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Original Research Article

Evaluation of Analgesic and Anti-Inflammatory Activities of hydro-alcoholic leaf extract of *Hypericum revolutum* in Mice

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Abstract

Background: Despite the progress that has been made in the past for the development of pain and inflammation therapy, there is a need for effective and potent medicinal agent with lower side effects. The aim of this study was to evaluate the analgesic and anti-inflammatory activities of 80% methanol leaf extract of *Hypericum revolutum* in mice.
Method: After extraction of the crude using 80% methanol, *Hypericum revolutum* extract was evaluated for analgesic and anti-inflammatory activity in hot plate test, acetic-acid induced writhing and carrageenan induced paw edema models. Extract was evaluated at 100, 200 and 400 mg/kg doses. The reference control groups were treated with morphine 5mg/kg or indomethacin 10mg/kg while vehicle, distilled water (10 ml/kg), treated animals were grouped as negative controls.
Result: In the hot plate method, all doses of the extract and the standard drug morphine significantly prolonged the latency time in the hot plate method ($p<0.05$ or $p<0.01$ or $p<0.001$) when compared to the negative control throughout the observation time. Greater increase in latency was observed at the 120 minute and greater on the other time intervals with 200mg/kg of the extract with a significant value ($p<0.001$). Similarly, all test doses of the extract significantly ($p<0.05$) reduced the acetic acid induced writhing in mice. The extract of HR produced a significant analgesic activity with 62.4, 47.1 and 55.3% inhibition of number of writhing at 100, 200 and 400 mg/kg dose levels, respectively although the inhibition was greater for the standard.
Moreover, for carrageenan induced paw edema test treatment with the extract (100, 200, and 400mg/kg) and the standard have shown significance ($p<0.01$ or $p<0.001$) suppression of edema at all-time points as compared to controls.
Conclusion: The 80% hydro-methanolic extract of *H. revolutum* exhibited analgesic and anti-inflammatory effects in mice models.

Keywords: Analgesic, Anti- inflammatory, Hypericum revolutum, Hot plate, Carrageenan.

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1. Introduction

Inflammation is a local defense response to tissue injury intended to remove or limit the spread of injurious agent or initiation of tissue healing process. It may involve symptoms and tissue injury consisting of edema formation, leukocyte migration and infiltration and granuloma formation. Pain and inflammation occur as nonspecific indicator of numerous disease conditions. During the inflammatory process different kinds of endogenous mediators such as prostaglandins, bradykinin, histamine and serotonin are released from inflammatory cells. Among which, Prostaglandins are ubiquitous during inflammation mediating cell modulations and tissue responses[1].

Over the last few years, reports indicate that about 20% of the world population suffering from chronic pain. Also, chronic inflammation is a primary contributing factor involved in multiple chronic diseases. Overall, the estimated prevalence of chronic inflammatory disease is about 5 to 7% in the western world. These figures may indicate that pain and inflammatory conditions have been a global public health concern and worse better attention to relieve the burden [2].

Presently, the management of pain and inflammation relies on either the use of narcotics or non-narcotics such as non-steroidal anti-inflammatory agents (NSAIDs) which have known adverse and toxic effects. Some of these adverse effects include gastrointestinal upset due to altered protective gastric mucosa, renal damage and respiratory depression in long-term use as well as possible dependence particularly with opioid analogues. Like NSAIDs, corticosteroids are also known for their debilitating side effects such as clouding of the lens in one or both eyes, elevation of blood sugar (trigger or worsen diabetes), increased risk of infections, osteoporosis and suppression of adrenal gland hormone production[3]. Additionally, if these drugs are taken for prolonged treatment duration, the risk for developing different complication will be very high.

Consequently, newer anti-inflammatory and analgesic agents lacking those adverse effects are of a potential research interest across the world. Phytochemicals from medicinal plants have been an active area of researchers because of their safety profile as well as their long-standing use across different sociocultural and folkloric traditional medicine of the world. Therefore, searching better analgesic and anti-inflammatory agents from plants with traditional claims should still be views as a logical research strategy in search of better newer agents.

