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 \textit{in vitro} 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 \textit{Capsicum chinenses} fruit extract has therapeutic potential against neuroblastoma. To carry out this study, capsaicin and dihydrocapsaicin extract were made from \textit{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 \textit{Capsicum chinenses} reduced neuroblastoma cell viability in a concentration-dependent manner with an IC\textsubscript{50} of 69.75 µg/mL. Our \textit{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 vanilliod receptor TRPV1 using molecular docking. In conclusion, these results illustrated that \textit{Capsicum chinenses} extract containing capsaicin and dihydrocapsaicin is effective in reducing viability of neuroblastoma cells \textit{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\textsuperscript{4}). These tumors are diagnosed in infants under the age of five (Woods \textit{et al.}, 2002\textsuperscript{2}). Over 700 new cases of neuroblastoma are reported yearly in the United States alone (Loachim and Mederios, 2009\textsuperscript{9}). 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\textsuperscript{4}). In spite of clinical and technical advances, neuroblastoma is still a huge challenge to medicine and research laboratories alike (Schwab \textit{et al.}, 2003\textsuperscript{5}). Existing treatments for neuroblastoma primarily consist of various chemotherapeutic regimens (Barrett and Rochelle, 2016\textsuperscript{6}; Grünwald and Ezziddin, 2010\textsuperscript{7}). For instance, cisplatin, doxorubicin, etoposide, and cyclophosphamide are used for treating neuroblastoma (Conter \textit{et al.}, 2014\textsuperscript{8}; Pearson \textit{et al.}, 2008\textsuperscript{9}; McGregor \textit{et al.}, 2005\textsuperscript{10}; Mishima \textit{et al.}, 2004\textsuperscript{11}; Keshelava \textit{et al.}, 1998\textsuperscript{12}). These drugs are highly toxic...
and have health and developmental effects on pediatric patients (Ries and Klastersky, 1986; Punt, 1997; Tacar et al., 2013; Aldossary, 2019). For instance, cyclophosphamide has been associated with the development of acute myeloid leukemia, bladder cancer, hemorrhagic cystitis, and permanent infertility (Kasper et al., 2005). 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). 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; Khan et al., 2008; Khan et al., 2012; Agbarya et al., 2014; Khan et al., 2018). One of such substance is an ingredient found in pepper, namely capsaicin (Bley et al., 2012; Clark and Seong-Ho, 2016). Capsaicin is the common name for the bioactive ingredient, 8-methyl-n-vanillyl-6 nonemide (a vanilliloid) (Porseva et al., 2014). It is derived from plants of the genus Capsicum (Othman et al., 2011; Friedman et al., 2019). The transient receptor potential vanilloid-1 (TRPV1) is the known capsaicin receptor that is highly expressed in sensory neurons (Koo et al., 2007). Capsaicin, heat, and protons activates this receptor to induce the influx of Ca2+ and Na+ ions (Porseva et al., 2014). Capsaicin presents a burning sensation and desensitizes sensory neurons after prolonged administration (Dray, 1992). Oh and Lim demonstrated that capsaicin or dihydrocapsaicin caused induction of p53, p21, and Go/Gi arrest. They also showed that dihydrocapsaicin induced autophagy, dilation of the ER and mitochondria (Oh and Lim, 2009). Qutenza (a high-dose capsaicin patch) is in the management of neuropathic pain from postherpetic neuralgia and HIV-associated neuropathy (Katz et al., 2015). The metabolism of capsaicin occurs in the liver and in the gut lumen (O’Neill et al., 2012). Studies by Reilly et al., 2013, using human hepatic microsomes and 59 fragments demonstrated that capsaicin is metabolized into 16-hydroxy capsaicin, 17-hydroxy capsain, and 16,17-hydroxycapsaicin, and vanillin and that cytochrome P450 is implicated in the hepatic capsaicin metabolism (Reilly et al., 2013).

