



Original Research Article

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## Comparative study of *in-vitro* and *ex-vivo* activities of a bioactive compound from *Aloe vera* plant - A therapeutic compound on zebra fish

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### Article Info

### Abstract

#### Keywords:

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*Aloe vera* is a medicinal plant with a traditional history, complex constituents, and various pharmacological activities. In India, it is found in Maharashtra, Gujarat, Rajasthan, Tamil Nadu, and Andhra Pradesh. *Aloe vera* is also called the wand of heaven, potted physician, wonder plant, plant of life, and heaven's blessing. *Aloe vera* contains vitamins A, B12, C, and E, choline, folic acid, minerals, sugars, amino acids, and sterols. The pulp gel of *Aloe vera* works as an antivirus, antibacterial, antifungal, anti-cancer, and wound healing, with the medicinal principles being phenolics and polysaccharide compounds. In this study, with the objective of the production of not only healthy but also standardized sizes of plantlets to be used in the subsequent controlled stages of the process, *Aloe vera* has been analyzed for various phytochemicals and Antioxidants and standardized for the initiation of *Aloe vera* through micropropagation where the produced *in-vitro* plants were tested for the presence of phytochemicals and antioxidants along with the efficiency of anti-microbial activity against the *ex-vivo* plants proving the quality and success of micropropagation. The zebrafish is an important model which has 84% of gene similarities and 70% of disease similarities. Here we use adult zebrafish as a model organism in research and it is widely used in testing and understanding the study compound's pharmacology and therapeutic efficacy. The mixture was tested across behavior, locomotor, and neuromotor responses to understand the activity compound. The complete assessment procedure entails exposing adult zebrafish to the given combination for a period of 21 days and its activity is analyzed.

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### Introduction

*Aloe vera* belongs to the Asphodelaceae (Liliaceae) family, and is a shrubby or arborescent, perennial, xerophytic, succulent, pea-green coloured plant. The plant has triangular, fleshy leaves with serrated edges, yellow tubular flowers, and fruits that contain numerous seeds. Each leaf is composed of three layers: 1) An

inner clear gel that contains 99% water and the rest is made of glucmannans, amino acids, lipids, sterols, and vitamins. 2) The middle layer of latex, which is the bitter yellow sap and contains anthraquinones and glycosides. 3) The outer thick layer of 15–20 cells is called the rind which has a protective function and synthesizes carbohydrates and proteins. Inside the rind are vascular bundles responsible for the transportation

of substances such as water (xylem) and starch (phloem).

Aloe vera is a stemless or very short-stemmed plant growing to 60–100 centimeters (24–39 inches) tall, spreading by offsets. The leaves are thick and fleshy, green to grey-green, and have phytochemicals such as lignans, phytosterols, poly-phenols, acetylated mannans, poly mannans, anthraquinone C-glycosides, anthrones, and other anthraquinones, (emodin and various lectins). The flowers are produced in summer on a spike up to 90 cm (35 in) tall, each flower being pendulous, with a yellow tubular corolla 2–3 cm (3/4–1+1/4 in) long. Aloe vera forms arbuscular mycorrhiza, a symbiosis that allows the plant better access to mineral nutrients in the soil.

Antioxidants play an important role in nutrition by lengthening the shelf life of food and reducing nutritional loss and formation of harmful substances. However, the safety of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) is now in doubt. Thus, attention is now increasingly paid to the development and utilization of more effective and non-toxic antioxidants of natural origin. A great number of natural medicinal plants have been tested for their antioxidant activities and results have shown that the raw extracts or isolated pure compounds from them were more effective antioxidants *in vitro* than BHT or vitamin E, so, medicinal plants can be a potential source of natural antioxidants. The high content of antioxidant polyphenolic compounds, such as catechin, ingested in the human diet represents an important source of non-nutritional antioxidants (Moein et al., 2008). Antioxidants inhibit and scavenge radicals, thus providing protection against infections and degenerative diseases. They can either directly scavenge or prevent the generation of ROS. The two most commonly used synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have begun to be restricted because of their toxicity and DNA damage induction. The plant species have been investigated in the search for novel antioxidants, but generally, there is still a demand to find more information concerning the antioxidant potential of plant species as they are safe and also bioactive (Rajamanikandan et al., 2011).

