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Evaluation of Antimicrobial activity of Methanolic Leaf Extracts of Selected *in vitro* and *in vivo* Grown Convolvulaceae Members

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Medicinal plants have been used virtually in all cultures as a source of medicine. The fresh leaves of *in vivo* grown and *in vitro* grown plants of *Evolvulus alsinoides, Evolvulus nummularius,* and *Merremia tridentata* were collected and examined for antimicrobial activity. Zone and turbidity methods are employed for antimicrobial assay. Methanolic extracts of the leaf parts of *in vivo* and *in vitro* grown cultures have exhibited antimicrobial activity. The extracts are found to be more effective against gram-positive bacteria and yeast (MIC of 0.05 to 0.25mg/ml) than gram-negative bacteria (MIC of 0.25 to 50 mg/ml). The extracts are found to be more effective against bacteria rather than filamentous fungi. The zone formation and MIC results from *in vitro* grown leaf extracts were less compared to the *in vivo* grown leaf extracts.

Key words: *In vitro*, *in vivo*, Convolvulaceae, Antimicrobial activity, MIC.

The practice of using medicinal plants for the treatment of various diseases started since the dawn of civilization[1]. Due to rapid studies in biotechnology and synthetic chemistry, the debate use of plants possessing several medicinal properties could be assessed due to the presence of therapeutic value[2].

Medicinal substances found in plants are the products of natural metabolic processes. Each species has its own genetic structure that governs the presence of chemical components or bioactive molecules[3]. Medicinal herbs are the local heritage with global importance. World is endowed with a rich wealth of medicinal herbs[4]. Medicinal herbs are moving from fringe to mainstream use as a greater number of people endeavor to opt for herbal formulations over the allopathic compounds, since these are devoid of side effects and cost effective[5].

Conservation of medicinal plants and capability to utilize them in a sustained manner are essential for the well being and continued survival of man[6]. The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants[7].

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The importance of plants extracts is profound, since they can be used as a potential source of antibiotics controlling various fungal and bacterial pathogens[8]. Administering chemotherapeutic compounds orally, are able to control a wide range of microbes but there is also a possibility that they may cause an imbalance in the gut microflora, allowing opportunistic pathogenic coliforms to become established in the gastrointestinal tract with resultant deleterious effects[8, 9].

The three taxa from *convolvulaceae* currently selected to study antimicrobial activity of *in vivo* and *in vitro* grown plants are *Evolvulus alsinoides, Evolvulus nummularius* and *Merremia tridentata.*

Evolvulus alsinoides (Common name: *Shankapushpi*; *Local name: Vishnu krantha)* is a perennial herb. Stems several to numerous, prostrate or ascending, slender, with appressed and spreading hairs. Plant parts are used in the treatment of nervous exhaustion, memory loss, general weakness, scrofula, brain tonic, aphrodisiac, dysentery, chronic bronchitis, asthma, anthelmintic and anti-phlogistic, childhood fever, nausea and vomiting associated with motion, sickness, sea sickness and pregnancy, jaundice, cold and the oil stimulates the growth of hair. *Evolvulus nummularius* (Common name: Kidney weed; Local name: *Lakshmi krantha*) is a perennial herb. Stems several, rooting at nodes, prostrate, 20-40 cm, slender, ± villous or scabrous. Plant parts are used in the treatment of bronchial asthma and as an anthelmintic agent. *Merremia tridentata* (Common name: *Mogra, Kong kong pasir*; Local name: *Sitasavaram, aeluka chevi aaku*) is a climbing weed with slim, grayish leaves, white flowers and seed capsules. Plant parts are used in the treatment of bone fracture and piles[10-14].

MATERIAL AND METHODS

Collection of plant materials

The plants are widely distributed in Gajuwaka region of Visakhapatnam District. Whole plant was collected from Visakhapatnam during rainy season and the experiments are conducted during August to November 2007-2008.

Explants

Healthy explants such as shoot tips of *Evolvulus alsinoides, E. nummularius* and *M. tridentata* were selected for tissue culture.

