

## Research Article



## Assessment of *In vitro* - *In vivo* Antimigratory and Anti-angiogenic Activity of *Curcuma longa* Linn. and *Tinospora cordifolia* Willd. Extracts in Cervical Cancer

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### ABSTRACT

Anti-angiogenic agents of plant origin have been identified as promising adjuncts to chemotherapy for better therapeutic effects. Such compounds may serve as oncopreventives against transformation of adjacent nontumour cells, reduce side effects and prevent emergence of drug resistance. *Curcuma longa* Linn and *Tinospora cordifolia* Willd., are also reported to have anticancer potential in many cancers at experiential, experimental and clinical levels. Aim of the present study was to evaluate curcuma and tinospora plant extracts/compounds for their anti-migratory activity on cervical cancer cells and for anti-angiogenic activity in chick embryo chorio-allantoic membrane model. Five interventional agents viz. curcumin, turmeric oil, polysaccharides from *Curcuma longa* and berberine and polysaccharides from *Tinospora cordifolia* and mixtures of the extracts were evaluated by scratch wound assay for anti-migratory activity with SiHa and C33a cell lines. Modulation of clonogenic property of the cancer cells by the agents was studied with soft agar colony formation assay. Chick embryo chorio-allantoic membrane model (CAM) was employed for assessment of antiangiogenic activity of the extracts. The results indicated that all 5 extracts could significantly reduce migration of cancer cells after 24 and 48 hrs. In addition significantly less and slow growing colonies formed, as compared to controls, in the soft agar assay. A dose dependent inhibition of new vessel formation was seen in the CAM, treated with all the agents, extracts and their mixtures. The study shows interesting potential of *Curcuma longa* and *Tinospora cordifolia*-their phytoactives, extracts and mixtures, individually and synergistically, to inhibit migration of cervical cancer cells *in vitro* and inhibit angiogenesis *in ovo*.

**Keywords:** Antiangiogenic, Antimigratory, *Curcuma longa* Linn, *Tinospora cordifolia* Willd., Cervical cancer, Phytopharmaceuticals.

### INTRODUCTION

Due to its prolonged pre-invasive state, accessibility for early detection, follow up and treatment, cervical cancer, as compared to other malignancies, provides a unique opportunity for prevention, early diagnosis and treatment. Unfortunately, notwithstanding wide spread screening programmes, HPV vaccination and treatment of early stage disease, many patients still present with cervical cancer at an advanced stage, or a recurrent and persistent lesion.<sup>1</sup> Tumor angiogenesis is a major prerequisite for rapid growth, progression, and metastasis. The degree of neoangiogenesis has been used to predict cervical cancer progression and control rate.<sup>2</sup>

In cervical cancer, vascular endothelial growth factor (VEGF) is the most important inducer of increased sprouting of new blood capillaries from the existent mature vascular network. The mechanisms for increased VEGF are: (1) HPV- 16 E-6 oncoprotein activates VEGF gene promoter independent of p53<sup>3</sup> (2) HPV-16 E7 oncoprotein is also found to be a contributory factor for angiogenesis. It is up regulated by eukaryotic translation initiation factor E (eIF4E), a factor progressively increasing with degree of cervical pathology.<sup>4</sup> (3) Up regulation of c-myc protooncogene, also increases VEGF (4) NF-κB activation leads to enhanced tumour growth by direct and indirect mechanisms. The indirect mechanism involves promotion of angiogenesis.<sup>5,6</sup> NF-κB works through proliferative and anti-apoptotic molecules.<sup>7</sup> For

the management of cervical cancer, metastatic or recurrent, search and identification of novel anti-angiogenic compounds is essential.