When incubated with red grape juice and red wine with a high content of condensed tannins, the poliovirus, herpes simplex virus, and various enteric viruses are

inactivated. In tissue-cultured cell assays, tannins have shown antiviral, antibacterial, and antiparasitic effects. Tannins isolated from the stem bark of *Myracrodruonurundeuva* may offer protection against 6-hydroxydopamine-induced toxicity. The tannins isolated from the stem bark also have anti-inflammatory and antiulcer activity in rodents, showing a strong antioxidant property with possible therapeutic applications. Foods rich in tannins can be used in the treatment of HFE hereditary hemochromatosis, a hereditary disease characterized by excessive absorption of dietary iron, resulting in a pathological increase in total body iron stores (Katkar et al., 2010).

Micropropagation facilitates the growth, storage, and maintenance of a large number of plants in small spaces, which makes it a cost-effective process. Micropropagation is used for germplasm storage and the protection of endangered species. Also, used for the genetic modification of a plant or simply to increase its yield. The cells of the plants can be genetically altered to produce plants with desirable characteristics. This technique utilizes the plant's ability to rejuvenate the tissues rapidly.

Bacterial resistance to antibiotics is increasingly becoming a concern for animal health. Currently, used antibiotic agents are failing to end many bacterial infections due to super-resistant strains. For this reason, the search is ongoing for new antimicrobial agents, either through the design and synthesis of new agents or through the search of natural sources for as-yet undiscovered antimicrobial agents. Herbal medications in particular have seen a revival of interest due to a perception that there is a lower incidence of adverse reactions to plant preparations compared to synthetic pharmaceuticals. Coupled with the reduced costs of plant preparation, this makes the search for natural therapeutics an attractive option.

## Materials and methods

### Extract preparation

The collected leaves were screened for contamination and thoroughly washed. The specimens were then shade-dried, ground in a mechanical mixer-grinder, and extracted with n-butanol by maintaining the powder: solvent ratio as 1:6 using a Soxhlet apparatus. The crude extract obtained was concentrated at 40°C under reduced pressure (72 mbar) with a Rotavapor. The dried

extract was weighed to determine the yield of soluble constituents and stored in a vacuum desiccator at room temperature until further use.

### Extract collection

The amount of extract was collected using various solvents for phytochemical analysis and quantitative analysis.

### Phytochemical screening

Semi-quantitative phytochemical screening of the extract was done. The extract was analyzed for the

presence of flavonoids, saponins, terpenoids, reducing sugars, cardiac glycosides, steroids, tannins, phlorotannins, anthraquinone, and oil individuals, and the results were tabulated.

### Phytochemical analysis

Phytochemical screening was done to analyze secondary metabolites that are responsible for curing ailments. The phytochemical screening of the plant extract was carried out in all different regions with collected leaf extracts such as alkaloids, saponins, glycosides, carbohydrates, tannins, flavonoids, steroids and fats and oils.

**Table 1.** Qualitative phytochemical analysis of *Aloe vera*.

Phytochemicals	Test Name	Procedure	Result
Alkaloids	Mayer's	1ml, Extract + 1ml, Mayer's reagent (Potassium mercuric iodide).	Whitish yellow/Cream colored precipitate.
	Wagner's	1ml, Extract + 1ml, Wagner's reagent (Iodine in potassium iodide).	Reddish brown precipitate.
Saponins		2ml, Extract (conc)+ 20ml, Distilled Water. Shake well for 15 mins.	Layer of foam formation (2cm).
Glycosides	Borntrager's	1ml, Extract + Few drops of diluted H <sub>2</sub> SO <sub>4</sub> + 1ml, Chloroform + 1ml, (10%) Ammonia Solution.	Formation of Pink color.
Carbohydrates	Molisch's	1ml, Extract + 1ml, (1%) $\alpha$ -naphthol solution + Few drops of H <sub>2</sub> SO <sub>4</sub> , side of the test tube.	Purple/Reddish violet color.
	Benedict's	1ml, Extract + 1ml, Benedict's reagent. Boil for 2 mins at 37°C and cool down.	Formation of Red precipitate.
Tannins		1ml, Extract + 1ml, (0.1%) Ferric chloride.	Dark blue/ Greenish black color.
Flavonoids	Shinoda's H <sub>2</sub> SO <sub>4</sub>	1ml, Extract + Few crystals, Sodium hydroxide. 1ml, Extract + 1ml H <sub>2</sub> SO <sub>4</sub> .	Formation of a Yellow color. Formation of a Orange color.
Fats and Oils		1ml, Sample on the filter paper. Stand for 15 minutes.	A Greasy spot/Layer.
Mucilage and Gums		1ml, Extract + 1ml, (100%) Ethanol.	Formation of White cloudy precipitate.
Phenolic Compounds	Ferric chloride	1ml, Extract + 1ml, (5%) Ferric chloride.	Dark Green/ Dark Violet color.
	Lead Acetate	1ml, Extract + 1ml, (10%) Lead Acetate.	Bulk White precipitate.
Proteins		1ml, Extract + 1ml, (10%) Sodium hydroxide. Boil at 30°C, 10 mins Add, 1ml, (2%) Copper Sulphate.	Formation of Violet color.
	Biuret	1ml, Extract + 1ml, (2%) Copper Sulphate+ 1ml, (100%) Ethanol.	Formation of Pink color.

