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In Vivo Anticancer Activity of *Snuhi Kshara* Against 1,2-Dimethyl Hydrazine Induced Colon Cancer

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ABSTRACT

Background- Colon cancer is the third most diagnosed cancer globally with incidence rate 1.15 million new cases. *Snuhi* (*Euphorbia neriifolia* Linn.) possesses anti-inflammatory, anti-ulcer, antioxidant, immunomodulatory, cytotoxic and anticarcinogenic properties as per previous studies.
Objective- The present study aimed to explore anticancer activity of *Snuhi Kshara* (SK), the formulation of *upavisha snuhi* against colon cancer in Wistar rats.
Materials and methods- 1,2-dimethylhydrazine (DMH) was used to establish colon cancer model in female Wistar Albino rats. The acute toxicity of *Snuhi Kshara* was tested in accordance with OECD guidelines. Change in body weight, hematological and biochemical parameter, colon length and weight, Aberrant Crypt Foci (ACF) count, histopathological findings with toxicity score were used to screen *In Vivo* anticancer activity.
Result- The oral LD_{50} cut off value of *Snuhi Kshara* was found to be 5000mg/kg body weight. *Snuhi Kshara* significantly ($p<0.05$) increase the body weight and colon length in treated group. *Snuhi Kshara* significantly ($p<0.05$) decrease the Total Cholesterol Level (TCL), colon weight, Aberrant Crypt Foci (ACF) count and histopathological toxicity score. *Snuhi Kshara* decreased the colonic injury induced by 1,2-dimethylhydrazine and helped to repair.
Conclusion- Based on the findings, it can be concluded that *Snuhi Kshara* possesses anticancer activity against DMH induced colon cancer. Further studies are required to determine the exact mechanism of action of *Snuhi Kshara*.

Keywords: *Snuhi* (*Euphorbia neriifolia* Linn.), *Snuhi Kshara*, Anticancer, Aberrant Crypt Foci (ACF)

INTRODUCTION

Cancer is a disease characterized by the uncontrolled division of abnormal cells. When this type of growth occurs in the colon or rectum, it is called as colorectal cancer (CRC). Colon cancer and rectal cancer are often grouped together due to many common features. Colorectal cancer is the third most diagnosed cancer globally with incidence rate 1,93,1590 (10%) and the second most deadly cancer with mortality rate 9,35,173 (9.4%).[1]

The advancement in awareness of CRC pathophysiology, increased treatment options and population-based screening of CRC have led to inhibition of cancer

progression and prolonged overall survival. Yet CRC remains most deadly cancer globally due to the unmet therapeutic strategy, screening programs and increasing incidence rates also exposure to environmental risk factors such as shifting lifestyle and diet towards westernization is attributed. These facts highlight the necessity of new researches on better prevention and treatment of CRC. [2]

Snuhi botanically identified as *Euphorbia neriifolia* Linn. is one among the *Upavisha*. *Snuhi* has been attributed with a number of synonyms depicting its morphological identifying characters and pharmacological activities. *Snuhi* possesses *Katu rasa, Katu vipaka, Ushna virya, Laghu- Tikshna guna, Kaphavatahara, Vedanasthapana, Lekhana, Tikshnavirechaka, Raktashodhaka, Shothahara, Kaphanissaraka, Twakado-shahara Karma*. [3] These Gunas are responsible for the pharmacological activities of *Snuhi*. About 462 formulations having *Snuhi* as an ingredient are used to combat almost 62 varied diseases such as *Gulma, Udara, Shotha, Vranashotha, Plihavikara, Dushivisha* etc., [4]

Snuhi extracts and isolated flavonoids possesses anticancer activity against HepG2 cytotoxicity, Murine F1B16 and B16F10 melanoma, K562, Panc-1, 81T and BE3, HCT116 (colon), MCF7, MDA-MB-231 (breast), K562 and HEL cell lines, EAC and DLA cancer, and renal and hepatocarcinoma in mice, which entitles *Snuhi* and its formulations towards the use as an anticancer drug. [5, 6, 7]

Snuhi Kshara (SK), a unique single drug formulation of *upavisha Snuhi*, described in classical texts *Rasatarangini* and *Ayurveda Sara Sangraha*. [8, 9] Despite the data, no study has been reported on the *In Vivo* anticancer activity of *Snuhi Kshara*, the formulation of *upavisha Snuhi* till date. Hence, present study was designed to evaluate the anticancer activity of *Snuhi Kshara* against colon cancer in Wistar rats.

