



Original Research Article (Experimental)

In vitro and *in vivo* evaluation of a standardized *Curcuma longa* Linn formulation in cervical cancer



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ABSTRACT

Background: The anti-cancer activity of phytochemicals present in turmeric or *haridra* (*Curcuma longa* Linn) extracts against cancer has been described in various '*in vitro* and *in vivo*' studies.

Objective: In the present study, *in vitro* and *in vivo* anti-cancer and chemo-preventive activity of a new standardized Supercritical Turmeric Oil Extract (SCTOE) NBFR-03 was evaluated in cervical cancer models.

Methods and materials: *In vitro* cytotoxicity of this formulation was assessed at 10, 20, 40, and 80 µg/ml concentrations, in three cervical cancer cell lines (HeLa, SiHa, ME180) using Sulforhodamine B assay. The *in vivo* anti-cancer activity was evaluated in two groups of female nude mice; the first one was with tumor xenograft implants and at the same time treatment was started with 96 µl/kg/day p.o. and 192 µl/kg/day p.o. NBFR-03 for three months. The second group was kept as chemoprevention group where mice were pre-treated with the formulation (96 µl/kg/day p.o.) for two weeks and injected with cancer cell suspension with continued treatment for three months.

Results: No cytotoxicity was seen in any cell line with the extract when compared to positive control (Adriamycin 10 µg/ml). In mice the first treatment group with tumor xenograft implants did not show any significant anti-tumor activity but showed a trend where higher dose group had smaller tumor volumes as compared to lower dose group and controls ($p = 0.37$ and $p = 0.34$ respectively). The chemopreventive group with pre-treated mice also showed smaller tumor size as compared to controls ($p = 0.163$).

Conclusion: NBFR-03 turmeric oil extract showed a promising trend in mice pre-treated with NBFR-03. There is a scope for further studying the potential of this extract as complementary therapy and as a chemopreventive.

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1. Introduction

Cervical cancer is a significant cause of morbidity and mortality among women in developing countries [1,2]. The recent cervical cancer statistics in India has shown that the mortality due to cervical cancer in the year 2018 was 63,000 women whereas in 2020 it was 70,000 [3]. In India, the treatment for cervical cancer is

expensive and beyond the reach of majority of women and it is often diagnosed in advanced stages. Currently in the screening programmes with Papanicolaou (Pap) smears or with other tests we can detect not only early stage cancer but also the pre-cancerous phases known as Cervical Intraepithelial Neoplasia (CIN) as designated in histological samples, or biopsy or known as Squamous Intraepithelial Lesions (SIL) in cytology by Pap smears, which could be of low grade or high grade [4]. Treatment of pre-cancerous conditions can be almost 100% successful and surgical or chemo-preventive pharmacotherapy can be used to prevent this disabling and destructive disease [1,5–7].

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There is no doubt that healthy sexual habits and use of Human Papilloma Virus (HPV) vaccine are the best measures for primary prevention of cervical cancer but these cannot be adopted in all public health programs because of high cost and poor compliance by the public. Hence, secondary prevention, by treatment of pre-cancerous conditions (SIL or CIN), is an important option to prevent cervical cancer. Moreover, available vaccines may prevent only 70% of cervical cancers, not all, because the vaccine may not be effective against all subtypes of carcinogenic HPV [8].

Phytochemicals from many medicinal plants have been studied for anti-cancer activity [9]. Natural phytoconstituents have been reported to block carcinogenesis and to inhibit tumor progression both *in vitro* and *in vivo* models [10]. These chemopreventive effects can be attributed to immunomodulation, growth arrest in cell cycle, induction of apoptosis, inhibition of oncogenic pathways like NF- κ B, kinases, DNA synthesis inhibition, and modulation of other signal transduction pathways. Chemoprevention by natural products, primarily affects transforming cells without causing any damage to normal cells. There are some clinical trials for cervical cancer chemoprevention with dietary supplements, micronutrients and other chemical agents but these are not effective in all cases or the chemical agents may cause unacceptable side-effects [11–14].

