Title: Chemopreventive Potential & Safety Profile of NBFR-03 (*Curcuma longa* oil) in Women with Cervical Precancer (Low- Grade Squammous Intraepithelial Neoplasia) in Papanicolaou Smears

Short Title: Chemopreventive Potential of NBFR-03 (*Curcuma longa* oil) in Women with Cervical Low- Grade Squammous Intraepithelial Neoplasia in Papanicolaou Smears

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Title: Chemopreventive Potential & Safety Profile of NBFR-03 (*Curcuma longa* oil) in Women with Cervical Precancer (Low- Grade Squammous Intraepithelial Neoplasia) in Papanicolaou Smears

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Abstract (200 words)

Objective: To determine whether *Curcuma longa* Linn oil extract, NBFR-03, could arrest Low – Grade squammous Intraepithelial Neoplasia (LSIL) in Papanicolaou (Pap) Smears from women, when given orally for 12 weeks.

Material & Methods: A total of 1473 women underwent Pap smear screening. Out of these 88 cases had LSIL. Only those with persistent LSIL (N =21) subsequent to antimicrobial therapy were included for clinical examination, Pap smears, colposcopy , clinical biochemistry, urinalysis and Serum IL-6, before and after treatment. Standardised NBFR-03 (0.2ml) capsule was administered, twice daily , for 12 weeks.

Results: None progressed to higher grade lesion as assessed by Pap smears and colposcopy. Sixteen cases regressed to Atypia, ASCUS or inflammatory pattern; 3 persisted as LSIL, 1 discontinued early because of itching. None had any significant abnormality clinically or biochemically.

Micrometry showed a significant reduction in nuclear diameter and N/C ratio after treatment ($p<0.02$, and < 0.05 respectively). Serum IL-6 levels showed a significant reduction (Mean 248± 156 (SEM) vs 27.7± 10.5 (SEM) pg/ ml; $p< 0.05$).

Conclusion: Use of NBFR-03 for 12 weeks was associated with arrest or regression of LSIL in Pap smears and colposcopy, and with reduction in circulating IL-6 levels.

Type of study: Open labeled exploratory clinical trial of NBFR-03 in cervical precancer (LSIL)

Key words: Chemoprevention, Cervical LSIL, NBFR-03, Turmeric oil, Pap smears ,

Introduction

Cervical cancer is a very common cause of death and morbidity in women, particularly in developing countries (Moore, 2010). Primary prevention by immunization against High Risk Human Papilloma Virus (HPV) subtypes 16/18 is possible and its safety and long term efficacy is being investigated. Even if it succeeds it will take another 25 years to cover the young target population, and can prevent only 70 % of cancers (Cuzick, 2010, Cuzick *et al*, 2010). Moreover already several millions of women are infected with HPV and are being screened by Papanicolaou smears / HPV DNA for cancer and for precancerous conditions or intraepithelial neoplasia (Sankaranarayanan *et al*, 2010). Treatment of precancer is more cost-effective than treatment of cancer, and more importantly, less painful and with a better quality of life for women. This study was directed to the diagnosis and potential treatment modality of the earliest phase of precancer detected in Papanicolaou (Pap) smears, technically called as Low – Grade Squammous Intraepithelial Neoplasia (LSIL)(NIH,2001). The gynecological expertise to treat LSIL is not likely to be available for millions of women in developing countries and even after conventional treatment like laser, CO2 vaporization, or loop excision, recurrence may occur in about 5-30% of cases (Ramos *et al*, 2008; Melnikow *et al*, 2009).

Cervical cancer is usually a slowly developing neoplasm and screening methods like the Pap smear and HPV DNA test are available for early detection of precancer and cancer, hence there is considerable scope for chemoprevention (Weinberg, 2007; Sasieni, 2006). Apart from nutrients and antihormones or Vitamin A analogues, COX-2 inhibitors, extracts of medicinal plants also have shown *in vivo* and *in vitro* anticancer activity and are evaluated for secondary chemoprevention or as complementary therapy in advanced cancer (Sasieni, 2006; Gullett *et al*, 2010; Hefler *et al*, 2006; Vaidya *et al* , 2010a).

