the surface of neuroblastoma cells. We demonstrated a stronger binding affinity of capsaicin to human D4 Dopamine receptor (DRD4) than to the known vanilloid receptor TRPV1 using molecular docking. In conclusion, these results illustrated that *Capsicum chinenses* extract containing capsaicin and dihydrocapsaicin is effective in reducing viability of neuroblastoma cells *in vitro* and may serve as a naturally derived treatment source for this pediatric cancer, secondly, capsaicin may have multiple targets, and its strong binding to human D4 Dopamine receptors may point to different pathways by which capsaicin exerts its cancer killing effects.

Keywords: Neuroblastoma; Capsaicin; TRPV1, Childhood Cancer

1. Introduction

Neuroblastomas are solid tumors that are derived from an embryonal malignancy of the sympathetic nervous system (**Mora and Gerald, 2004**[1]).   These tumors are diagnosed in infants under the age of five (**Woods et al., 2002**[2]). Over 700 new cases of neuroblastoma are reported yearly in the United States alone (**Loachim and Mederios, 2009**[3]). A coordinated worldwide study of childhood cancer incidence shows that neuroblastoma accounts for 6-10% of cancers in Caucasian populations. In the United States, children of African American parents shows an incidence of 8.5 per million whereas 11.5 is recorded for White children (**Stiller and Parkin, 1992**[4]). In spite of clinical and technical advances, neuroblastoma is still a huge challenge to medicine and research laboratories alike (**Schwab et al., 2003**[5]). Existing treatments for neuroblastoma primarily consist of various chemotherapeutic regimens (**Barrett and Rochelle, 2016**[6]; **Grünwald and Ezziddin, 2010**[7]). For instance, cisplatin, doxorubicin, etoposide, and cyclophosphamide are used for treating neuroblastoma (**Conter et al., 2014**[8] **Pearson et al., 2008**[9]; **McGregor et al., 2005**[10]; **Mishima et al., 2004**[11]; **Keshelava et al., 1998**[12]). These drugs are highly toxic

and have health and developmental effects on pediatric patients (**Ries and Klastersky, 1986**[13]**; Punt, 1997**[14]**; Tacar et al., 2013**[15]**; Aldossary, 2019**[16]**).** For instance, cyclophosphamide has been associated with the development of acute myeloid leukemia, bladder cancer, hemorrhagic cystitis, and permanent infertility (**Kasper et al., 2005**[17]). Stuart and colleagues demonstrated that long-term use of cisplatin in testicular cancer patients resulted in hearing, renal, and respiratory impairments (**N.S. Stuart, 1990** [18]). Given the long-term toxic effects of these chemotherapeutic drugs, it is critical to develop safer treatment regimens for neuroblastoma.

Over the past few years, dietary modifications and natural products derived from plants have been studied for their roles in the prevention and treatment of many cancers (**Reddy et al., 2003**[19]**; Khan et al., 2008**[20]**; Khan et al., 2012**[21]**; Agbarya et al., 2014**[22]**; Khan et al., 2018**[23]). One of such substance is an ingredient found in pepper, namely capsaicin (**Bley et al., 2012**[24]**; Clark and Seong-Ho, 2016**[25]). Capsaicin is the common name for the bioactive ingredient, 8-methyl-n-vanillyl-6 nonemide (a vanilloid) (**Porseva et al., 2014**[26]). It is derived from plants of the genus *Capsicum* (**Othman et al., 2011**[27]**; Friedman et al., 2019**[28]**)**. The transient receptor potential venilloid-1 (TRPV1) is the known capsaicin receptor that is highly expressed in sensory neurons (**Koo et al., 2007**[29]**).** Capsaicin, heat, and protons activates this receptor to induce the influx of Ca^{2+} and Na^+ ions (**Porseva et al., 2014**[30]). Capsaicin presents a burning sensation and desensitizes sensory neurons after prolonged administration (**Dray, 1992**[31]**)**. Oh and Lim demonstrated that capsaicin or dihydrocapsaicin caused induction of p53, p21, and G_0/G_1 arrest. They also showed that dihydrocapsaicin induced autophagy, dilation of the ER and mitochondria (**Oh and Lim, 2009**[32]**)**. Qutenza (a high-dose capsaicin patch) is in the management of neuropathic pain from postherpetic neuralgia and HIV-associated neuropathy (**Katz et al., 2015**[33]). The metabolism of capsaicin occurs in the liver and in the gut lumen (**O'Neill et al., 2012**[34]). Studies by Reilly et al., 2013, using human hepatic microsomes and S9 fragments demonstrated that capsaicin is metabolized into 16-hydroxycapsaicin, 17-hydroxycapsaicin, and 16,17-hydroxycapsaicin, and vanillin and that cytochrome P450 is implicated in the hepatic capsaicin metabolism (**Reilly et al., 2013**[35]).

Research focused on variety of cancers indicate that capsaicin and dihydrocapsaicin have clear roles in regulating the growth and proliferation of tumor cells (**Oh and Lim, 2009**[36]**; Díaz-Laviada and Rodríguez-Henche, 2014**[37]**).** In vitro and in vivo studies demonstrate that capsaicin potently suppresses the growth of cancer lines such as human prostate carcinoma cells and T-cell leukemia cells (**Dîaz-Laviada, 2010**[38]**; Brown et al., 2010**[39]**; Sánchez et al., 2007**[40]**).** However, the role of capsaicin on neuroblastoma cell viability has yet to be tested. Thus, the aim of this study was to investigate the effect of capsaicin and dihydrocapsaicin extracted from *Capsicum chinenses* fruits on SH-SY5Y neuroblastoma cells' viability, the potential receptors that mediate the interaction between capsaicin and these cancer cells and finally the potential thermodynamics of capsaicin-receptor interactions.

2. Materials and Methods

2.1. Description of Plant and Collection of Plant material

Capsicum *chinenses* originate from the Family Solanaceae, and they are well known for their exceptional heat. The appearance and characteristics of the plants include small, herbaceous, compact perennial bushes about 0.5 m in height. The flowers, as with most Capsicum species, are small and white with five petals. It has shallow roots. Their fruits can be harvested when green or can be allowed to reach full maturity when they exhibit a range of colors from yellow, orange to deep purple, depending upon the selection. Inside the fruit is a spongy ribbing that separates the internal space and gives attachment to several small-sized, round, and flattened seeds.

Capsicum chinenses fruits were obtained from the Tuskegee University School farms in Tuskegee, Alabama. All plants were cultivated under organic management conditions for high yield content purposes. Once mature (95 to 100 days), the fresh red fruits from the plant were collected into a black polyethene bag and transported immediately into the Department of Chemistry Laboratory, Tuskegee University where the fruits were washed with deionized water and frozen at -20 °C until extraction. A voucher specimen is deposited in the Department of Chemistry.

Only the red fruits were handpicked and were grinded to a thick, semi-solid paste which had the characteristic red color as of the fruit. The resulted fine paste was mixed with hexane and eluted. The elute was air-dried to obtain powdered extract.

2.2. Reagents

All chemicals (Hexane, methanol –LC-MS (≥ 99.9%), water, and ethanol absolute proof (≥ 99.5%) were all high-performance liquid chromatography (HPLC) grade from Sigma Aldrich, MO, USA. Capsaicin and dihydrocapsaicin standards were obtained from Santa Cruz Biotechnology Inc., CA, USA. The concentrations of the capsaicin and dihydrocapsaicin standards were evaluated using a stock solution of 6 mg/mL capsaicin and a stock solution of 5 mg/mL dihydrocapsaicin. The standards were dissolved completely 10:1 hexane-ethanol solution.

2.3. Extraction and Isolation

Red fruits of *C. chinenses* were removed from -20 °C and defrosted at room temperature. The procedure used by Abugri et al. was adopted with little modifications (2012). The *Capsicum chinenses* fruits of mass (10 g) were weighed into a mortal and then 10 mL hexane was added. The content was ground using a pestle to release the capsaicin and dihydrocapsaicin into the solvent. Samples were collected into a large pyrex culture test tube and vortexed for three min and incubated at 30 °C for 20 min with automated shaking at 100 rpm using a clinical bench top centrifuge (IEC Centra, CL2, International Equipment Company, Needham Heights, MA, USA). Extracts were cooled at room temperature for five min, filtered into a pre-weighed amber bottle, and then dried in an incubator at 37 °C (Fisher Scientific, Model 146E, USA). Finally, the dry weight was measured.