Haridra (*Curcuma longa* Linn), mentioned in classical Ayurvedic texts, and commonly known as turmeric or *haldi*, is being used traditionally for several disorders like dyspepsia, wound healing, bleeding disorders, diabetes, hemorrhoids, anemia, skin diseases, etc. There are many bioactive substances reported from turmeric, but the key active ingredients of *C. longa* include curcuminoids: curcumin (diferuloylmethane), demethoxycurcumin, monodemethoxycurcumin, cyclocurcumin, bisdemethoxycurcumin, and curcumene. Furthermore, volatile oils (turmerone, zingiberene, α -phellandrene, sabinene, cineol, borneol and sesquiterpenes) also have pharmacological activity [15–17].

The use of *haridra* extracts in *Arbuda* (cancer) has been described in *Brihatrayi* which are the most revered three text books or *granthas* (*Samhitas*) in Ayurveda written by the three great ancient *rishis* Charak, Sushrut and Vagbhat or Nighantus [18–21]. Cancer or *Arbuda* has been described in Ayurveda as below:

वृत्तं स्थिरं मन्दरुजं महान्तमनन्तमूलं चिरवृद्धयपाकम् ।
कुर्वन्ति मान्सोच्छ्रयमत्यगार्धं तदुर्बुदं शास्त्रविदो वदन्ति ॥ (सु. नि.)

ग्रन्थि अपचि अर्बुदं गलगण्डानाम निदानं /13

(A rounded, immobile, painless, deep rooted, growing continuously, and non-suppurative swelling, which erodes into the muscle with extensive destruction, is called as *Arbuda* by experts).

This description matches the description of cancer (solid malignant tumours) in allopathic or modern medicine [22].

There is extensive literature on Ayurvedic indications and usage of turmeric extracts in clinical practice supported by phytochemistry, clinical trials, and *in vitro* and *in vivo* experiments, but this is beyond the scope of this article. Its specific use in cancer has been elaborated in *Yogratnakar* as below [23].

हरिद्रा लोध्रपत्राङ्गः धूममनः शिला ।
मधुप्रगाढो लेपोऽयं मैदोर्बुदहरः परः॥ (यो. र.)

अर्बुद चिकित्सा / 6

(*Haridra*, *Lodhra*, *Patranga*, *Dhooma*, and *Manahsheela* along with honey can be used as thick local application in fleshy or fatty *arbuda*).

The use of turmeric oil is specifically mentioned for oral cancer in classical texts of Ayurved [24].

उभे हरिद्रे पिप्पल्यः सैन्धवं देवदरुच ।
विडङ्गं चित्रकं बिल्वं रोहिषस्य च पल्लवाः ॥
द्विगुणं च पयो दद्यात् तस्मिद्धं नश्यतां नयेत् ॥
श्लैष्मजं सन्निपातोत्थं शिरोरोगं नियच्छति ।
उपजिह्वां च मालाञ्च कण्ठशालूकमर्बुदम् ॥ (वङ्ग. सं.)

मुखरोगधिकार / 107, 109, 110

(Two types of turmeric, *pippali*, *saindhav*, *deodaru*, *vidanga*, *chitrak*, *bilwa*, leaves of *rohish* should be mixed with twice the amount of water and made *siddha* in *taila*, it is useful in *kapha* type *sannipatik* disease and *arbuda*, or tumor, of the head, throat, neck and palate).

We have previously demonstrated the safety and anti-cancer activity of different types of turmeric extracts [25]. Our earlier studies have shown that turmeric oil protected the cells from benzo [a]pyrene induced damage in experimental models. In the clinical study, it reduced the number of micronuclei in oral mucosal cells and peripheral blood lymphocytes in patients with oral submucous fibrosis, along with increased antioxidant activity [9]. We have also reported the safety and tolerability of turmeric oil in healthy human volunteers [26]. However, there was no study on its activity in different cervical cancer cell lines or cervical cancer animal models. Therefore, a standardised turmeric extract NBFR-03 was evaluated for its anti-cancer and chemopreventive activity in preclinical and clinical studies.

