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RESEARCH ARTICLE

A study on antidepressant-like effect of dihydroxy flavones in mice and their mechanisms involved

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ABSTRACT

Background: In the treatment of depression, drugs with a quick onset of action and high margin of safety are intensely being searched. Previous studies indicate flavonoids as a potential source for such drugs. Hence, in the present study, four dihydroxy flavones have been selected for investigation. **Aims and Objectives:** The aim of this study is to investigate the potential antidepressant effect and the mechanism of action of dihydroxy flavone derivatives in mice. **Materials and Methods:** Mice were subjected to forced swim test and tail suspension test for 6 min. The period of immobility in these animals was recorded after treatment with different doses of 3,7-dihydroxy flavone, 3,3'-dihydroxy flavone, 6,3'-dihydroxy flavone, and 5,6–dihydroxy flavone. Interacting drugs such as para chlorophenylalanine, alpha-methyl-para-tyrosine, L-arginine, and naloxone were employed to delineate the role of various mechanisms involved in the action of these dihydroxy flavones in forced swim test. **Results:** A dose-dependent reduction in the immobility period of mice was recorded in both forced swim test and tail suspension test for dihydroxy flavones indicating the antidepressant-like effect of these compounds. However, 6,3'-dihydroxy flavone was found to be less effective than the other three compounds. Various interacting drugs differentially modulated the reduction of immobility period produced by dihydroxy flavones in forced swim test. Monoaminergic, opioid, and nitric oxide pathways were evident in the action of dihydroxy flavones. **Conclusion:** The present study identified the potential antidepressant effect of a few dihydroxy flavone derivatives involving novel mechanisms.

KEY WORDS: Dihydroxy Flavones; Forced Swim Test; Tail Suspension Test; Antidepressant

INTRODUCTION

Among many psychiatric disorders, depression is one of the most common causes for ill health that affects nearly 121 million people worldwide. Depression also causes a significant economic burden to the society.[1] Depression is a heterogeneous disorder that affects a person's mood, behavior, and physical well-being. Even though a variety of drugs are currently available to treat depression, there is a therapeutic lag of about 3–4 weeks, but the emergence of side effects to these medications precedes the benefit of antidepressant therapy. Hence, new drugs with immediate efficacy and good safety profile are highly desirable.

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Flavonoids have been identified as active constituents of many medicinal plants such as *Hypericum perforatum* used in traditional system of medicine for the treatment of depression.[2,3] Many flavone compounds have been reported in literature to possess potent antinociceptive effect in different animal models.[4-6] A reciprocal association has been suggested between chronic pain and depression or anxiety disorders. Thus, chronic pain may lead to emotional disturbances for long periods. In fact, depression and anxiety may increase the perception of acute and chronic pain.[7,8] The

serotonergic and noradrenergic neuronal pathways have been clearly implicated in the etiology of depression as well as in pain modulation in the central nervous system. Many of the currently employed antidepressant drugs are therapeutically used in the management of neuropathic pain. In addition, the use of opioid analgesics such as oxycodone[9] and tramadol[10] in major depression also supports the contention that pain and depression are closely related. It was felt interesting whether flavone compounds which possess analgesic effect could be considered for their antidepressant effect in animal models. A few structurally related dihydroxy flavones have been found to exert opioid-mediated analgesic effect in mice.[5] These compounds (3,7-dihydroxy flavone, 3,3'-dihydroxy flavone, 6,3'-dihydroxy flavone, and 5,6-dihydroxy flavone) have been investigated in the present study for their antidepressant effect in mice using forced swim test and tail suspension test. In addition, the possible mechanisms that could be involved in their action were also considered worth investigating.

MATERIALS AND METHODS

Animals

Swiss albino mice weighing around 20–25 g of either sex were selected for the study. Animals were sourced from the institutional animal house facility. The animals were housed in a controlled environment, with food and water *ad libitum* on a natural day and night cycle. In all the experiments, each group consisted of 6 animals. To avoid circadian variation and to maintain uniformity, all the experiments were carried out between 9.00 and 14.00 h. The experimental protocol was approved by the institutional animal ethics committee. Maintenance of animals and experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India.

