Callistemon viminalis (Sol. ex Gaertn.) exhibiting anti-neuroblastoma activity via molecular interaction of ascorbic acid and 5-lipoxygenase: An *in vitro* and *in silico* study

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Abstract

Neuroblastoma is the extracranial of children characterized by diverse clinical manifestations. The present study aimed to investigate the anti-neuroblastoma activity of hydromethanolic extract of Callistemon viminalis (Sol. ex Gaertn.) flower. The anti-neuroblastoma activity of hydromethanolic extract of C. viminalis flower was analysed in SH-SY5Y cell line using MTT assay. The study also evaluated drug-likeness and pharmacokinetic properties of bioactive compounds of extract using SwissADME tool and conducted in silico analysis to determine the molecular interaction between ascorbic acid and 5-LO for explaining the anti-neuroblastoma effect. Significant cytotoxicity was observed in SH-SY5Y cells in a dose and time dependent manner.

The ADMET properties of the analysed bioactive compounds of C. viminalis showed them as a suitable drug candidate with appropriate pharmacokinetic properties.

In silico results revealed that ascorbic acid binds into the catalytic domain and interacted with Ser447, Tyr470 and Arg457 of 5-LO with a binding energy of -5.11 Kcal/mol. The total phenol and ascorbate content were found to be 18.71 mg CAE/gm and 16.8 mg/100gm of extract, respectively. Hydromethanolic extract of C. viminalis flower induced cytotoxic effect in human neuroblastoma SH-SY5Y cells via interaction of its constituent ascorbic acid with the 5-LO, one of the important mediators of Neuroblastoma.

Keywords: *Callistemon viminalis*, Anti-neuroblastoma activity, Flower extract, Phytochemical, SH-SY5Y cells.

Introduction

Neuroblastoma is the extracranial solid tumor most commonly occurring in children and characterized by a diverse clinical manifestation. The neuroblastoma at the metastatic stage has often poor prognosis.[12] Every year, there are more than 650 cases diagnosed in the United States alone in which the incidence of neuroblastoma is responsible for approximately 7% of all childhood cancers.[15,34] Neuroblastoma is responsible for 15% of deaths in children due to cancer.[28] There is a role of oxidative stress in the pathogenesis of neuroblastoma as reported in an animal study which showed time dependent increase in the oxidative DNA damage in peripheral blood leukocytes upon injection of cultured human neuroblastoma cells in nude mice.[26]

The neuroblastoma cells are characterized by elevated levels of arachidonic acid which is the substrate of lipoxygenases for eicosanoid biosynthesis.[30] Also, there is a high level of eicosanoids producing enzymes which generate a variety of mediators that may promote metastasis, anti-apoptosis and angiogenesis.[31] A previous study reported that there is an elevated expression of 5-Lipoxygenase (5-LO), leukotriene C4 synthase, leukotriene A4 hydrolase and leukotriene receptors in a majority of primary neuroblastoma tumors and in several cell lines like SH-SY5Y, IMR-32, SK-N-BE(2), SK-N-FI, SK-N-SH, SK-N-DZ and SK-N-AS.

It was also found that inhibition of 5-LO by chemical compound or using receptor antagonist results in G1-cell cycle arrest and apoptosis in neuroblastoma cells.[35] So, inhibition of 5-LO and associated pathway may be used to develop a promising therapeutic strategy for the management of neuroblastoma. A recent study has reported the inhibition of 5-LOX by naturally derived bioactive products.[13]

Medicinal plants possess a treasure of bioactive compounds with potential therapeutic values and provide novel leads compounds.[11] Considerable interest is received in the natural antioxidants from researchers due to their ability to protect the organism from oxidative stress and having vital pharmacological activities.[25] *Callistemon* is one of the promising genera of medicinal plants, it belongs to the family Myrtaceae and includes about 34 species that are reported for several pharmacological properties.[2] *Callistemon viminalis (Sol. ex Gaertn.)* (*C. viminalis*) is a member species that is found in most parts of the globe and is popularly used in folk medicine to treat gastroenteritis, skin infections, diarrhea, respiratory conditions and hemorrhoids.[32]

C. viminalis possessed a rich variety of secondary metabolites including phenolic compounds like the presence of tannins, flavonoids and phenolic acid which are responsible for the health-related properties of the plant.[4,14,34] The extracts of the aerial parts, bark, leaves, fruits and flowers of *C. viminalis* are shown to possess biological activities like antioxidant, antidiabetic, hepatoprotective, cytotoxic and analgesic.[1,3,4,17,33] Despite having significant antioxidant activity, the studies exploring the role of *C. viminalis* as an anti-neuroblastoma agent and underlying molecular mechanism do not exist.

