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Evaluation of *in vitro* antioxidant, anti-bacterial, cytotoxic and *in vivo* analgesic and neuro-pharmacological investigation of *alysicarpus vaginalis* available in Bangladesh

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Abstract
Purpose: The objectives of this research were to investigate the effects of *Alysicarpus vaginalis L.* (Alyce clover) leaf extracts prepared with ethyl acetate and methanol on *in vitro* activities like antioxidant, antimicrobial and cytotoxic, as well as on some *in vivo* activities like analgesic and neuropharmacological properties in an animal model.
Study Design: Phytochemical screening was used as the primary method of evaluation for both the ethyl acetate (EAEAV) and methanolic extract (MEAV) of *Alysicarpus vaginalis (L.)*. Because of the pharmacological interest in the plant's constituent components, it was decided to investigate its potential on *in vitro* activities, such as antioxidant, cytotoxic and antibacterial. Whether or if the observed effects on its *in vivo* analgesic and neuropharmacological properties in animal models are statistically significant.
Place and Duration of Study: This investigation was performed in Laboratory of Phytochemistry and Pharmacology, Department of Pharmacy and Laboratory of Microbiology, Stamford University Bangladesh, Dhaka between June 2022 to October 2022.
Methodology: Both EAEAV and MEAV was subjected for phytochemical screening using various test reagent and was also researched for its potential antioxidant, antibacterial, cytotoxic, analgesic, and neuropharmacological effects. The DPPH Free Radical Scavenging Assay, the Disk Diffusion Method and the Brine Shrimp Lethality Bioassay were all used to determine cytotoxic, antimicrobial and antioxidant activity, using different concentrations respectively. *Swiss albino* mice were subjected to acetic acid-induced writhing and hot plate tests to determine analgesic efficacy. cGMP pathway induced mechanism test was used to confirm the actual analgesic activity. Open field and hole cross tests were used to evaluate locomotor activity as part of the neuropharmacological experiment.
Results: Different phytochemical constituent were detected in the experimental result of EAEAV and MEAV's phytochemical screening. MEAV showed highest antioxidant activity with an IC_{50} value of 34.70 µg/mL where EAEAV showed IC_{50} value of 12.49 µg/mL and MEAV also showed higher antibacterial activity compared to EAEAV. EAEAV and MEAV showed most notable lethality with LC_{50} value of 3.89 µg/mL and 8.71 µg/mL compared to the conventional vincristine sulphate's LC_{50} value in the brine shrimp lethality bioassay. As opposed to this, EAEAV reduced peripheral nociception in the acetic acid-induced writhing nociceptive paradigm, with percent inhibitions of 42.48% and 47.00% where MEAV reduced 40.63% and 51.77% at the aforementioned dosages as opposed to conventional Diclofenac sodium. cGMP pathway mechanism confirmed the % inhibition of pain using both EAEAV which boosted the pain reduction into 59.62% and 71.12%. Additionally, a considerable analgesic effect was seen in the hot plate test. The extract, when compared to the usual Diazepam, was also shown to reduce motor coordination in the Open Field and Hole Cross Tests.
Conclusion: These results confirmed the conventional medicine claims of the *Alysicarpus vaginalis (L.)* plant's therapeutic virtues and suggest potential mechanisms of action.

Keywords: *Alysicarpus vaginalis*, antimicrobial, antioxidant, analgesic, cGMP, cytotoxic, neuropharmacological

1. Introduction
A large portion of the finite resource of primary health care demands in the developing countries comes from plants used for medicinal purposes. About 80% of people worldwide chose to believe on traditional medicines as their first treatment option, according to the World Health Organization (WHO). Over 3.5 billion people in the world's least developed countries rely on locally available herbal remedies and medicinal plants to treat a wide range of health complications [1]. Traditional medicine, particularly herbal remedies used by ordinary people, has lately attracted more attention all around the globe.

An ancient medical practice had been refined in many parts of the globe by people who were living in close proximity to their natural environments at the time. In the search for new chemotherapeutic drugs derived from plants, information provided by various ethnic groups about indigenous traditional herbal treatments has always played an extremely important role [2].