Anti-angiogenic agents of plant origin have been identified as promising adjuncts to chemotherapy for the better therapeutic effects. Several phytochemicals have also emerged as chemotherapeutic agents- paclitaxel (mitotic inhibitor), camptothecin (cytotoxicity effect), vinblastine (anti-microtubular agent) and combretastatin (inhibitor of tubulin polymerization).<sup>8,9</sup> But these too have the side effects due to cytotoxicity on non-cancer rapidly proliferative tissues. The quest is on for active phytochemicals in clinical study that inhibit tumour angiogenesis by inhibition of VEGF. Anti-angiogenic therapy with medicinal plant extracts may offer advantages of safety over conventional cytotoxic drugs used in cancer therapy. Such compounds may also serve as oncopreventives against transformation of other cells, reduce side effects and emergence of drug resistance.<sup>10</sup>

*Curcuma longa* Linn. (*C. longa*) and *Tinospora cordifolia* Willd. (*T. cordifolia*) are medicinal plants commonly used in Ayurved. The extracts of these plants have been reported to have anti-cancer activity *in vitro*, *in vivo* and at clinical levels.<sup>11-14</sup> These compounds (individual and mixtures) were also studied for *in vitro* anti-cancer activity on cervical cancer cell lines and had shown significant cytotoxic activity.<sup>15</sup>



The present study was undertaken to evaluate anti-angiogenic and antimigratory activity of the phytomolecules of the plants-curcumin, turmeric oil and curcuma polysaccharide from *C. longa*, and polysaccharide and berberine from *T.cordifolia*. In the present study, Chick embryo Chorio-Allantioic Membrane (CAM) model is used to evaluate anti-angiogenic activity of compounds. The CAM angiogenesis model is a useful tool for screening compounds for anti- or pro-angiogenic activity. The wound scratch method was used to assay the effects of the phytoactives on the migratory behavior of cervical cancer cells.

Cervical cancer is associated with Human Papilloma Virus (HPV) infections in >95% of cases but HPV DNA may be negative in a small percentage hence both HPV negative and HPV positive cell lines were used in the experimental studies.

## MATERIALS AND METHODS

### Cell culture

Cervical cancer cell lines C33a (HPV-ve) and SiHa (HPV +ve) were procured from NCCS, Pune. These cells were maintained in appropriate laboratory conditions. C33a was maintained in minimal essential medium, and SiHa in DMEM, supplemented with 10% (v/v) heat-inactivated fetal bovine serum, antibiotics, in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. Interventional studies were carried out with standard concentrations of compounds.

### Plant extracts

Curcumin was provided by Omni Active Pvt Ltd, Mumbai and berberine was procured from Sigma, curcuma polysaccharide was provided by Natural Remedies Pvt Ltd, Bengaluru and turmer one was provided by Nisarga Biotech Pvt Ltd., Satara. *Tinospora* polysaccharide was extracted from the stem of *Tinospora cordifolia* plant according to the method described by Sharma *et al.*<sup>16</sup> Cells were treated with Inhibitory Concentration 50 (IC<sub>50</sub>) doses of extracts in wound assay and soft agar colony formation assay. IC<sub>50</sub> of berberine, curcumin and turmeric oil in C33a cells were 30.1 µg/ml, 107 µg/ml and 117.4 µg/ml respectively. Higher concentrations of curcuma and tinospora polysaccharides were used (200 µg/ml). In SiHa cells IC<sub>50</sub> of berberine and curcumin were 25.11 µg/ml and 63.09 µg/ml respectively and turmeric oil (100 µg/ml), curcuma and tinospora polysaccharides (200 µg/ml) were used. Mixture (mixture of 25 µg/ml of each extract) was used in these experiments. Anti-angiogenic activity was assessed by CAM assay using 3 concentrations of the extracts (50, 100 and 200µg/ml) and two mixtures (mix 25 and mix 50: mixture of 25 µg/ml and 50 µg/ml of each extract respectively).