Considering the significant role of oxidative stress and lipoxygenase pathway in the pathophysiological progression of neuroblastoma and the utility of *C. viminalis* flower as a therapeutic antioxidant, this study was performed to assess the anti-neuroblastoma effect of *C. viminalis* floral extract using SH-SY5Y cell line. The study also analysed the phytochemical compounds, drug likeness, pharmacokinetics, total phenol and ascorbate content in the extract and conducted *in silico* analysis for explaining the anti-neuroblastoma effect of the extract.

Material and Methods

Collection and processing of plant materials: The plant *C. viminalis (Sol. ex Gaertn.)* was identified and flowers were collected from the garden of the Department of Biotechnology, Mohanlal Sukhadia University, Udaipur, Raj., India. Then, flowers were washed three times with distilled water to remove debris and put on filter paper for 15-20 min. to soak water. Flowers were now shade dried for two weeks followed by dicing and they were coarsely powdered using an electric blender Riigo W3 at medium speed for 10 min.

Floral extract preparation: The cold extraction method was used to prepare hydromethanolic extract of the *C. viminalis* flower. The floral powder was soaked in 80% hydrometholic solution at stirring condition for 72 hours. The supernatant was gradually aliquoted and the remaining precipitate was soaked in the fresh hydrometholic solution. The process was repeated till the extracted supernatant becomes light in color. The supernatant was sieved with Whatmann filter paper no. 1 and evaporated using a rotatory evaporator and the remaining fractions were kept under vacuum to eliminate any residual fraction of hydromethanol in the extract. The powdered extract was finally stored at -80˚C until use.

Quantitative Estimation

Total phenol content: The Folin-Ciocalteu's phenol reagent method was used to determine the total phenol content.[16] The floral extract was taken at the concentration of 100mg/mL for the analysis. The reaction volume was prepared using extract, distilled water (to make volume upto 2ml) and Folin-Ciocalteu reagent (0.4 ml) followed by incubation for 5 minutes in the dark at room temperature. Then, 7% sodium carbonate (2 ml) was added and the final volume was made 5 ml with distilled water followed by incubation in the dark at room temperature for 90 minutes. The optical density (O.D.) of the mixture in triplicate was recorded at 750 nm using a spectrophotometer (Chemiline, India). The linear regression from caffeic acid standard curve was used to determine the total phenolic content in the extract and expressed in terms of caffeic acid equivalent (mg of CAE/g of extract).

Estimation of ascorbic acid: The estimation of ascorbic acid was performed by the 2, 6-dichlorophenol indophenol (DCPIP) titration method according to a previous study with minor modification.[9] The standard ascorbic acid solution (1 mg/5ml) was prepared by dissolving 40 mg of ascorbic acid in 200mL of 3% metaphosphoric acid. Then 5 ml of the stock was pipetted out in a conical flask and titrated against 2,6-dichlorophenol indophenol dye till a light pink colour appeared in the solution which persisted for a few minutes. Similarly, the extract was diluted and titrated against the dye solution.

The ascorbic acid content present in the test samples was determined using the formula: Amount of ascorbic content (mg/100g) = 1 x V2 ml x Vs ml x 100/ V1 ml x Ws x 5 where 1 = mg of standard ascorbic acid taken for titration, V1 = Volume of dye consumed by 1 mg of standard ascorbic acid, V2 = Volume of dye consumed by 5 mL of the test sample, Vs= Total volume of the extract, 100= Ascorbic acid content/100g of the sample, Ws = Weight of sample taken for extraction and 5 = Volume of the test sample taken for titration

Cell Culture and Treatment: SH-SY5Y cells were cultured in Ham's F12 nutrient media (Thermo Fisher Scientific, USA) having 10 % (v/v) fetal bovine serum (Thermo Fisher Scientific, USA) and 1% antibiotic-antimycotic solution (CELLclone, India). The cells used in the experiment were of passage number 31–35 and authenticated by STR profiling. The cultured SH-SY5Y cells were maintained at 37 °C and 5% carbon dioxide under the humidified condition as a monolayer.

