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A comparative study between Anti-Clastogenic synergestic effect of Ursolic Acid, Beta sitosterol and Sitosterol-3-O glucoside (Drug I) and only Ursolic Acid, Beta sitosterol (Drug II) on human chromosomes.

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ABSTRACT
Introduction:
In the current context of globalization, cancer is costing millions of life worldwide, and commercially available allopathic anti-cancer drugs have many side effects. Search for a phyto-active (ayurvedic) component acting cornerstone for anticancer agent will be a boon to mankind. In our previous studies, we reported for the first time, potent anti-cancer properties in leaf extract from Barleria lupulina Lindl. Ursolic acid, beta-sitosterol andsitosterol-3-O-glucoside were found to be the active components. Even though a substantial work on ursolic acid and beta-sitosterol have been done by many investigators, but studies on sitosterol-3-O-glucoside is limited. The present comparative study was carried out to find the anti-caner property of only sitosterol-3-O-glucoside on human chromosomes in vitro.
Methods:
The formulation containing active ingredients - Ursolic acid , beta sitosterol and sitosterol-3-O-glucoside were mixed in different proportions and treated to gamma-irradiated human lymphocyte chromosomes in vitro. Percentages of chromosomal aberrations were elucidated at different time intervals. Various statistical tools were used to analyze the data.
Results & Discussion:
Convincing results were obtained, that synergistic effect of Ursolic acid , beta sitosterol and sitosterol-3-O-glucoside was demonstrating more amelioration effect (54.25% recovery) than only the combination of Ursolic acid and beta sitosterol. Even, the formulation was able to cure squamous cell carcinoma of human lungs, in vivo. This may stand out to be a potent anti cancer drug in near future.

Index Terms: *anti-cancer, human chromosome, mutagen, ayurveda, sitosterol-3-O-glucoside*

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I. Introduction

Cancer is a life threatening disease, and is the cause of death of millions of people all over the globe. According to the World Health Organization, in 2008 approximately 7.6 million people died worldwide due to cancer and in 2018 (after ten years) the mortality number is 9.6 million (current statistics) (WHO: Cancer: Overview). Anti-cancer drugs currently available as chemotherapeutic agents, not only destroy cancer cells, but also kill normal cells and leave a heavy toll on patients with severe side-effects (Krukiewicz K & Zak JK., 2016) Therefore, discovery of an anticancer drug with minimum side effects but satisfyingly potent preferably from plant origin needs immediate attention of researchers.

Chromosomal aberrations lead to tumor formation and cancer (Donna GA,ColinCo, FrankMcC, JoeWG.,2003). Chromosomal aberration is used as an index of cancer. The pioneering discovery by Muller(1927)on artificial mutagenesis in *Drosophila* opened a new horizon to the cytogenetic study of chromosomal aberrations.

In our previous works (Sur P.K. & Das P.K., 2012), we reported the anti-clastogenic, anti-cancer, radio-protective activities of leaf extract from *Barlerial upulina* Lindl. on laboratory animals as mice and fish. Further isolation of phyto-active molecules from the leaf extract, proves the presence of ursolic acid, beta-sitosterol, and sitosterol-3-O-glucoside as major anti cancer agents (Das P.K. & Sur P.K., 2012)

In the present invention, the author studied the anti-cancer effect of sitosterol-3-O-glucoside alone. A comparative study between anti-clastogenic &anti cancer-synergestic effect of UrsolicAcid+Beta

sitosterol+Sitosterol-3-O glucoside (Drug I) and only UrsolicAcid+Betasitosterol (Drug II) on human chromosomes (*in vitro*) was performed to study the convincing anti cancer role of only Sitosterol-3-O glucoside. Chromosomal aberrations were induced by 1.2Gygamma (γ) radiations *in vitro*.