The plant species *Alysicarpus vaginalis* (L.) DC, more widely known as Alyce clover, is a member of the Fabaceae family of plants. It has long been revered for both its ancient and modern medical applications. Plants of this genus are indigenous to Sri Lanka, India, Bangladesh, Malaysia, Yemen, Cambodia, Vietnam, Thailand, China, Nepal, Indonesia, Laos, the Philippines, the Maldives, Oman and Myanmar, as well as certain areas of Africa and Oceania. In traditional medical practice, the entire plant was administered to patients suffering from cytotoxicity, renal calculi, and sepsis [3, 4]. The extract from the leaves has been tried for treating eye problems and earaches [5]. In cases of renal disease, skin issues, leprosy, and respiratory difficulties, the roots of this plant are used as a diuretic [6]. A cough may be treated by drinking a decoction made from the roots of the plant. The root of this plant is often employed in the treatment of renal and urinary tract disorders, as well as leprosy and pulmonary conditions. It is possible that A. vaginalis has a very significant role in reducing and avoiding hepatotoxicity caused by Necrotic Body [7]. For medical purposes, the whole plant is utilized and it is effective in curing bone fractures and sword cuts.

The purpose of this study was to investigate the effects of *Alysicarpus vaginalis* L. (*Alyce clover*) whole plant extracts prepared with ethyl acetate and methanol on *in vitro* activities such as antioxidant, antimicrobial, and cytotoxic activities, as well as on some *in vivo* activities such as analgesic and Neuropharmacological activities in an animal model. Specifically, the objectives of this research were to investigate the effects of the plant extracts on which may help the researcher to use its phytochemicals to use in pharmaceutical products as well as cosmetic products.

2. Material and Methods

2.1 Plant materials

The sample plant *Alysicarpus vaginalis* was taken in April 2022 from Mahamaya Lake in Durgapur, Chittagong. The professionals at the Bangladesh National Herbarium in Mirpur, Dhaka, were able to correctly identify the plant (accession number: DACB87209). The whole plants were dried in the shade and then processed into a powder.

2.2 Drugs and Chemicals

Methanol, NaOH, dilute HCl acid, conc. H_2SO_4, acetic acid, and methyl blue were all purchased from Sigma Chemical Co., USA. Morphine sulphate was purchased from Gonoshasthaya Pharmaceuticals Ltd (Dhaka, Bangladesh). Vin Cristine Sulphate was brought from Celon Laboratories Pvt. Limited, India. We purchased some sterile saline solution from Orion Infusion Ltd. Square Pharmaceuticals Ltd. was the manufacturer of both diclofenac sodium injection and diazepam injection. The German company Merck supplied the DMSO.

2.3 Preparation of crude extract

19.87 gm of powdered particles were placed in a cleaned, flat-bottomed glass container and doused with 800 ml of ethyl acetate. This sealed container's contents were left undisturbed for 20 days, with only occasional shaking and stirring. The whole batch was then coarsely filtered using clean filter paper. Air drying process was used to hasten the drying process of the resulting filtrate (an extract of ethyl acetate). The resulting concentration was sticky and olive-green in hue. Ethyl acetate crude extract is the official name for the gooey concentration (1.6 gm). Then the remaining powder was steeped in 800ml of methanol for 15 days, sometimes shaken and stirred. The mixture was filtered using clean filter paper. The methanol filtrate was dried using a ceiling fan. It produced a greenish-olive sticky concentration. Gummy concentration was methanol extract (2.1 gm) [8].

2.4 Phytochemical analysis

Carbohydrate, Alkaloids, flavonoids, steroids, glycosides, saponins, gums, and tannins were among the compounds examined qualitatively in the newly generated crude extract. The tests were performed using various reagents [6]. 10% (w/v) solution of the extract was used in each of the tests, unless a different concentration was specified in an individual test.

2.5 Antimicrobial test

2.5.1 Test microorganisms

Pure cultures of Gram-positive (*Staphylococcus aureus*, *Bacillus cereus*) and Gram-negative (*Klebsiella pneumonia*, *Escherichia coli*, *Pseudomonas aeruginosa*) bacterial pathogens were obtained from the Department of Microbiology, Stamford University Bangladesh.