Anti-neuroblastoma activity: The anti-neuroblastoma activity was determined using 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. A plating density of 1 × 104 cells was used to seed cells in each well of 96-well plate (Corning Costar, USA) for 24 hours and then treated with floral extract at different concentrations (100, 250, 500, 750 and 1,000 µg/ml) and vehicle control for 24, 48 and 72 hours. After the incubation time, the cells were treated with 10 µl of MTT (5 mg/mL) for further 2-3 hours at 37°C and finally 100 µl of DMSO was added to each well to dissolve the precipitates. The percentage cell survival was calculated after measuring the OD at 572 nm using the formula: (O.D vehicle - OD samples/ O.D vehicle) x 100. In each experiment, triplicate wells were used for each concentration.

***In silico* analysis:** The molecular docking study was performed on Windows 10 (64-bit) operating systems with 4 GB RAM and 2.50 GHz Intel(R) Core(TM) i5-7200U processor. Autodock tools 4.2.6 and MGL tools version 1.5.7 were utilized to carry out molecular docking[24] which are made available for free by the Scripps Research Institute at https://autodock.scripps.edu/. The other software requirements have been specified along with the protocols used. The structure of stable-5-LO bound to 3-acetyl-11-keto-beta-boswellic acid (AKBA) (PDB ID: 6NCF) was chosen as target proteins for performing docking studies. The structure of the complex was downloaded from RCSB database and protein preparation was carried out using the Autodock wizard by deleting attached water molecules, bound heteroatoms/ligand, adding polar hydrogens, Kollman charges, spreading charge equally over all atoms and checking for missing atoms on residues.

The PDB files were then converted to the PDBQT format for executing the next step. Ligand structures were drawn on ChemBioDraw Ultra 14.0, minimized using MM2 forcefield in the Chem3DPro 14.0 software and saved as PDB files. The ligands were then prepared using Autodock tools, where non-polar hydrogens were merged, gasteiger charges added and torsions were set as specified one by one for each ligand. The ligand files were then saved in the PDBQT format.

For carrying out docking between prepared receptors and ligands, grid was generated by taking the center on attached ligand. The grid dimensions for PDB ID: 6NCF was 34.0835 x -25.595 x 33.435 with spacing0.375, keeping number of points as 126, 90, 114 in X, Y, Z direction respectively.

After the grid generation, docking was carried out using Lamarckian genetic algorithm with default genetic algorithm parameters and results were saved as docking log files for individual ligands. The conformations for each ligand were analysed and the best conformations were taken keeping binding energy as the criteria. The 3D and 2D interaction diagrams were created using Maestro visualizer.

Druggable attribute of *C. viminalis* bioactive compounds: The key bioactive compound of *C. viminalis* reported from previous studies was predicted for the drug-likeness and pharmacokinetic analysis on the baisis of absorption, distribution, metabolism, excretion and toxicity (ADMET) properties.[22,36] Further, the bioavailability of compounds was calculated using the physico-chemical structures properties.[6]

Results and Discussion

Phytochemical analysis

Total phenol content: The phenolic compounds are effective at neutralizing free radicals and perform antioxidant action. The total phenolic content of each of the extract was determined as caffeic acid equivalents using linear regression from the standard curve whose R^2 value came out to be 0.99. The total phenolic content in the hydromethanolic floral extract was found to be 18.71 ± 0.92 mg CAE/gm of extract (Figure 1).

Estimation of ascorbic acid: Ascorbic acid possesses significant anticancer activity and antioxidant power.[19] The ascorbic acid content (AAC) of the extract was determined by DCPIP titration method. The ascorbic acid content in the hydromethanolic extract of *C. viminalis* was found to be 16.8 ± 0.46 mg/100gm (Figure 1).