2.4. Validation and quantification of capsaicin in extract using HPLC/HPTLC

To identify the presence of capsaicin and dihydrocapsaicin in the extract, samples were resuspended into solution using 1mL of Hexane-ethanol and tested using high performance thin layer chromatography (HPTLC) as a qualitative validation (**Figures 1-4**). High performance thin layer chromatography (HPTLC) analysis of the extract was performed using a developing chamber (29 cm x 24.5 cm). The mobile phase constituted a 1:1 ratio of ethyl acetate-hexane (total volume was 20 mL). The mobile solvent was poured into the chamber and allowed for 20-30 min total time to saturate under a closed taped lid in a fume hood. A silica gel TLC DC-glasplatten-Kieselgel with fluorescence indicator at 254 nm (size = 2.25 µm, layer thickness = 0.25 nm medium pore diameter = 60 A, 10 x 20 cm glass plate) was used to run the sample. A 1 cm height was chosen as the reference point for spotting of compounds, and a straight line was marked across the plate. Samples were spotted in 1 cm interval gaps with that of capsaicin and dihydrocapsaicin standards on the same fluorescent TLC plate. Standards consisted of capsaicin and dihydrocapsaicin were obtained from Santa Cruz biotechnology Inc. CA, USA. The plates were then placed into the chamber and allowed to sit for approximately 16 min for total run time for complete separation of compounds. After development, retention factors were calculated for the pure capsaicin and dihydrocapsaicin in the *C. chinenses* fruits. Bands that were similar in the plate were further verified using high performance liquid chromatography (HPLC) to confirm the presence of capsaicin and dihydrocapsaicin.

Extracts were analyzed concurrently with external standards in the same sequential run. The samples were analyzed using Agilent 1100 series high performance liquid chromatography (HPLC) equipped with a diode array detector (DAD) coupled with UV –Visible spectrometer. The mobile phases used were 95% methanol and 5% HPLC water at a pH of 3.0, all HPLC grade. The injection volume and pressure was set up to 20 µL/mL and maximum pressure of 400bar. Compounds were detected at UV wavelengths of 254 and 280 nm with reference wavelength of 530 nm. Identification and quantification were done using external standards by comparison of the retention time of samples to standards using Agilent ChemStation software version B.03.01.

2.5. Cell culture

The neuroblastoma SH-SY5Y cells were purchased from Sigma, with a passage number of 24. Neuroblastoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 15% fetal bovine serum (FBS). SH-SY5Y neuroblastoma cells were cultured in T-25 flasks and maintained at 37 ºC with 5% CO_2. Cells were allowed to grow to 100% confluency. Next, cells were trypsinized and transferred onto six-well plates for microscopy analysis. Another subset of neuroblastoma cells were utilized for the trypan blue cell viability assay and were cultured in 60 mm petri dishes. Once wells or petri dishes were 70% confluent with SH-SY5Y cells, they were utilized for testing with different concentrations of *Capsicum chinenses* extract. The cell viability test was conducted in triplicate.

2.6. Extract administration

A 10 mg/mL concentration of the extract was initially prepared in 0.1% dimethyl sulfoxide (DMSO) as a stock solution. From this stock solution, 1 pg/mL, 1 ng/mL, 10 ng/mL, 1 µg/mL, 10 µg/mL, 100 µg/mL, 1 mg/mL, 10 mg/mL, and 100 mg/mL concentrations were prepared by adding the proper volume of 0.1% DMEM. Control

preparations were prepared by using equivalent volumes of the DMSO mixed with DMEM.   Twenty microliters of each extract concentration or control were administered to each well or petri dish.   Photomicrographs and cell viability measures were obtained three hours after treatment administration.

2.7. Microscopy

Differential interference contrast (DIC) microscopy was performed, and images of neuroblastoma cells were captured.   The photomicrographs were taken and digitized using an Olympus 1X2-UCB microscope and the 7.5 version of Metamorph software. Each plate of cells was viewed under the microscope at 100X magnification using the DIC setting. Next, three separate photomicrographs were taken from every well in different fields and stored as a TIFF file. All cell treatments and image capturing were performed in parallel.

2.8. Trypan blue assay

To measure cell viability, a trypan blue assay was performed.   Briefly, wells were trypsinized to create a cell suspension. Next, 0.1 mL of 0.4% trypan blue stain was added and mixed thoroughly with 0.5 mL of SH-SY5Y cells and then allowed to stand for 5 min at 25°C. Next, ten microliters of the resulting mixture were used to fill a hemocytometer (LW Scientific Hemacytometer Neubauer Bright Line, Double-Counting Chamber) for cell counting. All viability evaluations were conducted under the microscope at magnification of 100X.

2.9.   Molecular Docking of capsaicin to receptors

The crystal structure of squirrel TRPV1 (PDB ID: 7lqz), human TRPV2 (PDB ID: 2f37), human prohibitin 2 (PDB ID: 6iqe), human dopamine receptor DRD1-Gs signaling complex (DRD1) (PDB ID: 7ckz), human dopamine D3 receptor (DRD3) (PDB ID: 3pbl), and human D4 Dopamine receptor (DRD4) (PDB ID: 5wiv) were extracted from the RCSB website (https://www.rcsb.org/) for the docking analysis.   These receptors were used in the virtual screening process against capsaicin molecule (Pubchem CID: 1548943) using PyRx (an open-source software for performing virtual screening that combines AutoDock Vina, AutoDock 4.2, Mayavi, Open Babel etc.) [41,42, 43]. The receptor and the capsaicin molecule were prepared using AutoDock Vina wizard, and Biovia Discovery Studio software (version; 21.1.0.278). Both the receptors and the capsaicin structures were minimized and converted to a pdbqt format. In the case of the receptors, their bond orders were assigned, and charged hydrogen atoms added to the proteins. The structures minimizations were carried out using the AutoDock Vina wizard. The receptor grid box was generated in PyRx using the build-in Vina Wizard module, the dimensions of the grid boxes were maximized to covered entire molecule in each case. The docking process were carried out using the Auto-Dock wizard in-built in PyRx program with an exhaustiveness of 8. The best receptor-capsaicin complex that showed the highest relative free binding energy released was saved as a pdb file and exported into Biovia Discovery Studio software for specific atomic interaction analysis between the capsaicin and the receptors.

2.9. Data Analysis

Data obtained from trypan blue assays are displayed as mean ± S.E.M. An analysis of variance (ANOVA) was utilized for assessing statistical significance of the different treatment group. A Tukey's multiple comparison post-hoc test was performed if warranted.   The alpha value was set at < 0.05. Statistical comparisons and graphs of viability assays were prepared using GraphPad Prism version 6.0.

3. Results

3.1. Phytochemical analysis of C. chinenses fruits by HPTLC/ HPLC

Comparison of band heights for identification of capsaicin and dihydrocapsaicin from the extract were done based on matching extracts' retention values with external standards retention values using HPTLC. The retention factor (RF) values for the pure capsaicin, pure dihydrocapsaicin, and the *C. chinenses* extract were calculated to be 0.59, 0.61 and 0.57, respectively.

To further confirm for the presence of capsaicin and dihydrocapsaicin we ran a portion of the air-dried extract on a high-performance liquid chromatography apparatus equipped with a diode array detector. The verification of dihydrocapsaicin in *C. chinenses* extract was done by overlaying the chromatograms of both standard compounds with that of the extract.   These chromatograms were obtained by running the HPLC at 280 nm and 254 nm.   At

280 nm, the HPLC detected pure dihydrocapsaicin (blue line for dihydrocapsaicin standard in **Figure 4**) at a retention time of 4.45 min.   Running the extract samples at 280 nm revealed a retention time of 4.40 min (green line representing sample from extract in Figure **4**), indicating the presence of dihydrocapsaicin.   The extract sample (green line) also contained other peaks at retention times 5.75 min and 6.25 min.
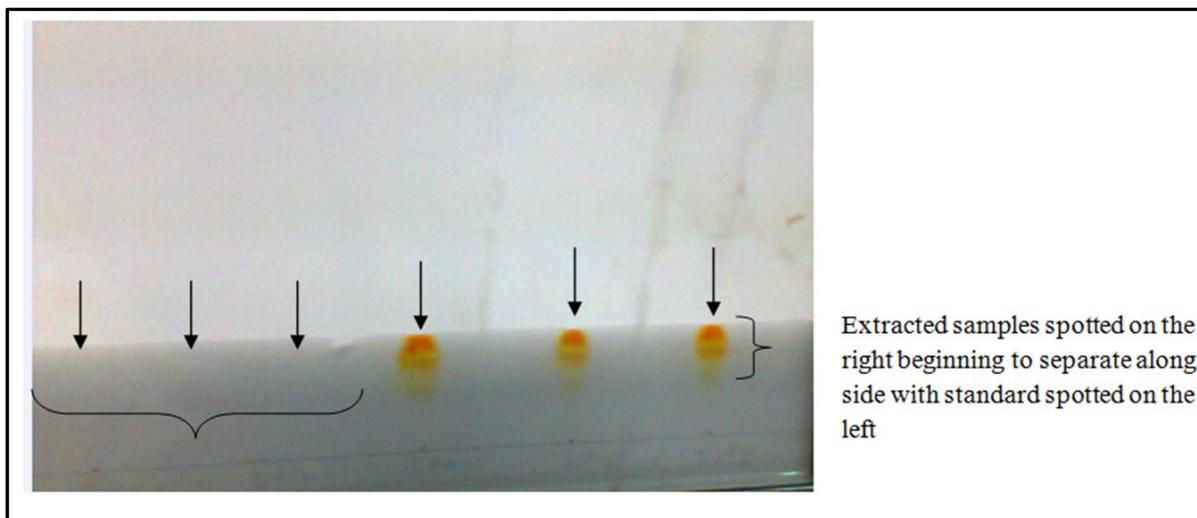