Surface sterilization

The explants were washed thoroughly under running water, followed by three washes with distilled water and immersed in 70% ethanol for three minutes. The explants were again rinsed three times in sterile, double distilled water and are then immersed in 5% sodium hypochlorite for 10 minutes. Finally the explants are rinsed thrice in sterile, double distilled water.

Indirect shoot regeneration

Shoot regeneration potential via callus phase of shoot tips of *E. alsinoides*, *E. nummularius* and *M. tridentata* were studied by culturing on MS (Murashige and Skoog) medium fortified with 1mg/l of 2,4-Dichlorophenoxyacetic acid (2, 4-D) and 1mg/l of 6-BenzylAdenine (BA) incubated at 25°C ± 2°C.

Growth and Maintenance of Plant Tissue and Cell Cultures

The callus and shoot cultures belonging to the taxa of the present study on solid media were sub cultured at regular intervals of 1-2 weeks.

Rooting of *in vitro* Shoots

For root induction in *E. alsinoides*, *E. nummularius* and *M. tridentata*, the shoots (> 3cm) were excised from primary cultures and cultured on semi solid MS medium supplemented with 1 mg/l of IAA.

Acclimatization and transfer of plantlets to field

The plantlets, regenerated through *in vitro* techniques, with healthy root and shoot systems were taken out from the culture medium and washed gently with sterile distilled water to remove all traces of medium from the plantlet. The washed plantlets were transferred to small plastic cups containing sterile sand. The pots were then covered with polythene bags to maintain high humidity and kept in plant growth chamber. The plantlets were moistened with water two times per day. After fifteen to twenty days, the polythene bags were removed and transferred to larger pots containing sterile sand and soil (1:1 ratio), and are kept under shade in the poly house for another two weeks.

Preparation of plant extracts

Both *in vivo* and *in vitro* grown fresh leaves were separately washed thoroughly under running water, shade dried and used for extraction. The plant materials were homogenized to a fine powder and stored in airtight bottles.

The methanolic extracts of plants were extracted using soxhlet extractor. About 25 g of leaf powders separately were carefully transferred into round bottom flask of soxhlet extractor. The plant materials were soaked in 2 litres of methanol for 24 hours at room temperature. The final extracts were filtered through whattman's filter paper no.1. The methanol present in the methanolic extract was evaporated under reduced pressure (Buchi vaporator) to yield the residue. The residue thus obtained was suspended in DMSO (Dimethylsulfoxide) to obtain different concentrations of crude extract. These extracts were evaluated for their antimicrobial activity and MIC (Minimum Inhibitory concentration).

Microorganisms and their maintenance

Four bacterial and two fungal cultures were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India and preserved in deep freezer at Department of Biochemistry, GITAM University, Visakhapatnam, India. The cultures employed for experimentation are *Escherichia coli* (MTCC No.118) and *Vibrio parahaemolyticus* (MTCC No 451) of gram-negative bacteria, *Staphylococcus aureus* (MTCC No. 96) and *Bacillus subtilis* (MTCC NO.121) of gram-positive bacteria, and *Saccharomyces cerevisiae* (MTCC No. 463) and Aspergillus *niger* (MTCC No.281) of fungi.

The above bacterial cultures and *S.cerevisiae (yeast)* were maintained on Muller Hinton Agar (MHA) and *A.niger* culture was maintained on Sabouraud's Dextrose Agar (SDA) at 4°C temperature until used for the study. Before use, the bacterial and fungal cultures were revived in Muller Hinton Broth (MHB) for bacteria and yeast, and Sabouraud's Dextrose Broth (SDB) for *A.niger*.

Procedure for Antimicrobial assay

Zone Method

MHA (for bacterial and yeast growth) and SDA (for *A.niger* growth) was weighed and mixed in distilled water based on the composition. The media was autoclaved for 20 minutes at 121°C (15 lbs pressure) and cooled to 45°C. The bacterial and fungal cultures with optical density of 0.6 were taken and 50 ml of inoculum was added per 500ml of MHA for bacteria and yeast, SDA for *A.niger*. To each petriplate, 20 ml of the media was poured and was kept for solidification. By using gel puncture (8 mm diameter) wells have been made in the plate (4 wells per plate) for the addition of plant extracts at different concentrations.