Drugs and Chemicals

3,7-dihydroxy flavone, 3,3'-dihydroxy flavone, 6,3'-dihydroxy flavone, and 5,6-dihydroxy flavone (Research Organics - Chennai, India), para chlorophenylalanine (PCPA) (Tokyo Chemical Industries, Japan), alpha-methyl-para-tyrosine (AMPT) (Sigma Aldrich, St. Louis. MO.USA), naloxone hydrochloride (Endo Labs, USA), L-arginine (Tokyo Chemical Industries, Japan), and carboxymethylcellulose sodium salt (Himedia, India) were used.

The dihydroxy flavones were prepared as a fine suspension in 2% carboxymethylcellulose and injected i.p in selected doses given below, 30 min before the procedure.

3,7-dihydroxy flavone (2.5 mg/kg, 5 mg/kg, and 10 mg/kg), 3,3'-dihydroxy flavone, 6,3'-dihydroxy flavone, and 5,6-dihydroxy flavone (5 mg/kg,10 mg/kg, and 20 mg/kg) were used.

The doses of above compounds were selected based on the results of pilot experiments.

Forced Swim Test[11]

In this test, the mouse is forced to swim inside a vertical open cylindrical container (height - 25 cm and diameter - 10 cm) with 19 cm of water column at 25°C ± 1°C. The mouse initially swims vigorously in circles and tries to climb the wall or dive to the bottom. After a minute, the active swimming starts to decrease and the animal tries to stay immobile or floating with only minimal movements to keep its head above the water level. The total duration of immobility is recorded for 6 min in which the 1st min is taken as the time for the animal to get adapted to the new environment. Hence, readings were not taken during the 1st min. A decrease in the immobility period is indicative of antidepressant effect. Different groups of mice received 3,3'-dihydroxy flavone, 6,3'-dihydroxy flavone, and 5,6-dihydroxy flavone - i.p in doses of 5, 10, and 20 mg/kg and 3,7-dihydroxy flavone in doses of 2.5, 5, and 10 mg/kg, 30 min before test. Imipramine 20 mg/kg i.p was used for comparison in a separate group of mice.

Tail Suspension Test[12]

A mouse is suspended from a rod of 10 cm length attached perpendicularly to the vertical iron stand, 50 cm above the floor level, by adhesive tape placed approximately 1 cm from the tip of the tail in a silent environment under dim light. The animal remains immobile after active and unsuccessful attempts to escape when suspended by tail. The animal is observed for 6 min and the period of immobility recorded. Effective antidepressants reduce the immobility period of the animals. 3,3'-dihydroxy flavone, 6,3'-dihydroxy flavone, and 5,6-dihydroxy flavone were administered i.p to different groups of mice in doses of 5, 10, and 20 mg/kg and 3,7-dihydroxy flavone in doses of 2.5, 5, and 10 mg/kg. Animals were subjected to the test, 30 min after administration of the test compound. Imipramine 20 mg/kg i.p was used as a standard for comparison.

Investigations on the Mechanism of Action

Further experiments were done to understand the mechanism by which these dihydroxy flavones exert their antidepressant activity. A single dose of 3,7-dihydroxy flavone (5 mg/kg), 3,3'-dihydroxy flavone (20 mg/kg), 6,3'-dihydroxy flavone (10 mg/kg), and 5,6-dihydroxy flavone (10 mg/kg) were selected for this purpose.

Serotonergic system

To assess the involvement of serotonergic pathway in the antidepressant action of these dihydroxy flavones, animals were pretreated with 5-hydroxytryptamine (HT) synthesis inhibitor - PCPA 100 mg/kg, i.p, for 4 consecutive days.[13] On the 5th day, i.e., 24 h after the last PCPA injection, the animals received

an injection of one of the dihydroxy flavones (3,7-dihydroxy flavone [5 mg/kg], 3,3'-dihydroxy flavone [20 mg/kg], 6,3'-dihydroxy flavone [10 mg/kg], or 5,6-dihydroxy flavone [10 mg/kg]) and were subjected to forced swim test after 30 min.

Adrenergic system

To elucidate the possible contribution of adrenergic system, mice were pretreated with AMPT, 100 mg/kg, i.p.[13] After 4 h, they received one of the flavone compounds 3,7-dihydroxyflavone(5 mg/kg), 3,3'-dihydroxy flavone (20 mg/kg), 6,3'-dihydroxy flavone (10 mg/kg), or 5,6-dihydroxy flavone (10 mg/kg) i.p and were subjected to forced swim test 30 min later.