II. Materials And Methods

a. **Preparation of the formulations:**

The present invention is concerned with the formulation of an anticancer drug Ursolic acid (≥90% purity), beta sitosterol (synthetic ≥ 95%) and sitosterol 3 O glucoside (analytical standard) were all purchased from Sigma-Aldrich®. They were mixed in different proportions each in a suitable solvent containing Water for Injections. Two formulations were prepared viz. Drug I= UrsolicAcid+Beta sitosterol+Sitosterol-3-O glucoside in the ratio 3:2:1 and Drug II= UrsolicAcid+Betasitosterol in equal proportions.

b. **Experimental Protocol:**

Human chromosomes were prepared from blood lymphocytes *in vitro*. The samples were divided into three groups SET I, SET II and SET III, and the experimental protocol is shown in Table 1 below

Table 1: Experimental Protocol

SET I	Lymphocytes post treated with DRUG I – γ radiation (1.2 Gy) from Cobalt ($_{27}Co^{60}$)
SET II (CONTROL) Cobalt ($_{27}Co^{60}$)	Lymphocytes exposed to γradiation (1.2 Gy) from
SET III	Lymphocytes post treated with DRUG II γ radiation (1.2 Gy) from Cobalt ($_{27}Co^{60}$)

Mitotic chromosomes from lymphocyte cells were studied for each set after 1 hr, 24hr, 48 hr and 1 week of treatment.

III. Results

a. Comparison between effect of DRUG I and DRUG II w.r.t. CONTROL

200 cells for each hour for each SET were studied in these series (total 800 cells and therefore 36800 chromosomes in each SET). Different types of structural chromosomal aberrations were studied and the study was done at various time intervals, viz: 1 hr, 24hr, 48 hr and 1 week [Table 2, Fig 1 and Fig 2].In case of CONTROL, the frequency of chromosomal aberrations increased from 1 hour to 24 hours, was maximum at 24thhour (66.28%) and then decreased there after upto 1 week. On the other hand, less frequency of aberrations was observed for treatment with DRUG I and DRUG II [Table 2, Fig 1, Fig 2]. At 24thhour, chromosomal aberration of 30.32% (54.25% recovery) was obtained with DRUG I whereas 48.07% with DRUG II (Table 2, Fig 1). Moreover, when compared with types of aberrations, much higher percentage of aberrations was observed with the CONTROL set (as 9.23% for sub chromatid gap, 6.69% with isochromatid gap, 3.05% with chromatid break, etc). But much less aberrations had been scored with DRUG II (as 7.97% with subchromatid gap, 3.74% with isochromatid gap, 1.53% with chromatid break; etc.) and even lesser were scored with DRUG I (as 4.45% with subchromatid gap, 1.15% with isochromatid gap, 1.35% with chromatid break, etc.) (Table 2, Fig 1). In every type of chromosomal aberration, it is observed that lesser aberrations are obtained with DRUG I than that compared with DRUG II and the CONTROL series (Table 2, Fig 1).

b. Statistical Analysis (Comparison between DRUG I and DRUG II treatment

To evaluate the effectiveness of sitosterol 3 O glucoside as an anti clastogenic and anti cancer agent, statistical analysis was done according to methods mentioned by Snedecor G.W. & Cochran W.G.(1967).

Analysis of the data reveals, that, in the pooled data, the Standard Error (SE), Critical Difference (CD) at5%, CD at 1% levels for DRUG I treated set are 68.25, 133.11 and 176.11 respectively. And that for DRUG II are 124.87, 244.74 and 322.16 respectively. **Therefore, the values shown by DRUG I are always less than the DRUG II which illustrates that less chromosomal aberrations were achieved by treatment with DRUG I, and it is more effective**. To compare the significance between treatment of the two drugs, t-Values, Chi-square values and r-Values are analyzed which are 110.11**, 36.984** and 0.971** respectively which are significant at 1% level (highly significant). Therefore, treatment with DRUG I reflects significantly high protection than that with DRUG II.

Moreover, Biopsy of lungs tissue revealed nests of polygonal cells and airways in between the tissues confirming carcinogenesis. Also, cells stacking together giving rise to non small cell lung cancer is prominent in the slides (Fig 3). It was a randomized, non-placebo treatment. DRUG I and DRUG II were administered twice

daily orally post prandialy, to two different groups. After approximately four months of treatment, hemoptysis decreased to once in alternate day, respiratory distress was much less and weight was also gained (from average 40 kg to average 60 kg), and among both the treatments DRUG I (ursolic acid: beta sitosterol: sitosterol 3 O glucoside- 3:2:1) was found to be the most effective composition. Tissue biopsy disclosed that the cells were normal in size, indicating cure (Fig 4).