Bioactive compounds from Turmeric or *Curcuma longa* Linn (*C longa*), specially curcumin, are known for chemopreventive activity (Kuttan *et al*, 1985; Ghaisas *et al*, 1994; Bhide & Jakhi, 1994; Nagabhushan & Bhide, 1987; Hastak *et al*, 1997; Garg *et al*, 2008; Ravindran *et al*, 2009). Earlier studies carried out by our team under the Reverse Pharmacology programme (Vaidya *et al* , 2010b) have demonstrated the activity and safety of Turmeric oil in experimental animals, rats and mice and in healthy human volunteers (Hastak *et al*, 1997; Pillai, 1997; Kayal *et al*, 1997; Ramachandran, 2000; Joshi *et al,* 2003). Other authors also have reported anticancer, antimicrobial, antifungal, and antioxidant activity of Turmeric or Turmeric oil (Kuttan *et al*, 1985; Jayaprakasha *et al*, 1999, 2002). Since LSIL is a common early precancerous lesion detected by Pap smears and cervical cancer can be prevented, we studied the potential chemopreventive activity of a standardized extract of Turmeric oil (NBFR-03), in women with LSIL. The study was approved by an independent intersystem ethics committee.

Subjects & Methods

Formulation development, standardization and quality control: An oral formulation was developed from the supercritical extract of turmeric oil (NBFR-03) with about 65% of turmerone. TLC and HPLC were carried out. The extract was tested for heavy metals, pesticide content, microbial load and aflatoxin as per national and WHO guidelines for natural products (WHO, 2007; CCRAS, 2008).

Accelerated stability and shelf life were studied for the formulation including variations in weight and extract content of the capsules. Gas chromatographic analysis of the extract from the formulation was carried out initially and after 12 months. The soft gelatin capsule contained 0.2 ml of supercritical extract of Turmeric oil. A healthy volunteer study was carried out with the new formulation for tolerability and acceptability.

Storage and Preservation: The capsules were preserved in brown bottles with silica gel packs under dry and cool conditions at room temperature, away from sunlight.

Healthy Volunteer study: This was carried out in 6 young healthy ambulatory subjects, aged 20 to 30 years under supervised conditions in the Ayurvedic Hospital. The volunteers were instructed to report any aberrations in diet and physical activity. A special case record form was used to record symptoms, signs and daily drug compliance. Haematology including platelets, organ function tests for liver, and kidney, lipid profile, fasting sugar, serum proteins and routine urine analysis for proteins, sugar and microscopy, were carried out initially and at the end of 1 month of capsule intake. The laboratory participates in the national Quality Control programme.

Blood pressure, weight and clinical examination were carried out at 0, 8, 24 and 48 hours and weekly thereafter. The dose was 1 capsule daily X 1 week, 1 capsule BD X 1 week and 1 capsule TDS X 2 weeks orally after breakfast or meals. Since our previous study with same dosage of turmeric oil, 0.2 ml capsules had shown safety upto 3 months and the earlier study in oral submucous fibrosis (OSMF) had also shown safety (Hastak *et al*, 1997; Joshi *et al*, 2003), this study with supercritical extract (NBFR-03) was restricted to 1 month.

Clinical study for chemopreventive activity in women with LSIL

Clinical setting: Women were enrolled from an outpatient Pap smear screening programme for women at the Medical Research Center- Kasturba Health Society and the joint leucorrhoea clinic at Ayurved Mahavidyalaya Hospital, Sion. After screening they were advised treatment of infections; partner treatment was ensured in sexually active women.