For many centuries, medicinal plants have been used to treat various disease conditions in various forms of preparations in Ethiopian traditional medicine. Similarly, many plants are claimed to have been effective and used to relieve pain or inflammation in folkloric medicine of Ethiopia. One of such plants is *H. revolutum* that it has been used to treat several ailments including malaria, skin infections, venereal diseases, gastrointestinal disorders, tumors, infertility and epilepsies. Its stem bark is usually boiled in water and administered either as steam bath or sorely for the treatment of malaria and other fevers while roots are known for to treat intestinal worms and dysentery[4]. Related to analgesic activity, *H. revolutum* was reported to treat rheumatism[5] and ear ache[6] in Ethiopia traditional medicine. A series of five benzopyran derivatives (1-5) have been isolated from a light petroleum ether extract of the twigs and leaves of *H. revolutum*[7].

Another supporting evidence for the candidature of *H. revolutum* for stated pharmacological screening comes from reports and findings of its family member, *H. perforatum.* Traditionally, extracts of the plant *H. perforatum* has been used in many parts of the world to treat a variety of health conditions such as nerve pain, malaria, viral, and bacterial infection; as a topical agent for wounds, burns, and insect bites; and internally as a sedative, anti-anxiety, and antidepressant. *H. perforatum* has also been used as/in an astringent, anti-inflammatory, immune support and menopause symptoms[8]. Based on these reports and findings, *H. revolutum* may have similar activities that require scientific validation. Therefore, the aim of this study was to evaluate the analgesic and anti-inflammatory activities of 80% methanol leaf extract of *Hypericum revolutum* in mice.

2. Materials and methods

2.1 Chemicals, Drugs and Reagents

Distilled water (DW) and Morphine (Ethiopian Pharmaceuticals Manufacturing Factory, Ethiopia), Carrageenan (Sigma Chemicals Co., St Louis, USA), Tween 80 (Atlas Chemical Industries Inc, USA), 0.9% normal saline IV solution (Anhui Easy way Medical Supplies Co., Ltd, China), Indomethacin (Cadilla Pharmaceuticals, Ethiopia) were obtained and used in the experiment.

2.2 Plant Material Collection

The leaves of *Hypericum revolutum* was collected from Ambo town in Oromiya, 120 km away from Addis Ababa, in December 2017. The collected plant specimen was identified and authenticated and a voucher specimen (collection number AE/001) of the plant was deposited for future references at the National Herbarium of College of Natural and Computational science, Addis Ababa University. Running water was used to wash and rinse the leaves gently and air-dried under shade. The leaves were then be pulverized to a coarse powder using mortar and pestle.

2.3 Experimental Animals

A total of 60 healthy Swiss albino mice of either sex having weights ranging from 20 to 35g, and six to eight weeks of age was either procured from Ethiopian Public Health Institute (EPHI) or bred in the animal house of the School of Pharmacy, Addis Ababa University was used for the experiment. All animals were fed with commercial pellets and have had free access to water *ad libitum*. Animals were fasted overnight and were weighed before each experiment. In addition, the animals were acclimatized for one week before commencement of the experiment. The care and handling of animals was according to accepted international guidelines [9].

2.4 Extraction

The plant was cleaned, shade dried and milled into fine powder. Accurately weighed powder was taken and macerated with 80% methanol (1:2) for 48 h. The contents were shaken during the first 6 h and allowed to remain with solvent for 18 h. After 24 h, the extract was filtered with cotton and then by suction filtration apparatus (Oakton, U.S.A) using Whatman filter paper (No. 1). The marc was re-macerated twice using the same volume of solvent to exhaustively extract the plant material. The filtrates were recombined and concentrated on rotavapour (Buchi, Switzerland) at 40^0C under reduced pressure. The resulting concentrate was freeze-dried in a lyophilizer. The yields

obtained with respect to the initial dried material were 17%. The dried extracts were reconstituted with water for oral administration.

2.5 Animal Grouping and Dose Selection

Mice were randomly divided into five groups with each group consisting of six mice. Group I served as a negative control were administered with 10ml/kg of distilled water. Group II served as positive control and treated with standard drugs; morphine (5mg/kg, s.c) for hotplate test, indomethacin (10 mg/kg, p.o) for carrageenan test. Groups III, IV and V were given 100, 200 and 400 mg/kg of the extract, respectively. Dose selection was carried out based on acute toxicity test done by Abebe Ejigu and Ephrem Engidawor[10].