Figure 1. Spotting of standard capsaicin, standard dihydrocapsaicin and *C. chinenses* extract samples on a TLC.
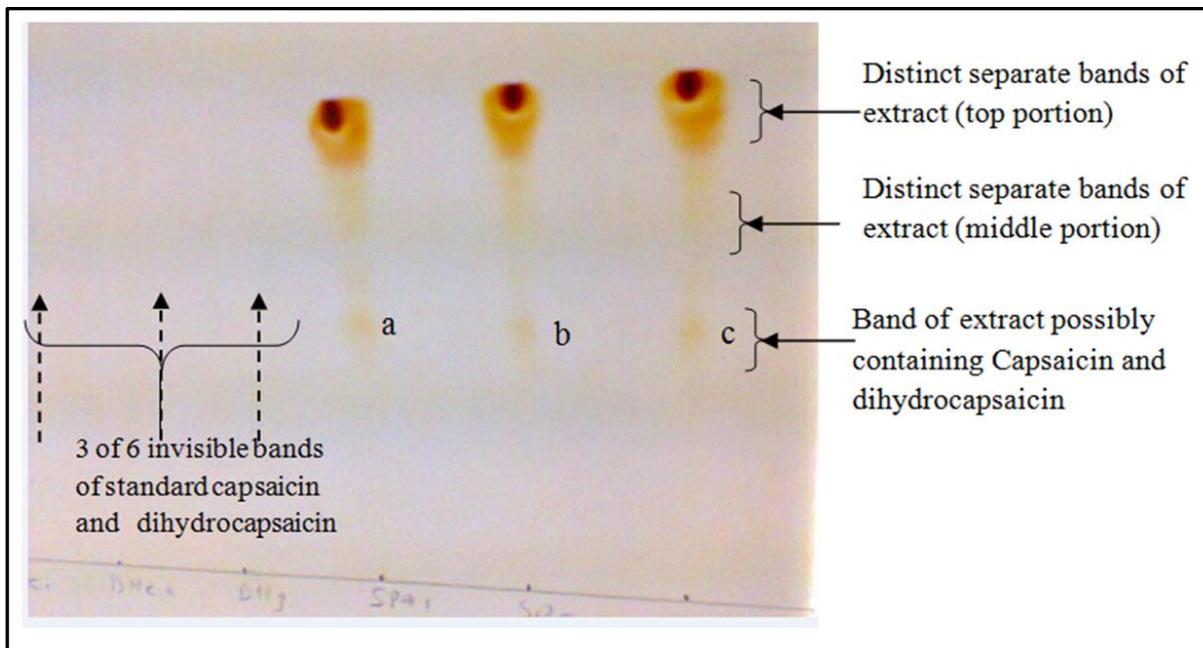

Figure 2. TLC results of extract revealing different spots indicating the presence of other   compounds including capsaicin and dihydrocapsaicin (**a, b,** and **c**).
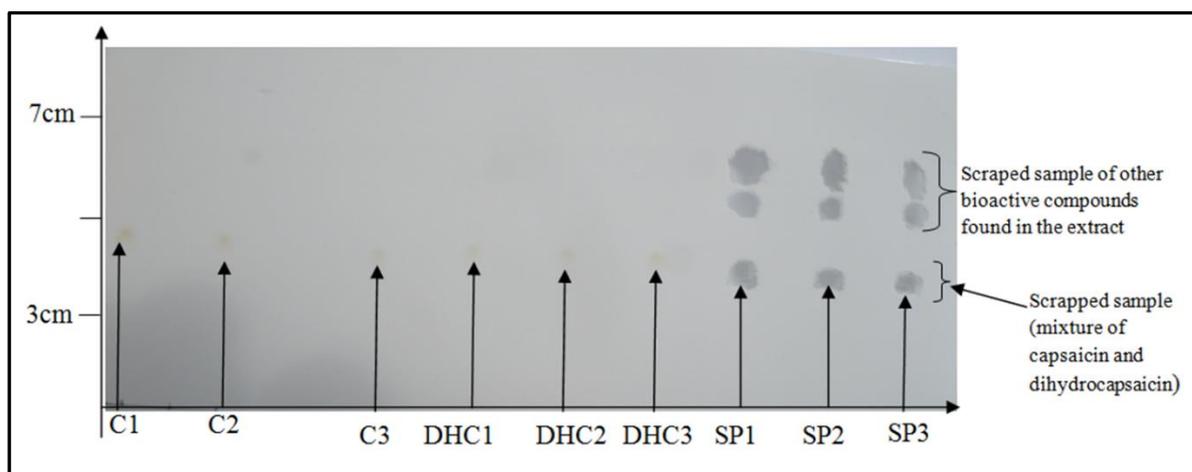
Figure 3. Scrapping of standard capsaicin, standard dihydrocapsaicin and *C. chinenses* extract samples from   TLC for HPLC analysis.
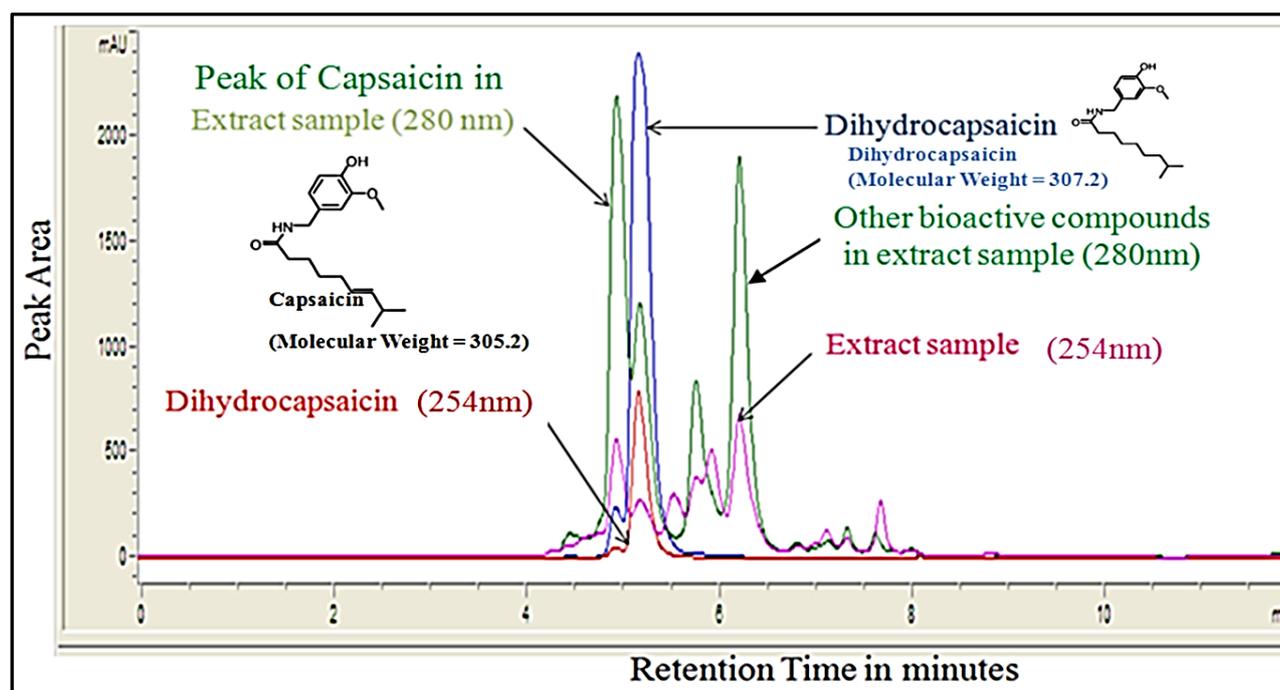

Figure 4. HPLC analysis of standard capsaicin, standard dihydrocapsaicin and *C. chinenses* extract samples from TLC. As shown the extract contain high amount of capsaicin and dihydrocapsaicin.