Collection of Pap smears, micrometry, colposcopy: Pap smears were collected from each women with a disposable spatula and endocervical brush and fixed immediately. Women with abnormal or unsatisfactory smears were advised repeat smears after treatment of genital infections. The Bethesda classification was followed for Pap smear diagnosis and infections were reported as has been described earlier (Joshi *et al*, 1993; Palayekar *et al*, 2000; Joshi *et al*, 2001; Paradkar *et al*, 2010). Colposcopy was carried out routinely in all cases with abnormal smears.

Micrometry was carried out in cases with persistent LSIL under oil immersion lens with the micrometer as used earlier and as described by other authors (Paradkar *et al*, 2010; Bollmann *et al*, 2001; Steinman *et al*, 2008).

Treatment of genital infections: This was as per the international guidelines modified for Indian population in a general hospital (CDC, 2006; NIRRH, 2006) and if repeat smears showed persistent LSIL (Joshi *et al*, 2010) they were interviewed for participation in the research project.

Twenty one women with inclusion/ exclusion criteria as given below, and giving an informed written consent participated in the study.

Inclusion criteria: 1) Persistent LSIL in Pap smears 2) Willingness to participate in the study and follow the schedule 3) Age between 25 and 65 years 4) Initial blood tests as described above for healthy volunteers, with additional serological tests for Human Immunodeficiency Virus antibodies, for serum *Treponema pallidum* antibody, and routine urinalysis within normal limits

Exclusion criteria: 1) High grade abnormal lesions in Pap smears 2) Pelvic inflammatory disease 3) Associated gynecological pathology like ovarian tumor, prolapse, polyp etc, 4) Uncontrolled systemic disease, 5) Food or drug allergies

Follow up: Examination was advised after every 2 weeks or anytime they had complaints, however if they were not able to meet the schedule they informed in advance and collected extra drug supply for 2 weeks. However the weekly examinations were not missed. Drug compliance was recorded. All participants did report at 4, 8 and 12 weeks with a margin of 3 - 5 days depending on their menstrual cyclicity.

Serum IL-6 : Serum IL-6 was measured using a commercial kit (Biosourse IL-6 EASIA kit; KAP 1261) as described earlier (Paradkar *et al*, 2010) in study cases before and after treatment with NBFR-03 using an enzyme immunoassay standardized in our laboratory with intra-assay and inter-assay coefficients of variation <5%.

Dose: I capsule with 0.2 ml of standardised Turmeric oil extract (NBFR-03) , twice daily, after breakfast and dinner for 12 weeks.

Results

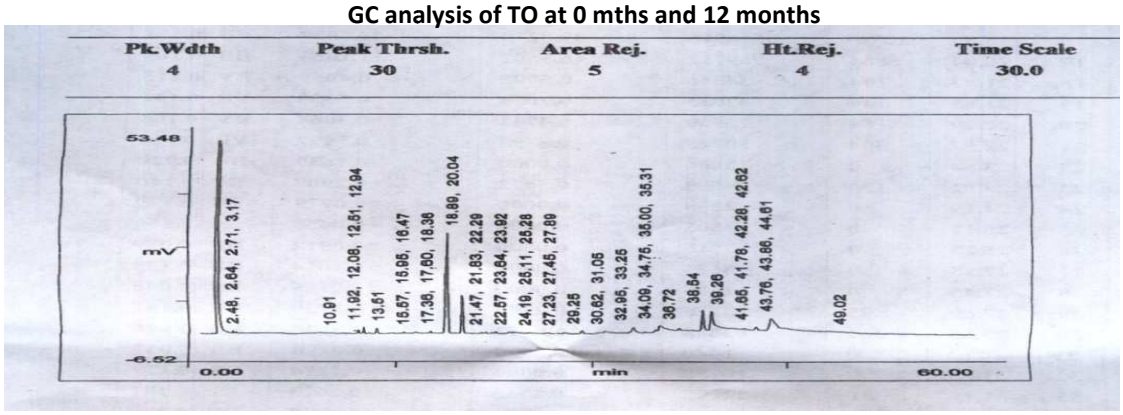
Formulation development & standardization

The heavy metal content (mercury, lead , arsenic, and cadmium) and 20 pesticides including those used commonly in the area of cultivation of the plant, *C. longa*, from where it was collected .The microbial load and aflatoxin were within the permissible limits (data not shown), as required by World Health Organization and Central Council for Research in Ayurveda and Siddha , so also the manufacture of capsules, shelf life and labeling as per Good Manufacturing Practices.