2.6 Test for Analgesic Activity

2.6.1 Hot Plate Test

Following administration according to respective grouping, mice were placed on a hot plate maintained at 55 ± 1 °C. Latency of nociceptive response such as licking, flicking of a hind limb or jumping was measured. Measurements were performed at time 0 before and 30, 60, 90 and 120 minutes after drug administration, with a cut-off time of 15 s to avoid lesions to the animals' paws. Maximum Possible Analgesia (percentage MPA), was calculated for each group as shown below[11].

$$\%\ \boldsymbol{Latency} = \frac{mean\ letency\ time\ (control\ group - treated\ group)}{Mean\ letency\ time\ of\ control\ group}\ x\ 100$$

2.6.2 Acetic acid-induce writhing test:

The method of Arul et al. was used with slight modification. Mice of either sex were divided into five groups with each consisting of six animals. Three groups were given different dose of the plant extract, while the control group was given a vehicle and the reference group was given 150mg/kg of aspirin just one hour before 0.6% acetic acid (10ml/kg, i.p.) administration. Five minutes after the acetic acid injection i.p., the number of writhes was counted to determine analgesic activity of HR 80% methanol extract. The animals were placed in a glass jar individually and the contractions of abdominal muscles together with stretching of the hind limbs were cumulatively counted over a period of 15 minutes. The percentage protection against writhing was taken as an index of analgesia and calculated using the following formula:

$$\%\ \boldsymbol{Analgesic\ activity} = \frac{mean\ writhing\ count\ (control\ group - treated\ group)}{Mean\ writhing\ count\ of\ control\ group}\ x\ 100$$

2.7 Test for Anti-Inflammatory Activity

2.7.1 Carrageenan Induced Paw Edema Test

The test was conducted according to the method of Winter *et al*., [12], with slight modification. In to the plantar side of the right hind paw, fifty microliters of 1% carrageenan suspended in saline was injected. Indomethacin (10 mg/kg, PO), the vehicle and different doses of the extract were administered one hour prior to carrageenan injection. The paw volume was measured at immediately, 1st, and 2nd hours after the injection using a plethysmometer.

2.8 Statistical Analysis

The experimental results obtained were expressed as mean ± standard error of mean (SEM) of responses. Analysis of results was done using Statistical Package for Social Sciences (SPSS) software version 21. The statistical significance was determined by using One-way Analysis of Variance (ANOVA) followed by Tukey post Hoc test. The analysis was performed with 95% confidence interval and the value, $p<0.05$, was considered as statistically significant.

3. Result

3.1 Analgesic Activity

3.1.1 Hot Plate Test

Table 1 depicts the effect of methanolic extract on the hot plate test. When compared to the negative control, all doses of the extract and the standard drug morphine significantly prolonged the reaction time in the hot plate method. The increase in latency time observed by the lower dose was not significantly different from the effect of the middle and the higher dose of the extract at all-time intervals. Although the increase in latency time was not significantly different between the test extract and the standard drug Morphine, highest increase in percentage compared to the latency time at time 0 was higher with the standard and the higher dose of the extract resulting in 262.7% and 245.4% rise in latency time at time 120 min respectively. There was a difference in the latency of the different treatment groups in time. At 30 min, the standard drug resulted in a higher effect than all doses of the extract, however for the rest of the time intervals although the standard drug resulted in higher effect than the extract that difference was much less.

Table 1: Effect of 80% methanol leaf extract of *H. revolutum* in hot plate test

Groups	Mean latency time (sec)				
	0 min	30 min	60 min	90 min	120 min
Neg. con (10ml/kg)	3.89± 1.47	3.70±0.81	4.28±0.40	4.28±0.22	5.19±0.67
MOR(10mg/kg)	3.86±0.96	9.38±0.81[a3] (143%)	10.73±2.23[a2] (177.9%)	12.62±1.54[a3] (226.9%)	14.00±0.7[a3] (262.7%)
100mg/kg	3.58±1.02	8.27±2.15[a1] (131%)	11.00±1.07[a3] (72.3%)	12.12±2.31[a2] (232.4%)	11.27±2.64[a2] (200.8%)
200mg/kg	4.60±1.50	8.60±0.73[a3] (86.9%)	10.67±2.11 [a2] (131.9.9%)	13.14±1.58 [a3] (185.6%)	13.8±2.22 [a3] (200%)
400mg/kg	3.92±0.31	9.28±0.65[a3] (136.7%)	10.55±1.32[a] (169.1%)	13.43±1.80[a3] (242.6%)	13.54±1.67[a3] (245.4%)