### Scratch wound assay

SiHa and C33a Cells were cultured to confluence or near confluence (>80%) in 6 well plates. On the bottom of each well a horizontal line was drawn with marker. Three

separate wounds were scratched using a sterile 200 µl pipette tip, through the cells moving perpendicular to the line drawn. The cells were gently washed with PBS and treated with the test compound in media. Pictures were taken of fields just above and just below the horizontal line using phase-contrast microscope and 10X. Plates were incubated and pictures were taken after 24, 48 and 72 hrs. The distance between the edges was measured and percent recovery was calculated using the following equation:

$$\%R = [1 - (\text{wound length at } T_n / \text{wound length at } T_0)] \times 100.$$
<sup>17</sup>

Percent recovery= (%R), wound length at 0 h= T<sub>0</sub>, wound length at n hrs = T<sub>n</sub>

### Soft agar colony formation assay

The soft agar colony formation assay was performed to analyze the clonogenic property of the cells.<sup>18</sup> Briefly, 1 × 10<sup>3</sup> cells in 1 mL of 0.35% agar with medium were layered on top of 1 mL of 1% agar with medium in a 6-well plate. Test compounds were added to the top agar at the desired concentration. MEM and DMEM (Himedia) for C33a and SiHa respectively supplemented with 10% FBS, 0.5 mM sodium pyruvate, 10 mM HEPES and 1% penicillin/streptomycin was added to the top agar the day after plating. After 21 days, the numbers of colonies were counted with TS View software. The data are means ± standard error (SE) of 3 independent wells.

### CAM assay

Fertilized eggs of white leghorn breed hens were acquired from local poultry farm. The development of the embryo was ensured using an egg candler. Eggs were carefully surface sterilized with 70% alcohol and incubated at 37°C, with 80% humidity. The eggs were inoculated with 10µl of standard/test drug and sealed with parafilm. They were kept in vertical position with the air sac upward, for incubation, to prevent inoculated drugs touching the shell membrane. An extra embryonic membrane formed on day 4 of incubation by fusion of the chorion and the allantois which is employed as *in vivo* model for angiogenesis.

On 12<sup>th</sup> day the eggs were broken gently from the sides of air sac and inner shell membrane was removed, carefully, using forceps. The CAM was dispensed out on a petri plate containing 2 ml normal saline. Stereomicroscopic images of four non-overlapping regions of CAM were taken using image analysis software. The images were then analyzed by counting the bifurcation and the average of the count was compared with that of control and standard drug using '3.1 Analysis Software'. The counting was done as follows: single intact blood vessel was considered as 1 and the bifurcation point of a single blood vessel as 2.<sup>19</sup> Adriamycin was used as a positive control.