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; Diaz-Laviada and Rodriguez-Henche, 2014). 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 (Diaz-Laviada, 2010; Brown et al., 2010; Sánchez et al., 2007). 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 polyethylene 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 ºC 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 mortar 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 ºC 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 ºC (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-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% CO2. 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 trypsin 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: 6ieq), 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). 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 AutoDock 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 dihydrocapsaicin 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.

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.
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 however in cells that were administered with greater than 100 µg/mL of the C. chinenses extract (Figure 6).

Figure 6. Microphotographs showing the morphology of cells post administration (PA) of extract or DMSO control for 3 hours.
Figure 7. SY5Y cells viability post treatment to capsaicin extract from *C. chinenses*. Data reported at Mean + standard deviation. *p value < 0.005.

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

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.
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 IC50s for standard capsaicin and dihydrocapsaicin were 69.31 µg/mL and 68.46 µg/mL, respectively. The IC50s for both standard treatments were significantly lower than the resulting IC50 for the C. chinenses extract (83.56 µg/mL, p<0.05).

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

Table 1. IC50s of standard capsaicin, standard DHC and extract treatments.

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). 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). 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).

### Table 2. Receptors and their interacting residues to capsaicin

| Receptor | ∆G 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 |
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 Ca²⁺-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., 2013⁷).

Capsaicin inhibits the degeneration of dopamine neurons by blocking glial activation and oxidative stress (Chung et al., 2017⁴⁸). The transient receptor potential potential receptor vanilloid type 1 (TRPV1), is a cation channel that is highly expressed in sensory neurons (Gunthorpe et al., 2018⁴⁹) and in the brain and plays a crucial role in the central nervous system (Kauer et al., 2009⁵⁰; Starowicz et al., 2008⁵¹). TRPV1 activation is showed to regulates

|       |         |                        |                        |
|-------|---------|------------------------|------------------------|
|       |         | Hydrophobic: Pi-Sigma  |                        |
| ILE98 | 3.52    |                        |                        |
| PHE95 | 3.78    |                        |                        |
| TRP80 | 3.83    |                        |                        |
| TRP80 | 3.79    |                        |                        |
| ILE98 | 4.08    | Hydrophobic: Alkyl     |                        |
| TRP80 | 4.26    |                        |                        |
| TRP90 | 5.25    |                        |                        |
| PHE92 | 4.99    |                        |                        |
| TRP99 | 5.26    |                        |                        |
| PHE156| 4.01    | Hydrophobic: Pi-Alkyl  |                        |
|       |         |                        |                        |
| DRD3  | -6.0    |                        |                        |
| THR369| 2.23    | Hydrogen Bond: Conventional Hydrogen Bond |                        |
| TYR373| 2.14    |                        |                        |
| GLU90 | 2.87    |                        |                        |
| SER366| 3.36    |                        |                        |
| VAL86 | 4.00    | Hydrophobic: Pi-Sigma  |                        |
| VAL86 | 3.94    | Hydrophobic: Pi-Sigma  |                        |
| LEU89 | 5.47    | Hydrophobic: Alkyl     |                        |
| LEU89 | 5.09    |                        |                        |
| PHE345| 4.73    |                        |                        |
|       |         |                        |                        |
| DRD4  | -8.3    |                        |                        |
| SER196| 2.54    | Hydrogen Bond: Conventional Hydrogen Bond |                        |
| ASP115| 2.44    |                        |                        |
| VAL193| 3.76    |                        |                        |
| VAL116| 3.55    | Hydrophobic: Pi-Sigma  |                        |
| PHE410| 5.32    |                        |                        |
| VAL87 | 5.23    | Hydrophobic: Alkyl     |                        |
| LEU111| 5.21    |                        |                        |
| VAL193| 4.70    | Hydrophobic: Alkyl     |                        |
| PHE91 | 5.18    |                        |                        |
| PHE411| 5.05    |                        |                        |
| CYS119| 5.08    |                        |                        |
neuroinflammation and contributes to mesencephalic dopaminergic neuronal survival by blocking oxidative stress (Park et al., 2012). 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). 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). TRPV2 has been demonstrated experimentally to respond to high-threshold noxious heat (Julius and Basbaum, 2001). 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). Capsaicin binds to prohibitin 2 and translocate it from the mitochondria to the nucleus (Kuramori et al., 2009). 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). Downregulation of prohibitin 2 shows a reduced parkin-mediated mitophagy and suppressed proliferation and migration of lung cancer cells (Zhang et al., 2020). 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.
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). 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 has been shown to be a potential anti-cancer agent in both in vitro and in vivo models (Min et al., 200466) by preventing cancer cells proliferation, metastasis (Xue et al., 201867). It has been revealed that capsaicin blocks VEGF-induced angiogenesis (Min et al., 200466), down-regulates p38 mitogen activated protein kinases (MAPK) and focal adhesion kinase (FAK) activation (Surh, 200268; Bhutani et al., 201169). It also induces the degradation of hypoxia inducible factor 1a (HIF1a), which is key transcription factor for cancer progression (Ristoiu et al., 201170; Chakraborty et al., 201170).