This formulation NBFR-03 contains supercritical extract of turmeric oil (SCTOE) which is a by-product of curcumin extraction, manufactured and standardised in collaboration with our centre, and coded by Nisarga Biotech Private Ltd (NBPL). Further, NBFR-03 was evaluated for chemopreventive activity and safety in women with Low - Grade Squamous Intraepithelial Lesion (LSIL), a pre-cancerous condition for cervical cancer [27,28]. The data presented in this article is a part of preclinical studies of above-mentioned formulation carried out during the year 2009 which remained unpublished [29]. The objective of this study was to determine whether NBFR-03 had any *in vitro* or *in vivo* anti-cancer activity in experimental studies in collaboration with Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Kharghar. The clinical trial was published earlier; however, the authors believe that in view of the importance of the positive potential chemopreventive activity of turmeric oil, demonstrated in this animal model, this data also needs to be documented in literature [27].

2. Materials and methods

The formulation was developed by MRC-KHS in collaboration with Nisarga Biotech Pvt Ltd, Satara, from the supercritical extract of turmeric rhizome (*C. longa* Linn) oil as described earlier [27]. It was manufactured according to the GMP requirement and tested for the heavy metals, pesticides, microbial load and aflatoxin, and standardized by gas chromatography and mass spectrometry. Dosage of the formulation was calculated as per the human dose used in previous studies and by clinicians [26]. A safety and toxicity study in healthy rats and rabbits was conducted by National Toxicology Centre in Pune (Unpublished data). The dosage was 0.2 ml of NBFR-03, twice a day in humans and was calculated as per the earlier clinical studies on traditionally used turmeric oil extract in volunteers and Oral Submucous Fibrosis. The *in vitro* and *in vivo* studies were planned in collaboration with the ACTREC, Kharghar.

The project was approved by the Institutional Ethics Committee at ACTREC.

2.1. *In vitro* anti-cancer activity

2.1.1. Cell culture

Cervical cancer cell lines ME180 (HPV negative), HeLa (HPV-16 positive), and SiHa (HPV-18 positive) were used for the study. ME180 was maintained in Minimal Essential Medium (MEM), and SiHa and HeLa in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% (v/v) heat-inactivated fetal bovine serum, [32] penicillin and streptomycin antibiotics and HEPES (Himedia) in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. The formulation was dissolved in dimethyl sulfoxide (DMSO) and intervention studies were carried out with 10, 20, 40 and 80 µg/ml concentrations.

2.1.2. Sulforhodamine B (SRB) assay

In vitro anti-proliferative activity of NBFR-03 was assayed by Sulforhodamine B assay [30]. 5×10^3 cells were seeded in 96 well-plate and were allowed to adhere and grow for a further 24 h. On day 2, fresh medium in different concentrations (10, 20, 40 and 80 µg/ml) of the formulation was added and incubated. Cells were fixed after 72 h of treatment with ice-cold 10% trichloroacetic acid (TCA) for 1 hr at 4 °C. The plates were washed 5 times in distilled water, stained with SRB dye and again washed with 1% v/v acetic acid. The dye was solubilised by adding 100 µl of 100 mM tris base (pH 10.5). The assay was performed in triplicates. The percent (%) growth was calculated from the optical density readings at 540 nm with reference to 690 nm. Appropriate media and solvent controls were kept. Adriamycin (10 µg/ml) was used as a positive control.

2.2. *In vivo* anti-cancer activity

Initially normal mice were used for gross toxicity with all doses.

2.2.1. Nude mouse human cervical cancer xenograft model

Female nude mice, 6–8 weeks old, weighing 18–22 g, were used to assess the anti-cancer and chemopreventive activity of NBFR-03. These are athymic mice which do not reject a subcutaneous implant of xenobiotic cancer tissue, removed from a cervical cancer patient [31]. The animals were kept at 23 ± 2 °C with relative humidity maintained between 50 and 60%. The mice were housed in transparent polycarbonate filter top cages and were fed with pelleted turmeric free feed. Mice selected for experiments were free from any other disease. Weight of these animals ranged from 16.5 to 20.8 gm. All study procedures employed were approved by the ACTREC institutional animal ethics committee.