Anti-neuroblastoma activity: The anti-neuroblastoma activity of the extract was performed by MTT assay. The extract exhibited a cytotoxic effect in a time and dose-dependent manner. There was considerable cell death observed at different concentrations of extract at 24, 48 and 72 hours of incubation, however, the maximum toxicity was found at 72 hours (Figure 2). The control cells were untreated and appropriate vehicle control was used.

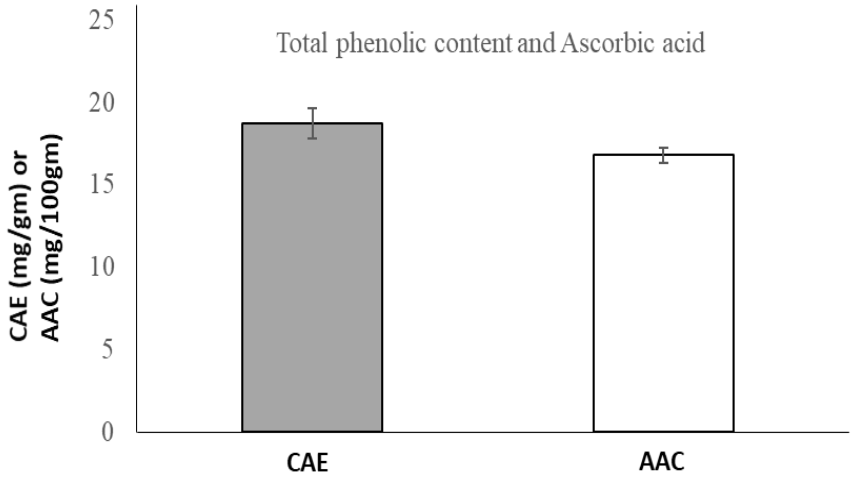

Figure 1: The total phenolic content of *C. viminalis* flower extract is represented as caffeic acid equivalents (CAE mg/g) and ascorbic acid content (AAC) as mg/ 100g. The experiments were carried out in triplicate and values are represented as mean ± SEM.

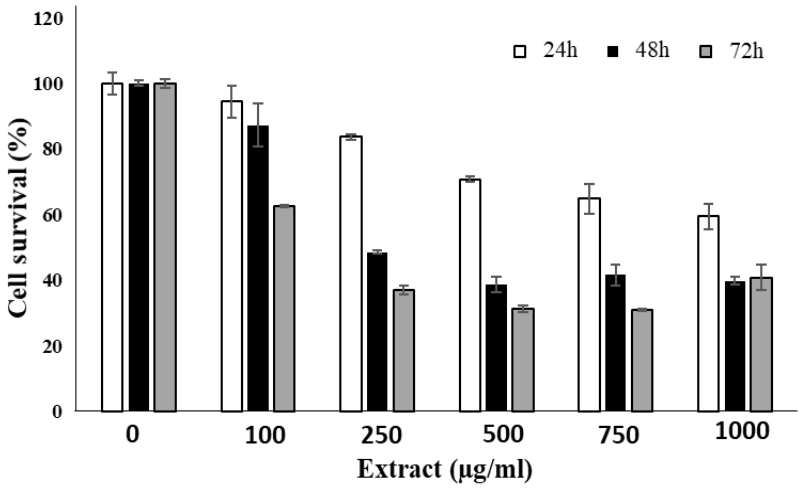

Figure 2: Cytotoxicity assay showing anti-neuroblastoma activity of *C. viminalis* floral extract at different doses for 24, 48 and 72 hours. The values are represented as mean ± SEM.