Plant-derived natural products have been an important subject to early medicine due to their repository benefits as therapeutic agents (Ncube et al., 200871). Various phytochemicals have been isolated and characterized in higher plant extracts, providing effective and safe treatment of diseases, disorders, and infections (Raskin et al., 200272; Nair et al., 200573; Ramya et al., 200874; Subedi et al., 202075). 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., 201076). 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., 201377). 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 (Diaz-Laviada, 201078). 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 Bilde, 198579; Singh et al., 200180). 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., 200681). 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., 200982). 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 substantial 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.

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References

1. Mora, J., & Gerald, W. L. (2004). Origin of neuroblastic tumors: clues for future therapeutics. *Expert Review of Molecular Diagnostics, 4*(3), 293–302.
2. Woods, W.G., Gao, R.N., Shuster, J.J., Robison, L.L., Bernstein, M., Weitzman, S., Bunin, G., Levy, I., Brossard, J., Dougherty, G. and Tuchman, M., 2002. Screening of infants and mortality due to neuroblastoma. New England Journal of Medicine, 346(14), pp.1041-1046.
3. Ioachim, H. L., & Medeiros, L. J. (2009). Ioachim’s lymph node pathology (H. L. Ioachim & L. J. Medeiros, Eds.).
4. Stiller, C.A. and Parkin, D.M., 1992. International variations in the incidence of neuroblastoma. International journal of cancer, 52(4), pp.538-543.
5. Schwab, M., Westermann, F., Hero, B. and Berthold, F., 2003. Neuroblastoma: biology and molecular and chromosomal pathology. *The lancet oncology, 4*(8), pp.472-480.
6. Barrett, David M., and Rochelle Bagatell. "The beginning of the end of package deal therapy for patients with high-risk neuroblastoma?." (2016): 2437-2439.
7. Grünwald, F. and Ezziddin, S., 2010, March. 131I-metaiodobenzylguanidine therapy of neuroblastoma and other neuroendocrine tumors. In *Seminars in nuclear medicine* (Vol. 40, No. 2, pp. 153-163). WB Saunders.
8. Conter, H.J., Gopalakrishnan, V., Ravi, V., Ater, J.L., Patel, S. and Araujo, D.M., 2014. Adult versus pediatric neuroblastoma: the MD Anderson cancer center experience. *Sarcoma, 2014*.
9. Pearson, A.D., Pinkerton, C.R., Lewis, I.J., Imeson, J., Ellershaw, C., Machin, D., European Neuroblastoma Study Group and Children’s Cancer and Leukaemia Group, 2008. High-dose rapid and standard induction chemotherapy for patients aged over 1 year with stage 4 neuroblastoma: a randomised trial. *The lancet oncology, 9*(3), pp.247-256.
10. McGregor, L.M., Rao, B.N., Davidoff, A.M., Billups, C.A., Hongeng, S., Santana, V.M., Hill, D.A., Fuller, C. and Furman, W.L., 2005. The impact of early resection of primary neuroblastoma on the survival of children older than 1 year of age with stage 4 disease: the St. Jude Children's Research Hospital Experience. *Cancer: Interdisciplinary International Journal of the American Cancer Society, 104*(12), pp.2837-2846.
11. Mishima, Y., Nagasaki, E., Terui, Y., Irie, T., Takahashi, S., Ito, Y., Oguchi, M., Kawabata, K., Kamata, S. and Hatake, K., 2004. Combination chemotherapy (cyclophosphamide, doxorubicin, and vincristine with continuous-infusion cisplatin and etoposide) and radiotherapy with stem cell support can be beneficial for adolescents and adults with esthesioneuroblastoma. *Cancer: Interdisciplinary International Journal of the American Cancer Society, 101*(6), pp.1437-1444.
12. Keshelava, N., Seeger, R.C., Groshen, S. and Reynolds, C.P., 1998. Drug resistance patterns of human neuroblastoma cell lines derived from patients at different phases of therapy. *Cancer research*, 58(23), pp.5396-5405.

