Synergistic Anticancer Activity of the Medicinal Plant Bioactives: Curcuma Longa Linn. and Tinospora Cordifolia Willd. in Cervical Cancer

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ABSTRACT
Anti-cancer effects of medicinal plant products are mentioned in the ancient texts of Ayurved. Currently they have been studied at experiential, experimental and clinical levels in Reverse Pharmacology mode. Curcuma longa Linn and Tinospora cordifolia Willd. the most widely used for their medicinal properties in Ayurved are also reported for having anticancer potential in many cancers. The aim of study is to explore anti-inflammatory and anti-proliferative potential and to understand the mechanism of action of these medicinal plants extracts individually or synergistically on cervical cancer cell lines. Anti-inflammatory activity of 5 extracts (curcumin, turmeric oil and polysaccharides from Curcuma longa and berberine and polysaccharides from Tinospora cordifolia) and mixture of the extracts was evaluated in vitro macrophage activation assay and modulation of TNF-α and IL-6 release. Extracts were assessed for their cytotoxicity by sulforhodamine B (SRB) assay on SiHa and C33a cervical cancer cell lines. Indole amine 2, 3-dioxygenase (IDO) assay for immune modulation and flow cytometry with propidium iodide (PI) staining for cell cycle analysis were also performed. The results indicated that curcumin, berberine and the mixture of all 5 extracts could significantly reduce cytokine release in the supernatant from lipopolysaccharide (LPS) activated macrophages. All the extracts showed cytotoxicity in a dose dependent manner in both cell lines. Mixture of the extracts showed better activity than individual extracts in all the experiments. Our study reveals that Curcuma longa and Tinospora cordifolia extracts synergistically demonstrate anti-inflammatory and anti-proliferative activity. There is therefore scope for using these plant bioactives as complementary therapy or for chemoprophylaxis.

Keywords: Cervical cancer, Curcuma longa Linn, Tinospora cordifolia.

INTRODUCTION
The plant kingdom is a rich source of compounds with diverse medicinal properties well known since ages. The anti-cancer effect of medicinal plant products mentioned in the ancient texts, has been studied at experiential, experimental and clinical levels and demonstrated its potential as a therapy with scientific approach.1,2 There is a vast range of phytochemicals, which can alone or synergistically inhibit cancer cell proliferation. Pharmacology, chemistry and clinical uses of these plants are studied in the Reverse Pharmacology mode. Curcuma longa and Tinospora cordifolia are among these widely studied plants from Ayurved.

Cervical cancer is a major cause of mortality and morbidity in women, despite extensive screening efforts. Each year 527,624 new cases and 265,672 deaths are reported worldwide. In India the annual incidence accounts for 122,844 and mortality for 67,477 cases.3,4 Cervical cancer usually allows sufficient period of time for early diagnosis and appropriate management. Eighty percent of the deaths occur in the developing countries due to the lack of widespread screening programs, with limited access to this time window.5

Chronic inflammation is the major culprit in the process of carcinogenesis. Persistent exposure of the inflammatory mediators like cytokines, free radicals, chemo kinesetc leads to the mutagenesis and normal cell proliferation control is lost.6 In case of cervical cancer, the main causal factor is persistent viral infection by Human Papilloma Virus (HPV) and there are many other infections that act as cofactors like Bacterial vaginitis, Herpes Simplex Virus 2, Chlamydia trachomatis, or Trichomonas vaginalis.7

Prevention by HPV vaccine has shown promising results, however in India it would take several years to implement the regular vaccination program. Moreover, these vaccines are against two high risk HPV types, 16 and 18, the most prevalent and may prevent only 70% of cervical cancers.8,9 Other cofactors such as use of hormonal contraceptives, smoking, nutritional deficiencies, sexual promiscuity, other Sexually Transmitted Diseases (STDs) and multiparity may also lead to the cervical lesion.10

Anti-cancer agents from medicinal plants have been identified as promising treatments that improve the therapeutic effects when used in combination with chemotherapy. The Ayurvedic medicine system applies a holistic approach for the treatment of any disease. These formulations contain multiple plant extracts and they collectively work on the disease and maintain homeostasis of the body. In the present study five extracts (curcumin, turmeric oil and polysaccharides from Curcuma longa Linn and berberine and polysaccharides from Tinospora cordifolia) and their mixture were evaluated for their anti-inflammatory and anti-proliferative activity.
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% CO2 at 37°C. Intervention studies were carried out with standard concentrations of compounds.