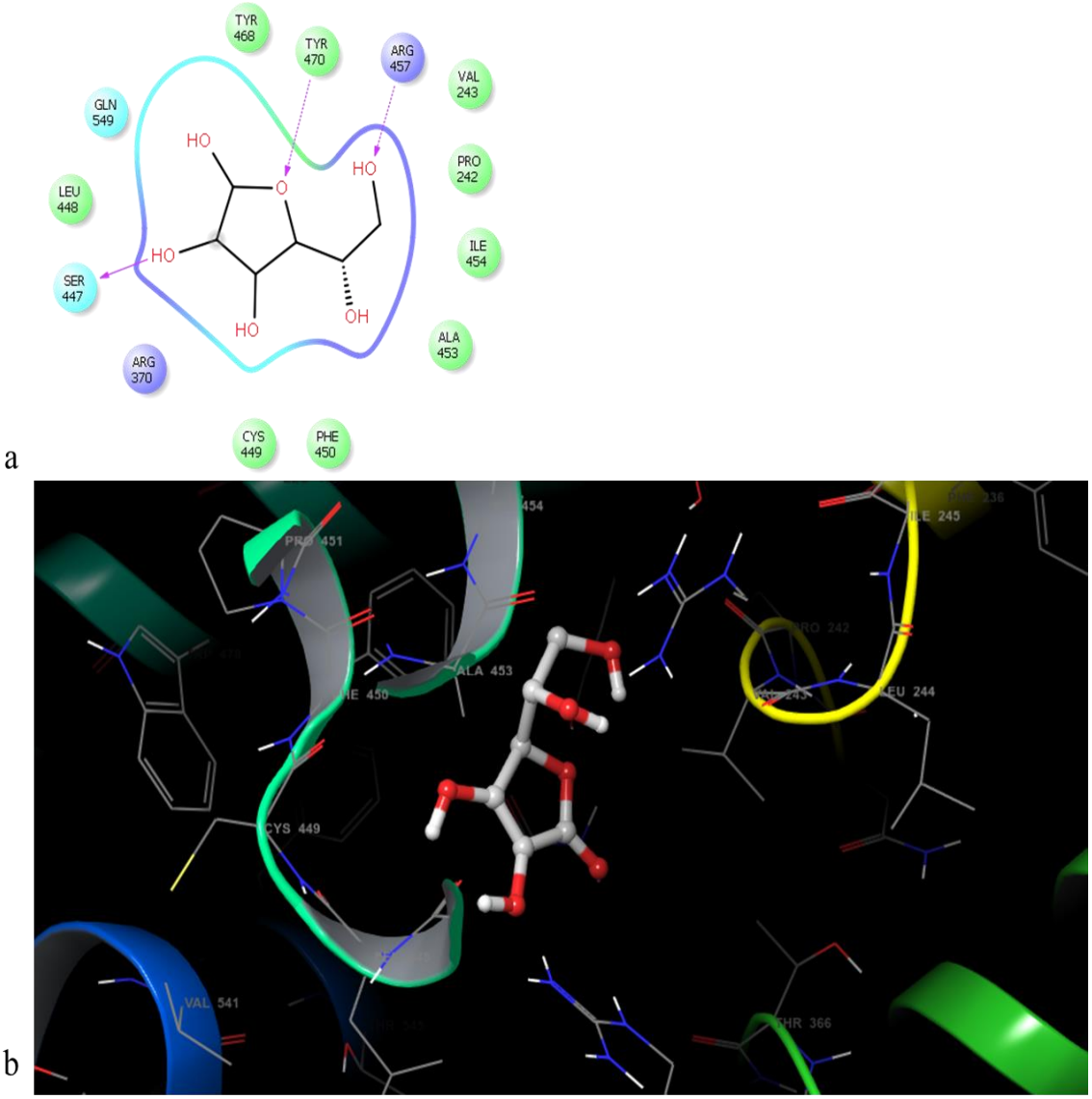

Figure 3: *In silico* analysis: a. 2D binding mode of Ascorbic acid on the structure of Stable-5-LO bound to AKBA (PDB ID: 6NCF) b. 3D binding mode of Ascorbic acid on the structure of Stable-5-LO bound to AKBA (PDB ID: 6NCF).

Molecular Docking: The docking results suggest that ascorbic acid interacted with the structure of stable-5-LO bound to AKBA (PDB ID: 6NCF) and displayed binding affinities -5.11 Kcal/mol**.** Ascorbic acid was properly positioned into the binding pocket constructed by polar Ser 447, Gln 549, hydrophobic Tyr 468, Tyr 470 Leu 448, Cys 449, Ile 454, Ala 453, Phe 450 positively charged Arg 370 and Arg 457 amino acids. Hydroxyl group of furan ring showed H-bond interaction with Ser 447, oxygen of furan ring with Tyr 470 and dihydroxyethyl of ascorbic acid showed H-bond interaction with Arg 457 (Figure 3). The details of interactions of all compounds have been presented in table 1.