MATERIALS & METHODS

The study for evaluation of anticancer activity of *Snuhi Kshara* (SK) against colon cancer in Wistar rats was carried out in following phases-

1. Pharmaceutical study
2. Analytical study
3. Experimental study

1. Pharmaceutical study

- Raw drug sample i.e., *Snuhi kashtha* was procured from field and authenticated at the Dravyaguna Department of the Institute and also at the Department of Botany (Rashtrasanta Tukdoji Maharaja Nagpur University, Nagpur, Herbarium No. 10563).
- Test drug i.e., *Snuhi Kshara* was prepared at *Rasashastra Evum Bhaishajya Kalpana* department in the Institute as per *Sharangadhara Samhita*. [10]

2. Analytical study

- Raw drug sample i.e., *Snuhi kashtha* was analyzed for Organoleptic parameters (color, odor, taste) and Physicochemical parameters (foreign matter, total ash, acid-insoluble ash, water and alcohol soluble extractive) as per API. [11]
- Test drug i.e., SK was analyzed for Organoleptic parameters (color, odor, taste), Physicochemical parameters (pH, loss on drying, acid-insoluble ash, sodium, potassium & iron assays) and Chromatographical parameter (TLC) as per API. [12]
- The study was performed at Qualichem Laboratories, Gokulpeth Market, Nagpur and Sheetal Analytical Laboratory, Sadashiv peth, Pune.

3. Experimental study

The study has been approved by Institutional Ethical Committee (IEC) and Institutional Animal Ethical Committee (IAEC) (CRY/2021/014)

prior to the commencement of animal experiment. The study was performed at Crystal biological solutions limited, Pune and accomplished according to CPCSEA guidelines (CPCSEA registration No. 2030/PO/RcBiBt/S/18/ CPCSEA).

Reagents and Chemicals

1, 2-Dimethylhydrazine (DMH) was procured from Sigma-Aldrich Chemicals Private Ltd., methylene blue solution and other solvents and chemicals were procured from standard laboratories.

Animal procurement

Female Wistar albino rats of 6-8 weeks and 150-200 g were procured from the animal house of the laboratory. Rats were housed in their cages for 7 days prior to commencement of dosage in the experimental room after veterinary examination. The animals were housed in sterile propylene cages with bedding of clean paddy husk and facilities for food and water bottle. The animals were maintained at room temperature 22±3°C, relative humidity 55±5% and illumination cycle set to 12 hours each light and dark for adaptation to the experimental environment.

Acute toxicity study

In order to assess the safety and to determine a safe dose of a drug, an acute toxicity analysis was carried out in compliance with the Organization for Economic Co-operation and Development (OECD No. 423). The fractions to be tested were given to female Wistar rats who had been fasted overnight. The SK was given as a suspension in carboxymethyl cellulose (CMC), and the dosage was determined based on the body weight of Wistar rats. After administration, the animals were constantly monitored for 30, 60, 120, 180 and 240 min, and once daily thereafter, for 14 days. The animals were then examined for gross behavioral changes, body weight and necropsy and pathology.

Experimental procedure

30 Wistar Albino Rats were used in the study. The rats were randomized and allocated into five groups with six animals each as follows:

Table No. 01: Animal groups and drug dose specification

Sr. No.	Animal group	Group name	Drug specification	Dose in rats
1.	Group-1	Normal Control (NC)	_	_
2.	Group-2	Disease Control (DC)	DMH	30mg/kg s.c. for 7 days.
3.	Group-3	Standard Group (STD)	DMH + 5-FU	30mg/kg s.c. for 7 days + 20mg/kg i.p. for next 14 days.
4.	Group-4	Experimental Group- 1 (SK-1)	DMH + SK	30mg/kg s.c. for 7 days + 22.5mg/kg p.o. for next 14 days.
5.	Group-5	Experimental Group- 2 (SK-2)	DMH + SK	30mg/kg s.c. for 7 days + 45 mg/kg p.o. for next 14 days.