Plant extracts

Curcumin (CurcuWIN™) was provided by Omni active Pvt Ltd, Mumbai and berberine were procured from Sigma Laboratories. Curcuma polysaccharide (Turmacin™) was provided by Natural Remedies Pvt Ltd Bangalore and Turmeric oil 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.11

Evaluation of anti-inflammatory activity of compounds

In vitro macrophage activation

In vitro macrophage activation technique was used for the evaluation of anti-inflammatory activity of extracts.12 Briefly healthy human volunteer blood was collected in heparin tubes and mononuclear cells were separated using density gradient centrifugation with Ficol (Sigma). Monocytes were washed and seeded in the culture plates with media containing GMCSF 50ng/ml. On the 7th day fresh media were added with test compounds, positive control (LPS: 50ng/ml) and negative control (Verapamil). After 24 hrs supernatant was collected and stored at -80°C till further analysis. Cytokines (TNF α and IL-6) estimation in the supernatant was carried out using Bio source ELISA kit.

Acridine orange and ethidium bromide assay

Cells were trypsinized and plated in 24 well plates with 10⁵ cell/well cell density and incubated for overnight. Next day cells were treated with the various concentrations of compounds with fresh media and incubated. Media was removed and EtBr and acridine orange staining solution was added to the well and mixed gently just prior to microscopy. Samples were evaluated immediately in a fluorescence microscope using a fluorescein filter and a 10X objective.13

SRB Assay

In vitro cytotoxicity was seen by Sulforhodamine B assay.14 Approximately 4x10⁵ cells were seeded in the 96 well-plate on day one and next day fresh media with different concentrations of five test compound was added and incubated. After 24, 48 and 72 hrs the cells were fixed with ice-cold 10% trichloroacetic acid (TCA) for 1hr at 4°C. The plates were stained with SRB and the dye was solubilised by adding tris base (pH 10.5). The test was performed in triplicates. The % survival was calculated from the optical density readings at 540 nm with reference to 690 nm.

Determination of indole amine 2,3-dioxygenase (IDO) enzymatic activity

Indole amine 2,3-dioxygenase enzyme activity was evaluated by measuring the change in kynurenine concentration in the macrophages culture supernatant.15 Monocytes were isolated from healthy human volunteer blood PBMC, and cultured for seven days. On eighth day cells were treated with IC50 concentrations of the extracts and incubated. After 24 hrs cells treated with100 µM tryptophan and incubated for four hours. Culture supernatant (100 µL) was treated with 50 µL of 30% trichloro acetic acid, and centrifuged at 10 000 rpm for five minutes. The supernatant was then added to an equal volume of Ehrlich reagent in a 96 well plate. Absorbance was measured at 492 nm, using a microplate reader (Biotek).

DNA damage

DNA fragmentation assay was used to determine the induction of apoptosis caused by extracts.16 In brief, after cancer cells were treated with the extracts for 24 hours, and treated with TENS buffer with Proteinase K for overnight. DNA was isolated using the chloroform, phenol extraction method. The DNA was precipitated with 100% ethanol and resuspended in TE buffer. DNA electrophoresis was carried out on 2% agarose gel containing 0.1 mg/ml ethidium bromide. After electrophoresis, DNA fragments were analyzed with a UV-illuminated camera.

Cell cycle analysis

Cell cycle analysis on the basis of cellular DNA content was done by flow cytometry at National Institute Immuno haematology (ICMR), Parel, Mumbai. In brief SiHa and C33a cells were seeded in 6 well plates and incubated. After 24 hrs of treatment cells were harvested fixed in cold 70% ethanol for 30 min at 4°C. Cells were treated with 50 µl of ribonuclease from 100 µg/ml stock and 200 µl of Propidium iodide from 50 µg/ml stock solution. The samples were run on flow cytometer (BD FACS Calibur) and analyzed on Mod Fit LT 4.1 software.17

Statistical analysis

The data was statistically analyzed using Student’s t test using the Graphpad Prism software Version 7.0. The results obtained with the extracts were compared with the media and solvent controls. Data is presented as mean ± SEM.
RESULTS

Anti-inflammatory activity of extracts

Monocytes treated with various concentrations of extracts showed morphology of activated macrophage. TNF-α release from monocytes treated with LPS and the extracts is inhibited in a dose dependent manner. Curcumin and berberine significantly reduced the levels of TNF-α as compared to LPS control (*p=0.003 and †p=0.006 respectively). However polysaccharides and turmerone also suppressed TNF-α release as compared to LPS (Fig 1a). Two mixtures of (25µg/ml and 50µg/ml of each extract) showed better activity than the individual extract at the same concentrations (*p=0.05 and †p=0.001) (Fig 1b). IL-6 levels in the media supernatant were also reduced significantly (p<0.05) by curcumin and berberine at 50µg/ml concentration and by all extracts at 100µg/ml concentration (p<0.02) (Fig 2a and 2b).