Druggable attribute of *C. viminalis* bioactive compounds: Due to strong binding affinity of ascorbic acid with 5-LO and significant ascorbate content present in the extract, the druggable properties of ascorbic acid along with other previously reported compounds of *C. viminalis* like 1,8-Cineole, α-pinene and α-terpineol were determined. The druggable properties of the bioactive compounds are shown in table 2. The parameters studied are the key attributes needed in screening novel therapeutics to prevent drug failure at the early clinical phase. All the analysed compounds were found to follow the Lipinski's rule with no violation in case of 1,8-cineole and ascorbic acid and just one violation in α-pinene and α-terpineol. The bioavailability value of ascorbic acid was found to be 0.56 and all other compounds were 0.55, indicating their suitability for oral administration.

The bioavailability radars showed the feasibility of the compounds for oral bioavailability (Figure 4a). While all the compounds were permeable through Blood–Brain Barrier (BBB), none of them were P-glycoproteins (P-gp) substrate. The GI absorption was high for 1,8-cineole, α-terpineol and ascorbic acid and low for α-pinene. Further, it was predicted that none of the compounds, except α-pinene (against CYP2C9), was able to inhibit the Cytochrome P450 (CYP) enzyme isoforms (CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4). The boiled (Brain or IntestinaL EstimateD permeation model) -egg plot showed the higher probability for BBB permeability of 1,8-cineole, α-pinene and α-terpineol while higher probability of GI absorption was confirmed in case of ascorbic acid (Figure 4b). The skin permeability values (Kp) of the tested compounds ranged from -3.95 to -8.54 cm/s showing moderate to low value.

Table 1
Docking Results of Ascorbic acid on the structure of Stable-5-LO bound to AKBA (PDB ID: 6NCF).

Compound	Binding Energy (Kcal/mol)	Inhibition Constant (Ki)	Hydrogen Bonding Interactions (Conventional)	Receptor Ligand Interactions
Ascorbic acid	-5.11	179.86 µM	Tyr 470, Arg 457, Ser 447	Tyr 468, Tyr 470, Arg 457, Val243, Pro 242, Ile 454, Ala 453, Phe 450, Cys 449, Arg 370, Ser 447, Leu 448, Gln 549

Table 2
Pharmacokinetics, lipophilicity and drug-likeness parameters of key *C. viminalis* bioactive compounds.

Compound		1,8-Cineole	α-Pinene	α-Terpineol	Ascorbic acid
Lipophilicity and Drug-likeness	Molecular weight	154.25	136.23	154.25	176.12
	TPSA (Å2)	9.23	0	20.23	107.22
	Consensus Log $P_{o/w}$	2.67	3.44	2.58	-1.24
	Lipinski's Rule	Yes	Yes	Yes	Yes
	Bioavailability Score	0.55	0.55	0.55	0.56
Pharmaco-kinetics	GI absorption	High	Low	High	High
	BBB permeant	Yes	Yes	Yes	No
	P-gp substrate	No	No	No	No
	CYP1A2 inhibitor	No	No	No	No
	CYP2C19 inhibitor	No	No	No	No
	CYP2C9 inhibitor	No	Yes	No	No
	CYP2D6 inhibitor	No	No	No	No
	CYP3A4 inhibitor	No	No	No	No
	Log Kp (cm/s)	-5.3	-3.95	-4.83	-8.54
	Leadlikeness	No	No	No	No
	Synthetic accessibility	3.65	4.44	3.24	3.47