Opioid mechanism

To investigate the participation of opioid system in the antidepressant action of dihydroxy flavones, mice were pretreated with naloxone (10 mg/kg i.p),[14] and after 15 min, the animals received an injection of 3,7-dihydroxy flavone (5 mg/kg) or 3,3'-dihydroxy flavone (20 mg/kg) or 6,3'-dihydroxy flavone (10 mg/kg) or 5,6-dihydroxy flavone (10 mg/kg). The antidepressant effect was recorded 30 min later by forced swim test.

Nitric oxide (NO) mechanism

Pre-treatment with NO precursor and L-arginine 500 mg/kg i.p 15 min before dihydroxy flavone administration was used to investigate the role of NO pathway in the antidepressant effect of dihydroxy flavones.[15] Test compounds were 3,7-dihydroxy flavone (5 mg/kg), 3,3'-dihydroxy flavone (20 mg/kg), 6,3'-dihydroxy flavone (10 mg/kg), and 5,6-dihydroxy flavone (10 mg/kg) i.p.

Statistical Analysis of the Data

Data obtained from various experiments were analyzed using ANOVA, followed by Dunnett's *t*-test and unpaired *t*-test (SPSS 16 Software). A probability value <5% was considered to be statistically significant. The results are represented as tables.

RESULTS

Effect of Dihydroxy Flavones on Forced Swim Test and Tail Suspension Test

The mean period of immobility in vehicle-treated mice was 193.5 ± 3.61 s in forced swim test and 199.0 ± 7.51 s in tail suspension test [Table 1]. In imipramine (20 mg/kg)-treated animals, there was a significant reduction in the immobility period in both forced swim test (8.0 ± 4.95 s) and tail suspension test (14.67 ± 1.71 s).

A dose-dependent reduction in the immobility period was observed in mice after treatment with 3,7-dihydroxy flavone (2.5,5 and 10 mg/kg, i.p) in both forced swim test and tail suspension test [Table 1]. Similarly, a significant and dose-dependent reduction in the immobility period was evident in both the tests, after treatment with 5,6-dihydroxy flavone in doses of 5, 10, and 20 mg/kg i.p [Table 1].

Table 1: Effect of dihydroxy flavones in forced swim and tail suspension tests on mice

Treatment-mg/kg, i.p	Period of immobility[a] (s)	
	Forced swim test	Tail suspension test
3,7-dihydroxy flavone		
2.5	144.3±3.02*	148±2.48*
5	45.83±2.55*	122.8±2.37*
10	14.33±2.60*	59.17±7.58*
3,3'-dihydroxy flavone		
5	83.33±13.10*	174.8±13.41*
10	73.50±9.97*	154.0±13.07*
20	45.50±6.63*	122.8±3.12*
6,3'-dihydroxy flavone		
5	137.2±10.91*	125.0±3.89*
10	114.5±2.01*	113.3±4.25*
20	105.8±2.09*	114.7±4.81*
5,6-dihydroxy flavone		
5	92.33±4.50*	135.7±2.16*
10	55.33±1.61*	103.2±2.14*
20	25.00±2.35*	45.5±2.78*

Vehicle-treated mice recorded an immobility period 193.5±3.61 s in forced swim test and 199±7.51 s in tail suspension test. In imipramine (20 mg/kg i.p)-treated group, the period of immobility was 8.0±4.95* s in forced swim test and 14.67±1.71* s in tail suspension test. [a]The period of immobility was recorded during a 6 min swimming/tail suspension session. Each value represents the mean±SEM of six observations. *P<0.05 compared to vehicle treatment (one-way ANOVA followed by Dunnett's *t*-test). SEM: Standard error of the mean

Treatment with 3,3'-dihydroxy flavone also resulted in a significant and dose-dependent reduction of immobility period in mice subjected to forced swim test and tail suspension test. However, there was a greater reduction of immobility period in forced swim test compared to tail suspension test in all the doses of 3,3'-dihydroxy flavone [Table 1].