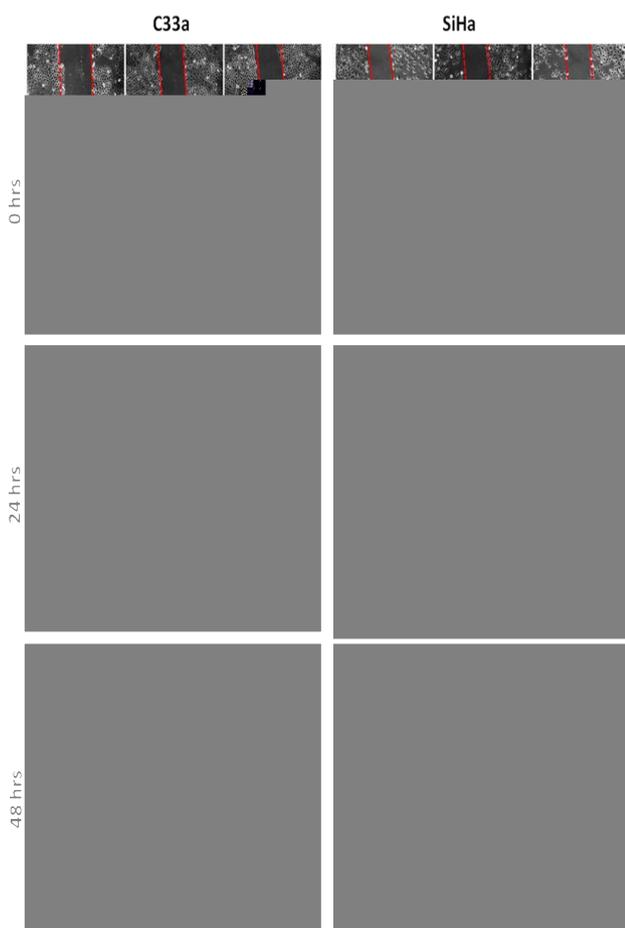
**Statistical analysis**

The data was statistically analyzed using ANOVA followed by Dunnett’s test using the GraphPad Prism software version 7.0. The results obtained with the extracts were compared with the media and solvent controls. Data are presented as means ± SEM

**RESULTS**

**Scratch wound assay**

C33a and SiHa cells were treated with the extracts for 0, 24, and 48 hours. It was observed that the migration of cells was inhibited by the treatment (Figure 1). Additionally, incubating cells with the mixture of extract suppressed cell migration more effectively than individual extracts ( $p < 0.001$ ). Quantitative determination of the gaps in terms of percent recovery showed a significant inhibitory effect after 24 hrs ( $p < 0.001$ ) in all the extracts and after 48 hours in curcumin, turmeric oil, berberine, curcuma polysaccharide ( $p < 0.001$ ), and tinospora polysaccharide ( $p < 0.01$ ) in C33a cells. SiHa cells also showed significant inhibition of migration when treated with curcumin, berberine, curcuma polysaccharide ( $p < 0.001$ ), turmeric oil ( $p < 0.01$ ) and tinospora polysaccharide ( $p < 0.05$ ).

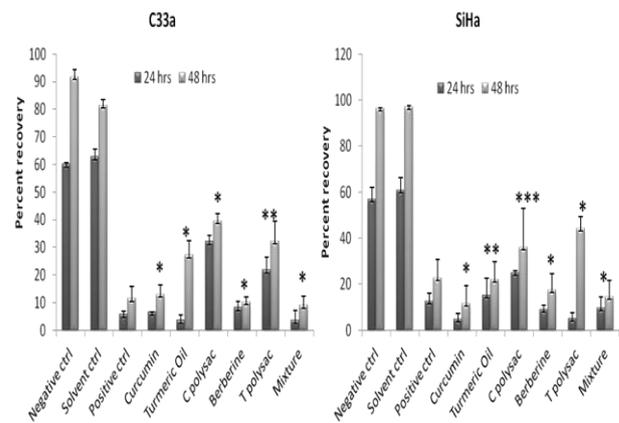


**Figure 1: A.** C33a and SiHa cells analyzed for scratch wound healing assay. Images of scratch wound at 0, 24 and 48 hrs.

**Soft agar colony formation assay**

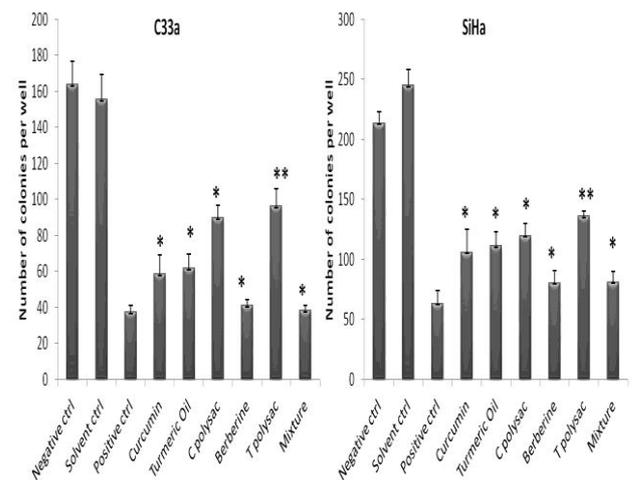
Soft agar colony formation assay was performed to evaluate the relative colony forming activities of the cells.