Solid human cervical tumour HPV positive tissue (earlier preserved in a frozen state), was cut into 2×2 mm size pieces, and these were implanted subcutaneously in mice to allow growth initiation. For this procedure, the mice were transferred to a laminar hood and anesthetized. The abdominal surface was cleaned with 95% ethanol. An incision (1 cm) was made in the lateral abdominal wall above the peritoneum. The cervical implant was inserted into a subcutaneous pocket created by blunt dissection in the adipose tissue. The wound was closed using 4-0 vicryl suture.

The mice were divided in 4 groups of 6 animals per group:

- Tumor bearing mice treated with oral intragastric tube feeds of vehicle control (sesame oil) for three months
- Tumor bearing mice treated with test drug NBFR-03 single dose (96 µl/kg/day p.o.) for three months

- Tumor bearing mice treated with oral intragastric tube feeds of test drug NBFR-03 double dose (192 µl/kg/day p.o.) for three months
- Pre-treated with oral intragastric tube feeds of NBFR-03 single dose (96 µl/kg/day p.o.) for 15 days followed by subcutaneous injection of cancer cell suspension [32] and continued treatment with test drug up to three months.

The mice were examined twice a week for weight and tumour growth. Macroscopic tumours were measured by height, width, and length using callipers. Tumour volume was calculated using the formula $\frac{1}{2} \times \text{length} \times \text{width} \times \text{height}$. Mice were housed for 3 months following transplantation until tumour growth was externally obvious and the mice were then euthanized.

Relative tumor volume (RTV) and test vs control ratio (T/C) was calculated as follows:

$$\text{RTV} = \text{TV}_n / \text{TV}_0,$$

where TV_n is the tumor volume on day n and TV₀ is the tumor volume on day 0.

$$\text{T/C} = \text{mean RTV of treated group} / \text{mean RTV of control group}.$$

2.3. Statistical analysis

The data represents mean \pm SEM. Student's t-test was applied to compare control and experimental groups. P value of < 0.05 is used for statistical significance.

3. Results and discussion

3.1. *In vitro* cytotoxicity

The study drug showed no significant cytotoxicity (Fig. 2) as compared to positive control is SRB assay. Maximum concentration of treatment (80 µg/ml) also could not inhibit the cell growth.

Our concentrations were based on our earlier experience with clinical studies [26]. Curcuma oil was used by Cheng et al. in human hepatocellular carcinoma cells and they found apoptotic activity at higher concentrations (100 µg/ml onwards) [33]. Li et al. also used similar extract containing curcuma oil in Hepa1-6 cells with concentrations up to 500 µg/ml and cell viability was reduced significantly [34]. This suggests that turmeric oil could be active at higher concentrations *in vitro* but in view of clinical safety it is important to study the organ safety of the extract.

3.2. *In vivo* anti-cancer activity of NBFR-03 in nude mice (Group A, B and C)

Gross toxicity of the formulation was evaluated in normal mice. There was no toxicity seen in the normal animals at a high dose also. *In vivo* anti-cancer activity of the extracts was studied in nude mice classified in four groups (Fig. 1). At the end of three months, survival of the animals in group A, B, and C was 33%, 50% and 50% respectively, indicating poor survival in untreated group. Average weight of mice in group A, B, and C did not show any significant difference (Fig. 3 a).