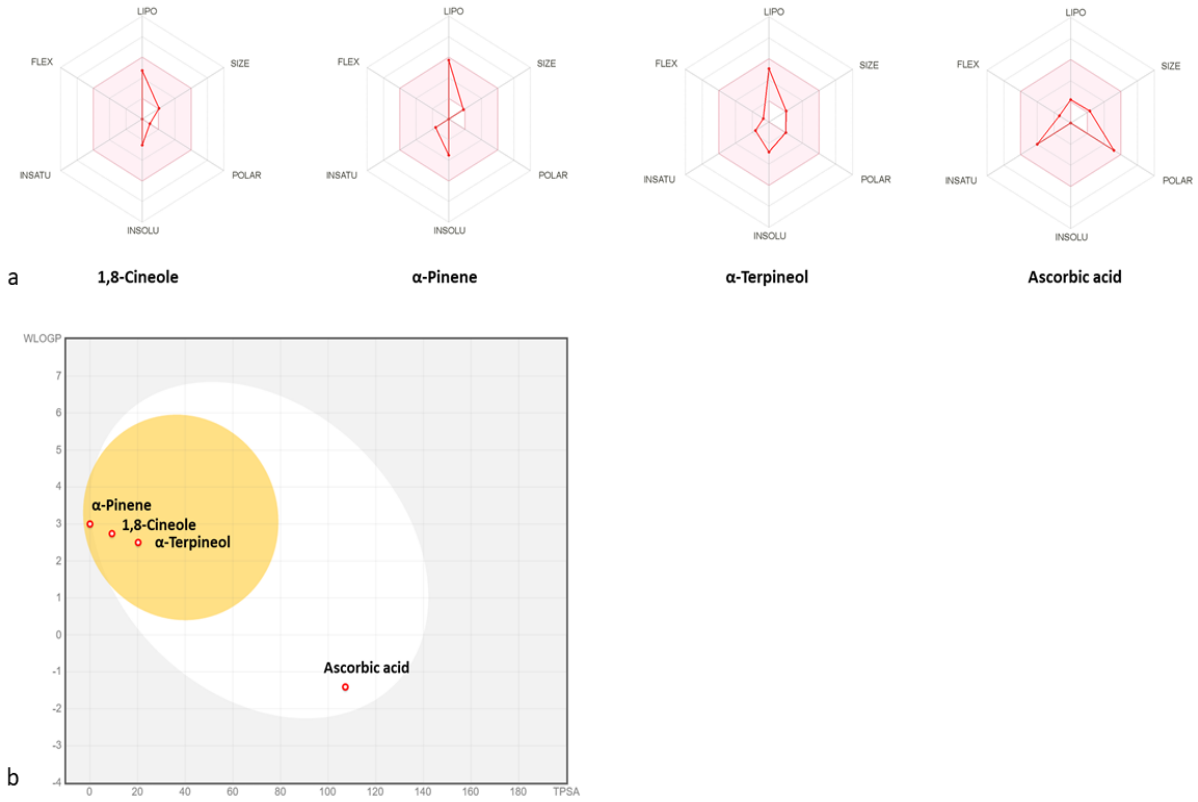

Figure 4: Druglikeness and pharmacokinetics of bioactive compounds of *C. viminalis* floral extract. (a) Bioavailability radar showing oral bioavailability attributes like Lipophilicity (LIPO), Molecular size (SIZE), Polarity (POLAR), Insolubility (INSOLU), Insaturation (INSATU), Flexibility (FLEX) where the pink colored area represent the most appropriate zone. (b) Boiled-egg plot (WLOGP Vs TPSA) showing the probability of compound entry through GI (white area) and BBB permeation as per their physico-chemical structure.

Neuroblastoma is one of the most common solid tumors and presents itself during early childhood and rarely during adolescence and young age. The precursor cells of the sympathoadrenal lineage from the neural crest give rise to neuroblastoma. The disease occurrence in Europe and North America is 10.5 per million children whose age lies between 0 and 14 years.[21] There are several treatment options available for high risk neuroblastoma including chemotherapy, myeloablative chemotherapy with autologous hematopoietic stem cell transplantation, surgery, immunotherpay and irradiation. However, the present therapeutic options have huge side effect which causes great discomfort to the patient both physically and mentally. So, there is a need for alternative therapy which may have fewer side effects, one of which is phytotherapy.