The prepared plant extracts with concentrations of 0.05mg/ml, 0.25mg/ml, 0.50mg/ml, 1mg/ml) (except for *A.niger* taken as 5mg/ml, 25mg/ml, 50mg/ml and 100mg/ml) were tested separately for their antimicrobial activity.

After addition of the plant extracts at different concentrations in agar wells, the plates were kept aside for 2 hours for diffusion. Bacterial and yeast cultures are incubated for 18 hours at 37°C and fungal culture (*A.niger*) was incubated for 3-4 days at 25°C. The result was obtained by measuring the zone diameter, an indication of growth of the microorganisms. The experiment was repeated three times and the mean values are presented.

Area of zone of inhibition is calculated based on the following equation:

$$\text{Area of zone of inhibition} = \pi/4((\text{Zone diameter})^2 - (\text{well diameter})^2)\ \text{mm}^2$$
$$= 3.14/4(D^2 - d^2)\ \text{mm}^2$$

Turbidity method

Minimum Inhibitory concentration (MIC) of the plant extracts was determined by broth dilution susceptibility test. The bacterial and fungal cultures with optical density of 0.6 were taken. 0.5 ml of inoculum is added to 5ml of MHB for bacteria and yeast, and SDB for *A.niger*. The prepared plant extracts with concentrations of 0.01mg/ml, 0.05mg/ml, 0.1mg/ml, 0.25mg/ml, 0.5mg/ml and 1mg/ml) (except for *A.niger* taken as 0.1mg/ml, 0.5mg/ml, 1mg/ml, 2.5mg/ml, 5mg/ml and 10mg/ml) were tested separately for their

MIC. The optical densities of cultures were determined using spectrophotometer (LaboMed, Inc.) at 600nm. The lowest concentration of extract that showed no growth of the test organism after 48 h of incubation in comparison with the control tube, which included 5ml of MHB for bacteria and yeast and SHB for *A.niger* was designated as (MIC).

RESULTS

The ethnobotanical screening tests of i*n vivo* leaf and *in vitro* grown leaf extracts of *E. alsinoides, E. nummularius* and *M. tridentata* (Fig. 1) in methanol as solvent against both human and plant pathogenic bacteria and fungi using zone technique are depicted in Tables 1 and 2. Two antibiotics (Penicillin and Streptomycin) were used as standards and the zone of inhibition was shown to be 14mm (*B.subtilis*), 18mm (*E.coli*) with penicillin and 26mm (*B.subtilis*), 17 mm (*E.coli*) with streptomycin and were listed in Table 3. MIC of all the taxas were recorded in Table 4. Based on results from area of zone of inhibition (Table 2), curved graphs are executed from excel and were provided in Fig. 2.

Evolvulus alsinoides

Methanolic extracts of the leaf parts of i*n vivo* and i*n vitro* grown cultures have exhibited antimicrobial activity. Methanolic leaf extract of *Evolvulus alsinoides* exhibited antibacterial and antifungal activity against standard strains of *S.aureus*, *E.coli*, V.*parahaemolyticus, B.subtilis, S.cerevisiae* and *A.niger*. The leaf extract of *Evolvulus alsinoides* in methanol showed antimicrobial activity at 0.05 mg/ml against all

Table 1. Antimicrobial activity Methanolic leaf extract of *in vivo* and *in vitro* plant (Zone of activity in mm including 8mm Well diameter)

Microorganism	Conc. (in mg/ml	Antimicrobial zone measured in millimeters					
		Evolvulus alsinoides		*Evolvulus nummularius*		*Merrimia tridentata*	
		in vivo	in vitro	in vivo	in vitro	in vivo	in vitro
B.subtilis	*0.05*	*22*	*15*	*16*	*10*	*10*	*10*
	0.25	23.5	16	17	12	11	11
	0.50	25	18	18	13	12	13
	1.00	26.5	19	20	14	13	14
S.aureus	0.05	20	16	15	-	10	-
	0.25	22	17	16	11	12	10
	0.50	24	18	17	12	13	10
	1.00	26	19	20	14	14	11
E.coli	0.05	10	10	-	10	-	-
	0.25	12	11	-	11	-	9
	0.50	14	13	-	12	-	10
	1.00	16	15	-	14	-	11
V.parahaemolyticus	0.05	12	9	11	-	-	-
	0.25	14	10	12	9	-	-
	0.50	18	11	14	10	-	9
	1.00	20	12	15	11	-	9
S.cerevisiae	0.05	23	13	18	10	10	-
	0.25	24	15	19	12	12	10
	0.50	25	17	20	13	14	11
	1.00	26	18	21	14	15	12
A.niger	5.00	17	9	10	-	-	-
	25.00	20	10	11	-	-	-
	50.00	22	11	13	9	-	-
	100.00	24	12	15	10	9	9