Data represent mean ± S.E.M (n = 6); 1=p<0.05, 2=p<0.01, 3=p<0.001; a: relative to control MOR - morphine 10 mg/kg; data in parenthesis show percentage increase in latency of licking relative to time zero within individual group.

The maximum possible analgesia of the extract is shown in table 2. As shown in table 2 the extract at a dose of 200 and 400 mg/kg has resulted in 207% and 213.8 % at 90 min respectively. In addition, the middle dose of the extract has resulted in MPA of 165.9% at 120 min. Moreover, the positive control morphine has caused similar analgesia effect at 90 min resulting in 194.8% of MPA but less than the middle and higher dose of the extract.

Table 2: The Analgesia effects of (MPA %) *H. revolutum* 80% methanol extract in hot plate test model

Groups	Mean Percent Analgesia (%)			
	30 min	60 min	90 min	120 min
Neg. CON	-	-	-	-
MOR	153.5	150.7	194.8	169.7
100mg/kg	123.5	157	183.2	117.1
200mg/kg	132.4	149.3	207	165.9
400mg/kg	150.8	146.5	213.8	160.9

Data represent mean percent analgesia (%) relative to the negative control group with respect to specific measurement time, (n = 6); MOR - morphine 10 mg/kg.

3.1.2 Acetic acid induced writhing test

As shown in table 3, all test doses of HR extract significantly (p<0.05) reduced the acetic acid induced writhing in mice. Compared to the higher dose levels, the 100 mg/kg dose found to have better analgesic effect against acetic acid induced writhing test. While MO resulted in the superior percent inhibition and significant inhibition and analgesic effect against all test doses of the extract as well as the negative control group based on acetic acid induced writhing test. The 80% methanol extract of HR produced a significant analgesic activity with 62.4, 47.1 and 55.3% inhibition of number of writhing at 100, 200 and 400 mg/kg dose levels, respectively.

Table 3: Analgesia effects of (MPA %) *H. revolutum* 80% methanol extract in acetic acid induced writhing test model

Groups	Mean	% inhibition
Neg. CON	14.17±0.48	-
MO	3.17±0.48[a1b1c1d1]	77.6
100mg/kg	5.33±0.80[a1]	62.4
200mg/kg	7.50±0.85[a1]	47.1
400mg/kg	6.33±0.49[a1]	55.3

Data represent mean ± S.E.M and percent inhibition of number of writhing with respect to the negative control, (n = 6); 1=p<0.05, 2=p<0.01, 3=p<0.001; a: relative to control b: relative to standard c: relative to 100 mg/kg; MO - morphine 10 mg/kg

3.2. Anti-Inflammatory Activity

3.2.1. Carrageenan Induced Paw Model

The anti-inflammatory activity of 80% methanol extract of *H. revolutum* using carrageenan induced paw edema is summarized in Table 4.Treatment with the extract (100, 200, and 400mg/kg) and the standard have shown significance (p<0.01 or p<0.001) suppression of edema at all-time points as compared to controls. Accordingly, treatment with 200 mg/kg and 400 mg/kg resulted in reduction of edema by about 61.4% and 51.5%, respectively at 2 h. The percent reduction in paw volume after 1 hr was greater with the higher dose of the extract resulting in almost 64% reduction. However the standard drug Indomethacin, 200 mg/kg and 100 mg/kg of the extract resulted in 60%, 55% and 46% reduction in paw volume respectively. On the other hand, percent reduction with 400 mg/kg was lower than the other 200 mg/kg and the standard drug at 2 hr. Although the standard used produced maximum reduction in edema formation at 2 hr, the changes observed were not significantly different to that produced by the all doses of the extract.