* s.c.- Subcutaneous, i.p.- Intraperitoneal, p.o.- *per os*, 1,2-dimethylhydrazine (DMH)- an inducer for induction of colon cancer, 5-Fluorouracil (5-FU)- standard drug used in the experiment, SK-1- *Snuhi Kshara* at dose 22.5mg/kg, SK-2- *Snuhi Kshara* at dose 45mg/kg

After 21 days, blood samples of rats were collected through retro-orbital route for hematological & biochemical parameters and colon samples were collected for ACF determination and Histopathology of colon under anesthesia.

ASSESSMENT PARAMETERS

Body weight

The body weight changes of all the groups were measured throughout the study. The rats were weighed at the beginning of the experiment and then subsequently once a week and final before sacrifice.

Hematological parameters

Blood samples were collected into vacutainer sterile tubes coated with EDTA

as an anticoagulant for determination of- hemoglobin, RBC, WBC and platelet.

Biochemical parameters

Blood samples were collected into vacutainer sterile tubes coated with EDTA as an anticoagulant and samples were centrifuged in cooling centrifuge for about 10 mins at 5000 rpm to separate plasma from blood. This plasma was used for determination of Total Cholesterol Level (TCL) with the help of biochemistry analyzer and cholesterol kit.

Colon length and weight

Colons were excised and cleaned with potassium phosphate buffered saline (0.1M, pH 7.2) and then assessed for colon length and weight.

Aberrant Crypt Foci (ACF)

Isolated colons were split open longitudinally and placed on strips of filter paper with their luminal surfaces open and exposed. The colons were then fixed in 10% buffered formalin for 24hrs. Each of the fixed colons was cut into proximal and distal portions of equal lengths and portion was further cut into 2cm long segments. Each segment was stained with 0.2% methylene blue solution for 2min.The segment were examined using a light microscope at 10X to score the total number of ACF.

ACF were distinguished from normal crypts by their thicker, darker-stained, raised walls with elongated slit-like lumens and significantly increased distance from the lamina to basal surface off cells. [13]

Histopathology

All the preserved colon tissues from all the groups were processed routinely and fixed in 10% formalin. The specimen was dehydrated in ascending grades ethanol, cleared in xylene, and embedded in paraffin. The sections of 3-5 µ thickness were cut and stained with hematoxylin-eosin stain and observed under 40x. Histopathology examination with toxicity score of all the organs were carried out and noted down.

STATISTICAL ANALYSIS

Collected data was entered into Microsoft Excel spreadsheet. All quantitative variables were presented as mean ± SD. Statistical significance was calculated by One way ANOVA (Analysis of variance) F-test for overall comparison and Bonferroni multiple comparison test for pair wise comparison for all the parameters. p Value <0.05 was considered statistically significant. All statistical tests were carried out using statistical software STATA, version 10.1, 2011.

RESULT

Acute toxicity study

In the acute oral toxicity study, there were no signs of clinical pathogenesis and mortality in female Wistar Albino rats at a dose of 2000- 5000 mg/kg body weight. Hence, *Snuhi Kshara* was found to be safe as per OECD 423 guideline.

Assessment parameters

Significant changes were seen in body weight, TCL, colon length & weight, proximal and distal ACF and histopathological toxicity score of experimental rats when compared study drug with Disease Control (DC) group. No significant changes were seen in Hb, RBC, WBC and platelet when compared study drug with DC.

Table No. 02: Comparison of change in mean body weight before and after treatment across the groups and between the group

Overall comparison			Pairwise comparison of TEST vs other groups			
Groups	**Mean**	**SD**		**Mean difference**	**p Value**	**Result**
NC	24.67	2.87	SK-1 vs DC	31.83	**0.001**	S
DC	-13	3.95	SK-2 vs DC	34.5	**0.001**	S
STD	16.67	1.37	SK-2 vs SK-1	2.67	1.000	NS
SK-1	18.83	3.54	Bonferroni's multiple comparison test			
SK-2	21.5	10.82				
p Value	**0.0001**, S					