13. Ries, F. and Klastersky, J., 1986. Nephrotoxicity induced by cancer chemotherapy with special emphasis on cisplatin toxicity. *American journal of kidney diseases*, 8(5), pp.368-379.

14. Punt, C. J. A., Voest, E. E., Tueni, E., Van, O. A. T., Backx, A., de Mulder, P. H. M., Hecquet, B., Lucas, C., Gerard, B., & Bleiberg, H. (1997). PhaseIb study of doxorubicin in combination with the multidrug resistance reversing agent S9788 in advanced colorectal and renal cell cancer. *British Journal of Cancer*, 76(10), 1376–1381.

15. Tacar, O., Sramornsak, P. and Dass, C.R., 2013. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. *Journal of pharmacy and pharmacology*, 65(2), pp.157-170.

16. Aldossary, S.A., 2019. Review on pharmacology of cisplatin: clinical use, toxicity and mechanism of resistance of cisplatin. *Biomedical and Pharmacology Journal*, 12(1), pp.7–15.

17. Kasper, D. L., Fauci, A. S., Hauser, S. L., Longo, D. L., Jameson, J. L., & Loscalzo, J. (n.d.). *Harrison’s Principles of Internal Medicine: Vol. Vol. 1 & Vol. 2* (20th ed.). McGraw Hill Professional.

18. Stuart, N. S. A., Woodroffe, C. M., Grundy, R., & Cullen, M. H. (n.d.). Long-term toxicity of chemotherapy for testicular cancer—the cost of cure. *British Journal of Cancer*, 61(3), 479–484.

19. Reddy, L.A.I.N.I., Odhav, B. and Bhoola, K.D., 2003. Natural products for cancer prevention: a global perspective. *Pharmacology & therapeutics*, 99(1), pp.1-13.

20. Khan, N., Afaq, F. and Mukhtar, H., 2008. Cancer chemoprevention through dietary antioxidants: progress and promise. *Antioxidants & redox signaling*, 10(3), pp.475-510.

21. Khan, S.I., Aumsuwan, P., Khan, I.A., Walker, L.A. and Dasmahapatra, A.K., 2012. Epigenetic events associated with breast cancer and their prevention by dietary components targeting the epigenome. *Chemical research in toxicology*, 25(1), pp.61-73.

22. Agbarya, A., Ruimi, N., Epelbaum, R., Ben-Arye, E. and Mahajna, J., 2014. Natural products as potential cancer therapy enhancers: A preclinical update. *SAGE open medicine*, 2, p.2050312114546924.

23. Khan, M.I., Rath, S., Adhami, V.M. and Mukhtar, H., 2018. Targeting epigenome with dietary nutrients in cancer: Current advances and future challenges. *Pharmacological research*, 129, pp.375-387.

24. Bley, K., Boorman, G., Mohammad, B., McKenzie, D. and Babbar, S., 2012. A comprehensive review of the carcinogenic and anticarcinogenic potential of capsaicin. *Toxicologic pathology*, 40(6), pp.847-873.