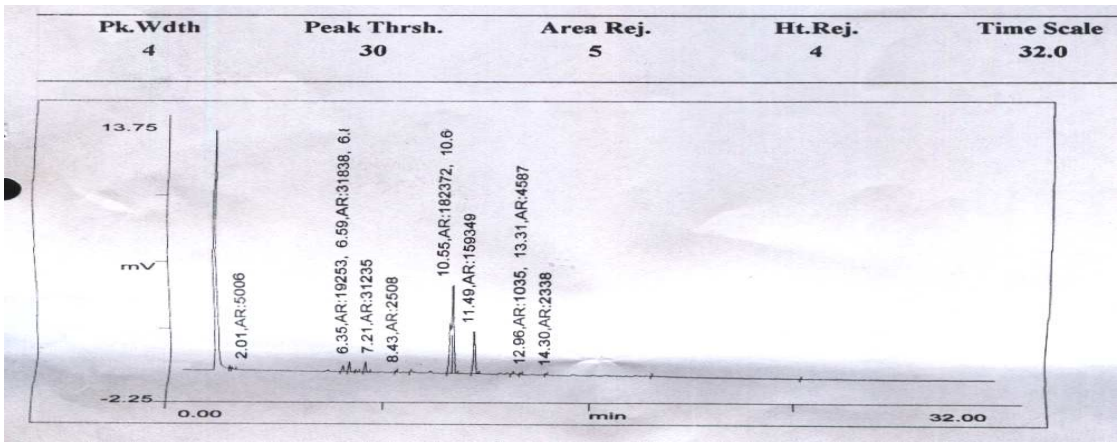
Figure 1. Gas chromatographic analysis of NBFR-03 capsule extract initially and after 12 months.

0 mths

GC analysis of TO at 0 mths and 12 months



12 months



Healthy Volunteer Study

There were no significant symptoms or signs or any biochemical alterations in 6 healthy volunteers due to NBFR-03 intake. The mean (±SD) weight of the volunteers was 60.6±9.39kg before starting treatment and 60.08±9.06kg after 4 weeks of treatment. The mean systolic and diastolic blood pressures of volunteers were 115/76.7 mm Hg and 108/75 mm Hg respectively (paired t test; NS). There were no significant differences between the haematological parameters, liver functions, renal functions, lipid profile, serum proteins, fasting sugar and urinalysis in volunteers before and after NBFR-03 treatment.

Results of screening programme

A total of 1473 women were participated in the screening programme. Prevalence of Low Grade Squammous Intraepithelial Lesions (LSIL) was 6.49 %, High -Grade Lesions (HSIL), 1.19 % and invasive Squammous Cell Carcinoma, 0.63%. Out of these 88 cases with LSIL only those with persistent LSIL after

antimicrobial therapy as described earlier (Joshi *et al*, 2010) were invited to participate in the study and 21 were included for study as per the selection criteria. Methods included clinical examination, Pap smears, colposcopy , clinical biochemistry, urinalysis, and serum IL-6 measurement, before and after treatment. Standardised NBFR-03 (0.2ml) capsule was administered, orally twice daily after breakfast and dinner, for 12 weeks.

Clinical assessment

Mean (SEM) age and parity of these cases were 41±2.5 and 1.78±0.020. They all belonged to the lower socioeconomic group. There was no significant difference in the mean blood pressure and weight of these cases before and after treatment (paired t test;NS).