A significant reduction of immobility period in mice was recorded after treatment with 6,3'-dihydroxy flavone in both the tests [Table 1]. However, a maximum reduction was observed with 10 mg/kg treatment, beyond which there was no further reduction in the immobility period by increasing the dose.

Effect of Interacting Drugs

The above results on forced swim test and tail suspension test indicated a potential antidepressant-like effect of the

investigated dihydroxy flavones. It was considered interesting to identify the probable mode of action of these compounds. For this purpose, the effect of dihydroxy flavones on forced swim test was carried out after pre-treatment with various interacting drugs.

In the doses employed, the interacting drugs *per se* did not significantly modify the immobility period compared to vehicle-treated mice (data not shown).

PCPA was included in the paradigm to delineate the role of serotonergic pathways in the action of dihydroxy flavones. Varying results were obtained for different dihydroxy flavone in animals pretreated with PCPA. The reduction in immobility period produced by 3,3'-dihydroxy flavone was significantly attenuated in PCPA-pretreated animals [Table 2].

In contrast, a further significant reduction in immobility period was evident for 6,3'-dihydroxy flavone and 5,6-dihydroxy flavone in PCPA-pretreated animals. However, the reduction in immobility period observed with 3,7-dihydroxy flavone was not altered by PCPA pre-treatment.

Pre-treatment with a tyrosine hydroxylase inhibitor and AMPT significantly attenuated the reduction in immobility period produced by 3,7-dihydroxy flavone, 3,3'-dihydroxy flavone, and 5,6-dihydroxy flavone. However, the reduction in immobility period produced by 6,3'-dihydroxy flavone remained unaltered by AMPT pre-treatment [Table 2].

L-arginine pre-treatment significantly attenuated the reduction in immobility period recorded with 3,7-dihydroxy flavone, 3,3'-dihydroxy flavone, and 5,6-dihydroxy flavone. On the contrary, the reduction in immobility period observed with 6,3'-dihydroxy flavone was further potentiated by L-arginine pre-treatment [Table 2].

The reduction in the immobility period effected by 3,7-dihydroxy flavone, 3,3'-dihydroxy flavone, and 5,6-dihydroxy flavone was significantly attenuated by naloxone pre-treatment. However, the reduction in the immobility period observed with 6,3'-dihydroxy flavone was not significantly altered by pre-treatment with naloxone [Table 2].

DISCUSSION

Even though depression has been recognized as a proliferating health problem worldwide, it is considered that the current antidepressant treatment strategies are far from adequate.[16] The intolerable side effects associated with the use of currently employed antidepressant drugs also necessitate the development of new drugs with minimal adverse effects. The current understanding of the etiology of depression has also widened the scope for novel antidepressant drugs. In addition to the reuptake mechanisms of monoamines, other neuronal pathways such as N-methyl-D-aspartate receptors and L-arginine-NO pathway and even the role of opioid system have been suggested to be potential targets for newer antidepressant drugs.[9,17,18]

The search for antidepressant drugs from nature has been motivated by the traditional use of *H. perforatum* for the treatment of depression.[2] Flavonoids have been identified as active constituents of *H. perforatum*, and many other plants suggested for depression in Indian system of medicine and investigated for their effects in experimental animals.[3] Hence, in the present study, it was considered interesting to investigate the potential antidepressant effect of structurally related dihydroxy flavones.

The results of the present study indicate that the investigated dihydroxy flavones (3,7-dihydroxy flavone, 3,3'-dihydroxy flavone, 6,3'-dihydroxy flavone, and 5,6-dihydroxy flavone) significantly decreased the immobility period of mice in both forced swim test and tail suspension test. A dose-dependent reduction in the immobility period was evident for all the compounds except 6,3'-dihydroxy flavone [Table 1]. This observation reveals the potential antidepressant effect of the above dihydroxy flavones. An earlier study[5] reported the antinociceptive effect of the above dihydroxy flavones in mice and also reported that these compounds did not alter the locomotor activity (open field apparatus) and motor performance (rotarod) of mice. Flavonoids such as rutin,[13]

Table 2: Effect of interacting drugs on dihydroxy flavones induced reduction in immobility period in forced swim test