3.2. Effects of C. chinenses extract containing capsaicin and DHC on neuroblastoma cell morphology

We tested whether capsaicin and dihydrocapsaicin extracted from the *C. chinenses* would affect the structure of neuroblastoma cells. Immediately after administration, the cells treated with the extract were not visually distinct from cells treated with vehicle control. However, within 24 hours, cells administered the capsaicin and dihydro-capsaicin extract had differential changes in morphology (**Figure 5**). Cells treated with vehicle control exhibited a cell confluency of approximately 95% with no clear disruption of cellular morphology.   All these cells were well adhered to the culture plate.   Cells treated with either pure capsaicin (1mg/mL) or the extract (1 mg/mL) had 0% confluency measurements within 24 hours. Moreover, these cells were rounded and had no extensions

(compared to vehicle controls; see **Figure 5**).   After washing with media to remove floating dead cells, we confirmed that the extract and standard capsaicin- or dihydrocapsaicin-treated samples contained no living cells.
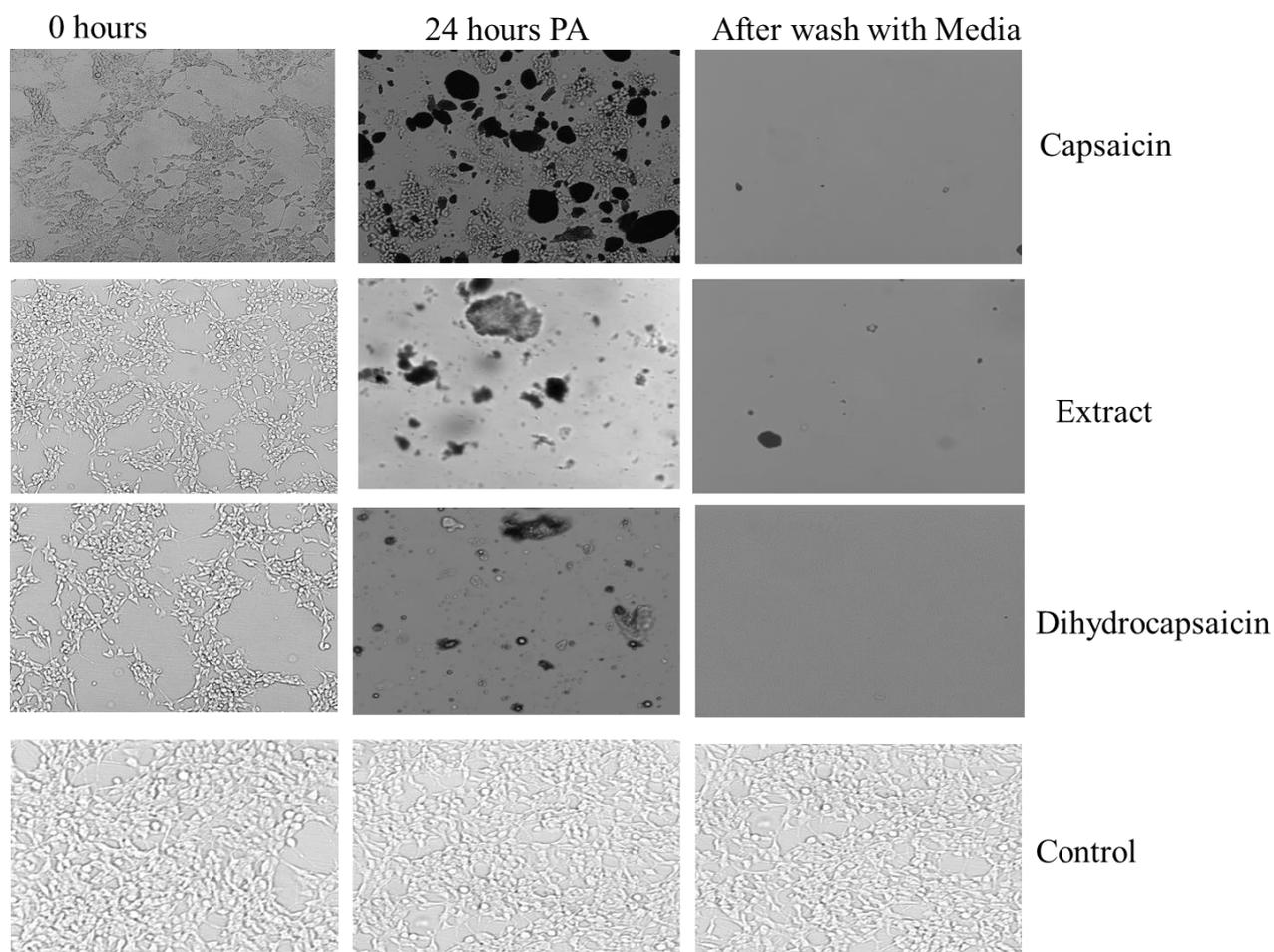
Compound Microscope, magnification 10x

Figure 5. SY5Y Cells treated with standard capsaicin (1mg/mL), standard dihydrocapsaicin(1mg/mL) or the *C. chinenses* extract (1 mg/mL) for 24 hours.

Figure 6. Microphotographs showing the morphology of cells post administration (PA) of extract or DMSO control for 3 hours.

3.3. Response of increasing concentrations of standard capsaicin, standard dihydrocapsaicin and C. capsicum extract on neuroblastoma cell viability

After detecting that the *C. chinenses* extract killed all cells within 24 hours, we focused on determining the effects of the extract, standard capsaicin and standard dihydrocapsaicin on a shorter time scale (3 hours) and at various concentrations. The SH-SY5Y cells treated with vehicle control were confluent, spread, fibroblastic in appearance and contained neurite extensions associated with healthy neuroblastoma cells. Cells treated with 1 ng/mL and 1 μg/mL of the extract had similar cellular morphologies to those given vehicle. Cytopathology was evident how-ever in cells that were administered with greater than 100 μg/mL of the *C. chinenses* extract (**Figure 6**).
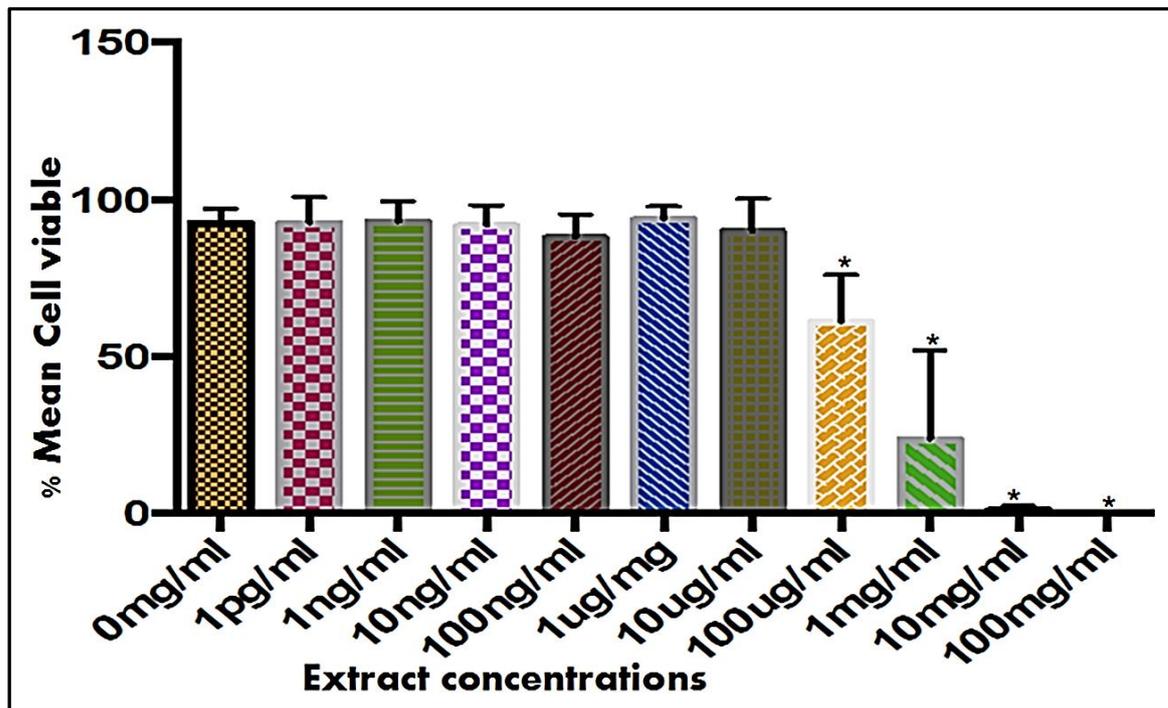
Figure 7. SY5Y cells viability post treatment to capsaicin extract from *C. chinenses.* Data reported at Mean + standard deviation. *p value < 0.005.
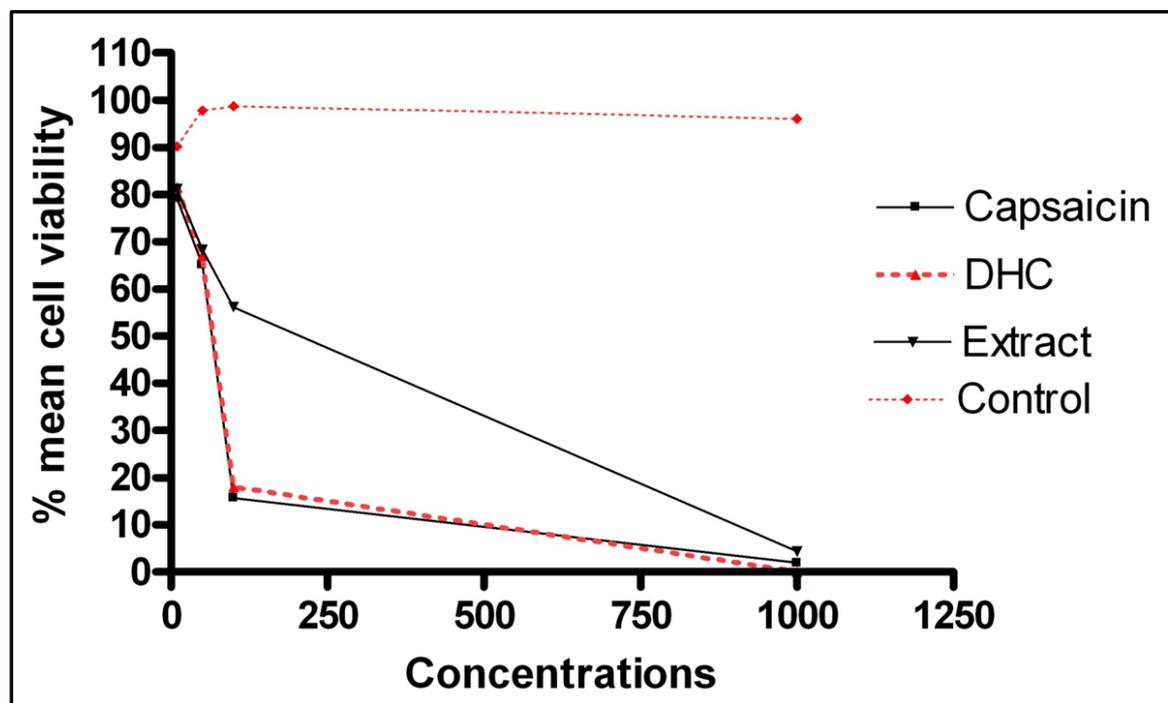
Figure 8. SY5Y cells % mean viability post treatment to standard capsaicin, standard dihydrocapsaicin, and extract from *C. chinenses* for 24 hours. Data reported at Mean + standard deviation.