**Figure 1a:** TNF-α release (Mean±SEM) in supernatant of macrophage culture, stimulated with LPS and treated with the extracts.

LPS: lipopolysaccharide, VPM: verapamil, Scur: Soluble Curcumin, C poly: Curcuma polysaccharide, TO: turmeric oil, T polysac: tinospora polysaccharide. (*p<0.05, †p<0.01, ‡p<0.001)

**Figure 1b:** TNF-α release (Mean±SEM) in supernatant of macrophage culture, stimulated with LPS and treated with mixtures of the extracts.

* p=0.05 and †p=0.001, Mix 25: mixture of 25µg/ml of each extract, Mix 50: mixture of 50µg/ml of each extract

**Figure 2a:** IL-6 release (Mean±SEM) in supernatant of macrophage culture stimulated with LPS and treated with the extracts.

LPS: lipopolysaccharide, VPM: verapamil, Scur: Soluble Curcumin, C poly: Curcuma polysaccharide, TO: turmeric oil, T polysac: tinospora polysaccharide. *p<0.05, †p<0.02, ‡p<0.003

**Figure 2b:** IL-6 release (Mean±SEM) in supernatant of macrophage culture, stimulated with LPS and treated with the extracts

* p=0.005 and †p=0.003, Mix 25: mixture of 25µg/ml of each extract, Mix 50: mixture of 50µg/ml of each extract

Acridine Orange and Ethidium Bromide assay

This assay was used for testing apoptosis by visualizing nuclear changes in the cells and formation of necrotic and apoptotic bodies. As visible in micrographs viable cells emitted uniform fluorescent green color in negative control. Cells treated with curumin, berberine and T. polysaccharide and mixture of compound are showing apoptosis and have condensed chromatin, nuclear fragments and stain orange (Fig 3). Necrotic cells also stain orange but with intact nuclear morphology.

SRB assay

Cytotoxicity of the compounds was evaluated with SRB assay on C33a and SiHa cell lines with Podophyllotoxin used as a positive control and DMSO solvent control. Both cells were treated with four concentrations of all compounds ie 25, 50, 100 and 200 µg/ml, as well as two mixtures of compounds containing 25 and 50µg/ml of each extract. As reflected in the graphs test compounds showed cytotoxic activity in cervical cancer cells in a dose dependent manner. Both cell lines showed different responses to the extracts.
Figure 3: Acridine Orange and Ethidium Bromide assay in C33a and SiHa cell lines treated with individual extracts (25, 50, 100 and 200µg) and mix 25 (mixture of 25µg/ml of each extract).

Berberine, curcumin and Turmeric oil showed maximum cytotoxicity in C33a cells (IC50: 30.1, 107 and 117.4 µg/ml respectively) whereas Curcuma and Tinospora polysaccharides showed less cytotoxicity as compared to the controls. In SiHa cells berberine and curcumin showed maximum cytotoxicity (IC50: 25.11 and 63.09 µg/ml respectively). Mixture of the extracts inhibited the cell growth comparable to positive control (Fig 4a and 4b).

Figure 4a: Cytotoxicity of plant extracts (µg/ml) in C33a cell line using SRB assay expressed in absorbance at 540nm (Mean±SEM). (Mix 25: mixture of 25µg/ml of each extract, Mix 50: mixture of 50µg/ml of each extract)
Figure 4b: Cytotoxicity of plant extracts (25, 50, 100 and 200µg/ml) in SiHa cell line using SRB assay expressed in absorbance at 540nm (Mean±SEM). (Mix 25: mixture of 25µg/ml of each extract, Mix 50: mixture of 50µg/ml of each extract)

IDO enzymatic activity

Media supernatant of macrophages untreated and treated with extracts was analyzed for change in kynurenine concentration. Standard curves for kynurenine were plotted and experimental values obtained using the colorimetric assay were compared. Reduced levels of kynurenine, in the supernatant, showed inhibition of IDO enzyme activity. Figure 5 shows that all the extracts and their mixture significantly suppressed the enzyme activity as compared to negative and solvent controls.