25. Clark, Ruth, and Seong-Ho Lee. "Anticancer properties of capsaicin against human cancer." *Anticancer research* 36, no. 3 (2016): 837-843.

26. Porseva, V.V., Shilkin, V.V. and Masliukov, P.M., 2014. Capsaicin treatment in studying of peripheral pain processing. *FOOD SOURCES, MEDICAL USES AND HEALTH IMPLICATIONS*, p.99.

27. Othman, Z. A. A., Ahmed, Y. B. H., Habila, M. A., & Ghafar, A. A. (2011). Determination of Capsaicin and Dihydrocapsaicin in Capsicum Fruit Samples using High Performance Liquid Chromatography. *Molecules*, 16, 8919–8929.

28. Friedman, J.R., Richbart, S.D., Merritt, J.C., Brown, K.C., Denning, K.L., Tirona, M.T., Valentovic, M.A., Miles, S.L. and Dasgupta, P., 2019. Capsaicinoids: multiple effects on angiogenesis, invasion and metastasis in human cancers. *Biomedicine & Pharmacotherapy*, 118, p.109317.

29. Koo, J.Y., Jang, Y., Cho, H., Lee, C.H., Jang, K.H., Chang, Y.H., Shin, J. and Oh, U., 2007. Hydroxy-α-sanshool activates TRPV1 and TRPA1 in sensory neurons. *European Journal of Neuroscience*, 26(5), pp.1139-1147.

30. Porseva, V.V., Shilkin, V.V. and Masliukov, P.M., 2014. Capsaicin treatment in studying of peripheral pain processing. *FOOD SOURCES, MEDICAL USES AND HEALTH IMPLICATIONS*, p.99.

31. Dray, A., 1992. Mechanism of action of capsaicin-like molecules on sensory neurons. *Life sciences*, 51(23), pp.1759-1765.
32 Oh, S.H. and Lim, S.C., 2009. Endoplasmic reticulum stress-mediated autophagy/apoptosis induced by capsaicin (8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin is regulated by the extent of c-Jun NH2-terminal kinase/extracellular signal-regulated kinase activation in WI38 lung epithelial fibroblast cells. *Journal of Pharmacology and Experimental Therapeutics*, 329(1), pp.112-122.

33 Katz, N.P., Mou, J., Paillard, F.C., Turnbull, B., Trudeau, J. and Stoker, M., 2015. Predictors of response in patients with postherpetic neuralgia and HIV-associated neuropathy treated with the 8% capsaicin patch (Qutenza). *The Clinical journal of pain*, 31(10), pp.859-866.

34 O’Neill, J., Brock, C., Olesen, A.E., Andresen, T., Nilsson, M. and Dickenson, A.H., 2012. Unravelling the mystery of capsaicin: a tool to understand and treat pain. *Pharmacological reviews*, 64(4), pp.939-971.

35 Reilly, C.A., Henion, F., Bugni, T.S., Ethirajan, M., Stockmann, C., Pramanik, K.C., Srivastava, S.K. and Yost, G.S., 2013. Reactive intermediates produced from the metabolism of the vanilloid ring of capsaicinoids by p450 enzymes. *Chemical research in toxicology*, 26(1), pp.55-66.

36 Oh, S.H. and Lim, S.C., 2009. Endoplasmic reticulum stress-mediated autophagy/apoptosis induced by capsaicin (8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin is regulated by the extent of c-Jun NH2-terminal kinase/extracellular signal-regulated kinase activation in WI38 lung epithelial fibroblast cells. *Journal of Pharmacology and Experimental Therapeutics*, 329(1), pp.112-122.

37 Díaz-Laviada, I. and Rodríguez-Henche, N., 2014. The potential antitumor effects of capsaicin. *Capsaicin as a Therapeutic Molecule*, pp.181-208.