2.5.2 Antimicrobial susceptibility test

The disk diffusion technique was used to conduct *in vitro* susceptibility testing against the plant extracts. In general, sterile cotton swabs were used to apply a homogeneous layer of 0.1% bacterial suspension on the surface of solid Mueller-Hinton agar medium. When the inoculum was completely dry and absorbed, we put impregnated 6 mm sterile filter paper discs to the surface of the inoculated plates for 5 minutes to allow for diffusion, and then we incubated the plates for 24 hours. Finally, a calibrated scale accurate to the millimeter was used to quantify the inhibition zones that had developed around the discs [9]. The antibacterial and antifungal disc standard was 30 mg of the antibiotic kanamycin. Antimicrobial testing was done in triplicate to ensure accuracy.

2.6 Antioxidant activity analysis

2.6.1 DPPH free radical scavenging assay

Each concentration of plant extracts and the standard (500, 250, 125, 62.5, 31.25, 15.62 and 7.81 mg/mL) was placed in a separate 1 mL test tube and labeled accordingly. Each test tube then had 2 mL of a 0.004% DPPH solution in the solvent added to bring the total volume to 3 mL. For 30 minutes, the mixture incubated at room temperature in the dark. We then took an absorbance reading at 517 nm [10, 11]. The following formula was used to calculate the radical scavenging activity of DPPH. The IC_{50} value was determined by a linear regression analysis.

2.7 Cytotoxicity analysis

2.7.1 The brine shrimp lethality bioassay

The lethality bioassay using brine shrimp is an excellent method for isolating bioactive chemicals in plant materials. Brine shrimp (*Artemia salina* Leach) eggs were obtained and hatched in an aquarium maintained at 25 °C with a steady

supply of oxygen. The nauplii were given two days to grow after hatching. The extracts were dissolved in a measured volume of pure DMSO and sea salt water to make stock solutions of the samples. The desired concentrations of 1, 5, 10, 20, 50, 100, 200 and 500µg/ml were achieved by transferring predetermined amounts of stock solution to the test tubes. To ensure consistency, the same amount of DMSO was used in both the sample and control tubes. Using a Pasteur pipette, ten nauplii were transferred to each test tube. Very low concentrations of vincristine sulfate (10, 5, 1, 0.5, 0.25, 0.125 and 0.06µg/ml) were tested as a positive control. The number of nauplii that had survived in each test tube was counted after 24 hours of observation [12]. The Abbott formula [13] was used to adjust the proportion of dead brine shrimp nauplii at each extract concentration. Fenny probit analysis was used to get the LC_{50} value after the % mortality was adjusted [14].

2.8 Experimental animals

2.8.1 For analgesic and Neuropharmacological activity

We utilized young, healthy Swiss albino mice weighing between 19 -26 g each as our test subjects. Jahangirnagar University in Saver, Dhaka, Bangladesh, is where we got our mice. Maintaining a baseline state was a priority. Temperature and humidity of 25 °C (77°F) relative humidity of 55-65%, and a 24-hour light/12-hour dark cycle is all considered to be typical environmental conditions. For 8 days following collection, the aforementioned circumstances are kept constant. In accordance with Jahangirnagar University's guidelines, we provided mice with a diet of appropriate food and purified water to help them recover from the water and food deprivation they had during transport and acclimate to the laboratory setting before using them in any experiments. Mice were ready for experiment after 5 days of rest.