3.3. Tumor volume

Tumor volume of mice in all groups was measured twice a week. The mice, treated with NBFR-03 192 µl/kg/day (group C) showed reduced tumor volume as compared to the control, however the difference was not statistically significant (p value: 0.34). After 3

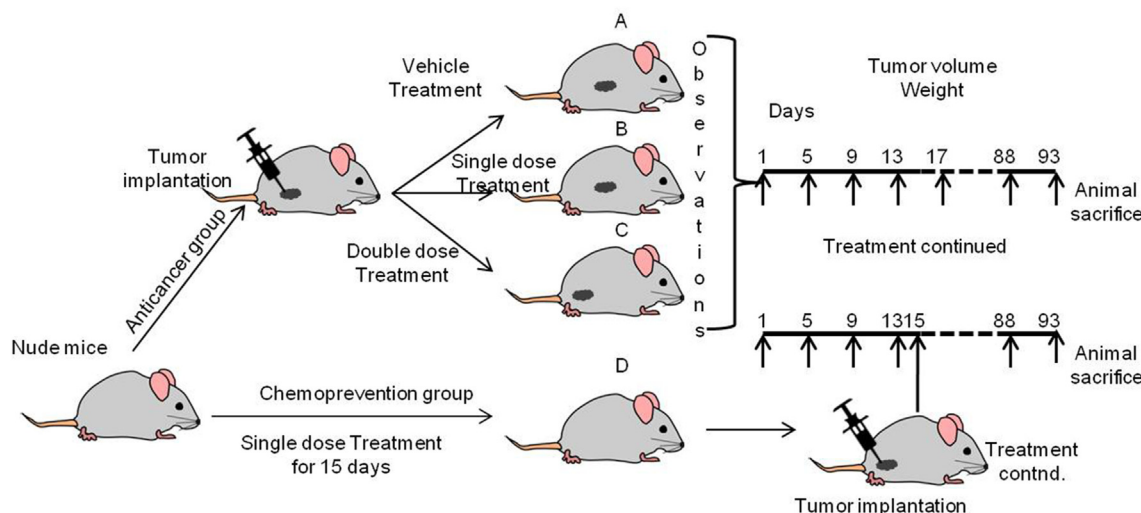


Fig. 1. Schematic view of flow of activities for *in vivo* experiment.

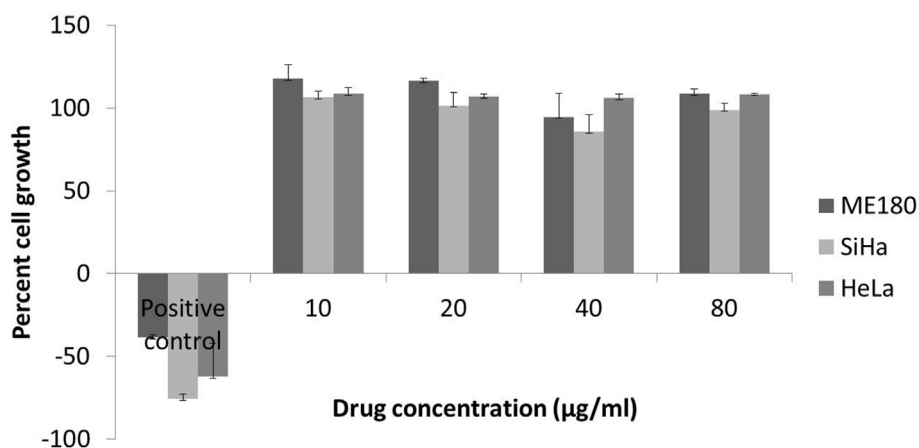


Fig. 2. Percent growth of ME180, SiHa, HeLa cells after treatment with different concentrations of turmeric oil and positive control (10 µg/ml Adriamycin).

months, mean ± SEM tumor volume of group A, B, and C were 10.1 ± 2.96 , 12.34 ± 5.23 , and 6.36 ± 1.61 CC respectively.

T/C value shows the quantitative efficacy of a test drug on tumor volume. T/C value below 0.2 is considered as strong anti-cancer activity whereas T/C value below 0.45 confirms intermediate anti-cancer activity of the drug [35,36]. Although the T/C values for group B and C are above the cut-off point (0.86 and 0.52 respectively), there is a noticeable trend towards decrease in T/C as a function of time and concentration of NBFR-03 (Fig. 3 b). RTV data also shows that after 15 days of treatment, tumor growth rate was slower in group C than control (Fig. 3 c). The macroscopic images of tumor in control and test group animals also show a trend of reduction after 3 months of treatment (Fig. 4).