C33a and SiHa cells were grown on soft agar for 3 weeks.



**Figure 1: B** Percent recovery of the wound with and without treatment after 24 and 48 hrs in C33a and SiHa cells. Mixture: mixture of 25µg/ml of each extract (\* $p < 0.001$ , \*\* $p < 0.01$ , \*\*\* $p < 0.05$ )

Cells treated with all the extracts formed significantly less and slow growing colonies as compare to controls ( $0 < 0.001$ ). The assay revealed the potential of the extracts, to suppress anchorage-dependent growth in cancer cells.



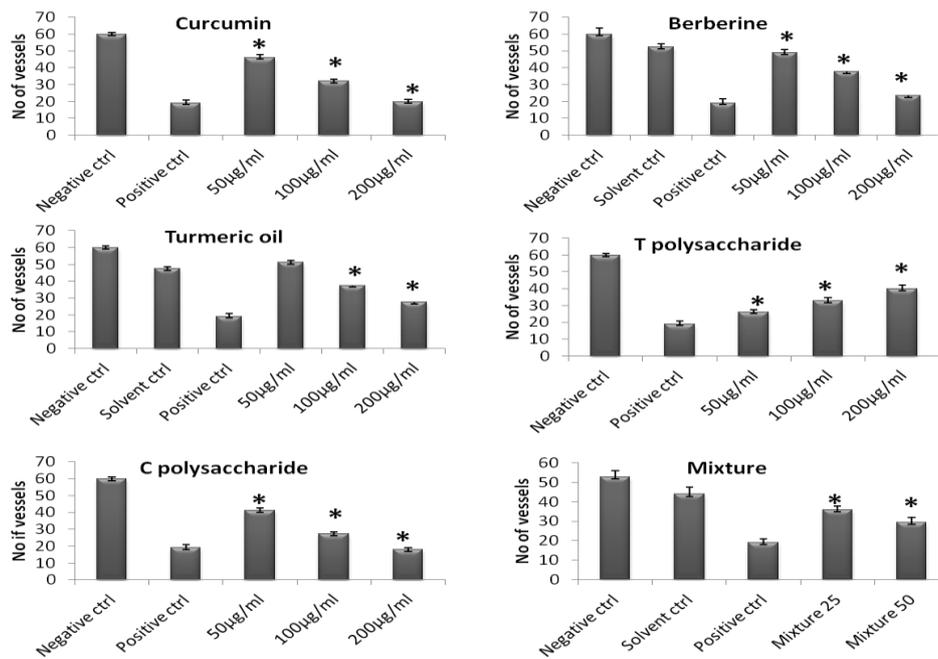
**Figure 2:** Soft agar colony formation assay: Number of colonies (means ± SEM) formed in the cells treated by the extracts. (\* $p < 0.001$ , \*\* $p < 0.01$ ), Mix 25: mixture of 25µg/ml of each extract