2.9 Analgesic studies

2.9.1 Hot plate test

The hot plate technique was used to evaluate the analgesic activity in line with the approach described earlier, but with some slight modifications [15]. Each group of mice (there were a total of twelve) served as a control, a positive control, or a test group for the experiment. Test animals were given test samples at 200 and 400 mg/kg body weight, positive control animals were given morphine at 10 mg/kg body weight, and vehicle control animals were given distilled water at a dosage of 10 mL/kg body weight (B/W). To conduct this experiment, Eddy's hot plate was heated to 51±0.5 ºC, where the animals were placed. 30 minutes before the start of the experiment, both the test samples and the reference medication were given. Mice were monitored pre-and post-injection, as well as 30, 60 and 90 minutes afterwards. Before, 30, 60 and 90 minutes following oral administration of the samples, reaction times were observed when the animals licked their fore or hind paws or leapt. Twenty seconds was set as the limit to prevent paw injury. The latency of the anti-nociceptive reaction was measured from the moment the animal's paws were placed on the plate until the moment they licked them.

2.9.2 Acetic acid induced writhing method

The technique provided by Dharmasiri JR *et al*. was applied, with some adjustments made, to determine whether or not the plant extract had analgesic effects [16]. Diclofenac sodium (10 mg/kg), plant extract (200 and 400 mg/kg), and normal saline solution (10 mL/kg) were orally administered to each group of four mice in a separate experiment. The control group received the normal saline solution. After waiting another 30 minutes, an intraperitoneal (i.p.) injection of a solution containing 0.7% acetic acid (10 mL/kg) was given to all of the animals that had been divided into the various groups. The number of writhes, or abdominal constrictions, that occurred between 5-15 minutes following an injection of acetic acid was measured and counted. An analgesic response was regarded to have occurred when the animals that were evaluated writhed much less as compared to the animals that were in the control group.

2.10 Neuropharmacological activity

2.10.1 Hole cross test

Specifically, the protocol laid forth by Shahriar *et al*. (2015) was followed to conduct the experiment [17]. The cage was divided in half by a 30 x 20 x 14 cm steel divider. At the exact middle of the cage, at a height of 7.5 cm, a hole 3 cm in diameter was cut. The duration of the test was 5 min, during which time the animals were allowed to pass through the opening between the two chambers. Data was collected at 0, 30, 60 and 90 minutes after orally administering 200 and 400 mg/kg of body weight of the test medications and 1 mg/kg of B/W of the reference drug Diazepam.

2.10.2 Open field test

In this study, we employed a methodology similar to that described by Shahriar *et al*., 2015, with a few minor adjustments [17]. Four mice made comprised each of the control, positive control and test groups I and II. Oral administration of 200 and 400 mg/kg body weight of *Alysicarpus vaginalis* extract was given to the experimental group, while the vehicle (distilled water) was given to the control group and 5 mg/kg B/W of Diazepam was given to the positive control group. The grassy ground of a half-square-meter open space was sectioned up into a grid of black and white squares. A 40-centimeter-tall wall around the device. Mice were divided into two test groups, I and II and their square counts were recorded for five minutes at 0 minutes, 30 minutes, 60 minutes and 90 minutes and then compared to those of mice in a positive control group.

2.11 Investigation for analgesic activity mechanism(s)

2.11.1 Involvement of cyclic guanosine monophosphate (cGMP) pathway

The animals were given a pretreatment of methylene blue, an inhibitor of guanylyl cyclase and/or NO, at a concentration of 20 mg/kg of body weight (i.p.). After 15 minutes, mice were given either vehicle (0.2% tween 20, p.o., control group) or MEAV at a dose of 400 mg/kg. To investigate the potential involvement of the cGMP pathway in the observed antinociceptive effects of MEAV, the animals were given 0.6% acetic acid (v/v, 10 mL/kg, i.p.) 30 minutes after the administration of MEAV, and abdominal writhes were counted for 30 minutes beginning 5 minutes after the injection of acetic acid [18, 19].

2.12 Statistical analysis

Bioassay measurements were conducted in triplicate, and all tabular data is presented as the mean standard deviation. Excel were used to run the statistical tests.

3. Result

3.1 Phytochemical screening

Both EAEAV and MEAV were subjected to preliminary phytochemical analysis, which indicated the presence of glycosides, tannins, reducing sugar, flavonoids, gums, carbohydrates and alkaloids where saponin and gum were only present in MEAV and steroid is absent in both extract (Table 1).

