Summary & Conclusion
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Neuroblastoma is one of the major extra-cranial tumors observed in childhood. NCI, SEER database report 7.5% of all newly diagnosed childhood cancers as Neuroblastoma with estimated 2000 new cases of Neuroblastoma emerging in India every year. The etiology involves genes like MYCN, NRAS, ALK, TRK (A,B,C,), Bcl-2, Survivin, etc. The animal model selected for the present study was Swiss Albino Mice. The method of Neuroblastoma induction involved creation of Xenograft using N2A cell lines. The compound/molecule selected was Phytochemical Piperine to evaluate its anti-cancer efficacy based on tradition and large number of alkaloids turning out to be good ant-cancer agents. The primary gene cum protein targets involved in this study were TRK-A, Bcl-2 and Survivin. Piper longum.L has been traditionally used in the preparation of Ayurveda and Siddha formulations, in specific, thirikaduku. The active principle Piperine, an alkaloid, present in Piper longum.L, has been studied as an anti-cancer agent. The litmus test to prove Piperine as a probable inhibitor of the chosen targets (TRK-A, Bcl-2, Survivin). Prior to wet lab work, the compound was evaluated using CADD (Computer Aided Drug Design), in specific Docking (in-silico analysis). Based on the in-silico results, the compound Piperine and Piper longum.L extract were carried forward for in-vitro & in-vivo studies. The in-vitro studies involved the propagation of N2A cell lines and carrying out suitable assays and molecular studies, after Piper longum.L extract and Piperine treatment. The in-vivo studies were carried out by dividing the animals into 5 groups; Group 1 (Control), Group 2 (N2A Cell induced), Group 3 (N2A + Piper longum.L Extract 50mg/kg bw), Group 4 (N2A + Piperine 5mg/kg bw), Group 5 (Drug Control). The treatment regimen was carried out for a period of 30 days from the day of tumor confirmation, using HVA as a tumor indicator.
Effect of *Piper longum*.L extract and Piperine on N2A cell lines and N2A induced Xenograft model were as follows:

- The Docking studies carried out for Piperine as an inhibitor against Trk-A, Bcl-2 and Survivin showed very high affinity of Piperine binding against Trk-A and Survivin, while free energy values for Bcl-2 proved too positive for a fruitful interaction.

- A methanolic extract of *Piper longum*.L was prepared and phytochemicals were analysed using Spectrophotometry, AAS, TLC, HPTLC and HPLC confirming the presence of Piperine.

- N2A cell lines were treated with both Piperine and *Piper longum*.L extract. The Trypan blue assay and MTT assay, both confirmed the cytotoxicity of Piperine and *Piper longum*.L extract, with pure Piperine exhibiting higher cytotoxicity.

- Imaging studies were carried out using Light Microscopy and Scanning Electron Microscopy (SEM) to analyse morphological and anti-proliferative changes against N2A cell lines using Piperine as well as *Piper longum*.L extract. Both exhibited anti-proliferation activity with Piperine showing maximal activity. SEM examination especially threw light on Piperine’s ability to induce Apoptosis based cell death extensively.

- Comet assay performed on N2A cell lines after treatment with varying concentrations of Piperine and PL extract showed tailing, a proof of apoptosis, specially long tailing with respect to Piperine treatment. Apoptosis was exhibited at 50μg concentration of PL extract whereas Piperine exhibited prominent DNA damage at 25μg.

- RT-PCR results for the primary targets, *Trk-A, Bcl-2* and *Survivin* post PL extract and Piperine treatment demonstrated a progressive decrease in gene expression in treated cells as compared to tumor control, in case of *Bcl-2* and *Survivin* but remained unchanged in case of *Trk-A*. The Immunofluorescence (IF) and Western Blot analysis...
however showed reduced expression of TRK-A, Bcl-2 and Survivin suggesting a degradation of TRK-A post translation.

- Chronic toxicity in Swiss albino mice was assessed at 50mg/kg bodyweight PL Extract over a period of 60 days. The results showed almost nil toxicity.

- ILS percentage for PL extract treated group was 30.87% and for Piperine was 37.5% as compared to tumor control. The treated groups showed a 60% and 70% gain in Bodyweight for PL Extract and Piperine respectively when compared to tumor control group. There was extensive progress in behavioral activity of PL Extract and Piperine treated groups as compared to tumor control.