Symptomatology: These cases were already treated with antimicrobials and were generally asymptomatic. Out of 21 cases enrolled for the study, 1 discontinued within 1 week on her own due to mild itching, mainly in genital area, without any rash. Another case left for native place due to personal emergency and so did not start the medication. In 1 case treatment was discontinued after 8 weeks as she too had to go out of station. Two cases discontinued after 4 weeks due to haemorrhoids, or suspected allergic reaction. The remaining 16 cases completed 12 weeks of treatment. No abnormal clinical changes were observed in the general examination, systemic examination and gynecological check up. Three cases reported occasional aroma of turmeric in eructations , and 3 cases reported improvement in skin complexion after 4-8 weeks.

Compliance: Except in cases who discontinued for reasons described above, drug compliance was more than 95% as assessed by history and counting of the balance number of capsules returned at each visit.

Clinical biochemistry: The mean haemoglobin was 11.7±0.36gm/dl and 11.6±0.30gm/dl before and after study (paired t test NS). The blood counts and organ function tests did not change significantly during the treatment period (data not shown).

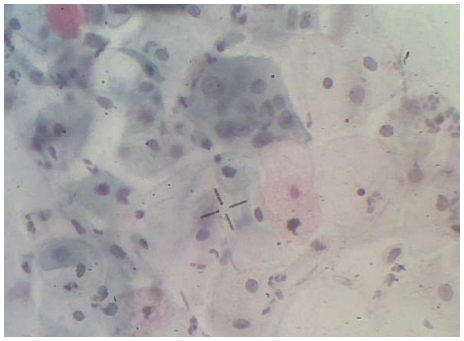
Pap smears, colposcopy and cytometry

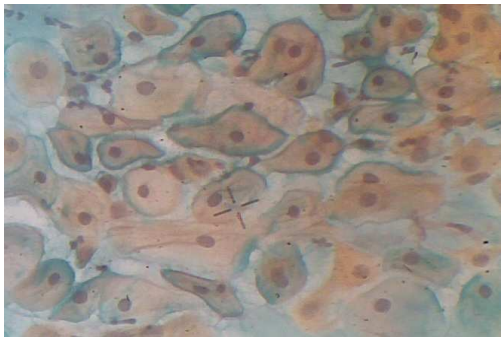
None of the cases, who had persistent LSIL after antimicrobial treatment, progressed to HSIL or cancer during 12 weeks of therapy as assessed by Pap smear and colposcopy (Table 1). Out of the 19 cases who had evaluation before and after therapy 16 regressed to lower degree of abnormality or negative status Three showed persistence of LSIL but of a minimal borderline nature. Colposcopy showed changes of CIN 1 in 10 out of 19 suggestive before treatment, and in 4 out of 19 cases after treatment (Figure 2 a,b).

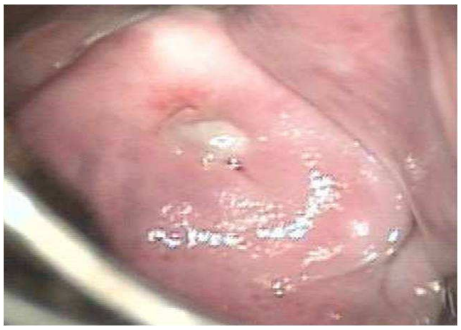
Figure 2: Pap smear & colposcopy in Case no 2 a) Persistent LSIL after antimicrobial treatment of infection; b) ASCUS after treatment with NBFR-03

a) Before treatment with NBFR-03

b) After treatment with NBFR-03







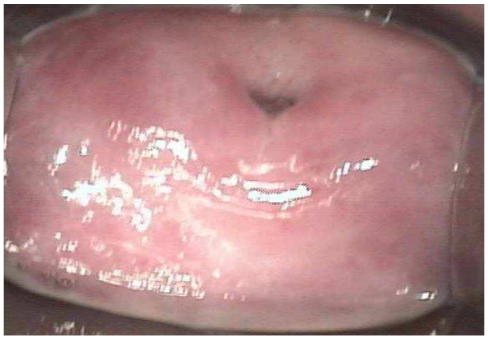


Table 1: Pap smear and colposcopy in 19 cases before and after treatment with NBFR-03

	Pap smear*			Colposcopy**	
Report	**Before treatment**	**After treatment**	**Report**	**Before treatment**	**After treatment**
LSIL	19	4	**CIN II**	—	—
ASCUS	—	4	**CIN I**	10	4
Atypia	—	5	**? CIN I**	3	6
Inflammatory	—	5	**Negative**	6	7

* One patient discontinued after 1 week.