- = zone not formed

A) *Evolvulus alsinoides*

B) *Evolvulus alsinoides* with scale reading

C) *Evolvulus nummularius*

D) *Evolvulus nummularius* with scale reading

E) *Merrimia tridentata*

F) *Merrimia tridentata* with scale reading

Fig. 1. Mother plants selected for the present study

tested bacteria and yeast. Antimicrobial activity against *A.niger* showed from 5mg/ml. The extracts are found to be more effective against bacteria rather than filamentous fungi. The extracts are found to be more effective against gram-positive bacteria and yeast rather than gram-negative bacteria.

The *in vitro* grown methanolic leaf extracts have also exhibited antimicrobial activity and the results are similar with extracts from aerial parts of the *in vivo* plant. The zone formation and MIC results from *in vitro* grown leaf extracts were less effective compared to the *in vivo* grown leaf extracts. The extracts are found to be more effective against bacteria (MIC 0.01 to 1 mg/ml) rather than filamentous fungi (MIC ≥1 mg/ml).

Table 2. Antimicrobial activity methanolic leaf extract of *in vivo* and *in vitro* plant(Area of zone of inhibition in mm² to plot Graph)

Microorganism	Conc. (in mg/ml	Antimicrobial zone measured in mm²					
		Evolvulus alsinoides		*Evolvulus nummularius*		*Merrimia tridentata*	
		in vivo	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*
B. subtilis	0.05	329.7	126.38	150.72	28.26	28.26	28.26
	0.25	383.27	150.72	176.62	62.8	44.74	44.74
	0.50	440.38	204.1	204.1	82.42	62.8	82.42
	1.00	501.02	233.14	263.76	103.62	82.42	103.62
S. aureus	0.05	263.76	150.72	126.38	-	28.26	-
	0.25	329.7	176.62	150.72	44.74	62.8	28.26
	0.50	401.92	204.1	176.62	62.8	82.42	28.26
	1.00	480.42	233.14	263.76	103.62	103.62	44.74
E. coli	0.05	28.26	28.26	-	28.26	-	-
	0.25	62.8	44.74	-	44.74	-	13.34
	0.50	103.62	82.42	-	62.8	-	28.26
	1.00	150.72	126.38	-	103.62	-	44.74
V. parahaemolyticus	0.05	62.8	13.34	44.74	-	-	-
	0.25	103.62	28.26	62.8	13.34	-	-
	0.50	204.1	44.74	103.62	28.26	-	13.34
	1.00	263.76	62.8	126.38	44.74	-	13.34
S. cerevisiae	0.05	365.02	82.42	204.1	28.26	28.26	-
	0.25	401.92	126.38	233.14	62.8	62.8	28.26
	0.50	440.38	176.62	263.76	82.42	103.62	44.74
	1.00	480.42	204.1	295.94	103.62	126.38	62.8
A. niger	5.00	176.62	13.34	28.26	-	-	-
	25.00	263.76	28.26	44.74	-	-	-
	50.00	329.7	44.74	82.42	13.34	-	-
	100.00	401.92	62.8	126.38	28.26	13.34	13.34

- = zone not formed

Table 3. Antimicrobial activity of penicillin and streptomycin (at 0.25mg/ml)