As shown in **Figure 7**, the mean viability of cells post extract administration at different concentrations was calculated using the trypan blue assay. The percentages of viable cells were the following: 93.37% (vehicle control), 94.03% (1 pg/mL), 93.82% (1 ng/mL), 92.12% (10 ng/mL), 89.06% (100 ng/mL), 93.91% (1 µg/mL), 90.81% (10

μg/mL), 60.42% (100 μg/mL), 32.32% (1 mg/mL), 1.23% (10 mg/mL), and 0% (100 mg/mL). The results demonstrated significant decreases in cell viability among the control treatment and the 100 μg/mL, 1 mg/mL, 10 mg/mL, and 100 mg/mL concentrations of the *C. chinenses* extract (**Figure 7**).

Next, as shown in **Figure 8,** we compared cell viabilities between pure capsaicin and dihydrocapsaicin treatments with that of the *C. chinenses* extract. After three hours of the various treatments, cell viability was quantified via the trypan blue assay.   Pure capsaicin produced the following cell viabilities: 75.37% (10 μg/mL), 64.70% (50 μg/mL), 12.43% (100 μg/mL), and 1.25% (1 mg/mL). Standard dihydrocapsaicin resulted in 73.95% (10 μg/mL), 62.45% (50 μg/mL), 15.35% (100 μg/mL) and 0.5% (1 mg/mL) cell viabilities. The extract treatment yielded the following mean viabilities: 77.92% (10 μg/mL), 67.22% (50 μg/mL), 50.31% (100 μg/mL), and 5.21% (1 mg/mL). SH-SY5Y neuroblastoma cells treated with vehicle control (DMEM) had mean viabilities all greater than 95% in all treatments (**Figure 8**). While there were no statistically significant differences between the standard dihydrocapsaicin and capsaicin treatments at any of the concentrations, cell viabilities for the two pure treatments and the *C. chinenses* extract were statistically significant from the vehicle control group at all concentrations ($p < 0.05$). At 100 μg/mL, both pure standards reduced neuroblastoma cell viability at a greater degree than the extract ($p < 0.05$).   There were no significant differences among cell viabilities of cells treated with the pure standards or the extract at 1 mg/mL.

As shown in **Table 1**, the $IC_{50}s$ for standard capsaicin and dihydrocapsaicin were 69.31μg/mL and 68.46 μg/mL, respectively. The $IC_{50}s$ for both standard treatments were significantly lower than the resulting IC_{50} for the *C. chinenses* extract (83.56 μg/mL, $p < 0.05$).

	Standard Capsaicin treatment	Standard DHC treatment	Extract treatment
IC50	*69.31 ± 2.69μg/mL*	*68.46 ±2.97μg/mL*	*83.56 ± 3.59μg/mL*

Table 1. $IC_{50}s$ of standard capsaicin, standard DHC and extract treatments.
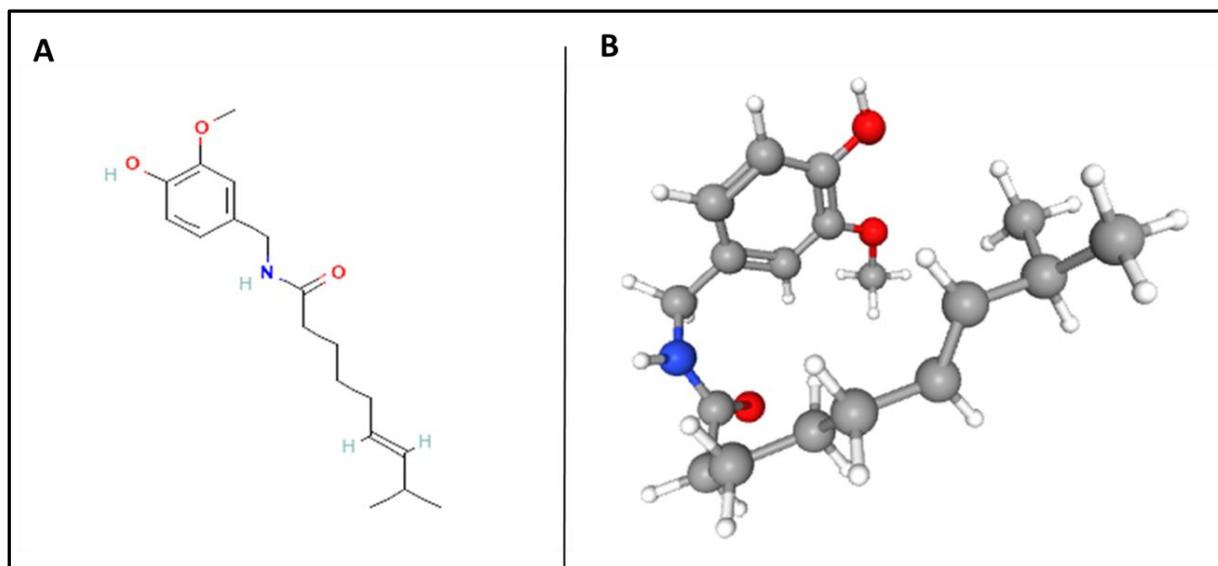
Figure 9. Chemical structure of capsaicin. (A) 2D molecular structure of capsaicin. (B) 3D molecular structure of capsaicin. IUPAC name: (*E*)-*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methylnon-6-enamid. Molecular formular: $C_{18}H_{27}NO_3$. (Source; Pubchem database; Pubchem CID 1548943).

3.4. Molecular docking of capsaicin to receptors

The chemical structure of capsaicin is shown in Figure 9. Capsaicin has a molecular weight of 305.4 Da, an XLogP3_AA of 3.6, a rotatable bond count of 9, hydrogen bond acceptor count of 3, hydrogen bond donor count of 2, heavy atom count of 22, and a topological surface of 58.6 Å².

Capsaicin-receptor interactions were studied in this work using docking simulations similar to works carried out by Wu et al (**Wu et al., 2021**[44]**).** The binding sites of capsaicin in these receptors were identified after blind docking process. The conformation that showed the strongest binding energy in each receptor was chosen for further ligand-receptor interaction analysis. As shown in **Figures 10, 11, 12, 13, 14** and **15** as well as in **Table 2**, the 3D and 2D structural depiction of the strongest binding conformation of capsaicin to the respective receptors studied are shown. Whereas **Figure 10** shows the interaction between capsaicin and the TRPV1 receptor which produced a net negative enthalpy of -6.3 kcal/mol, the TRPV2 receptor capsaicin interaction produced a comparatively less strong affinity to capsaicin (-5.9 kcal/mol; **Figure 11**). Prohibitin 2 and capsaicin interaction resulted in the release of -4.8 kcal/mol of free binding energy (**Figure 12**). DRD1 and DRD3 interaction with capsaicin recorded -6.6 and -6.0 kcal/mol respectively (**Figure 13** and **14** respectively). The interaction between capsaicin and DRD4 was the strongest (-8.3 kcal/mol). Capsaicin formed three strong hydrogen bonds with DRD4 by binding to SER196, ASP115 and VAL193 of DRD4. Hydrophobic Pi-sigma bonding was registered between VAL116 and capsaicin, whereas pi-pi T-shaped hydrophobic interaction was recorded between amino acid PH410 and capsaicin. Other hydrophobic interactions depicted occurred between capsaicin and the amino acids VAL87, LEU111, VAL193, PHE91, PHE411 and CYS119 (**Table 2**).

Wu et al., 2021 demonstrated that capsaicin binds and inhibits lipase in vitro (**Wu et al., 2021**[45]**).** In their structural analysis of lipase post capsaicin treatment, they demonstrated that capsaicin disturbed the secondary structure of lipase as well as inhibits the activity of lipase (**Wu et al., 2021**[46]).