Table 1: Presence of Phytochemicals in EAEAV and MEAV

Phytochemical constituent	EAEAV	MEAV
Flavonoid	-	+
Tannin	+	+
Alkaloid	+	+
Steroid	-	-
Saponin	-	+
Carbohydrate	+	+
Glycoside	+	+
Reducing Sugar	+	+
Gum	-	+

Here, (+) indicates the presence of the phytochemical and (-) indicates the absence of the phytochemical.

3.2 DPPH free radical scavenging assay: The antioxidant catalyzes the conversion of the stable free radical DPPH to the more reactive 1,1-diphenyl-2-picryl hydrazine. Absorbance at 517 nm was evaluated to determine DPPH's scavenging efficacy. The IC_{50} values were determined using DPPH and the percentage of inhibition it had using ascorbic acid, EAEAV and MEAV. The absorbance value was utilized to determine which of these two extracts employed a more effective solvent for its extraction process, and the results indicated that the MEAV included more antioxidants with IC_{50} value of 34.70 than the EAEAV with IC_{50} value of 12.49 (Table 2). Why MEAV has enhanced antioxidant activity is not understood although it may be related to increased extraction of flavonoid and phenolic compounds. Several studies have demonstrated a clear correlation between high flavonoid, alkaloid and phenolic content and high antioxidant activity [20, 21, 22].

Table 2: *In vitro* free radical scavenging effect of EAEAV and MEAV

Samples	Concentrations	% Inhibition	IC_{50} in DPPH radical scavenging analysis (µg/mL)
Ascorbic Acid	7.81	55.72	21.86
	15.625	59.67	
	31.25	70.57	
	62.5	73.70	
	125	81.60	
	250	88.55	
	500	93.86	
EAEAV	7.81	33.09	12.49
	15.625	35.25	
	31.25	51.79	
	62.5	68.34	
	125	80.57	
	250	86.33	
	500	90.64	
MEAV	7.81	38.12	34.70
	15.625	38.84	
	31.25	51.07	
	62.5	58.27	
	125	68.34	
	250	87.05	
	500	89.20	

3.3 Antibacterial activity

Both *Alysicarpus vaginalis* extracts were investigated for antibacterial activity against five different bacteria using disk-diffusion techniques. We tested our methanolic and ethyl acetate plant at different concentrations (3 mg/disc, 5mg/disc and 7 mg/disc) shown in Table 3 and Table 4. Inhibition zones against gram-negative and gram-positive bacteria were found to be distinct (varying from 6.0 to 14 mm). Maximal zone of inhibition was seen for both extracts, however, at 7 mg/disc (14 mm). Some bioactive constituents found in plant extracts, including secondary metabolites, may have antibacterial effects. Similar results were published in a prior research, suggesting that polar compound extracts are more promising as antibacterial agents than non-polar compound extracts [23, 24, 25]. Zones of inhibition against *Staphylococcus aureus* and *Bacillus* were determined to be the widest (between 10 and 13 mm), whereas those against *Escherichia coli* and *Klebsiella pneumonia* were the lowest (between 6 and 9 mm). Antibiotic resistance may have developed in these bacteria because of the limited area of inhibition they show when exposed to conventional ciprofloxacin. The results of this investigation show that both EAEAV and MEAV marginally suppress the development of various human infected viruses, confirming their potential for may use with other extracts in the pharmaceutical industry.

Table 3: Inhibition zone of *A. vaginalis* methanolic extracts against microorganisms

Diameter of Zone of Inhibition (mm)				
Test organisms	**EAEAV (300 µg/disc)**	**EAEAV (500 µg/disc)**	**EAEAV (700 µg/disc)**	**Ciprofloxacin**
Gram Positive Bacteria				
Bacillus cereus	10	11	13	25
Staphylococcus aureus	09	10	10	26
Gram Negative Bacteria				
Escherichia coli	06	07	08	25
Pseudomonas aeruginosa	06	07	08	27
Klebsiella pneumonia	08	09	09	24

Table 4: Inhibition zone of *A. vaginalis* ethyl acetate extracts against microorganisms