3.4. Group D

Mice in group D were pre-treated with single oral dose (96 µl/kg/day p.o) of the extract for 15 days before tumor injection. There was 100% survival in control and test group animals. There was no significant difference in animal weights of test group and control group. At the end of study, average tumor volume of chemopreventive group D was found to be lesser (0.035 ± 0.013 CC) as compared to controls (0.128 ± 0.067) (Fig. 5 a). The minimum T/C

value of group D was 0.54, which is higher than the cut-off point (Fig. 5 b). Relative tumor volume of group D shows slower growth as compared to control group (Fig. 5 c). The macroscopic image also shows the difference in the tumor size of control and test animals at the end of study (Fig. 5 d).

Results of *in vivo* experiments clearly show that NBFR-03 did not show any major toxicity in nude mice; moreover, their weight increased significantly during the treatment.

The formulation used in our study primarily contained turmerone and ar-turmerone [27,37]. Like curcumin, turmerone is also reported to be an important bioactive compound present in *C. longa* rhizomes. The chemoprevention showed by turmerone is mainly due to its antioxidant and anti-inflammatory activity. Ar-turmerone affects the cancer cell by causing apoptosis and cell cycle arrest similar to curcuminoids. Turmerone also increases the bioavailability of curcumin and potentiates the anti-cancer activity [17,38].

In a subsequent study, curcuma oil has shown chemopreventive and hepatoprotective activity against hepatocellular carcinoma in mice. It was found to be active against implanted hepatoma and showed anti-inflammatory, antioxidant, and anti-cancer activities [34]. However, our study with turmeric oil in oral submucous fibrosis was conducted in 1997, the clinical study in cervical pre-cancer was initiated in 2007, and was reported in 2011 [9,27].

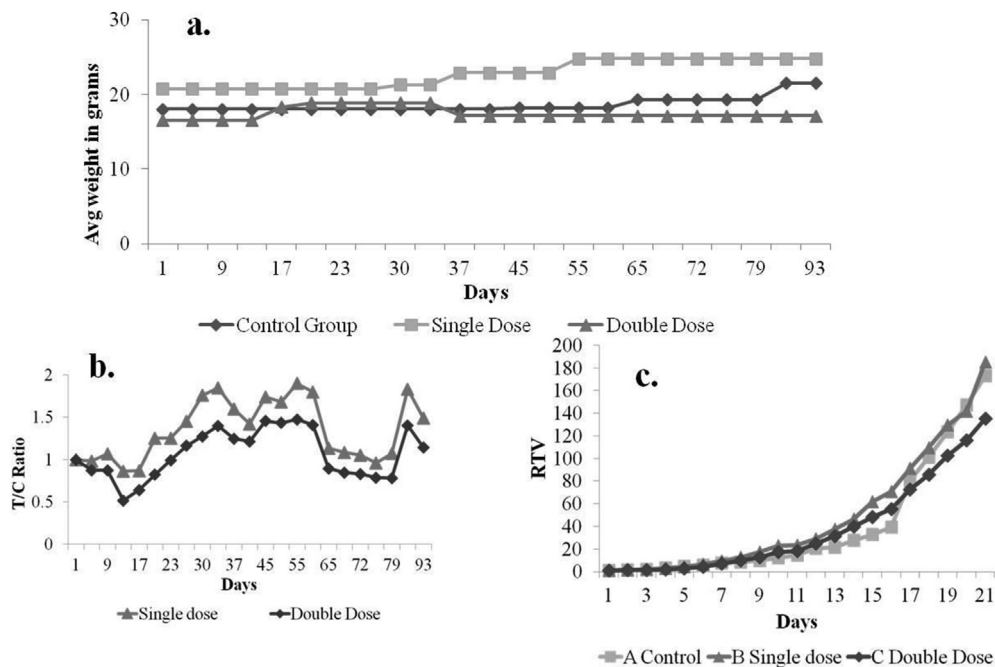


Fig. 3. *In vivo* study group A, B and C. a. Weight of animals in control and test group, b. T/C ratio of single (96 µl/kg/day p.o) and double dose (192 µl/kg/day p.o) groups, c. Relative tumor volume of control and test groups.