Treatment (mg/kg i.p)	Immobility period[a] (s) after pre-treatment with				
	Vehicle	PCPA[b]	AMPT[c]	L-arginine[d]	Naloxone[d]
3,7-dihydroxy flavone-5	45.83±2.55	51.17±5.86	110±7.06*	70.33±2.79*	92.00±5.06*
3,3'-dihydroxy flavone-20	45.50±6.63	120.67±8.48*	112.3±4.72*	138.0±8.93*	76.17±4.14*
6,3'-dihydroxy flavone-10	114.5±2.01	66.5±4.69*	114.7±5.70	58.83±2.95*	108.5±4.19
5,6-dihydroxy flavone-10	55.33±1.61	35.17±5.13*	103±3.96*	107.3±11.43*	90.67±4.67*

[a]The period of immobility was recorded during a 6 min swimming session. Each value represents the mean±SEM of six observations. *$P<0.05$ compared to immobility period recorded with vehicle pre-treatment (unpaired *t*-test) [b]Animals were treated with PCPA (PCPA 100 mg/kg i.p) for 4 consecutive days. Immobility period after treatment with different flavones was tested on the 5th day. [c]The animals received AMPT (AMPT 100 mg/kg i.p) 4 h before the dihydroxy flavone injection. [d]L-arginine (500 mg/kg i.p) or naloxone (10 mg/kg i.p) was administered 15 min before dihydroxy flavone treatment. PCPA: Para chlorophenylalanine, AMPT: Alpha-methyl-para-tyrosine, SEM: Standard error of the mean

quercetin,[19] naringenin,[20] and baicalein[21] have been shown to exert antidepressant effect in animal models. The results of the present study are in agreement with these earlier reports and suggest the potential use of dihydroxy flavones in depression. When compared to 6,3'-dihydroxy flavone, other three investigated compounds appear to be more effective in reducing the immobility period in both the tests [Table 1].

Mechanisms Involved

The recorded antidepressant effect of the dihydroxy flavones prompted an investigation on the possible mechanisms involved. Serotonergic system has been implicated in the pathogenesis of anxiety and depression. Most of the currently employed antidepressant drugs such as selective serotonin reuptake inhibitors prevent the uptake of serotonin (5-HT) and increase its concentration at synapses. PCPA is an inhibitor of the enzyme tryptophan hydroxylase. On continuous administration for 4 days in mice, it is reported to deplete the endogenous stores of serotonin by about 60%, without altering the levels of norepinephrine (NA) and dopamine (DA).[22] Pre-treatment with PCPA in mice has been shown to alter the response to fluoxetine and citalopram.[23] PCPA pre-treatment significantly attenuated the reduction in immobility period observed with 3,3'-dihydroxy flavone suggesting a role for serotonergic mechanism in the antidepressant effect of 3,3'-dihydroxy flavone [Table 2]. Flavonoids such as liquiritin and isoliquritin,[24] vitexin,[25] rutin,[13] and flavonoids from *H. perforatum*[2] have been shown to involve serotonergic pathways in mediating their antidepressant effect. The present results on 3,3'-dihydroxy flavone also indicate such a possibility. However, the effect of 3,7-dihydroxy flavone in reducing the immobility period of mice in forced swim test was not altered by PCPA pre-treatment, ruling out the involvement of serotonergic mechanism in the action of 3,7-dihydroxy flavone. The results observed after 6,3'-dihydroxy flavone and 5,6-dihydroxy flavone in PCPA-treated animals are more intriguing. Further significant reduction in immobility period was noticed in these animals when compared to flavonoid treatment alone. This observation may suggest that the antidepressant effect of 6,3'-dihydroxy flavone and 5,6-dihydroxy flavone may persist even after depletion of serotonin and additional mechanisms may be involved in the antidepressant effect of these compounds.