Diameter of Zone of Inhibition (mm)				
Test organisms	**MEAV (300 µg/disc)**	**MEAV (500 µg/disc)**	**MEAV (700 µg/disc)**	**Ciprofloxacin**
Gram Positive Bacteria				
Bacillus cereus	10	11	14	25
Staphylococcus aureus	07	08	12	26
Gram Negative Bacteria				
Escherichia coli	07	09	09	25
Pseudomonas aeruginosa	09	10	11	27
Klebsiella pneumonia	08	08	09	24

3.4 Cytotoxic Activity

The cytotoxic activity of EAEAV and MEAV crude was determined using a brine shrimp lethality test. The toxicity of EAEAV and MEAV was tested in a 24-hour test on *Artemia salina*, following Abbott method. The cytotoxic efficacy of the extracts was measured by contrasting them with a dimethyl sulfoxide-only control and a positive-control group (vincristine sulphate). Table 5 illustrates the proportion of brine shrimp that died as a result of the plant extracts at seven various conc. of the extracts, ranging from 7.81 to 500 µg/mL. It was demonstrated beyond a balance of probabilities that the concentration of the extracts was directly proportionate to the percentage of mortality. The bioassay using brine shrimp has been validated as a reliable, applicable, and cost-effective method for investigating the bioactivity of both synthetic compounds and plant extracts [12, 26]. The significance of this bioassay as a pre-screening tool for antitumor drug development is emphasized by the National Cancer Institute's (NCI, USA) demonstration of a link between the Brine shrimp test and *in vitro* inhibitory effects of human solid tumor cell line development [27]. To determine if a metabolite or plant extract is toxic to brine shrimp, the LC_{50} value must be more than 100 µg/mL to be considered non-toxic, between 500 and 1000 µg/mL to be considered slightly toxic, and less than 500 µg/mL to be considered toxic [28]. In accordance with the National Cancer Institute (NCI), the circumstances and criteria of cytotoxic activity for the crude extracts is an LC_{50} value ≤ 20, which is regarded to be extremely cytotoxic. This value indicates that the crude extracts are very effective in killing tumor cells [29]. Phytochemicals include flavonoids, alkaloids, glycosides, tannins, polysaccharides and saponins may be responsible for the toxicity of EAEAV and MEAV.

Table 5: Result of Brine Shrimp Bio-assay and LC_{50} value of EAEAV and MEAV

Sample name	Concentrations	Mortality %	LC_{50} value
Vincristine Sulphate	7.81	40	20.57
	15.625	40	
	31.25	50	
	62.5	60	
	125	100	
	250	100	
	500	100	
EAEAV	7.81	50	3.89
	15.625	70	
	31.25	80	
	62.5	80	
	125	90	
	250	100	
	500	100	
MEAV	7.81	70	8.71
	15.625	80	
	31.25	90	
	62.5	100	
	125	100	
	250	100	
	500	100	

3.5 Analgesic evaluation

3.5.1 Hot plate Method

Table 6 displays the hot plate test results for the crude *Alysicarpus vaginalis* extract. At dosages of 200 and 400mg/kg BW, the plant extract greatly sped up the response time of heat sensation in mice, and the percentage protection was almost same at both levels. The extract significantly raised the response time of heat feeling in the one and half hour of the research, with dosages of 200 and 400mg/kg B/W increasing it respectively, compared to the standard drug's activity, these findings were statistically significant. Both MEAV and EAEAV were evaluated for their anti-nociceptive effects on the hot plate test, which measures the brain's sensitivity to painful stimuli at the supra-spinal level. Specifically, the effectiveness of centrally acting analgesics may be determined with the use of this model. The response time to a heat stimulus was significant by the MEAV and EAEAV to a greater extent than with Diclofenac Sodium. The dosage at which MEAV or EAEAV are administered has a considerable influence on their ability to exert a central anti-nociceptive effect. In fact, investigation utilized an animal models of the hot plate test found that central analgesic medicines made from an alkaloid-rich extract worked well at doses of at least 200-400 mg/kg p.o. [30, 31].