**CAM assay**

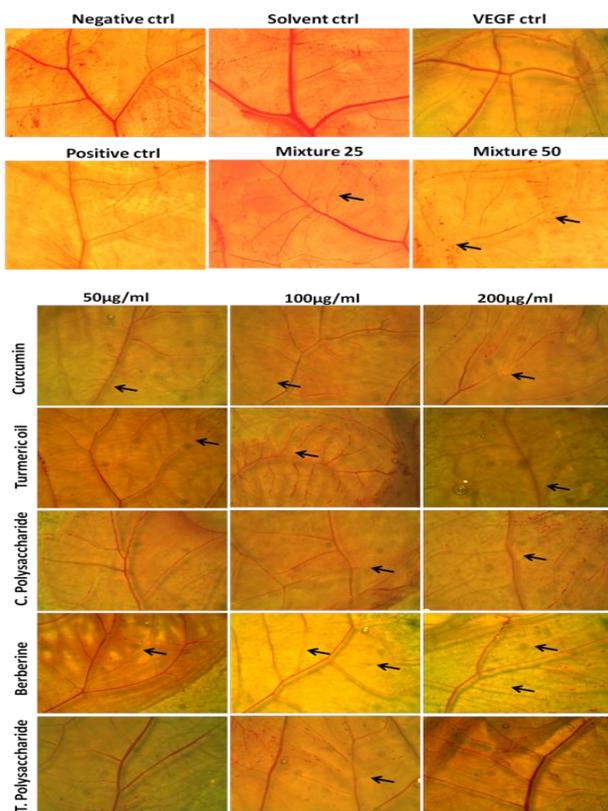
The anti-angiogenic potential of curcuma and tinospora extracts was explored using the CAM model by quantitative estimation of the blood vessels by stereomicroscopy. In untreated CAM the normal vascularisation with primary, secondary and tertiary micro blood vessels was observed. A dose dependent inhibition of vessel formation was seen in the CAM

treated with Curcumin, turmeric oil Curcuma polysaccharides and berberine. They showed distorted vascularisation as well as disturbed existing vasculature. However at high concentration, tinospora polysaccharides

demonstrated a minor increase in the number of blood vessels as compared to other extracts but, it was significantly less than normal control.



**Figure 3 A:** Antiangiogenic activity of extracts evaluated by number of total number of blood vessels (means ± SEM ) on Chick embryo chorioallantoic membrane assay (\*p<0.001) Mix 25: mixture of 25µg/ml of each extract, Mix 50: mixture of 50µg/ml of each extract



**Figure 3B:** Antiangiogenic activity of extracts using Chick embryo chorioallantoic membrane assay (Mixture 25:

mixture of 25µg/ml of each extract, Mixture 50: mixture of 50µg/ml of each extract)

**DISCUSSION**

The present study was intended to determine anti-angiogenic and anti-migratory activities of phytoactives of *Curcuma longa* and *Tinospora cordifolia* extracts-individually and in mixtures. This was studied by analyzing the most common attributes of tumor angiogenesis i.e. migration and clonogenic properties of cancer cells and blood vessel formation on CAM.

Turmeric (*Curcuma longa*) is a regular item of Indian diet as a spice, besides being used as a common Ayurvedic medicine. *Curcuma longa* extracts have demonstrated anti-cancer activity *in vitro* as well as *in vivo* at three stages: chemoprevention, angiogenesis, and tumor growth.<sup>20, 21, 22</sup> Anti-angiogenic activity of curcumin is reported in intestinal, hepato cellular, ovarian, and lung cancers.<sup>23, 24</sup> It has been shown to inhibit several proangiogenic factors (including VEGF and COX-2) in hepatocellular carcinoma. Chintana *et al* has shown that high dose of curcumin downregulated expression of VEGF, COX-2 and EGFR resulting in suppression of tumour growth and angiogenesis in CaSki implanted mice. Studies have shown that anti-angiogenic effects of curcumin in ovarian cancer are likely mediated by NF-κB via other transcription and

angiogenic growth factors.<sup>25</sup> Other signaling pathways by which curcumin affects angiogenesis include prostanoid production, Akt, tumor necrosis factor, activator protein (AP-1), cell surface adhesion molecules, mitogen-activated protein kinase, lipoxygenase, inducible nitric-oxide synthase, urinary plasminogen activator, chemokines, matrix metalloproteinase-2 (MMP2) and cyclin D1.<sup>26,27</sup>