Table 4: Effect of 80% methanol leaf extract of *H. revolutum* on carrageenan induced paw model

Groups	Mean paw volume (ml)		
	0hr	1hrs	2hrs
Neg. CON	0.94±0.08	1.66±0.34	1.34±0.06
INDO	0.73±0.32	0.67±0.18[a2] (59.6%)	0.44±0.20 [a3] (67.2%)
100mg/kg	0.81±0.19	0.90±0.27 [a2] (45.8%)	0.70±0.24[a2] (47.8%)
200mg/kg	0.74±0.14	0.75±0.23[a2] (54.8%)	0.49±0.21[a3] (63.4%)
400mg/kg	0.92±0.22	0.60±0.26[a3] (63.9%)	0.65±0.13[a3] (51.5%)

Data represent mean ± S.E.M (n = 6); 1=p<0.05, 2=p<0.01, 3=p<0.001; a: relative to control; INDO; Indomethacin (10mg/kg)

4. Discussion

With increasing morbidity and associated economic burden, pain and inflammation along with their management become sensitive and public health issue. Therefore, drug discovery and the search for a potential antinociceptive and anti-inflammatory agent is an active are

of research. This study evaluated the analgesic and anti-inflammatory effect of *H. revolutum* in animal models as the plant is widely accepted and used folk medicine in Ethiopia for earache and rheumatism disease conditions [5, 6]. In due course, the findings of this study have revealed that the 80% methanol extract of *H. revolutum* is endowed with analgesic and anti-inflammatory effect evidenced by increase in pain latency time and attenuation of edema formation in the animal models.

The hot plate test was used to test supra-spinal nociception and determine the involvement of central antinociceptive mechanism. The exposure of animal paws to thermal stimuli in the hot plate test leads to the development of non-inflammatory, acute nociceptive response. Pain induced by thermal stimulus of the hot plate and thermal radiation of tail flick is specific for centrally mediated activity. In this model, a hot plate is used with a constant temperature (maintained at 55±1°C) usually to measure two key behavioral integrity components, namely paw licking and flicking and jumping, based on their reaction time. Paw licking and flicking is the behavioral manifestation primly observed in the initial phase. Then, when the pain induced by the hot plate continues, the animal will start to create a mechanism to escape from the previous stimulus [11]. This model is advantageous in that it requires less time, causes limited tissue damage, very sensitive to strong analgesics and measurements are accurate[13,14].

In the hot plate test, the extract of *H. revolutum* leaves showed significant central analgesic effect. This is demonstrated by increase in latency time or increase in threshold of pain by thermal stimuli. There was greater increase in the latency time at 120 min of interval compared to the other time intervals except for the 100 mg/kg of the extract. This might imply that there is a time dependent increase in activity of the extract. The extract may have exerted an antinociceptive effect through central mechanisms as the hot plate test specifically demonstrates central antinociceptive effects. The plant extract might do so by acting at the periaque ductal gray matter of the central nervous system[15]. The different doses of the extract took longer time (peak time) to attain for maximal effect, which for all doses was 120 min. This delay might be explained by a probable time lag for the drug entry to the central compartment and distribution into the target site or formation of active metabolites that are endowed with analgesic activity with better half-life. A relatively better action of 400 mg/kg of the extract compared to morphine especially in the 1sthr suggests that there may be other constituents that contribute for the analgesic activity of the extract in addition to opioid like constituents.

Maximum possible analgesia was determined from the hot plate test which reflects a percentage of how much the administered substance protected pain sensation. As a group, the 200mg/kg dose of the extract showed a relatively higher MPA after 120 minutes. Even this dose level produced a better analgesic effect compared to morphine.

To evaluate the peripheral antinociceptive activity of the extract, acetic acid induced writhing test was employed as it is able to detect antinociceptive effects, sensitively [16]. The *H. revolutum* extract at all test doses significantly reduced the writhing response while the lower dose was superior in its action compared to the other doses. This may be explained by the difference in the pharmacokinetics of constituents of the plant extract. So the higher dose has more central distribution and has greater central effect but the lower dose has greater peripheral effect. It could also be explained by difference in the pharmacodynamics profile of the extract components. However, the effect of the lower dose was less than the standard drug Indomethacin. Acetic acid irritates the peritoneal cavity leading to stimulation of local nociceptors and induces liberation of endogenous substances such as 5-HT, histamine, prostaglandins (PGs), bradykinins and substance P, from peripheral sensory nerve endings[17]. This suggests that the extract of *H. revolutum* might work via suppressing the release of these endogenous substances and inflammatory mediators to produce peripheral analgesic activity. The writhing test shows good sensitivity, even it detects effects from weak analgesics, but this test alone may not postulate the involvement of central or peripheral activity[18,19].