DNA damage assay

Agarose gel electrophoresis method was employed for the assessment of DNA damage in the form of ladder. C33a and SiHa cells, treated with the extracts, show smearing of DNA due to apoptosis. Curcumin, turmeric oil, curcuma polysaccharide and berberine caused apoptosis and produced DNA fragments in the treated cells. Increased DNA fragmentation is seen in the mixture of the extracts which is comparable to positive control. Apparently DNA damage in SiHa cells was lesser than C33a cells (Fig 6). The size of the DNA fragments range from 700 to 100 base pairs.

Cell cycle analysis

Results of cell cycle analysis reveal that the cells treated with IC50 concentrations of extracts express main character of apoptosis i.e. cleavage of nuclear DNA into multiple fragments that is reflected in G0/G1 and S-G2/M phase together with an increase in sub-G1 phase corresponding to apoptotic cells. A considerable increase in the cells accumulating within the G2/M phase of the cell cycle was observed after treatment with the IC50 dose of curcumin, turmeric oil and berberine whereas curcuma and tinospora polysaccharides showed no significant difference from the control group in C33a cells (Fig 7a). SiHa cells also showed increased population of G2M after treatment with all the extracts. These results show that the mixture of the extracts mediated inhibition of C33a and SiHa cells viability and also arrest cells in the G2/M phase (Fig 7b).
Figure 6: Agarose gel electrophoresis assay for DNA damage in C33a and SiHa cells treated by IC50 concentration of the extracts (µg/ml) Mix 25: mixture of 25µg/ml of each extract


Figure 7a: Flow cytometric analysis of the cell cycle distribution with PI staining in C33a cells after treatment with extracts (IC50 concentration (µg/ml)) Mix 25: mixture of 25µg/ml of each extract

Figure 7b: Flow cytometric analysis of the cell cycle distribution with PI staining in SiHa cells after treatment with extracts (IC50 concentration) Mix 25: mixture of 25µg/ml of each extract
DISCUSSION

*Curcuma longa* and *Tinospora cordifolia* are considered as Rasayanas in Ayurved which have immune modulatory activity. *Curcuma longa* is an extensively studied plant for various types of diseases and especially for cancers. It is the modulator of many molecular pathways involved in cancer such as inactivation of NF-κB inactivation, cell cycle arrest, induction of apoptosis, inhibition of VEGF, down regulation of oncogenes etc. In *vitro*, *in vivo* and clinical studies in oral and cervical pre-cancer have shown chemo preventive potential of curcumin, a key component of *Curcuma longa*. Curcumin is demonstrated as an inhibitor of cancer at all stages, initiation, promotion and progression stages of development in the studies by Nagabhushan M, et al. Deshpande et al have shown the chemo preventive activity of turmeric, ethanolic turmeric extract and curcumin-free aqueous turmeric extract in mice with DMBA-induced mammary tumorigenesis. Curcumin also suppressed proliferative response of estradiol in cervical cancer cells, and induced apoptosis. Curcumin used in the present study is a polymer based formulation which is water soluble. It excludes the issue of curcumin bioavailability.

Turmeric oil includes ar-turmerone, turmerone, zingeriberene and other sesqui terpenes as its biologically active ingredients. A pilot clinical study of women with low-grade squamous intraepithelial neoplasia (LSIL), with supercritical extract of turmeric oil administered as oral capsules has shown potential for cervical cancer chemopreventive activity. The treatment also significantly reduced the serum IL-6 levels in the subjects.

Curcuma polysaccharide is reported for its diverse pharmacological activities, in conditions such as cancer, diabetes, depression, infections etc. Anti-cancer activity of polysaccharide extract has been shown *in vitro* as well as *in vivo* by Lakshmi et al and Kim et al. respectively. *Tinospora cordifolia* is a known immune modulator with various active compounds such as lactones, alkaloids, phenolics, diterpenoid, steroids, glycosides, sesqui terpenoid and polysaccharides. Many *in vitro* and *in vivo* studies have reported anti-cancer activity of isolated active compounds from this plant.

Berberine is an iso quinoline alkaloid found in tinospora and in other plants also. *In vitro* and preclinical studies of berberine have been reported for many pharmacological actions. Inhibition of transcriptional activity of activator protein 1(AP1) and cyclooxygenase-2 (Cox-2) has been shown *in vitro* with berberine. Inhibition of AP-1 also facilitates effective suppression of HPV transcription and thus inhibition of E6 and E7 oncogenes required for cellular transformation. It has also been shown to exhibit the anti-proliferative activity by inhibiting DNA topoisomerase II.