IV. Discussion

Ursolic acid is a penta-cyclic triterpenoid compound and is a major component of traditional medicinal herb as *Hedyotisdiffusa*, *Eribotrya japonica* and *Ligustrumlucidum*(Lai *et al.,* 2007). This triterpenoids have shown hepato-protective (Liu 1995), antiallergic (Bannoe*t al.,* 2004), anti-ulcer (Ovesná*et al.,* 2004), cardioprotective (Senthil*et al.,* 2007), antimicrobial (Ngouela*et al.,* 2005), anti-inflammatory, analgesic (Vasconcelos*et al.,* 2006) and anti oxidant (Huang *et al.,* 1994) activities. It has potent anti tumor activity also (Liu 2005; Ovesná*et al*., 2006, Pengcheng L., *et. al*. 2020). Even, in 2015, Chen H.and colleagues. had reviewed the structural diversity and evolution in chemistry of ursolic acid and emphasized on the future direction on research on this potent natural anti cancer agent. In our previous studies, the presence of ursolic acid in our extract of *Barleria lupulina* has shown anti-clastogenic, anti-tumor, anti-cancer and radio-protective activities on fish and mice (Das and Sur 2012).

β-sitosterol is one of the phytosterols that has structure similar to cholesterol. This phytosterol is active against numerous types of cancer (Novotny L., Abdel-Hamid M.E., Hunakova L. (2017)) as prostate cancer, lung cancer, colon cancer, breast cancer, ovarian cancer and even leukemia, by interfering various types of cell cycle pathways as apoptosis, metastasis, etc. (Bin Sayeed M.S. & Ameen S.S. (2015))Even, this sterol is used in the treatment of benign prostatic hyperplasia (BPH) (Bergese*t al.,* 1995) and prostatic carcinoma (Stephen *et al*.,2005). βsitosterol was also found to be present in leaves of *Barlerial upulina* of our previous work (Das P.K. and Sur P.K. 2012).

But, work on anti cancer property of sitosterol-3-O-glucoside is limited. Maiyo and colleagues (MaiyoaF. *et.al.* 2016) reported the anti cancer property of sitosterol-3-O-glucoside (isolated from *Prunusafricana*) on two human cancer cell lines (hepato-cellular carcinoma (HepG2) and colorectal carcinoma (Caco-2). Mulukuri and associates reported the anti-leukemic effect of β-sitosterol-3-O-glucoside isolated from *Evolvolusalsinoides* in2017 (SirishaMulukuri N.V.L., Singh O., Baenerje S. (2017)). Gohar*et al*., (2009) had stated that methanolic extracts of seeds of *Ceratoniasiliqua* L. has a rich source of natural anti oxidants, which contains β sitosterol-3-O-glucoside along with other flavonol glycosides.

In the present study, a comparison between the anti-clastogenic synergestic effect of Ursolic Acid, Beta sitosterol and Sitosterol-3-O glucoside (Drug I) and only Ursolic Acid, Beta sitosterol (Drug II) on human chromosomes was done to evaluate the effectiveness of only Sitosterol-3-O glucoside. To compare the significance between treatment of the two drugs, t-Values, Chi-square values and r-Values are analyzed which are 110.11**, 36.984** and 0.971** respectively which are significant at 1% level (highly significant). (Table 3). Moreover, frequency of chromosomal aberrations has reduced from 66.28% to 30.32% (54.25% recovery) after treating with DRUG I at 24 hrs time interval, which is highly significant. Such recovery was not obtained with DRUG II. (Fig2). When compared with different types of chromosomal aberrations, DRUG I showed quite higher recovery, than DRUG II (Fig 1).

Therefore it may be declared that synergistic effect of Beta seto sterol + ursolic acid + sitosterol 3 O glucoside has significantly more anti-clastogenic effect than Beta seto sterol + ursolic acid, and that sitosterol 3 O glucoside plays a major role here.

Patent had already been applied (Patent No: 268/KOL/2015) and it is under process.