38 Díaz-Laviada I. (2010). Effect of capsaicin on prostate cancer cells. *Future oncology (London, England)*, 6(10), 1545–1550. https://doi.org/10.2217/fon.10.117

39 Brown, K. C., Witte TR, Hardman WE, Luo H, Chen YC, Carpenter AB, Lau JK, & Dasgupta P. (2010). Capsaicin displays anti-proliferative activity against human small cell lung cancer in cell culture and nude mice models via the E2F pathway. *PLoS One*, 5(4).

40 Sánchez A. M., Malagarie-Cazenave, S., Olea, N., Vara, D., Chilocheches, A., & Díaz-Laviada, I. (2007). Apoptosis induced by capsaicin in prostate PC-3 cells involves ceramide accumulation, neutral sphingomyelinase, and JNK activation. *Apoptosis*, 12(11).

41 Welcome to the PyRx Website. 2021. Pyrx.sourceforge.io, https://pyrx.sourceforge.io/home.

42 Spike protein recognizer receptor ACE2 targeted identification of potential natural antiviral drug candidates against SARS-CoV-2 Pokhrel S, Bouback TA, Samad A, Nur SM, Alam R, Abdullah-Al-Mamun M, Nain Z, Imon RR, Talukder MEK, Tareq MMI, Hossen MS, Karpinski TM, Ahammad F, Qadri I, Rahman MS. Int J Biol Macromol. 2021 Sep 27;S0141-8130(21)02075-4.

43 Dallakyan S., Olson A.J. *Chemical Biology*. Humana Press; New York, NY: 2015. Small-molecule library screening by docking with PyRx; pp. 243–250.

44 Wu, D., Duan, R., Tang, L., Zhou, D., Zeng, Z., Wu, W., Hu, J. and Sun, Q., 2021. In-vitro binding analysis and inhibitory effect of capsaicin on lipase. *LWT*, p.112674.

45 Wu, D., Duan, R., Tang, L., Zhou, D., Zeng, Z., Wu, W., Hu, J. and Sun, Q., 2021. In-vitro binding analysis and inhibitory effect of capsaicin on lipase. *LWT*, p.112674.

46 Wu, D., Duan, R., Tang, L., Zhou, D., Zeng, Z., Wu, W., Hu, J. and Sun, Q., 2021. In-vitro binding analysis and inhibitory effect of capsaicin on lipase. *LWT*, p.112674.

47 Rohm, B., Holik, A. K., Somoza, M. M., Pignitter, M., Zaunschirm, M., Ley, J. P., Krammer, G. E., & Somoza, V. (2013). Nonivamide, a capsaicin analog, increases dopamine and serotonin release in SH-SY5Y cells via a TRPV1-independent pathway. *Molecular nutrition & food research*, 57(11), 2008–2018. https://doi.org/10.1002/mnfr.201200846
sient receptor potential vanilloid subtype 1 contributes to mesencephalic on reactions: forces contributing to stability.

Gunthorpe, M. J., & Szallasi, A. (2008). Peripheral TRPV1 receptors as targets for drug development: new molecules and mechanisms. *Current pharmaceutical design, 14*(1), 32–41. https://doi.org/10.2174/13816120878330754

Kauer, J. A., & Gibson, H. E. (2009). Hot flash: TRPV channels in the brain. *Trends in neurosciences, 32*(4), 215–224. https://doi.org/10.1016/j.tins.2008.12.006

Starowicz, K., Cristiano, L., & Di Marzo, V. (2008). TRPV1 receptors in the central nervous system: potential for previously unforeseen therapeutic applications. *Current pharmaceutical design, 14*(1), 42–54. https://doi.org/10.2174/13816120878330790

Park, E. S., Kim, S. R., & Jin, B. K. (2012). Transient receptor potential vanilloid subtype 1 contributes to mesencephalic dopaminergic neuronal survival by inhibiting microglia-originated oxidative stress. *Brain research bulletin, 89*(3–4), 92–96. https://doi.org/10.1016/j.brainresbull.2012.07.001