The present study, evaluated the anti-neuroblastoma potential of hydromethanolic extract of *C. viminalis* flower and also evaluated the total phenol and ascorbic acid content. Further, an *in silico* analysis to determine the molecular interaction between ascorbic acid and 5-LO was performed and druggable attribute of ascorbic acid and other constituent key compound reported in previous studies like 1,8-cineole, α-pinene and α-terpineol was described.

The secondary metabolites present in the callistemon have a diverse pharmacological role.[29] Flavonoids present in the extract act as a strong water-soluble antioxidant and free radical scavenger, which have anti-apoptotic, anti-aging, anti-carcinogen, anti-inflammatory, anti-atherosclerotic and cardioprotective activity.[27] Saponins and tannins have anti-inflammation and stringent activity, respectively.[10] Steroids have cholesterol-reducing and immunomodulatory properties. So, the medical properties of *C. viminalis* may be attributed to the presence of rich levels of total phenolic and ascorbate content. The total phenol and ascorbate content in the examined extracts was found to be 18.71 mg CAE/gm and 16.8 mg/100gm of extract respectively.

The higher levels of total phenol might be responsible for the anti-neuroblastoma activity by inducing a strong antioxidant response against the excessive free radical generation or by direct pathway modulatory effect. Moreover, the ascorbic acid is shown to interact and inhibit 5-LO activity by the *in silico* analysis which is involved in the biosynthesis of leukotrienes which has been extensively reported to have a major role in the pathogenesis of neuroblastoma.[35]

In a previous study, it has been shown that inhibition of 5-LO results in lower levels of downstream leukotrienes.[20] 5-LO is a monomeric protein having 673 amino acids and structurally possessed a C-terminal catalytic domain, a N-terminal C2-like domain, a PLAT domain within its C2-like domain, an ATP binding site and a SH3-binding domain.

The AKBA binds to 5-LO in a groove between the amino-terminal and catalytic domain and the polar groups of AKBA interact with Arg101, His130, Thr137 via H-bonds. However, polar group of ascorbic acid showed hydrogen bonding interaction with polar hydrophobhic and positively charged amino acids.

This indicates that ascorbic acid interacted in a balanced manner and binds with the catalytic domain of the protein. This explains, atleast partly, the underlying molecular mechanism behind the anti-neuroblastoma activity of *C. viminalis* floral extract by the interaction of its constituent ascorbic acid to 5-LO.

The drug-likeness and pharmacokinetics properties of bioactive compounds of the *C. viminalis* floral extract were analysed using ADMET properties. Our results suggested that all the analysed compound were in aggrement of Lipinski's rule of five and suitable for oral administration as indicated by bioavailability score.[18] The compounds does not shown any substrate specificity with P-glycoproteins that pump drugs out of cells. Apart from ascorbic acid, all the compounds were able to cross the BBB which was further confirmed by the boiled-egg plot (Figure 4a). The white and yellow region in boiled egg plot corresponds to the probability of being absorbed by the GI tract or brain permeation on the basis of physico-chemical space of molecules.[8]

The key isoforms of enzyme cytochrome P450 (CYP) include CYPs 3A4, 2D6, 2C19, 2C9 and 1A2 which play important role in regulating important drug attributes like interaction, metabolism and excretion.[7] So, if any compound inhibits any of these enzymes, then it may lead to impaired drug metabolism and adverse reaction.[5] In this study, none of the compounds were predicted to inhibit any of the CYP isoforms and also showed high GI absorption for 1,8-cineole, α-terpineol and ascorbic acid. Further, a low to moderate range of skin permeability (Kp) was found with all the compounds as more negative values corresponding to lower skin permeability.[23] The ADMET prediction has suggested that all the phytochemical compounds are suitable drug candidates with adequate pharmacokinetics.

The *in vitro* and *in silico* investigation in this study would help to explain the mechanisms of anti-neuroblastoma activity of *C. viminalis* clinically. However, the study lacks experimental confirmation using animal models treated with the extract to further validate the cell line and *in silico* findings.

Conclusion

The promising anti-neuroblastoma activity of the extract suggests that *C. viminalis* flower possesses several important therapeutic molecules which inhibit key mediators of neuroblastoma pathophysiology. This aspect must be further explored in future studies to develop a lead therapeutic molecule for the management of neuroblastoma.

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