Table 2: Various chromosomal aberrations for treatment with DRUG I and DRUG II at different time intervals

Time interval	Animal SET	No. of cells	No. of chromosomes	Types of aberrations									Total Aberrations	Percentage of Total Aberrations
				chromatid break	iso chromatid gap	iso chromatid break	sub chromatid gap	centromeric dissociation	translocation	ring chromosome	rabbit ear chromosome	rabbit ear chromosome with gap		
1 hour	DRUG I	200	9200	54	39	428	212	28	34	17	38	30	880	9.57 %
	CONTROL	200	9200	68	72	263	421	216	64	24	51	48	1227	13.34 %
	DRUG II	200	9200	49	48	116	416	112	42	21	21	20	845	9.18 %
24 hour	DRUG I	200	9200	171	203	529	1105	524	121	47	64	25	2789	30.32 %
	CONTROL	200	9200	529	1227	570	1531	1217	195	112	201	516	6098	66.28 %
	DRUG II	200	9200	239	1186	532	1216	728	136	124	115	146	4422	48.07 %
48 hour	DRUG I	200	9200	137	164	172	217	111	18	21	171	176	1187	12.90 %
	CONTROL	200	9200	250	981	482	1270	198	57	84	221	227	3770	40.98 %
	DRUG II	200	9200	186	118	182	1165	168	87	129	208	101	2344	25.48 %
1 week	DRUG I	200	9200	134	19	48	105	65	97	53	39	97	657	7.14 %
	CONTROL	200	9200	276	182	27	174	351	94	37	86	76	1303	14.16 %
	DRUG II	200	9200	88	24	27	136	54	28	36	69	108	570	6.20 %
Total Aberrations	DRUG I	800	36800	496	425	1177	1639	728	270	138	312	328	5513	14.98 %
	CONTROL	800	36800	1123	2462	1342	3396	1982	410	257	559	867	12398	33.69 %
	DRUG II	800	36800	562	1376	857	2933	1062	293	310	413	375	8181	22.23 %
Percentage of Total Aberrations			DRUG I	1.35	1.15	3.20	4.45	1.98	0.73	0.38	0.85	0.89		
			CONTROL	3.05	6.69	3.65	9.23	5.39	1.11	0.70	1.52	2.36		
			DRUG II	1.53	3.74	2.33	7.97	2.89	0.80	0.84	1.12	1.02		

DRUG I = Beta seto sterol + ursolic acid + sitosterol 3 o glucoside **DRUG II = Beta seto sterol + ursolic acid**

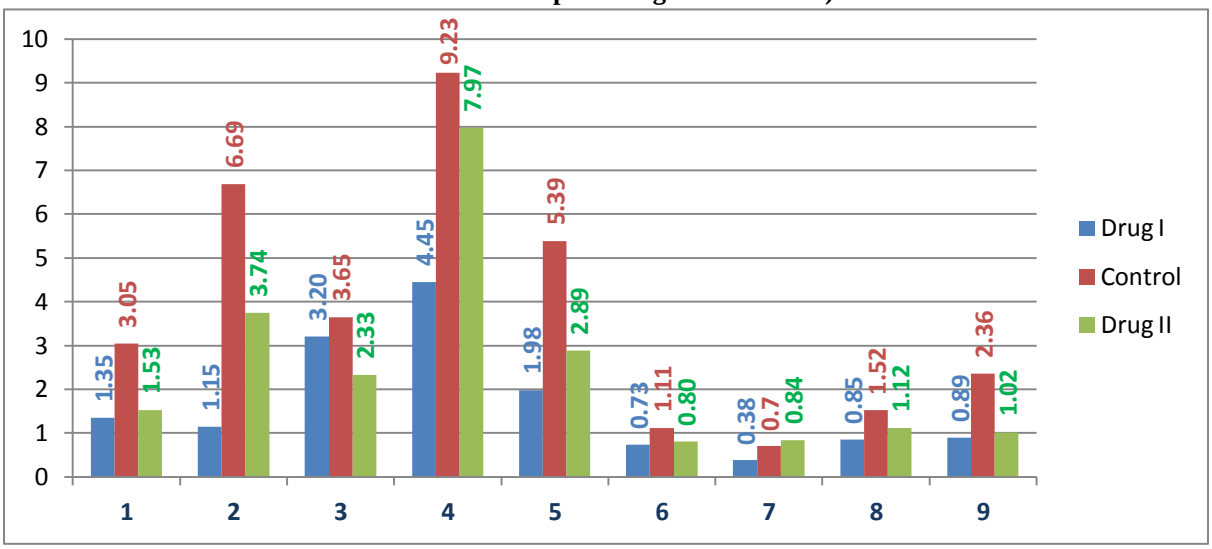
Table 3: Statistical Analysis to compare between DRUG I and DRUG II treated chromosomes