S- Significant, NS- Non-Significant

Table No. 03: Comparison of Hb across the group and between the group

Overall comparison			Pairwise comparison of TEST vs other groups			
Groups	**Mean**	**SD**		**Mean difference**	**p Value**	**Result**
NC	19.98	3.29	SK-1 vs DC	2.4	1.000	NS
DC	21.02	2.47	SK-2 vs DC	1.25	1.000	NS
STD	14.45	5.00	SK-2 vs SK-1	1.15	1.000	NS
SK-1	18.62	2.08	Bonferroni's multiple comparison test			
SK-2	19.77	3.21				
p Value	**0.0219**, S					

S- Significant, NS- Non-Significant

Table No. 04: Comparison of RBC across the group and between the group

Overall comparison			Pairwise comparison of TEST vs other groups			
Groups	**Mean**	**SD**		**Mean difference**	**p Value**	**Result**
NC	7.73	1.18	SK-1 vs DC	0.97	1.000	NS
DC	8.49	0.90	SK-2 vs DC	0.29	1.000	NS
STD	6.25	2.08	SK-2 vs SK-1	0.68	1.000	NS
SK-1	7.52	0.98	Bonferroni's multiple comparison test			
SK-2	8.19	1.08				
p Value	**0.0612**, S					

S- Significant, NS- Non-Significant

Table No. 05: Comparison of WBC across the group and between the group

Overall comparison			Pairwise comparison of TEST vs other groups			
Groups	**Mean**	**SD**		**Mean difference**	**p Value**	**Result**
NC	10.78	1.49	SK-1 vs DC	1.83	1.000	NS
DC	10.97	4.03	SK-2 vs DC	0.2	1.000	NS
STD	5.7	1.65	SK-2 vs SK-1	1.63	1.000	NS
SK-1	9.13	0.93	Bonferroni's multiple comparison test			
SK-2	10.77	1.04				
p Value	**0.0010**, S					

S- Significant, NS- Non-Significant

Table No. 06: Comparison of Platelet across the group and between the group

Overall comparison			Pairwise comparison of TEST vs other groups			
Groups	**Mean**	**SD**		**Mean difference**	**p Value**	**Result**
NC	443.5	91.12	SK-1 vs DC	12.33	1.000	NS
DC	417.17	167.16	SK-2 vs DC	23.5	1.000	NS
STD	418.33	98.58	SK-2 vs SK-1	11.17	1.000	NS
SK-1	429.5	81.69	Bonferroni's multiple comparison test			
SK-2	440.67	154.49				
p Value	0.9932, NS					

S- Significant, NS- Non-Significant

Table No. 07: Comparison of Total Cholesterol Level (TCL) across the group and between the group

Overall comparison			Pairwise comparison of TEST vs other groups			
Groups	**Mean**	**SD**		**Mean difference**	**p Value**	**Result**
NC	39.69	2.17	SK-1 vs DC	52.04	**0.001**	S
DC	123.21	6.78	SK-2 vs DC	62.90	**0.001**	S
STD	70.65	6.18	SK-2 vs SK-1	10.86	**0.037**	S
SK-1	71.17	8.09	Bonferroni's multiple comparison test			
SK-2	60.30	4.23				
p Value	**0.0001**, S					

S- Significant, NS- Non-Significant

Table No. 08: Comparison of Colon length across the group and between the group

Overall comparison			Pairwise comparison of TEST vs other groups			
Groups	**Mean**	**SD**		**Mean difference**	**p Value**	**Result**
NC	18.17	1.00	SK-1 vs DC	6.52	**0.001**	S
DC	10.92	0.77	SK-2 vs DC	6.85	**0.001**	S
STD	14.05	1.46	SK-2 vs SK-1	0.33	1.000	NS
SK-1	17.43	0.59	Bonferroni's multiple comparison test			
SK-2	17.77	0.65				
p Value	**0.0001**, S					

S- Significant, NS- Non-Significant

Table No. 09: Comparison of Colon weight across the group and between the group

Overall comparison			Pairwise comparison of TEST vs other groups			
Groups	**Mean**	**SD**		**Mean difference**	**p Value**	**Result**
NC	1.55	0.28	SK-1 vs DC	0.19	1.000	NS
DC	2.10	0.21	SK-2 vs DC	0.48	**0.011**	S
STD	1.94	1.11	SK-2 vs SK-1	0.28	0.398	NS
SK-1	1.90	0.24	Bonferroni's multiple comparison test			
SK-2	1.62	0.25				
p Value	**0.0012**, Significant					