Table 2. Receptors and their interacting residues to capsaicin

Receptor	ΔG kcal/mol	Residue	Bond distance /Å	Bond type
TRPV1	-6.3	TYR489	5.33	Hydrophobic: Pi-Pi Stacked
		TYR446	5.52	Hydrophobic: Pi-Pi Stacked
		PHE490	5.22	Hydrophobic: Pi-Pi T-shaped
		VAL442	4.77	Hydrophobic: Alkyl
		LEU445	4.86	Hydrophobic: Alkyl
		LEU482	4.97	Hydrophobic: Alkyl
		TYR446	5.24	Hydrophobic: Pi-Alkyl
		TYR489	5.03	Hydrophobic: Pi-Alkyl
		VAL442	5.24	Hydrophobic: Pi-Alkyl
TRPV2	-5.9	ASP144	2.78	Hydrogen Bond: Conventional Hydrogen Bond
		ASP144	4.24	Hydrophobic: Amide-Pi Stacked
		PRO95	5.13	Hydrophobic: Alkyl
		PRO137	4.39	Hydrophobic: Alkyl
		ILE141	5.42	Hydrophobic: Alkyl
		LEU91	4.88	Hydrophobic: Alkyl
Prohibitin 2	-4.8	ALA196	2.05	Hydrogen Bond: Conventional Hydrogen Bond
		ALA196	4.23	Hydrophobic: Alkyl
		VAL197	5.34	Hydrophobic: Alkyl
		ALA199	3.97	Hydrophobic: Alkyl
		ALA203	4.04	Hydrophobic: Alkyl
		PHE189	4.31	Hydrophobic: Pi-Alkyl
		TYR193	4.16	Hydrophobic: Pi-Alkyl
		LYS200	4.11	Hydrophobic: Pi-Alkyl
		ALA203	5.15	Hydrophobic: Pi-Alkyl
DRD1	-6.6	GLN160	2.65	Hydrogen Bond: Conventional Hydrogen Bond

		ILE98	3.52	Hydrophobic: Pi-Sigma
		PHE95	3.78	Hydrophobic: Pi-Sigma
		TRP80	3.83	Hydrophobic: Pi-Sigma
		TRP80	3.79	Hydrophobic: Pi-Sigma
		ILE98	4.08	Hydrophobic: Alkyl
		TRP80	4.26	Hydrophobic: Pi- Alkyl
		TRP90	5.25	Hydrophobic: Pi- Alkyl
		PHE92	4.99	Hydrophobic: Pi- Alkyl
		TRP99	5.26	Hydrophobic: Pi- Alkyl
		PHE156	4.01	Hydrophobic: Pi- Alkyl
DRD3	-6.0	THR369	2.23	Hydrogen Bond: Conventional Hydrogen Bond
		TYR373	2.14	Hydrogen Bond: Conventional Hydrogen Bond
		GLU90	2.87	Hydrogen Bond: Conventional Hydrogen Bond
		SER366	3.36	Hydrogen Bond: Carbon Hydrogen Bond
		VAL86	4.00	Hydrophobic: Pi-Sigma
		VAL86	3.94	Hydrophobic: Pi-Sigma
		LEU89	5.47	Hydrophobic: Alkyl
		LEU89	5.09	Hydrophobic: Alkyl
		PHE345	4.73	Hydrophobic: Pi- Alkyl
DRD4	-8.3	SER196	2.54	Hydrogen Bond: Conventional Hydrogen Bond
		ASP115	2.44	Hydrogen Bond: Conventional Hydrogen Bond
		VAL193	3.76	Hydrogen Bond: Carbon Hydrogen Bond
		VAL116	3.55	Hydrophobic: Pi-Sigma
		PHE410	5.32	Hydrophobic: Pi-Pi T-shaped
		VAL87	5.23	Hydrophobic: Alkyl
		LEU111	5.21	Hydrophobic: Alkyl
		VAL193	4.70	Hydrophobic: Alkyl
		PHE91	5.18	Hydrophobic: Pi- Alkyl
		PHE411	5.05	Hydrophobic: Pi- Alkyl
		CYS119	5.08	Hydrophobic: Pi- Alkyl

3.5. *Capsaicin interactions with its receptors*

Rohm et al., 2013 demonstrated that nonivamide (an analog of capsaicin) at sub micro molar concentrations stimulated the Ca^{2+}-dependent release of serotonin and dopamine in SH-SY5Y cells. They showed that nonivamide-treated SH-SY5Y cells upregulated the expression of dopamine D1 and D2 receptors. As shown in **Figure 13, 14 and 15**, we demonstrate by molecular docking analysis the binding of capsaicin to dopamine D1, D3, and D4 receptors (labeled as (DRD1, DRD3 and DRD4 respectively). Also in **Table 2**, we showed the specific residues involved in the binding of capsaicin to these receptors. They revealed that that capsaicin induced serotonin and dopamine release (**Rohm et al., 2013**[47]).

Capsaicin inhibits the degeneration of dopamine neurons by blocking glial activation and oxidative stress (**Chung et al., 2017**[48]**).** The transient receptor potential receptor vanilloid type 1 (TRPV1), is a cation channel that is highly expressed in sensory neurons (**Gunthorpe et al., 2018**[49]) and in the brain and plays a crucial role in the central nervous system (**Kauer et al., 2009**[50]; **Starowicz et al., 2008**[51]). TRPV1 activation is showed to regulates

neuroinflammation and contributes to mesencephalic dopaminergic neuronal survival by blocking oxidative stress (**Park et al., 2012**[52]) and modulates neuronal function (**Kauer et al., 2009**[53]). In a rat   model of Parkinson's disease, nandamide was demonstrated to activate TRPV1 and modulates dopamine transmission in basal ganglia (**Morgese   et al., 2007**[54]). In this study, we demonstrated that capsaicin strongly binds to TRPV1 (**Table 2** and **Figure 10**).

TRPV2 is a capsaicin receptor homologue and is highly expressed in primary sensory neurons **(Tamura et al., 2005**[55]**).** TRPV2 has been demonstrated experimentally to respond to high-threshold noxious heat (**Julius and Basbaum, 2001** [56]**).** In this work via molecular docking we showed capsaicin can bind to TRPV2 (**Figure 11**) and hence could be a molecular target of capsaicin.