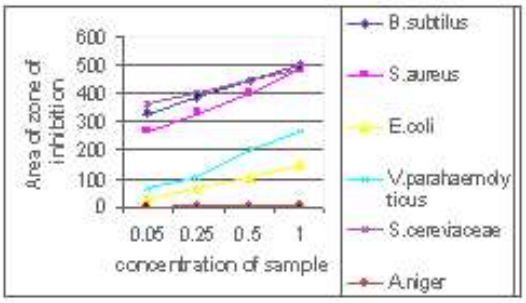
Microorganism	Penicillin		Streptomycin	
	Zone of activity(in mm)	Area of zone of inhibition(in mm²)	Zone of activity(in mm)	Area of zone of inhibition(in mm²)
B. subtilis	14	103.62	26	480.42
E. coli	18	204.10	17	176.62

The extracts are found to be more effective against gram-positive bacteria (15-19 mm zone formation) and yeast (13 to 18 mm zone formation) rather than gram-negative bacteria (9-15mm zone formation).

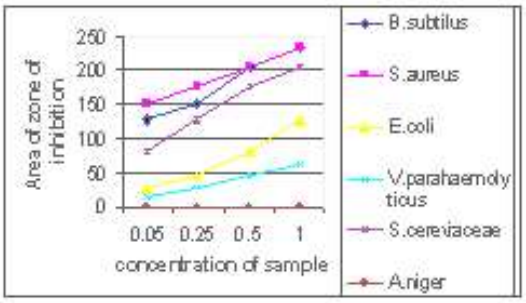
Evolvulus nummularius

Methanolic extracts of the leaf parts of *in vivo* plant and leaf parts from *in vitro* grown cultures exhibited antimicrobial activity. *Evolvulus nummularius* exhibited antibacterial and antifungal activity against standard strains of *S.aureus*, *E.coli*, V.*parahaemolyticus, B.subtilis, S.cerevisiae* and *A.niger*. The leaf extract of *Evolvulus nummularius* in methanol showed MIC of 0.05 - 1 mg/ml against all tested bacteria and yeast. MIC of *A.niger* was observed as 10mg/ml for *in vivo* and 50mg/ml for *in vitro* plant extracts. The extracts are found to be more effective against bacteria rather than filamentous fungi. The extracts are found to be more effective against gram-positive bacteria and yeast (MIC of 0.05 to 0.25mg/ml) rather than gram-negative bacteria and *A.niger* (MIC of 0.25 to 50 mg/ml).

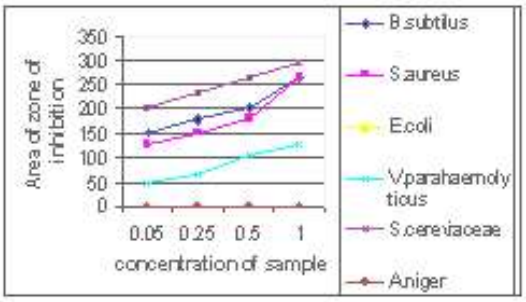
The zone formations in *in vitro* leaf extracts were less compared to *in vivo* plants. The extracts are found to be more effective against



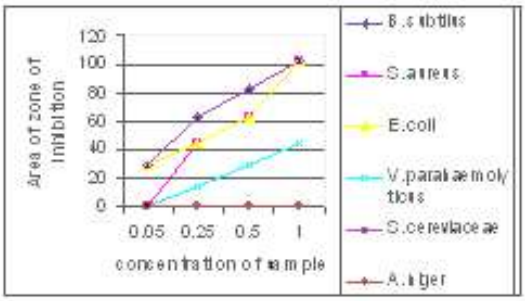
Area of zone of inhibition in *in vivo* grown leaf extract



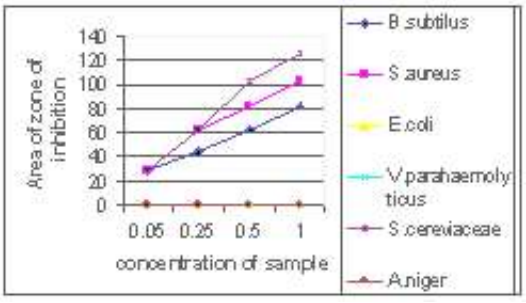
Area of zone of inhibition in *in vitro* grown leaf extract