** Not possible in one case after treatment

There was a significant reduction in nuclear diameters ($p<0.02$), Nucleocytoplasmic (N/C) ratios ($p<0.05$) and percentage of cells with coarse chromatin ($p<0.01$) in these cases after treatment with NBFR-03(Table 2).

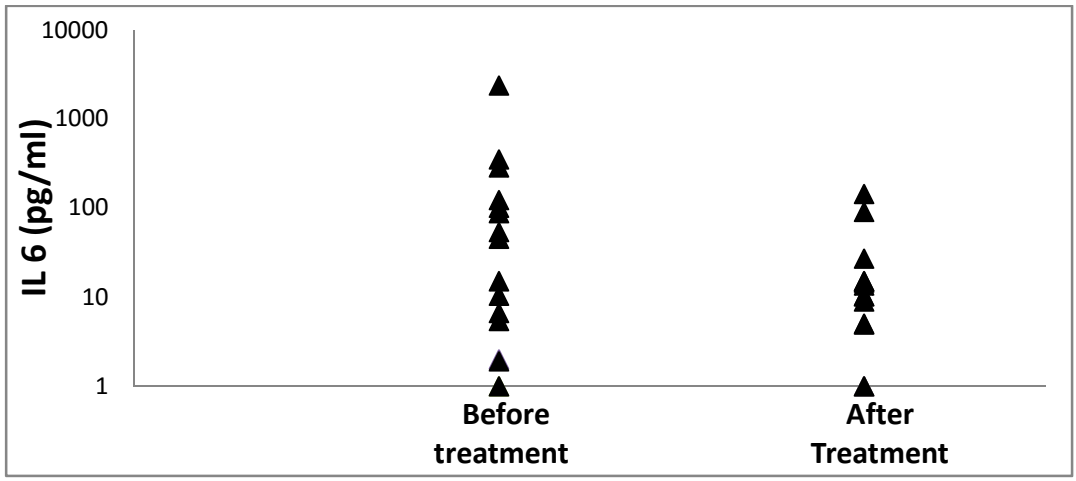
Table 2: Nuclear diameter, Nucleocytoplasmic (N/C) ratio and % cells with coarse chromatin before and after treatment (N=18)

	Nuclear diameter*		N/C Ratio**		% cells with Coarse chromatin***	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
Mean	13.52	12.14	0.28	0.23	12.6	6.89
SD	1.96	1.72	0.06	0.05	9.43	7.65
SEM	0.46	0.41	0.01	0.01	2.22	1.80

*Paired t test: p value *<0.02; **<0.05; *** p<0.01*

Serum IL-6 levels: The mean serum levels were 248.5 ± 156.4 (SEM) pg /ml before treatment and 27.7 ± 10.5 (SEM) pg /ml after treatment with NBFR-03 in LSIL cases (N=16) ($p< 0.05$, Wilcoxan Rank Test, 2 tailed). However in 8 LSIL cases serum levels were within normal limits initially. In one of these cases there was a rise of 50 pg/ml in 3 months.

Figure 2:. Serum IL-6 levels in 16 cases of LSIL before and after treatment with NBFR-03, 0.2 ml BD up to 3 months



Before vs After- $p<0.05$, Wilcoxan rank test, two tailed.

Long term follow up: Out of 19 cases 13 have come for follow up from 6 to 36 months after discontinuation of NBFR-03 treatment. They have not had any other treatment in the intervening

period. Their Pap smears show that 9 cases have remained in the regressed stage, 3 continued to be in the LSIL stage, and 1 regained LSIL status, however none progressed to higher grade. These findings were confirmed by colposcopy.