The polysaccharide fraction of *Tinospora* extracts is known to modulate immune system. A purified polysaccharide known as arabinogalactan is shown to increase proliferation of B-cells by exhibiting polyclonal mitogenic activity. Another study revealed its immune modulatory activity by inducing tolerance in mice against endotoxic shock by modulation of cytokines and nitric oxide.

There are studies of *Curcuma* and *Tinospora* active constituents, as adjuvant with chemotherapy drugs but in *vitro* studies of synergistic anti-cancer activity of these active constituents are lacking. The present study was intended to explore the individual and synergistic anti-cancer potential of extracts of these two rasayanas used in Ayurved.

The anti-inflammatory activity of traditional medicines is well known. It is due to the polyphenols which are metabolites of most natural products. They exert their anti-inflammatory activity through the modulation of MAPK, Akt and pathways, by inhibition of NF-κB phosphorylation thus transcriptional inactivation of pro-inflammatory cytokines and chemokines.

In the present study modulation of inflammation caused by active compounds is measured in terms of reduction or increase in cytokine release. PBMC derived Macrophage activation technique employs Lipo-polysaccharide (LPS) induced activation of monocytes, which causes the release of inflammatory cytokines.

The results show dose dependent inhibition of TNF-α and IL-6 significantly by curcumin and to some extent by turmeric oil, and polysaccharides. The mechanism of action of curcumin is reviewed by Aggarwal et al. However turmerone could not suppress NFκB expression in chronic myeloid leukemia cell line according to the previous report by the same group.

Whereas Liju et al showed significant reduction of inflammation by turmeric oil in carrageenan, dextran-induced acute inflammation, and formalin-induced chronic inflammation. Immunostimulatory activity of curcuma polysaccharides on un stimulated PBMCs was reported by Yue et al. Similarly the polysaccharide extract used in the present study inhibited IL-6 production by LPS stimulated macrophages.

Berberine significantly reduced TNFα and IL-6 release in LPS stimulated macrophages in this study. Similarly Chen et al showed PPAR gamma mediated reduction of inflammatory cytokine release in acetylated low-density lipoprotein (AcLDL) stimulated macrophages. It also reduced acute inflammation in carrageenan- and zymosan-induced paw oedema model.

*Tinospora polysaccharide* extract did not inhibit the cytokine release in this study but Sharma et al have shown immunostimulatory activity of the same extract using neutrophil phagocytosis model with *Candida albicans*. The compounds present in this polysaccharide extract were identified as columbin,
Modulation of IDO enzyme activity was also demonstrated by these extracts. All the extracts and their mixture reduced the IDO activity significantly as compared to controls. Curcumin affects multiple molecules in the signaling cascade upstream of IFN-γ induced IDO expression. Primarily it alters the IDO-mediated immune regulation by inhibiting the JAK-PKCS9 STAT1 signaling pathway. Although there are no studies reported for IDO inhibition with the other compounds used in the present study, phytochemicals with 2-phenyl-1-benzopyran-4-one backbone (flavones) are reported to suppress the expression of IDO-1 protein significantly. Mittal et al have found correlation between HPV-associated cervical intraepithelial neoplasia (CIN) 2/3 and higher expression of IDO1 and IFN-γ in a murine model. It suggests that the host immunity is invaded by over expression of IDO1 in HPV infected skin. In a clinical study of 251 cervical cancer patients’ high levels of kynurenine in serum were associated with advanced disease stage, lymph node metastases and worse survival in cervical cancer.

SRB assay demonstrated significant cytotoxicity by curcumin and berberine at lower concentrations in both the cell lines. C33a cells were more sensitive to the extracts as compared to SiHa cells. Cells treated with turmeric oil also showed growth inhibition at higher concentration. Curcuma and Tinospora polysaccharide showed activity at higher concentration and took a longer period of time, but did not show DNA damage in the cell. In addition, the mixture of all these compounds comes out as potent anti-proliferative compound. In view of these results, the extracts induced apoptosis in cells led to cell cycle arrest at G2M phase. When the DNA damage was caused at this stage, it could not be repaired and subsequently causes cell death.

We have demonstrated that the curcuma and tinospora active compounds modulate the immune system and cause apoptosis in cancer cells. These compounds work better when used in a synergistic manner. However, their composition in a formulation needs to be studied in detail. Future in vivo and clinical studies are necessary to evaluate the therapeutic effects of these compounds.

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