Carrageenan-induced hind paw edema model was employed to evaluate the anti-inflammatory activity of the extract. This model has been widely used for the discovery and evaluation of anti-inflammatory drugs [11,20,21] that particularly interfere with the production of PGs and reactive oxygen species. Carrageenan is a sulphated polysaccharide obtained from sea weed and is commonly used to induce acute inflammation. The time course of edema development in carrageenan-induced paw edema model in mice is generally represented by three distinct phases. The first phase of inflammation occurs between 0 and 1.5 h of carrageenan injection and is partly attributed to trauma of injection and also to histamine, and serotonin components. The second phase (1.5-2.5 h) is associated with the production of bradykinin and protease. The third phase is mediated by prostaglandin and lysosomes. Maximal vascular response as determined with leukocyte migration to the inflamed area, also reaches its maximum level in the third phase. Prostaglandins play a major role in the development of the third phase of inflammatory reaction which is measured from 2.5 to 6 h post-carrageenan injection[22–24].

At all test doses the extract showed significant inhibition of edema formation after carrageenan injection dose dependent manner with higher activity at 400mg/kg and lowest at 100mg/kg. This may be due, at least in part,

to the presence of different chemical components which might interfere with anti- inflammatory activity of the extract. Also, the concentration of active components of the extract may vary from dose to dose. The extract was effective at earlier phases (1-2h) indicating that the anti-inflammatory activity is likely to be attributed to inhibition of histamine and 5-HT release.

A number of studies recorded that several plant extracts showed analgesic and anti-inflammatory effect in animal models and their effects have been attributed to the presence of triterpenoids, alkaloids, glycosides, tannins and sterols[15,28–31]. A preliminary phytochemical test done by A. Ejigu and E. Engida work showed that, *H. revolutum* detected to contain alkaloids, saponins, phenolic compounds, and flavonoids[10]. Therefore, it may be due to the presence of these phytoconstituents or their combined effects that *H. revolutum* showed analgesic and anti-inflammatory activities. Flavonoids and saponins are well known for their ability to inhibit pain perception as well as anti-inflammatory properties due to their inhibitory effects on enzymes involved in the production of the chemical mediator of inflammation[12]. Flavonoids also inhibit inflammatory processes by inhibiting phosphodiesterases that are involved in cell activation [26]. Similarly, flavonoids are involved in induction of endogenous serotonin secretion or interaction with 5-HT2A and 5-HT3 receptors involved as a probable mechanism of central analgesic activity[32]. On the other hand, various studies reported that alkaloids isolated from various plants have shown inhibitory effect on eosinophil recruitment, leukotriene production in the pleural cavities, as well as inhibiting in the production of nitric oxide mediators which result in anti-inflammatory effect [25]. In addition, polyphenols exert their anti-inflammatory properties through inhibition of the production of inflammatory cytokines and chemokines and suppressing the activity of cyclooxygenase (COX)) and inducible nitric oxide synthase (iNOS) and thereby decreasing the production of reactive oxygen and nitrogen species (ROS/RNS) [27]. Thus, it is possible that at least part of the analgesic and anti-inflammatory activity showed in this study by the hydroalcoholic extracts of *H. revolutum* may be due to the presence of these polyphenolic substances.

5. Conclusion

The 80% hydro-methanolic extract of *H. revolutum* exhibited analgesic and anti-inflammatory effects in mice models. This may imply and support the traditional claim of the crude extract of *H. revolutum* leaves for relieving pain and inflammation in Ethiopian folklore medicine.

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Disclosure of Conflict of Interest

The authors have not declared any conflict of interests.

Ethics approval and consent to participate

The study was approved by Ethical Review Board of School of Pharmacy, Addis Ababa University. However, no consent was needed for this study.

Consent for publication

All co-authors have consented for publication of this manuscript.

Availability of data and materials

The data is available in public library of Addis Ababa University in a form of student thesis.

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