Discussion

Cervical cancer is the terminal event in the process of carcinogenesis which usually starts in the basal epithelium of cervix, commonly at the squammocolumnar junction. There are multiple changes which may occur sequentially or simultaneously which cause dedifferentiation and uncontrolled proliferation before the invasive stage is attained. In majority of cervical cancers this process involving initiation, promotion and invasion may take from 10 to 13 years (Weinberg, 2007). There is therefore ample opportunity for chemoprevention. Although several agents have been used in chemoprevention trials such as retinoic acid derivatives, indole-3 carbinol, α-difluoromethylornithine (DMFO), refocoxib, resveretrol, cisplatin, panex-ginseng-3 extract, curcumin, selenium, 5-fluorouracil, epigallocatechin and folic acid, none has shown total arrest or reversal (Bell *et al*, 2000; Cheng *et al*, 2001; RelaFollen *et al*, 2002; Alvarez *et al*, 2003; Abu *et al*, 2005; Kim *et al,*2005; Sasieni,2006; Hefler *et al*, 2006;Gullett *et al*, 2010, Bar – Sela *et al*,2010).

The mechanisms through which these agents may act are varied and extend from the stage of initiation to invasion and many agents are also used for palliation or for complementary therapy to prevent side effects of chemotherapy or radiotherapy. Turmeric products have been shown to have multiple mechanisms such as antioxidant, anti-inflammatory activity, inhibition of NFkB pathway, inhibition of telomerase , isotopomerase and several other pathways contributing to genetic mutation, inhibition of apoptosis and uncontrolled mitosis (Hastak , *1997;* Ravindran, 2007; Garg *, 2008* ; Pardo-Govea, 2005). Recently HPV specific pathways such as HPV transcription and AP-1 activity and downregulation of viral oncogenes have been shown to be inhibited by curcumin (Prusty and Das*,* 2005; Divya , 2006).

In the present study none of the cases progressed to a higher degree of abnormality during 12 weeks of therapy. This is very encouraging. Additionally there was regression in 16 cases which was maintained in the post-therapy follow up except in 2 cases, thus indicating that the pretreatment of genital infections followed by NBFR-03 therapy is likely to have a long lasting effect. In 5 cases there was total regression to a mild inflammatory pattern, and in 5 cases, mild Atypia, which was maintained post-therapy. Colposcopy findings corroborated the cytology findings, particularly in excluding a higher grade lesion which require a cervical biopsy for evaluation.

Interleukins-6

The role of various Interleukins such as IL-8 and IL-10, IL-12 has been elaborated by several authors, particularly in cervical cancer cell lines. The interleukins act directly on cancer cell receptors to promote mitosis, to inhibit apoptosis, and promote angiogenesis by attracting other leucocytes which produce growth factors. Thus they can influence each stage of carcinogenesis including infiltration and

metastasis. IL-6 is a 26 KD glycoprotein encoded by the gene on chromosome 7p21-p14. Elevated levels of IL-6 are associated with a poor prognosis in lymphomas and multiple myeloma and a decline is associated with partial remissions (Murooka,2009). Serum levels of IL-6 are reported to be elevated in gynecological cancers and precancer. IL-6 levels are known to be elevated in Pap smears, in cervicovaginal fluid and in circulation, in cervical biopsy specimens cervical intraepithelial neoplasia and in LSIL as well as HSIL (Tjiong MY *et al*;1999; Pardo-Govea *et al*,2005; Heikkila *et al*,2008; Paradkar *et al* ,2010). In the rat model of chemically induced cervical carcinogenesis it is shown that IL-6 is increased in cervical biopsy sections and in circulating blood as the degree of precancer advances and as the lesion becomes invasive (Bustamam *et al*,2008). In our previous study the mean ± SEM level of serum IL-6 in cases with inflammatory Pap smears was 15.9 ± 17.05 pg/ml and 14.2 ± 8.44 before and after treatment of infections with antimicrobials (Paradkar *et al*, 2010). In the present study it was observed that the circulating IL-6 levels in LSIL cases were high, 248.5 ± 156.4 (SEM) pg /ml, even after treatment of genital nonviral infections. Significant reduction was however observed after treatment with NBFR-03, bringing them to the range of cases with inflammation alone. This may signify a reversal of the progressively chronic inflammatory process in these cases. NFkB is activated in carcinogenesis. It induces the expression of IL-6 which is an autocrine factor secreted by transforming cells for their growth and survival. Additionally NFkB also plays an important role in the antiapoptotic signaling in a number of cancer cell types. Inhibition of NFkB could be a major contributory mechanism of anticancer activity of bioactive turmeric compounds. NFkB also activates TNF-alpha gene which is a growth factor. COX-2 inhibition is another possible mechanism of anticancer activity, as is shown with curcumin. These pathways need to be investigated for elucidating the exact mechanism of action (Divya and Pillai,2006, Ravindran *et al*, 2007).