Another molecular target of capsaicin is prohibitin 2 which is localized to the inner mitochondrial membrane (**Wei et al., 2017**[57]). Capsaicin binds to prohibitin 2 and translocate it from the mitochondria to the nucleus (**Kuramori et al., 2009**[58]). Prohibitin 2 has been revealed to play a crucial role in the maintenance of mitochondrial morphology and in governing apoptotic processes of the cell (**Zhang   et al., 2020**[59]). Downregulation of prohibitin 2 shows a reduced parkin-mediated mitophagy and suppressed proliferation and migration of lung cancer cells (**Zhang   et al., 2020**[60]). In **Figure 12**, we showed prohibitin 2 bound to capsaicin at the active binding site with residues ALA196, VAL197, ALA199, ALA203, PHE189, TYR193, LYS200 and ALA203.
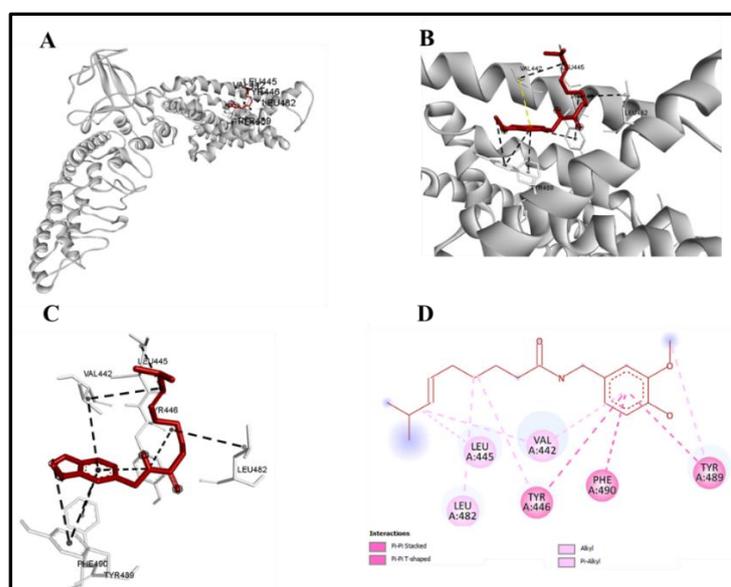
Figure 10. TRPV1 molecule interaction with capsaicin.   (**A**) TRPV1 molecule (grey) bound to capsaicin (red). (**B**) Close up view of capsaicin in the binding site of TRPV1. (**C**) Capsaicin-TRPV2 complex showing capsaicin bound to the interacting amino acids of TRPV1. (**D**) 2D representation of capsaicin bound to the TRPV1 molecule. Interacting amino acids, and their respective bond distances are depicted in **Table 2**.
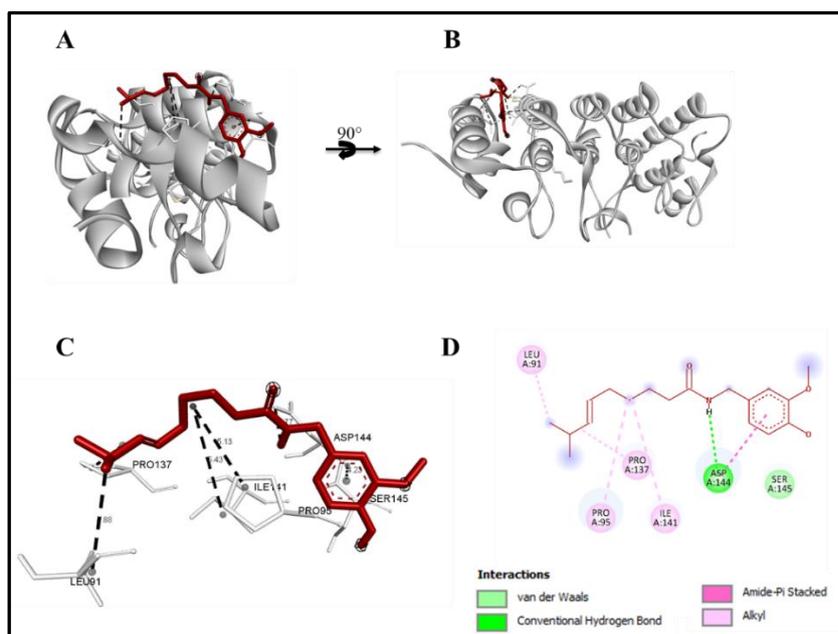
Figure 11. TRPV2 molecule interaction with capsaicin.   (**A**) TRPV2 molecule (grey) bound to capsaicin (red). (**B**) Close up view of capsaicin in the binding site of TRPV2. (**C**) Capsaicin-TRPV2 complex showing capsaicin bound to the interacting amino acids of TRPV2. (**D**) 2D representation of capsaicin bound to the TRPV2 molecule. Interacting amino acids, and their respective bond distances are depicted in **Table 2**.
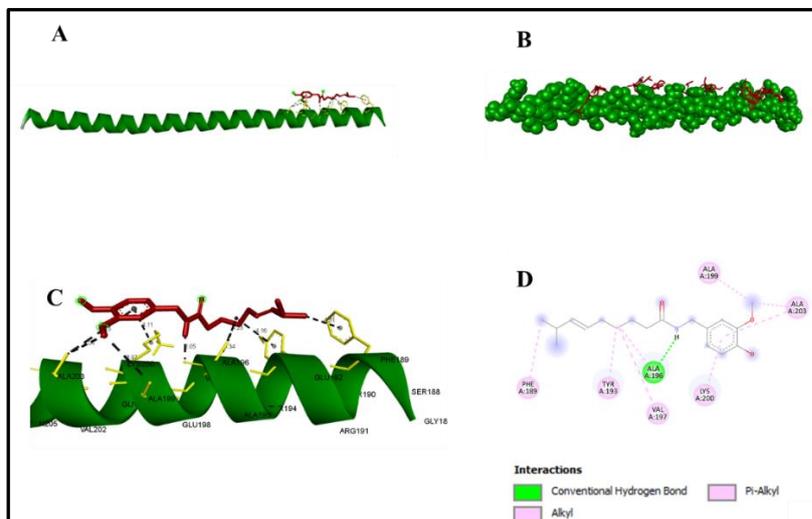
Figure 12. Prohibitin 2 molecule interaction with capsaicin.   (**A**) Prohibitin 2 molecule (green) bound to capsaicin (red). (**B**) Solid structural depiction of eight capsaicin molecules bound unto the surface of Prohibitin 2. (**C**) Capsaicin- Prohibitin 2 complex showing capsaicin bound to the interacting amino acids of Prohibitin 2. (**D**) 2D representation of capsaicin bound to the Prohibitin 2 molecule. Interacting amino acids, and their respective bond distances are depicted in **Table 2**.
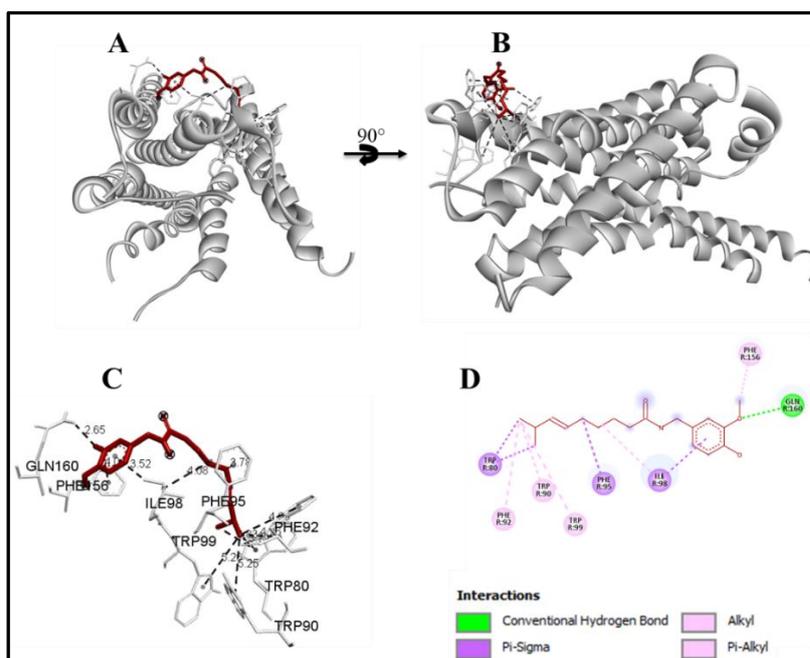
Figure 13. DRD1 molecule interaction with capsaicin. (**A**) Front view of DRD1 molecule (grey) bound to capsaicin (red). (**B**) Side view of capsaicin in the binding site of DRD1. (**C**) Capsaicin- DRD1 complex showing capsaicin bound to the interacting amino acids of DRD1. (**D**) 2D representation of capsaicin bound to the DRD1 molecule. Interacting amino acids, and their respective bond distances are depicted in **Table 2**.

3.6. Virtual analysis of thermodynamic parameters and type of binding forces

Capsaicin interactions with the cell surface receptors such as TRPV1, TRPV2, Prohibitin 2, DRD1, DRD3 and DRD4 were analyzed via in silico method. These interactions are characterized by forces such as hydrogen bonding, hydrophobic interactions, van de Waals forces and other electrostatic interactions which ultimately determines the thermodynamics of the interaction events (**Ross and Subramanian, 1981**[61]). The capsaicin solvent penetration of hydration layers possibly caused slight disordering of the solvent inaccessible regions of the proteins, subsequent short-range interactions of capsaicin with the receptors further exacerbates this process. In the final event, the net ΔG for the short-range interactions between capsaicin and these receptors produced negative enthalpy change (**Table 2**), hinting at thermostable complexes between the receptors and capsaicin. As indicated in **Table 2**, the strengthening of hydrogen bonds in the binding pocket and hydrophobic interactions between capsaicin and these receptors led to the observed negative enthalpies. The highest net negative enthalpy was observed between capsaicin binding to DRD4 receptor (-8.3 kcal/mol). The interaction between capsaicin and its receptor (the vanilloid receptor TRPV1) produced (-6.3 kcal/mol). The 2.0 kcal/mol energy differences between these two interactions possibly indicates that capsaicin might interact with higher affinity to DRD4 compared to TRPV1 than previously understood. This new finding highlights a novel approach that could be explored as capsaicin target in drug formulation process. This study however is not substantive enough to completely inform of the interaction between capsaicin and all receptors studied in this work, hence this results only provide a hint at the direction and starting point for *in vitro* binding kinetic studies, activity assays etc. that can be conducted between capsaicin and these receptors.
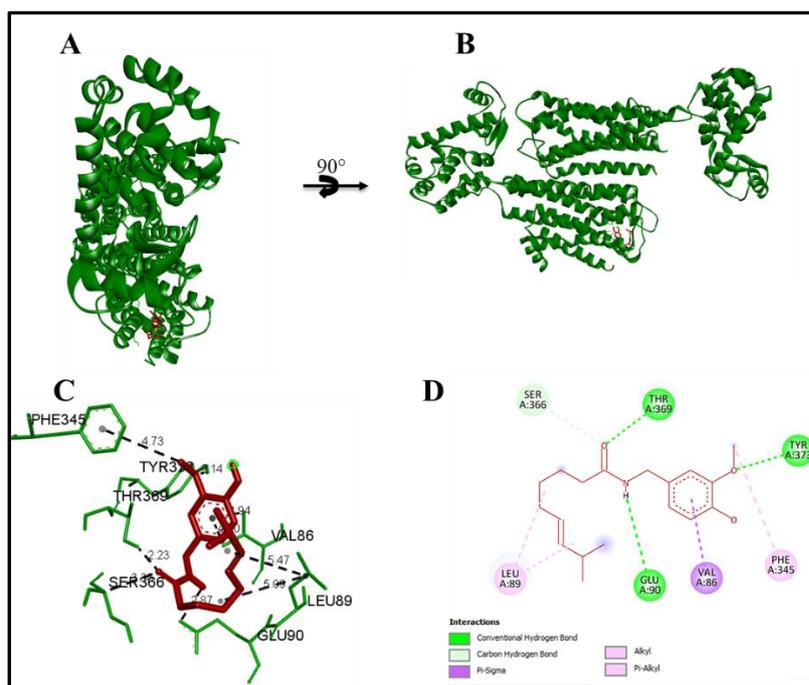
Figure 14. DRD3 molecule interaction with capsaicin.   (**A**) Front view of DRD3 molecule (green) bound to capsaicin (red). (**B**) Side view of capsaicin in the binding site of DRD3. (**C**) Capsaicin- DRD3 complex showing capsaicin bound to the interacting amino acids of DRD3. (**D**) 2D representation of capsaicin bound to the DRD3 molecule. Interacting amino acids, and their respective bond distances are depicted in **Table 2**.
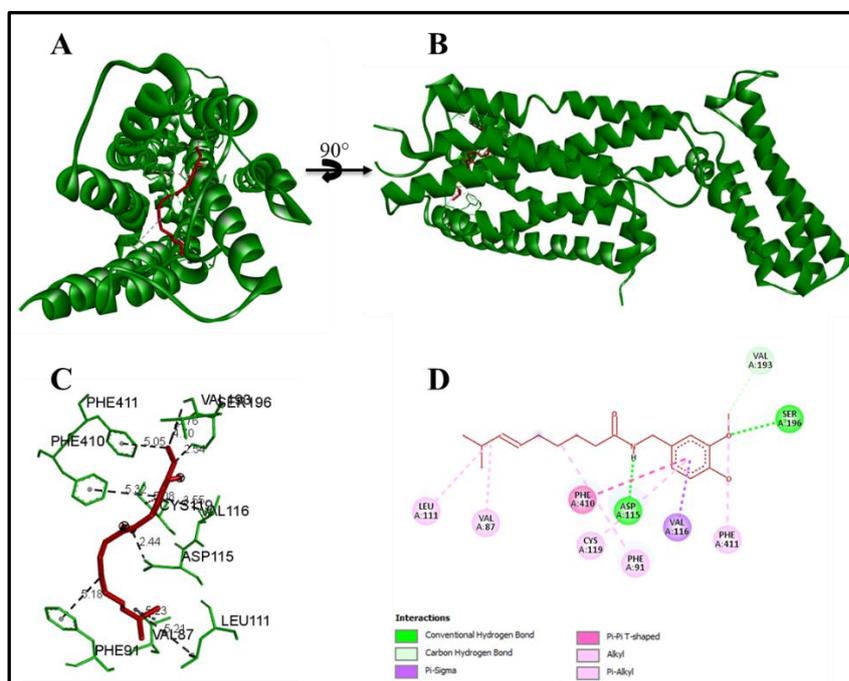