Aberration Statistics	Chromatid break	Iso chromatid gap	Iso chromatid break	Sub chromatid gap	Centromeric dissociation	Translocation	Ring chromosome	Rabbitcar chromosome	Rabbitcar with gap	Pooled
DRUG I ± S.E.	3.51	6.43	15.74	32.98	16.31	3.49	1.28	4.47	5.01	68.25
DRUG II ± S.E.	6.18	39.79	15.64	38.23	22.05	3.45	4.03	5.63	3.75	124.87
DRUG I C.D. at 5%	6.87	12.61	40.61	64.64	31.95	6.84	2.51	8.75	9.81	133.11
DRUG II C.D. at 5%	12.11	77.99	30.65	74.93	43.22	6.77	7.89	11.04	7.74	244.74
DRUG I C.D. at 1%	9.05	16.61	40.61	85.08	42.05	9.01	3.31	11.52	12.91	176.11
DRUG II C.D. at 1%	15.94	102.66	40.35	98.63	56.89	8.91	10.39	14.54	9.67	322.16
t - Values	43.63**	29.53**	29.36**	21.26**	196.64**	7.13**	0.54	76.31**	2.82*	110.11**
Chi-Square (χ^2)Values	14.298**	47.737**	19.121	34.122**	18.455**	16.028**	21.385**	14.066**	17.903**	36.984**
r - Values	0.854**	0.757**	0.755**	0.657*	0.991**	0.331	0.035	0.941**	0.156	0.971**

* means significant at 5% level.

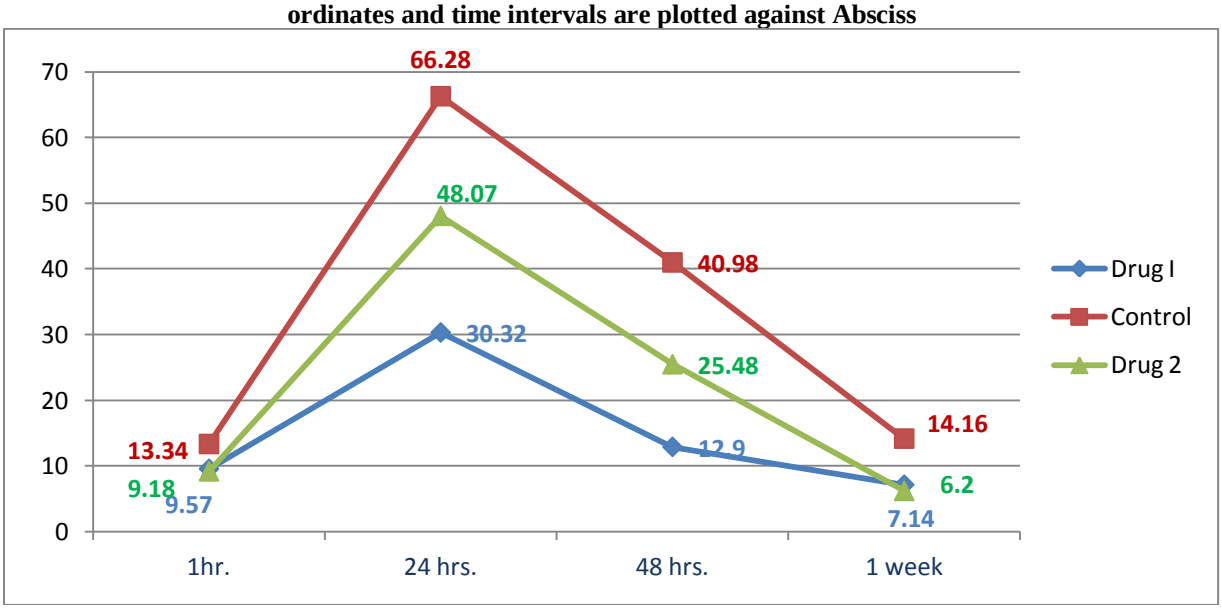
** means significant at 1% level- highly significant.