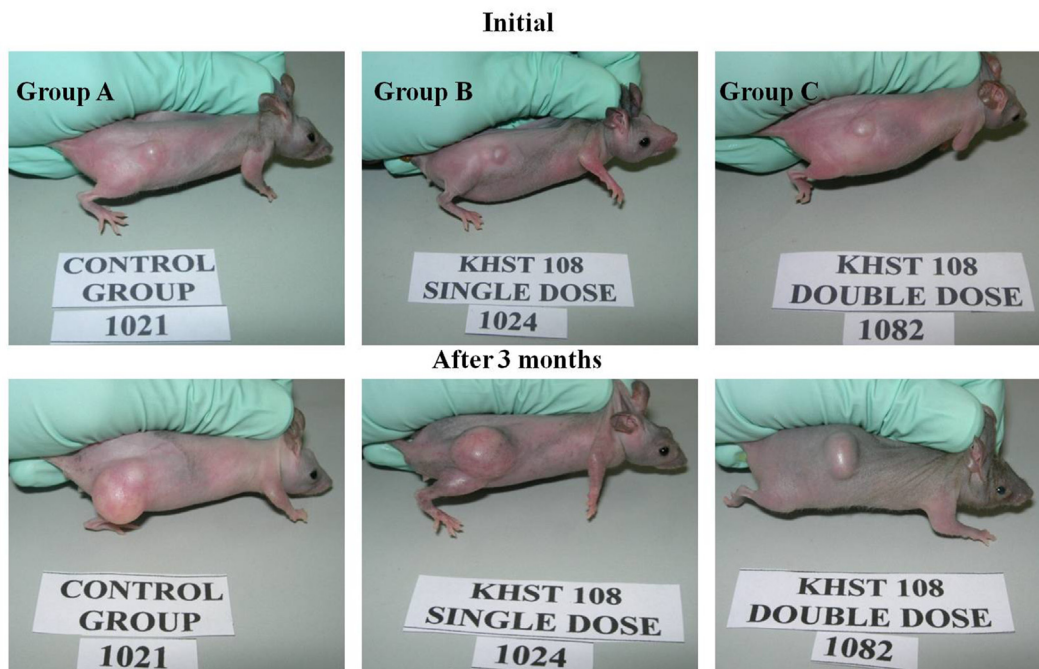


Fig. 4. Images of control and test group nude mice with xenograft implantation before treatment and after 3 months of treatment with NBFR-03 in 2 doses.

In our recent *in vitro* study NBFR-03 showed anti-proliferative activity at higher concentrations. It also demonstrated immunomodulatory activity by significant reduction in TNF- α and IL-6 levels in lipopolysaccharide (LPS) stimulated monocyte culture [39]. Moreover, a significant anti-angiogenic activity was seen in Chorio-Allantoic Membrane (CAM) model by reducing blood vessel formation [40].

In the present study, reduced tumour size in mice pre-treated with NBFR-03 indicated the potential chemopreventive role of the

extract and encouraged us to undertake the clinical study [27]. In the clinical study, 21 cases were enrolled, with cervical LSIL detected in Pap smears, for integrative treatment with anti-microbials followed by oral turmeric oil treatment for 12 weeks, and some of these were followed up to 3 years. In the clinical study, 16 out of 19 women showed regression from LSIL to normal and 3 women had arrest of lesion in Pap smear, after treatment with oral NBFR-03 formulation. It also reduced serum IL-6 levels in some cases indicating possible inhibition of NF κ B activity as reported by