Kauer, J. A., & Gibson, H. E. (2009). Hot flash: TRPV channels in the brain. *Trends in neurosciences, 32*(4), 215–224. https://doi.org/10.1016/j.tins.2008.12.006

Morgese, M. G., Cassano, T., Cuomo, V., & Giuffrida, A. (2007). Anti-dyskinetic effects of cannabinoids in a rat model of Parkinson's disease: role of CB(1) and TRPV1 receptors. *Experimental neurology, 208*(1), 110–119. https://doi.org/10.1016/j.expneurol.2007.07.021

Tamura, S., Morikawa, Y. and Senba, E., 2005. TRPV2, a capsaicin receptor homologue, is expressed predominantly in the neurotrophin-3-dependent subpopulation of primary sensory neurons. *Neuroscience, 130*(1), pp.223-228.

Julius, D. and Basbaum, A.I., 2001. Molecular mechanisms of nociception. *Nature, 413*(6852), pp.203-210.

Wei, Y., Chiang, W.C., Sumpter Jr, R., Mishra, P. and Levine, B., 2017. Prohibitin 2 is an inner mitochondrial membrane mitophagy receptor. *Cell, 168*(1-2), pp.224-238.

Kuramori, C., Azuma, M., Kume, K., Kaneko, Y., Inoue, A., Yamaguchi, Y., Kabe, Y., Hosoya, T., Kizaki, M., Suematsu, M. and Handa, H., 2009. Capsaicin binds to prohibitin 2 and displaces it from the mitochondria to the nucleus. *Biochemical and biophysical research communications, 379*(2), pp.519-525.

Zhang, H., Yin, C., Liu, X., Bai, X., Wang, L., Xu, H., Ju, J. and Zhang, L., 2020. Prohibitin 2/PHB2 in parkin-mediated mitophagy: a potential therapeutic target for non-small cell lung carcinoma. *Medical science monitor: international medical journal of experimental and clinical research, 26*, pp.e923227-1.

Zhang, H., Yin, C., Liu, X., Bai, X., Wang, L., Xu, H., Ju, J. and Zhang, L., 2020. Prohibitin 2/PHB2 in parkin-mediated mitophagy: a potential therapeutic target for non-small cell lung carcinoma. *Medical science monitor: international medical journal of experimental and clinical research, 26*, pp.e923227-1.

Ross, P.D. and Subramanian, S., 1981. Thermodynamics of protein association reactions: forces contributing to stability. *Biochemistry, 20*(11), pp.3096-3102.

Min, J.K., Han, K.Y., Kim, E.C., Kim, Y.M., Lee, S.W., Kim, O.H., Kim, K.W., Gho, Y.S. and Kwon, Y.G., 2004. Capsaicin inhibits in vitro and in vivo angiogenesis. *Cancer research, 64*(2), pp.644-651.

Xu, S., Zhang, L., Cheng, X., Yu, H., Bao, J. and Lu, R., 2018. Capsaicin inhibits the metastasis of human papillary thyroid carcinoma BCPAP cells through the modulation of the TRPV1 channel. *Food & function, 9*(1), pp.344-354.

Min, J.K., Han, K.Y., Kim, E.C., Kim, Y.M., Lee, S.W., Kim, O.H., Kim, K.W., Gho, Y.S. and Kwon, Y.G., 2004. Capsaicin inhibits in vitro and in vivo angiogenesis. *Cancer research, 64*(2), pp.644-651.

Surh, Y.J., 2002. Anti-tumor promoting potential of selected spice ingredients with antioxidative and anti-inflammatory activities: a short review. *Food and Chemical Toxicology, 40*(8), pp.1091-1097.
66 Bhutani, M., Pathak, A.K., Nair, A.S., Kunnumakkara, A.B., Guha, S., Sethi, G. and Aggarwal, B.B., 2007. Capsaicin is a novel blocker of constitutive and interleukin-6-inducible STAT3 activation. Clinical Cancer Research, 13(10), pp.3024-3032.