Figure 15. DRD4 molecule interaction with capsaicin.   (**A**) Front view of DRD4 molecule (green) bound to capsaicin (red). (**B**) Side view of capsaicin in the binding site of DRD4. (**C**) Capsaicin- DRD4 complex showing capsaicin bound to the interacting amino acids of DRD4. (**D**) 2D representation of capsaicin bound to the DRD4 molecule. Interacting amino acids, and their respective bond distances are depicted in **Table 2**.

4. Discussion

Capsaicin inhibits various oncogenic signaling pathways and have been shown to be a potential anti-cancer agent in both *in vitro* and *in vivo* models (**Min et al., 2004**[62]) by preventing cancer cells proliferation, metastasis (**Xu et al., 2018**[63]). It has been revealed that capsaicin blocks VEGF-induced angiogenesis (**Min et al., 2004**[64]), down-regulates p38 mitogen activated protein kinases (MAPK) and focal adhesion kinase (FAK) activation (**Surh , 2002**[65]**; Bhutani et al., 2011**[66]). It also induces the degradation of hypoxia inducible factor 1α (HIF1α), which is key transcription factor for cancer progression (**Ristoiu et al., 2011**[67]**; Chakraborty et al., 2011**[68]).

Plant-derived natural products have been an important subject to early medicine due to their repository benefits as therapeutic agents (**Ncube et al., 2008**[69]). Various phytochemicals have been isolated and characterized in higher plant extracts, providing effective and safe treatment of diseases, disorders, and infections (Raskin et al., 2002[70]; **Nair et al., 2005**[71]**; Ramya et al., 2008**[72]**;** Subedi **et al., 2020**[73]). In this work, the results from the chromatography experiments demonstrated that *C. chinenses* is a natural source of the heat-producing, anti-cancer compounds, capsaicin and dihydrocapsaicin. The trypan blue assay and morphological study revealed that the *C. chinenses* extract containing capsaicin and dihydrocapsaicin were effective in reducing the viability of neuroblastoma cells.

Thoennissen and colleagues demonstrated that capsaicin blocked breast cancer cell migration *in vitro* and significantly decreased the size of MDA-MB231 breast cancer tumors growing in immune-deficient mice (**Thoennissen et al., 2010**[74]**).** This present data also corroborate findings in MG63 human osteosarcoma cells studies in which capsaicin treatment prompted cellular morphology changes that were indicative of apoptosis (**Cho et al., 2013**[75]). Capsaicin has also been shown to induce apoptosis, ceramide accumulation and endoplasmic reticulum stress in androgen-sensitive prostate cancer cells at concentrations over 200 µM, induces (**Díaz-Laviada, 2010**[76]). Our findings indicate that capsaicin potently inhibited neuroblastoma cells growth *in vitro*.

To decipher the mechanism by which capsaicin inhibited neuroblastoma cell growth, we hypothesized that capsaicin reduces neuroblastoma viability by the induction of DNA damage, activation of oxidative stress mechanism, or by strongly binding to the TRPV1 and DRD4 receptors leading to MAP kinase activation and subsequently apoptosis. Capsaicin has been associated with DNA damage (**Nagabhushan and Blide, 1985**[77]**; Singh et al., 2001**[78]). In the presence of Cu (II) and molecular oxygen, capsaicin was reported to cause strand excision in DNA through an oxidative stress mechanism (**Oikawa et al., 2006**[79]). For example, capsaicin has been revealed to induce apoptosis by generating reactive oxygen species and disrupting mitochondrial transmembrane potential in human colon cancer cell lines. (**Yang et al., 2009**[80]). This could imply that capsaicin may also be able to induce neuroblastoma cell death either through oxidative stress or blocking transcription and translation by DNA excision.

The interaction between capsaicin and its receptor (the vanilloid receptor TRPV1) as well as the interaction between capsaicin and the dopamine receptors showed comparable enthalpies, indicating the possibility that capsaicin might interact potently with other receptors of neuroblastoma cells that could potentiate the apoptotic cascades of these cells than previously understood. These new findings highlight novel approaches that could be explored as capsaicin targets in drug formulation processes. This study however is preliminary and not substantive enough to completely inform of the in-depth actual interaction between capsaicin and all receptors studied in this work, hence this work can only provide the starting point for *in vitro* binding kinetic studies, activity assays etc. that can be conducted between capsaicin and these receptors.

While future studies are required to determine the mechanism by which the *C. chinenses* extract kills neuroblastoma cells, the present work indicates that the natural extract is effective in reducing neuroblastoma cell viability. Neuroblastoma's affinity to pediatric patients highlights the need to develop treatment methods that have few side effects and more importantly fewer developmental consequences. Capsaicin in its natural state is commonly used as food additives and for spices in food preparation. Thus, it could serve as an ideal candidate for nutritional medicine to prevent neuroblastoma. Moreover, coupling capsaicin and dihydrocapsaicin-containing extracts of *C. chinenses* with innovative medicinal delivery systems may serve well to treat neuroblastoma, a cancer primarily of the peripheral nervous system. Overall, our results point to *C. chinenses* extract with capsaicin and dihydrocapsaicin as a potential therapy for neuroblastoma, a pediatric cancer.

4.2. Conclusion

In conclusion, our data revealed that *C. chinenses* red fruits extracts are effective in limiting the growth of pediatric cancer cells (neuroblastoma) *in vitro*. Furthermore, the study supports that capsaicin and dihydrocapsaicin obtained from *C. chinenses* fruits could be a leading source of natural therapies for preventing or treating of neuroblastoma, a pediatric cancer.

Future study plans includes testing the mechanism(s) of *C. chinenses* extract-induced inhibition of neuroblastoma cells in order to understand the antitumor effects of capsaicin. Secondly, further studies to understand the signaling pathways implicated in capsaicin induction of cell death will be undertaken, by looking at the relationship between capsaicin administration and the induction of STAT3 phosphorylation. This will help decipher if capsaicin is implicated in the STAT3 activation pathway, which is known to have potential role in the prevention and treatment of multiple myeloma and other cancers. Hopefully, the relevant molecular mechanisms underlying capsaicin induction of cell death would be fully assessed to enhance our understanding of the effects and mechanisms of capsaicin on neuroblastoma. Finally, we also hope to explore specific cellular targets of capsaicin for therapeutic application.

Acknowledgements

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