Table 6: Primary Data Table for Hot Plate Test for Plant Extract of MEAV and EAEAV

Reaction time at different time intervals (in sec)					
Group	**Average wt. of mice (g)**	**0 min**	**30 min**	**60 min**	**90 min**
Control	21-25	6.4	7.6	06	5.4
Morphine (5mg/kg)		8.6	9.4	11	6.6
EAEAV (200mg/kg)		5.6	10.2	8.8	7.1
EAEAV (400mg/kg)		6.8	11.8	12	8
MEAV (200mg/kg)		6.65	8.6	6.75	5.8
MEAV (400mg/kg)		7.6	12	9.6	6.2

3.5.2 Acetic acid-induced writhing test

Table 7 represents the results of an acetic acid-induced writhing test in mice and the effect of the test drugs on the mice's licking reaction. The acetic acid-induced abdominal writhing in mice were considerably reduced after oral administration of either dosage of *A. vaginalis* plant extract. The EAEAV inhibited the writhing response by 42.48% and 47.00% at 200 and 400 mg/kg and MEAV inhibited 40.63% and 51.77% using the same doses respectively, while the conventional diclofenac sodium (10 mg/kg) demonstrated 79.61% inhibition compared with the control. When acetic acid is injected into a mouse's peritoneal cavity, the animal reacts by contracting its abdominal muscles, extending its forelimbs, and stretching its body. Prostaglandin pathways are hypothesized to be responsible for these symptoms [32]. The abdominal muscles writhe because acetic acid triggers the release of certain endogenous physiological chemicals. These molecules include histamine, serotonin, bradykinin and several prostaglandins. These substances activate the neurons in the spinal cord and brain that are sensitive to pain [33].

Table 7: Analgesic Activity of MEAV and EAEAV on Mice by Writhing Test

Administered Substance	Dose	% Writhing	% of Inhibition
Control	10mL/kg	100	0.00
Diclofenac sodium	10mg/kg	20.40	79.61
EAEAV	200mg/kg	57.52	42.48
EAEAV	400mg/kg	53.00	47.00
MEAV	200mg/kg	59.36	40.63
MEAV	400mg/kg	48.23	51.77

3.5.3 Involvement of cyclic guanosine monophosphate (cGMP) pathway

Table 8. Shows that the pre-treating the mice with the cGMP inhibitor methylene blue let us determine whether or not the cGMP pathway was involved (MB). Although acetic acid elicited nociceptive behavior, 20 mg/kg of MB alone did not alter this behavior. It was demonstrated that when given at a dose of 400 mg/kg, EAEAV and MEAV had very potent analgesic efficacy, which was augmented by the pre-treatment with MB, which elevated the percentage of inhibition from 47.00% to59.62% and 51.72% to 72.12% respectively. The antinociceptive effects of cGMP-blocking agents have thus far been widely recognized [19, 34, 35]. Methylene blue, an inhibitor of the cyclic guanosine monophosphate (cGMP) pathway, was shown to increase the potency of nociception induced in ants by EAEAV and MEAV. That EAEAV and MEAV might interact with the nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) pathway was established. Although the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) pathway has been shown to modulate various K^+ channels, it has been found that NO/cGMP-mediated activation of ATP-sensitive K^+ channels suppress action potential generation by hyperpolarizing the peripheral terminal of afferent neurons.

Table 8: Analgesic Activity of EAEAV and MEAV through cGMP pathway by Writhing Test

Administered Substance	Dose	% Writhing	% of Inhibition
Control	20mg/kg	100	0.00
EAEAV	400mg/kg	40.38	59.62
MEAV	400mg/kg	27.88	72.12

3.6 Neuropharmacological activity

3.6.1 Hole cross test

Because of the significant demand in the West for novel compounds of natural origin with depressive-sedative activity, this research employed a technique for the evaluation of plant extracts with impact on the CNS, more particularly a methodology utilized to assess depressive activity. Mice were administered both MEAV and EAEAV extracts intraperitoneally and their ability to complete crossing the hole diminished with time. Maximum inhibition of locomotor activity was observed between 200 and 400 mg/kg, doses of the both extract of *A. vaginalis* compared to the standard medication diazepam (Table 9). Specifically, when evaluating the effects of extracts at 200 and 400 mg/kg of B/W, the 400 mg/kg dosage significantly reduced locomotor activity. During the second period of observation in the hole cross experiment, the depressive effect of the both extracts at dosages of 200 and 400 mg/kg body weight was seen in the experimental animals. The maximum antidepressant impact was seen during the third (30-minute) and fourth (90-minute) observation periods. These findings were likewise statistically significant and dose-dependent.