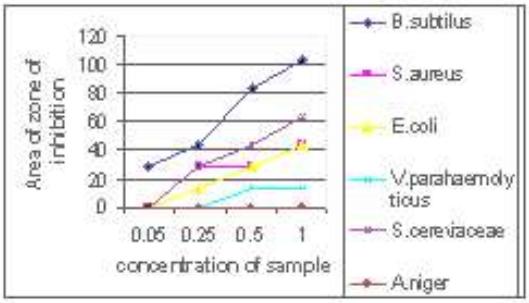
Area of zone of inhibition in *in vivo* grown leaf extract



Area of zone of inhibition in *in vitro* grown leaf extract



Area of zone of inhibition in *in vivo* grown leaf extract



Area of zone of inhibition in *in vitro* grown leaf extract

Fig. 2. Antimicrobial activity of *Evolvulus alsinoides, Evolvulus nummularius* and *Merremia tridentata*

bacteria and yeast (MIC 0.05 to 1mg/ml) than filamentous fungi (MIC 10 to 50 mg/ml). MIC of various bacterial samples differed and shown more for gram-negative bacteria (MIC of *E.coli* -1 mg/ml, *V.parahaemolyticus* -0.25 mg/ml in *in vivo* extracts) than gram-positive bacteria (MIC of *B.subtilis* - 0.05mg/ml, *S.aureus* - 0.05 mg/ml in *in vivo* extracts). *in vivo* samples on fungal species showed MIC of 0.05 mg/ml (for *S.cerevisiae*) and 50 mg/ml (for *A.niger*).

Merremia tridentata

Methanolic extracts of the leaf parts of *in vivo* and *in vitro* grown plants were tested for antimicrobial activity. The test revealed antibacterial activity against standard strains of *S.aureus, E.coli, V.parahaemolyticus, B.subtilis, S. cereviaseae* and *A.niger*.

The methanolic leaf extracts of *in vitro* grown plants have also exhibited antimicrobial activity. The extracts are found to be more effective against bacteria (MIC 0.25- 1.00 mg/ml) rather than multicellular fungi (MIC > 50 mg/ml). The extracts are found to be more effective against gram-positive bacteria (10-14 mm zone formation) and yeast (10 to 12 mm zone formation) rather than gram-negative bacteria (9-11mm zone formation).

Table 4. Minimum inhibitory concentration (mic) methanolic leaf extract of i*n vivo* and i*n vitro* plants

Microorganism	MIC in mg/ml					
	Evolvulus alsinoides		*Evolvulus nummularius*		*Merrimia tridentata*	
	in vivo	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*
B.subtilis	0.01	0.05	0.05	0.25	0.25	0.25
S.aureus	0.01	0.05	0.05	0.25	0.25	0.5
E.coli	0.25	0.25	1.00	0.25	1.00	0.5
V.parahaemolyticus	0.1	0.5	0.25	0.5	1.00	0.5
S.cerevisiae	0.01	0.1	0.05	0.25	0.25	0.5
A.niger	1.00	10	10	50	>50	>50

DISCUSSION

The potential for developing antimicrobials from plants appears rewarding, as it will lead to the development of a phytomedicine to act against microbes. Plant-based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials. India is a varietal emporium of medicinal plants and is one of the richest countries in the world with regard to genetic resources of medicinal plants. It exhibits a wide range in topography and climate, which has a bearing on its vegetation and floristic composition. Moreover, the agro-climatic conditions are conducive for introducing and domesticating new exotic plant varieties [15].

Methanolic leaf extracts of *Evolvulus alsinoides*, *Evolvulus nummularius* and *Merremia tridentata* from i*n vivo* and i*n vitro* grown plants has exhibited antimicrobial activity. The extracts are found to be more effective against bacteria rather than fungi. The extracts are found to be more effective against gram-positive bacteria and yeast rather than gram-negative bacteria. Previous studied plant extracts *Caesalpinia pulcherrima, Euphorbia hirta* and *Casuarina equisetifolia* were most active against gram-positive bacteria *B. cereus*[16], *Mitracarpus scaber. Leaves* formulated *at a minimum inhibitory concentration of 75mg/ml on E.coli*[17], *R. tetraphylla* and *P. minima* inhibited bacterial and fungal growth. Methanol extract showed MIC of 0.25 to 100 mg/ml against bacterial pathogens and 0.5 to 100 mg/ml against fungal pathogens[18].

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