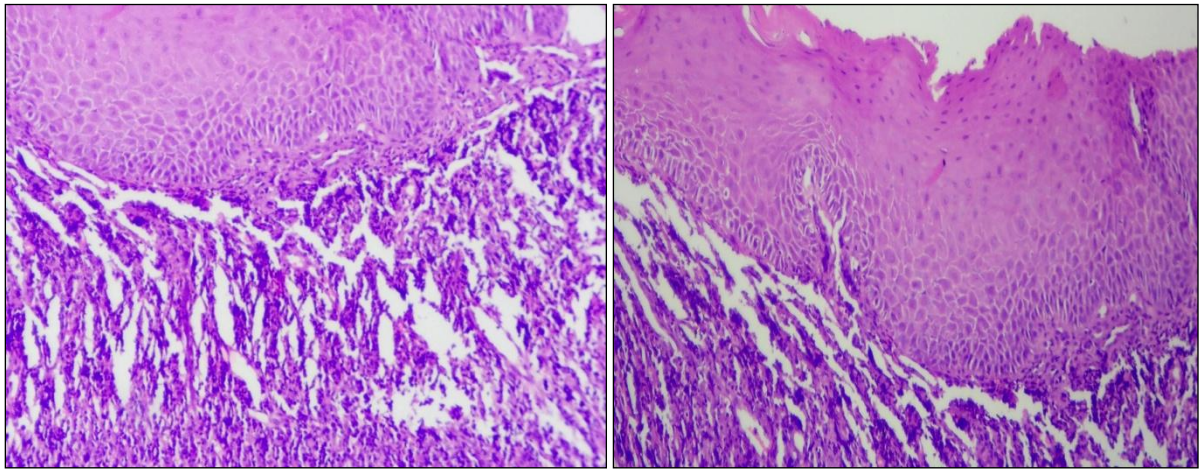
Fig 1: Comparative analysis of chromosomal aberrations after treatment with DRUG I, DRUG II and untreated CONTROL set (percentage of aberrations are plotted against the ordinates and types of aberrations are plotted against Abscissa)



1= Chromatid break, 2= Isochromatid gap, 3= Isochromatid break, 4= Subchromatid gap, 5= Centromeric dissociation, 6= Translocation, 7= Ring chromosome, 8= Rabbit-ear-chromosome, 9= Rabbit-ear chromosome with gap

Fig2: Comparative analysis of chromosomal aberrations after treatment with DRUG I, DRUG II and untreated CONTROL set w.r.t. different time intervals (percentage of aberrations are plotted against the ordinates and time intervals are plotted against Absciss





Nests of polygonal cells and airways in between the tissues

Cells stacking together giving raise to non small cell lung cancer

Fig 3: Squamous Cell Carcinoma of lungs

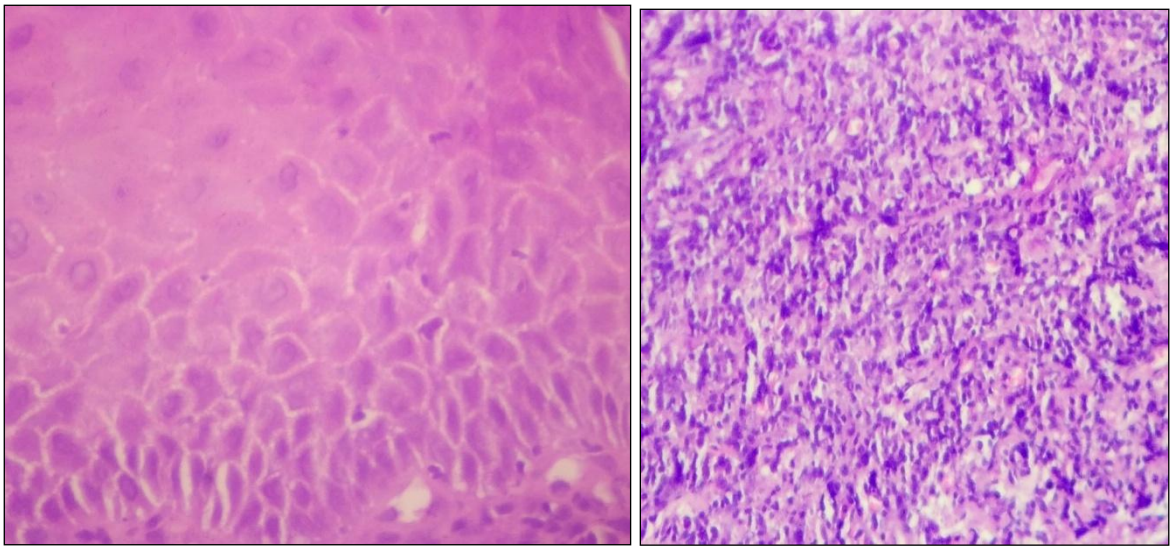


Fig 4: Healthy cells indicating cure

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