- Homovanillic acid is an end product in metabolism of L-DOPA, Dopamine and epinephrine, in the condition of Neuroblastoma. The Homovanillic acid urine content of PL extract and piperine treated animal showed significant reduction in HPLC analysis, when compared to those of tumour control group. Tumor control has an area of 56.84%, PL Extract 44.78% and Piperine treated 35.03%.

- Glucose levels within the tumor tissue increased with treatment by PL extract and Piperine because of the death of cells, therefore decreasing consumption. Whereas total protein, Urea and Uric Acid increased with treatment compared to tumor control.

- As with most cases the lipid profile for total cholesterol, free fatty acid, Phospholipids and Triglycerides increased to close to normal levels after treatment against malignancy by PL extract and Piperine when compared to tumor control as tumor cells suppress lipid metabolism to drive glucose metabolism.

- With increased adrenal mass, increase in adrenaline production is observed because of hyper metabolism of catecholamines with respect to the tumor control. The Adrenaline levels drop to near normal levels after treatment with PL extract and Piperine.
Metabolic and Pathophysiological enzymes, Creatine Kinase, LDH, Glu-6-PDH and 5’Nucleotidase are hyper-regulated in the case of tumor control especially, 5’Nucleotidase plays a critical role in drug resistance.

Elevated levels membrane bound ATPases, Na+/K+ ATPase, Ca2+ ATPase and Mg2+ ATPase are observed in Tumor Control. Upon treatment by PL Extract and Piperine the levels were reverted back.

Enzymatic and non-enzymatic antioxidant levels drop in serum during malignancy as they get utilized in the tumor tissue. In the present study, enzymatic antioxidant levels, SOD & CAT increase in serum after treatment with PL extract and Piperine, while LPO decreases. GST, GPX and GSH all three related antioxidants increase after being treated by PL extract and Piperine, drastically in Piperine treated.

The histopathological analysis of adrenal tissue showed restoration of cellular organelles towards normal in case of PL Extract and Piperine Treated animal groups when compared to that of Group 1 exhibiting enlarged nucleus and infiltration of Neuroblastoma tumor.

Immunhistological analysis of Adrenal Tissue for TRK-A, Bel-2 and Survivin showed reduced expression of the target proteins in treated groups (3 & 4) as compared to Group 2. Group 1 and 5 show normal level of protein expression. Piperine exhibits better efficacy at suppressing protein expression when compared to PL Extract.

Examination of tissue samples under Scanning Electron Microscope (SEM) and for an external ultra structural change in Neuroblastoma induced animal adrenal tissue reveal abnormal tissue architecture with the protuberant and rough surface. The abnormalities in the tissue architecture reverted back to near normal in group 3 and 4. Group 1 and 5 have a well defined ultra-structure.
Transmission Electron Microscope (TEM) results show abnormal intracellular architecture and enlarged nucleus for group 2 animal adrenal tissue when compared to Group 3 and 4, which show pro-apoptotic and apoptotic stages with the condensation of nucleus and subsequent breakdown, more pronouncedly viewed in Group 4. Group 1 and 5 showed well defined normal intracellular structure.

Consistent to the results from in-vitro RT-PCR, the expression of TRK-A did not change among the 5 groups whereas Bcl-2 and Survivin show progressively reduced expression approaching normal when compared to Group 2. Piperine treated group (Group 4) showed more pronounced reduction in Bcl-2 and Survivin expression.

Western blot analysis shows reduced protein expression for all the three target proteins, TRK-A, Bcl-2 and Survivin, approaching normal in case of treated groups (3&4) as compared to group 2.

Based on the results obtained, this study hypothesizes that Piperine inhibits TRK-A expression directly, thereby suppressing the expression of Bcl-2 and Survivin. As TRK-A and TRK-B protein expressions are interdependent, inhibition of TRK-A at the translational level, may affect TRK-B transcription which subsequently may cause rapid degradation of TRK-A, though transcribed.

In conclusion, the above study concludes that the Piperine alkaloid in the *Piper longum*.L Extract is scientifically validated for its anti-cancer, anti-proliferative, genotoxic, pro-apoptotic activity in Neuroblastoma (both cell line as well as animal model) with TRK-A, Bcl-2 and Survivin as the targets. This emphasizes Piperine as a novel drug and *Pipr longum*.L as a novel drug source against Neuroblastoma.