Chronic infection with High Risk HPV is a known carcinogenic initiator for cervical cancer. However all cases with HR HPV do not undergo transformation. Gangawar *et al* (2009) have also shown the association of IL-6 gene muatation with cervical cancer and this may explain why some cases with HR-HPV progress to cancer whilst others do not. The suppression of IL-6 can be one of the mechanisms of action of NBFR-03. In *in vitro* studies with cancer cell lines it is shown that there is loss of soluble receptor in transformed cells hence it is possible that IL-6 inhibiting agents like NBFR-03 may not be useful in advanced or invasive cancer, but the receptors are likely to be intact in the preinvasive phase as shown by the suppression of circulating IL-6 levels in the present study thus indicating the possibility of chemoprevention. Like curcumin NBFR-03 may also act through other mechanisms which need further exploration. Recently anticancer activity of essential oil of *Curcuma longa* or Turmerone has been reported *in vitro or in vivo* (Aratanechemuge *et al*, 2002; Li *et al*, 2009; Lim *et al*, 2010; Yue *et al*, 2010; Chen *et al*, 2011).

The follow up in 13 cases shows that the combined effect of treatment of STIs and NBFR-03 is likely to persist and indicates to investigate this compound further in cervical cancer chemoprevention.

Limitations of the present study

Although it is desirable to study local IL-6 levels this was not possible in the present study as this requires expensive equipment and standardization. On the other hand measurement of circulating IL-6 is standardized in our laboratory. No cervical biopsy was carried out in study subjects as repeat Pap smears after treatment with antimicrobials, in all of them showed persistent LSIL confirmed by colposcopy. Further , after treatment with NBFR-03 for 12 weeks, there was arrest or regression of the Pap smears in all cases.

Conclusions:

The administration of NBFR-03 was associated with arrest of progression in all 19 LSIL cases who took the treatment for 1 to 3 months, and with regression in 16 cases , thus indicating potential chemopreventive activity. An anti-inflammatory activity associated with reduction in circulating levels of IL-6, a potential biomarker for chemopreventive clinical trials, could be one of the mechanisms of action of the NBFR-03 Turmeric oil extract.

Acknowledgments: We thank 1) Kasturba Health Society, Wardha, Maharashtra, for infrastructure support, 2) Department of Biotechnology, Govt of India , for research project grant and 3) Ayurvidya Prasarak Mandal , Sion for Ayurvedic collaboration

Authors' Declaration: The center received a Research Grant from the Department of Biotechnology and the Turmeric oil capsules were supplied by Nisarga Biotek Private Limited, Satara. Part of the data was presented at the 4rth World Ayurveda Congress (WAC4), Benagaluru, India, in December , 2010 and is accepted for presentation in the Asia Pacific Organization for Gynecological Infections & Neoplasia (Indian Chapter), November 2011, Mumbai.

Conflict of interest: The centers received a research grant from the Department of Biotechnology , Government of India. Nisarga Biotech Pvt Ltd provided the formulation required for the study.

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