67 Ristoiu, V., Shibasaki, K., Uchida, K., Zhou, Y., Ton, B.H.T., Flonta, M.L. and Tominaga, M., 2011. Hypoxia-induced sensitization of transient receptor potential vanilloid 1 involves activation of hypoxia-inducible factor-1 alpha and PKC. PAIN®, 152(4), pp.936-945.

68 Chakraborty, S., Adhikary, A., Mazumdar, M., Mukherjee, S., Bhattacharjee, P., Guha, D., Choudhuri, T., Chattopadhyay, S., Sa, G., Sen, A. and Das, T., 2014. Capsaicin-induced activation of p53-SMAR1 auto-regulatory loop down-regulates VEGF in non-small cell lung cancer to restrain angiogenesis. PLoS one, 9(6), p.e99743.

69 Ncube, N.S., Afolayan, A.J. and Okoh, A.I., 2008. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. African journal of biotechnology, 7(12).

70 Raskin, I., Ribnicky, D.M., Komarnytsky, S., Ilic, N., Poulev, A., Borisjuk, N., Brinker, A., Moreno, D.A., Ripoll, C., Yakoby, N. and O’Neal, J.M., 2002. Plants and human health in the twenty-first century. TRENDS in Biotechnology, 20(12), pp.522-531.

71 NAIR, R., KALARIYA, T. and Chanda, S., 2005. Antibacterial activity of some selected Indian medicinal flora. Turkish Journal of biology, 29(1), pp.41-47.

72 Rajasekaran, C., 2008. In vitro evaluation of antibacterial activity of phytochemical extracts from leaves of Aegle marmelos (L.) Corr.(Rutaceae). Ethnobotanical leaflets, 2008(1), p.148.

73 Subedi, L., Lee, S.E., Madiha, S., Gaire, B.P., Jin, M., Yumnam, S. and Kim, S.Y., 2020. Phytochemicals against TNFα-mediated neuroinflammatory diseases. International journal of molecular sciences, 21(3), p.764.

74 Thoennissen, N.H., O’kelly, J., Lu, D., Ivanski, G.B., La, D.T., Abbassi, S., Leiter, A., Karlan, B., Mehta, R. and Koeffler, H.P., 2010. Capsaicin causes cell-cycle arrest and apoptosis in ER-positive and-negative breast cancer cells by modulating the EGFR/HER-2 pathway. Oncogene, 29(2), pp.285-296.

75 Cho WH, Lee HJ, Choi YJ, Oh JH, Kim HS, and Cho HS. 2013. Capsaicin induces apoptosis in MG63 human osteosarcoma cells via the caspase cascade and the antioxidant enzyme system. Mol Med Rep. 8(6). 1655–1662.

76 Díaz-Laviada, I., 2010. Effect of capsaicin on prostate cancer cells. Future oncology, 6(10), pp.1545-1550.

77 Nagabhushan, M. and Bhide, S.V., 1985. Mutagenicity of chili extract and dihydrocapsaicin in the presence of Cu (II). Environmental mutagenesis, 7(6), pp.881-888.

78 Singh, S., Asad, S.F., Ahmad, A., Khan, N.U. and Hadi, S.M., 2001. Oxidative DNA damage by capsaicin and dihydrocapsaicin in the presence of Cu (II). Cancer letters, 169(2), pp.139-146.

79 Oikawa, S., Nagao, E., Sakano, K., Kawanishi, S., Oikawa, S., Nagao, E., Sakano, K. and Kawanishi, S., 2006. Mechanism of oxidative DNA damage induced by capsaicin, a principal ingredient of hot chili pepper. Free radical research, 40(9), pp.966-973.

80 Yang, Kyung, Jong Pyo, Gyu-Yeol Kim, Rina Yu, Seong Ju, Won Kim, and Byung-Sam Kim. “Capsaicin induces apoptosis by generating reactive oxygen species and disrupting mitochondrial transmembrane potential in human colon cancer cell lines.” Cellular and Molecular Biology Letters 14, no. 3 (2009): 497-510.