*Tinospora* is reported to have anti-cancer activity in various cancers, which is attributed to the presence of alkaloids, diterpenoid lactones, glycosides, steroids, sesqui terpenoid, phenolics, aliphatic compounds or polysaccharides.<sup>28</sup> Berberine is a benzylisoquinoline alkaloid present in various plant species including *Tinospora cordifolia*. It has been studied as an anti-cancer agent. It exhibits direct anti-proliferative, anti-angiogenic and anti-metastatic activities in various types of cancer cell lines. It also boosts the efficacy of chemotherapy and radiotherapy drugs when used as an adjuvant.<sup>29</sup> Chu *et al* have analysed in vitro anti-angiogenic potential of berberine in cervical cancer in detail.<sup>30</sup> They found that berberine could inhibit the invasion of cervical cancer cell and reverse EMT, by E-cadherin induction, as well as by suppressing transcription of MMP-2 and u-PA and VEGF down regulation.

Polysaccharide fraction of *Tinospora cordifolia* has been shown to be effective in suppressing the metastasis caused by B16F-10 melanoma cells in mice.<sup>31, 32</sup> Later they reported that animals with induced angiogenesis when treated with *tinospora* extract the cytokines and growth factors such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , GM-CSF and VEGF were differentially regulated. Thus anti-angiogenic activity of the plant *T. cordifolia* was associated with the regulation of the levels of cytokines and growth factors in the blood of the angiogenesis-induced animal. The plant has been reported to reduce neutropenia of chemotherapy in swiss albino mice by an increase in GM-CSF and shown to increase survival time.<sup>33</sup>

Thippe swamy *et al* reported the anti-angiogenic and proapoptotic potential of *T. cordifolia* hexane fraction.<sup>34</sup> It caused growth inhibition and induction of apoptosis in a dose-dependent manner in ascites tumour-bearing animals. Further they demonstrated the molecular mechanism causing inhibition of ascites tumour growth by the purified octacosanol in CAM, rat cornea and peritoneum of Ehrlich ascites tumour bearing mice by inhibiting VEGF gene expression. Inhibition of proliferation of endothelial cells is considered as an important factor in suppression of tumour-induced neo vascularisation caused by octacosanol.<sup>35</sup> Similarly aqueous ethanolic extract of *Tinospora cordifolia* has been reported to have anti-proliferative, differentiation-inducing and anti-migratory/anti-metastatic activity in glioma cells and also affects signalling pathways engaged in its mechanism of action.<sup>36</sup>

Although the use of various chemotherapeutic compounds have been shown to have potential against cervical cancer, the drug resistance, limited efficacy and systemic toxicity demand development of new therapeutic agents. A potential benefit of phytochemicals derived from Ayurvedic medicinal plants is that they may act through multiple cell-signaling pathways and exert their effect on cancer cells without killing the normal cells.<sup>37</sup>

Although chemo preventive potential of Turmeric oil has been shown there are not many studies on the mechanisms of action of turmerone in cervical cancer.<sup>13, 14</sup>

The data from the present study suggests that all these extracts, curcumin, turmeric oil and curcuma polysaccharide from *C. longa*, and polysaccharide and berberine from *T. cordifolia* and the mixture of these extracts have anti-migratory potential targeting different signalling pathways in addition to inhibition of cell proliferation and promotion of apoptosis. Further as seen in CAM model, developing embryos, treated with the extracts formed a vascular zone and formation of new blood vessels was also reduced significantly, suggestive of potential anti-angiogenic activity. The mixtures have shown larger effect than single extracts. Recently in another study the anticancer effect of curcumin was shown to be greater after addition of turmerone and our study has confirmed this benefit of combining bioactives.<sup>38</sup>

Further expression studies and in vivo research will be useful for understanding the exact mechanism of action of these extracts and phytoactives. Additionally we can explore whether mixtures will be favorable for clinical trials and can be developed as adjuvant therapy with anti-neoplastic drugs.

In view of the availability of standardized formulations of *C. longa* and *T. cordifolia*, these can be studied as adjuvant to chemotherapy through Reverse Pharmacology path while more basic studies are being under taken.<sup>39,40</sup>

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