Table 9: The primary data table for the Hole Cross Test for the EAEAV and MEAV

Number of movements					
Group	**Average wt. of mice (g)**	**0 min**	**30 min**	**60 min**	**90 min**
Control	21-25	23.8	24.4	23.8	19.4
Diazepam		9.67	6.67	3.67	2.33
EAEAV (200mg/kg)		13.4	6.0	3.6	1.2
EAEAV (400mg/kg)		13.6	4.2	2.6	0.6
MEAV (200mg/kg)		8.4	4.2	3.2	1.2
MEAV (400mg/kg)		6.8	4.8	0.8	0.00

3.6.2 Open field test

Decrement of locomotor activity was observed at a dosage of 400 mg/kg of both EAEAV and MEAV, which is on par with the conventional medication diazepam in terms of its anxiolytic effects. There was a substantial and dose-dependent decrease in activity from 0 to 90 minutes in an open field test including groups treated with *A. vaginalis* (200 and 400 mg/kg body weight) (Table 10). At 200 and 400 mg/kg B/W, *A. vaginalis* plant extract substantially decreased the number of squares walked by the mice.

Table 10: The primary data table for the open field test for the EAEAV and MEAV

Number of movements					
Group	**Average wt. of mice (g)**	**0 min**	**30 min**	**60 min**	**90 min**
Control	20-25	89.6	39	30.8	22.8
Diazepam		87.2	62	36.2	18.8
EAEAV (200mg/kg)		86	38.2	29.8	21.2
EAEAV (400mg/kg)		82.2	37.7	28.6	20.6
MEAV (200mg/kg)		70.6	32.8	31.2	23.6
MEAV (400mg/kg)		60.8	27.8	25.2	16.4

An essential step in assessing both EAEAV and MEAV action on CNS is to examine its influence on locomotors activity of the animal. The extracts greatly lowered the locomotor activity as demonstrated by the results of the open field and hole cross tests (Table 9) (Table 10). The locomotor activity reducing impact was noticeable from the 2nd observation (30 min) and lasted up to the 4th observation period (90 min). Both EAEAV and MEAV were shown to have CNS depressive action in this study's experimental animals' models. When comparing the extracts, the dose dependent MEAV had more depressive action than EAEAV. The extent of sedation that results from depression of the central nervous system is a measure that may be obtained from the locomotor activity. This activity is a measure of the excitability of the central nervous system [36]. The effects varied with dosage and were statistically significant. This study adds credence to the idea that *A. vaginalis*'s usage in ethnomedicine is attributable to an effect on the central nervous system. Yet further research is needed to identify the precise Phyto-constituents and mechanism of action that contribute to the biological activities of the EAEAV and MEAV.

4. Conclusion

The present work demonstrated the pharmacological capabilities of methanolic and ethyl acetate extracts of the *A. vaginalis* plant, which were shown to augment the effects of neuropharmacological activity by reducing locomotor activity in a mouse model. The strongest neuropharmacological effects were seen in the 400 mL/kg group when given the methanolic extract of the *A. vaginalis* plant. Although *A. vaginalis* extracts have been shown to have antioxidant, cytotoxic, antibacterial, neuropharmacological and analgesic activities, further research is needed to determine the likely mechanisms of action responsible for these benefits. Using its isolated compounds pharmacological capabilities, the research presented *A. vaginalis* as would be pharmaceutical or cosmetic products API.

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6. Competing interests

The authors have confirmed that they have no conflicts of interest.

7. Funding statement

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8. Ethical Approval:

All experiments were reviewed by a relevant ethics committee and executed in conformity with all ethical guidelines, as the authors thus attest.

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