S- Significant, NS- Non-Significant

Table No. 10: Comparison of proximal colon ACF count across the group and between the group

Overall comparison			Pairwise comparison of TEST vs other groups			
Groups	**Mean**	**SD**		**Mean difference**	**p Value**	**Result**
NC	0	0	SK-1 vs DC	4.69	**0.001**	S
DC	16.68	0.76	SK-2 vs DC	6.86	**0.001**	S
STD	14.41	0.72	SK-2 vs SK-1	2.17	**0.001**	S
SK-1	11.98	0.55	Bonferroni's multiple comparison test			
SK-2	9.81	0.72				
p Value	**0.0001**, S					

S- Significant, NS- Non-Significant

Table No. 11: Comparison of distal colon ACF count across the group and between the group

Overall comparison			Pairwise comparison of TEST vs other groups			
Groups	**Mean**	**SD**		**Mean difference**	**p Value**	**Result**
NC	0	0	SK-1 vs DC	3.56	**0.001**	S
DC	19.59	1.28	SK-2 vs DC	7.34	**0.001**	S
STD	18.29	0.46	SK-2 vs SK-1	3.78	**0.001**	S
SK-1	16.04	0.76	Bonferroni's multiple comparison test			
SK-2	12.25	1.03				
p Value	**0.0001**, S					

S- Significant, NS- Non-Significant

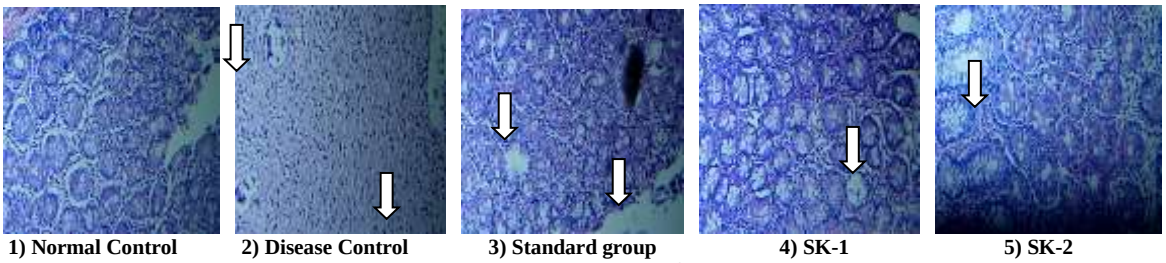
Table No. 12: Comparison of Histopathological toxicity score across the group and between the group

Overall comparison			Pairwise comparison of TEST vs other groups			
Groups	**Mean**	**SD**		**Mean difference**	**p Value**	**Result**
NC	0	0	SK-1 vs DC	1.34	**0.001**	S
DC	2.67	0.52	SK-2 vs DC	2.17	**0.001**	S
STD	1.17	0.41	SK-2 vs SK-1	0.83	**0.035**	S
SK-1	1.33	0.52	Bonferroni's multiple comparison test			
SK-2	0.5	0.55				
p Value	**0.0001**, S					

S- Significant, NS- Non-Significant

Table No. 13: Histopathological findings of colon tissue

Sr. No.	Groups	Histopathological observations
1	NC	Normal Control group rats exhibited no observable histopathological changes in colon.
2	DC	Disease control group revealed high colonic damage. Presence of necrosis, fibrosis, hyperemia, epithelial damage, ulceration, infiltration and sub mucosal abscesses were observed.
3	STD	Standard group significantly prevented the colonic injury. Presence of mild infiltration and ulceration was observed.
4	SK-1	Colon tissue shows reduced inflammation with mild infiltration, abscesses and ulceration in the mucosa.
5	SK-2	SK-2 group reveals significant reduction in gross colonic injury. Colon tissue shows negligible infiltration, reduced inflammation and mild ulceration & abscesses.



1) Normal Control **2) Disease Control** **3) Standard group** **4) SK-1** **5) SK-2**

Figure 1: Histopathology of colon

DISCUSSION

The present study evaluates the anticancer activity of *Snuhi Kshara* (SK) against 1,2-dimethylhydrazine (DMH) induced colon cancer in Wistar rats. Change in body weight, hematological & biochemical parameter, colon length & weight, ACF count, histopathological toxicity score and histopathological findings were used to screen *In Vivo* anticancer activity.