Another target for antidepressant activity is noradrenergic system.[26] Depressed individuals were found to have increased levels of α_1-adrenoreceptor in the prefrontal cortex,[27] downregulation of α_2 receptors,[28] and reduced levels of monoamines (NA, DA, and 5HT) in the cortical and limbic areas. Drugs such as duloxetine, venlafaxine, and desvenlafaxine inhibit the reuptake of serotonin and NA is presently used in the management of depression. AMPT is employed to study the role of adrenergic system in the effect of antidepressant drugs. AMPT can reduce the levels of DA and NA to the extent of 57% and 53%, respectively, in mice without altering the levels of serotonin.[29]

Pre-treatment with AMPT significantly reversed the decrease in immobility period of mice produced by 3,7-dihydroxy flavone, 3,3'-dihydroxy flavone, and 5,6-dihydroxy flavone suggesting a role for adrenergic system in the antidepressant effect of these dihydroxy flavones [Table 2]. Previous studies on flavonoids such as rutin,[13] vitexin,[25] liquiritin and isoliquritin,[24] and flavonoids from *H. perforatum*[2] indicated an involvement of adrenergic mechanism in their antidepressant effect. The present results on dihydroxy flavones are in agreement with the earlier reports. However, the reduction in immobility period observed with 6,3'-dihydroxy flavone was not altered by AMPT pre-treatment, thus excluding the role of adrenergic system in the action of 6,3'-dihydroxy flavone.

Apart from the classical monoamine reuptake inhibitors, alternative targets are being identified to treat depression. Many evidences indicate that opioid system also plays a significant role in the development of depression and serum β-endorphin levels are decreased in patients with severe depression.[30] The effectiveness of oxycodone, oxymorphone, and buprenorphine in patients with major depression refractory to other drugs has been reported.[9] Tramadol exhibited antidepressant effect in many animal models[31,32] and is therapeutically used in refractory major depression[10] and severe suicidal ideation.[33] The above evidences indicate a potential role for opioid system in the development of depression. Moreover, the dihydroxy flavones investigated in the present study were found to exert opioid-mediated analgesic effect in mice.[5] Hence, it was considered interesting to investigate possible role played by opioid mechanisms in the antidepressant effect of dihydroxy flavones. The results of the present study reveal that pre-treatment with an opioid antagonist naloxone reversed the reduction in immobility period produced by 3,7-dihydroxy flavone, 3,3'-dihydroxy flavone, and 5,6-dihydroxy flavone without altering the effect of 6,3'-dihydroxy flavone [Table 2]. This observation suggests a possible role for opioid system in the antidepressant effect of the above dihydroxy flavones except 6,3'-dihydroxy flavone. Depression and pain are comorbidities and agents that are useful in suppressing the manifestation of depression and alleviating pain will be highly desirable in therapeutics. Flavonoids may be considered ideal candidates with such useful properties.

A role for endogenous NO system has also been suggested in the pathogenesis of depression.[34,35] It has been proposed that the antidepressant effect of imipramine and venlafaxine involves the suppression of NO synthesis.[36] Flavonoids like hesperidin exerted antidepressant effect involving NO-cyclic guanosine 3'5'-monophosphate pathway[37] and L-arginine pre-treatment could reverse the protective effect of rutin against the immobilization stress-induced

anxiety-like behavior.[38] In the present study, pre-treatment with a NO donor L-arginine significantly attenuated the reduction of immobility period in forced swim test produced by 3,7-dihydroxy flavone, 3,3'-dihydroxy flavone, and 5,6-dihydroxy flavone [Table 2]. The above observation suggests a definite role for NO pathway in the antidepressant effect of dihydroxy flavones. However, a further reduction in immobility period was observed by L-arginine pre-treatment in 6,3'-dihydroxy flavone-treated mice.

CONCLUSION

In summary, the results of the above observations indicate a potential antidepressant activity of the investigated dihydroxy flavones. The effect of 3,7-dihydroxy flavone and 5,6-dihydroxy flavone appears to be more prominent in both forced swim test and tail suspension test compared to 3,3'-dihydroxy flavone and 6,3'-dihydroxy flavone. Whether the hydroxylation at 3'position of flavone nucleus might reduce the antidepressant efficacy remains a possibility. The present study also identified the multiple mechanisms that may be involved in the antidepressant effect of dihydroxy flavones. Evidence for the involvement of adrenergic mechanism in the antidepressant effect of three dihydroxy flavones was revealed. Surprisingly, serotonergic mechanism was found to play a role in only one of the dihydroxy flavones. In addition, opioid and NO pathways were also found to operate in the antidepressant effect of these compounds. In conclusion, the result of the present study has identified few dihydroxy flavones with potential antidepressant-like effect acting by novel mechanisms.

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