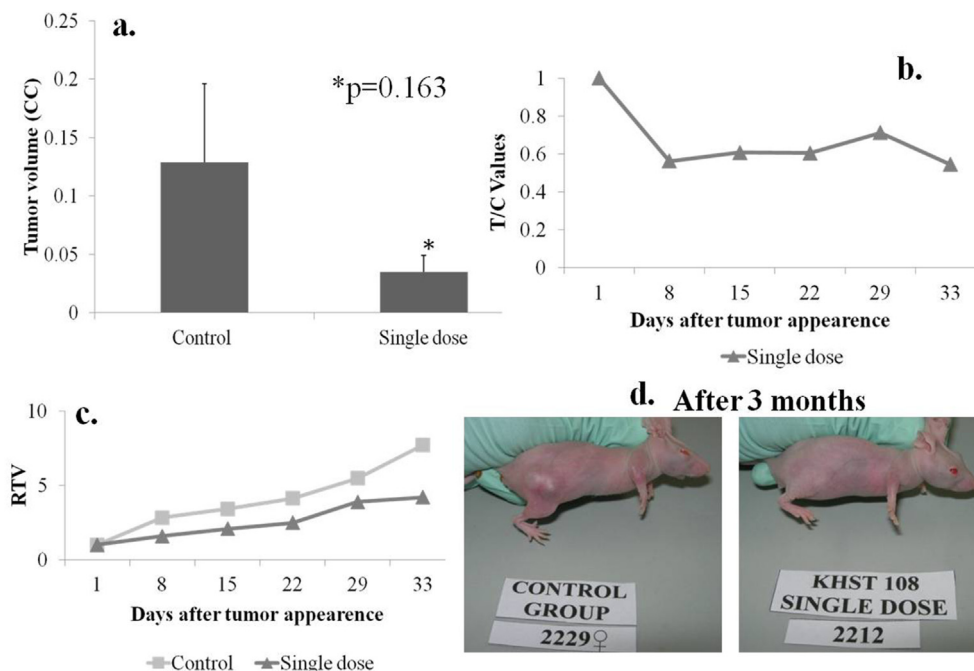


Fig. 5. *In vivo* study group D. a. Tumor volume of animals in control and test group, b. T/C ratio of D group, c. Relative tumor volume of control and test groups, d. Images of vehicle control and test group animals after 3 months.

other authors [27,41,42]. Out of these, 10 cases were available for follow-up by Pap smears/colposcopy from 6 to 36 months and none progressed to high grade lesion up to 3 years after discontinuation of treatment [28]. In another recent clinical study by our group, a different standardised oral extract of turmeric (Haldone® 600 mg BD) with curcumin, turmeric oil, and curcuma polysaccharides, was evaluated in women with cervical LSIL. Within 10 weeks of treatment 18 out of 20 cases showed regression in Pap smear and no major side-effects were observed and 2 were arrested i.e., did not progress to higher grade. The reduced nuclear diameter and nucleocytoplasmic ratios by micrometry were observed in both clinical studies [43].

4. Conclusion

Reverse Pharmacology path allowed us to conduct our experimental and clinical studies to be planned in a meaningful way with limited resources [25]. In conclusion, we can say that ‘*in vitro*’ experiments may give better results with pure compounds and cervical CIN (pre-cancer) cell lines could be used in future to assess the chemopreventive activity. In experimental animals, the use of pre-treatment with the chemopreventive therapy is likely to give more insights than simultaneous administration of the drug with tumor xenograft. More recent ‘*in vivo*’ models also display a cervical dysplasia or pre-cancer change before cancer develops and these may be good for prediction of chemopreventive efficacy of new agents [44].

These results indicate that whilst the turmeric extracts alone may not cure invasive or advanced cancer, they have the potential to prevent cancer when used in integrative chemoprevention therapy. These extracts also possibly reduce the side-effects of cancer therapy and modify the progress of cancer when used as complementary therapy [45].

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Conflict of interest

None.

Author contributions

P.H. Paradkar: Conceptualization, Writing - Original Draft, **A.S. Juvekar:** Methodology, Resources, Validation, **M.S. Barkume:** Methodology, Formal analysis, **A.J. Amonkar:** Conceptualization, Supervision, **J.V. Joshi:** Conceptualization, Supervision, Writing - Review & Editing, Project administration, Funding acquisition, **G. Soman:** Resources, Formal analysis, **A.D.B. Vaidya:** Conceptualization, Supervision.

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