Snuhi Kshara (SK) did not cause any clinical pathogenesis and mortality in the treated rats. LD50 value of SK, according to the OECD Guideline, 423 and under provided laboratory conditions was found to be in GHS Category 5 i.e., > 2000 - 5000 mg/kg body weight, with a LD50 cut off at 5000 mg/kg body weight.

Change in body weight showed significant changes in all groups may be due to alterations in the parameter. Test group SK-1 and SK-2 showed significant increase in body weight when compared to DC suggestive of anticancer activity of study drug SK at both doses (Table No. 2).

Hb and RBC showed significant changes in all groups which may be due to alterations in the parameter. Test group SK-1 and SK-2 revealed non-significant difference when compared with DC suggestive of no effect of study drug SK against colon cancer with the parameter (Table No. 3, 4).

WBC count showed significant changes in all groups which may be due to inflammatory response (Table No. 37). Test group SK-1 and SK-2 revealed non-significant difference when compared with DC suggestive of no effect of study drug SK on colon cancer with the parameter (Table No. 5).

A low platelet count might be a sign of certain cancers or infections. PLT count showed non-significant changes in all groups which may be due to minimum alterations in the parameter. Test group SK-1 and SK-2 showed non-significant difference of platelet count when compared to DC suggestive of no effect of study drug SK on colon cancer with the parameter (Table No. 6).

All cells have cell membrane which is made up of lipids i.e., cholesterol and hence in colon cancer as cells are multiplying or increasing, TCL also increases. TCL showed significant changes in all groups which may be due to alterations in the parameter. Test group SK-1 and SK-2 showed significant reduction of TCL when compared to DC suggestive of anticancer activity of study drug SK at both doses (Table No. 7).

Colon length and weight of all the rats were measured after the dissection. Colon length and weight showed significant changes in all groups which may be due to alteration in the parameters. Test group SK-1 and SK-2 showed significant increase in colon length when compared to DC suggestive of anticancer activity of study drug SK at both doses. Test group SK-2 showed significant decrease in colon weight when compared to DC suggestive of anticancer activity of study drug SK at 45mg/kg i.e., at higher dose (Table No. 8, 9).

At the end of the experiment proximal and distal colon samples of all rats were analyzed for Aberrant Crypt Foci (ACF) count. Proximal and distal colon ACF count showed significant changes in all groups which may be due to alteration in the parameters. Test group SK-1 and SK-2 showed significant decrease in proximal & distal colon ACF when compared to DC suggestive of anticancer activity of study drug SK at both doses may be due to antiproliferative activity of chemical markers euphol & nerifoliene (isolated triterpenoids from *Euphorbia neriifolia* Linn.) (Table No. 10, 11). [14]

Histopathological toxicity score showed significant changes in all groups which may be due to alterations in the parameter. Test group SK-1 and SK-2 showed reduction in Histopathological toxicity score when compared to DC suggestive of anticancer activity of study drug SK at both doses. Histopathological toxicity score and Histopathological observations both indicate that the administration of SK in both doses decreased the colonic injury induced by DMH (Table No. 12).

Test group SK-2 showed significant difference when compared to SK-1 suggestive of increased anticancer activity with the increase of dose by- TCL, proximal & distal colon ACF count and histopathological toxicity score.

Disruption of *samprapti* is the basic line of treatment instigated in Ayurveda which

causes *dosha shamana* and eventually cure the disease. Development of cancer is due to *mandagni*, *aama*, vitiation of *Vata*, *Pitta*, *Kapha* and *Saptadhatu dushti*. *Snuhi Kshara* possesses *katu, tikta, tikshna, vanhi deepana, ushna, Lekhana, pachana, darana, shodhana, Kapha-Vataghna* and *Tridoshaghna guna* thus *Snuhi Kshara* may attribute to anticancer activity.

CONCLUSION

Snuhi Kshara possesses significant (p Value <0.05) anticancer activity against DMH induced colon cancer in Wistar Albino rats as evidenced by reversal of altered total cholesterol level, colon length & weight, proximal and distal colon ACF count and histopathological findings. Further experimental and clinical studies with extended spectrum are required to know the exact mechanism of action of *Snuhi Kshara*.

Declaration by Authors

Ethical Approval: Approved

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