### Quantitative analysis

#### Determination of antioxidant potential

Antioxidants play an important role in nutrition by lengthening the shelf life of food and reducing

nutritional losses and the formation of harmful substances. Antioxidants inhibit and scavenge radicals, thus providing protection against infections and degenerative diseases. They can either directly scavenge or prevent the generation of ROS (Reactive Oxygen Species).

### Reducing power of extract

The ability of the extract to reduce  $\text{Fe}^{3+}$ –  $\text{Fe}^{2+}$  was analyzed. 25, 50, 100, 200, and 400  $\mu\text{g}$  of the extract were mixed with 2.5 ml of 0.2 M phosphate buffer and 2.5 ml of 1% potassium ferricyanide followed by incubation at 50°C for 30 min. 2.5 ml of 10% trichloroacetic acid was later added and the tubes were centrifuged at 3000 rpm for 10 min. 2.5 ml of the upper layer solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Absorbance was measured at 700 nm. An increase in absorbance of the reaction mixture indicated increased reducing power.

### Free radical scavenging activity

The stable 1,1-diphenyl-2-picryl hydroxyl radical (DPPH) was used for the determination of the free radical scavenging activity of the extract. Different concentrations (25, 50, 100, 200, and 400  $\mu\text{g}$ ) of the leaf extract were added, at an equal volume, to the ethanolic solution of 3 mL of DPPH (0.1mM). After 30 min of incubation in the dark at room temperature, the absorbance was recorded at 517 nm using a UV-Vis Spectrophotometer. Ascorbic acid was used as a standard. The percentage inhibition (I %) was calculated using the formula,

$$I \% = [\text{Abs (Control)} - \text{Abs (Sample)}] / \text{Abs (Control)} \times 100.$$

### Quantification of polyphenolics

The total phenolic content of the extract was determined using the Folin-Ciocalteu reagent method described by Lister and Wilson (2001). To the 50  $\mu\text{l}$  of each extract concentration (25, 50, 100, 200, and 400  $\mu\text{g}$ ), 2.5 ml of Folin- Ciocalteu reagent (1/10 dilution) and 2 ml of 7.5%  $\text{Na}_2\text{CO}_3$  (w/v) were added and mixed well. The mixture was incubated at 45°C for 15 min. The absorbance was measured at 765 nm using UV-Vis spectrophotometer (Varian, Inc., CA, USA) with  $\text{Na}_2\text{CO}_3$  solution (2 ml of 7.5%  $\text{Na}_2\text{CO}_3$  in 2.55 ml of distilled water) as blank. Gallic acid was used as a standard, and results were expressed as GAE (gallic acid equivalence) in  $\mu\text{g}$ .

### Determination of total flavonoid content

Flavonoids are secondary metabolites that are recognized as the characteristic red, blue, and purple anthocyanin pigments of plant tissues that are involved

in their physiological roles in the plants. Flavonoids are considered to be an important component of the human diet.

The flavonoid content was determined by the use of a slightly modified colorimetry method described. A 0.5 ml aliquot of appropriately (2 mg/2 ml) diluted sample solution was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5 %  $\text{NaNO}_2$  solution. After 6 min, 0.15 ml of 10%  $\text{AlCl}_3$  solution was added and allowed to stand for 6 min, and then 2 ml of 4%  $\text{NaOH}$  solution was added to the mixture. Immediately, water was added to bring the final volume to 5 ml, and then the mixture was thoroughly mixed and allowed to stand for another 15 min. The absorbance of the mixture was determined at 510 nm versus water blank. The analysis was performed in triplicate and the results were expressed as rutin equivalent.

### Explants sterilization

The explants were soaked in an antifungal and antibacterial solution containing carbendazim (0.1%) and streptomycin (0.1%) for 15 minutes. The sterilization is followed by the treatment of detergent, Polysorbate-20 for 20 minutes. The explants were washed with sterile water three times to ensure the complete wash of detergent. The sterilization is further carried out inside a laminar airflow chamber; explants were treated with Ethanol (70%) for 30 seconds and Mercuric chloride (0.1%) spanned for 8, 9, 10, 11, and 12 min. The explants were removed from the mercuric chloride solution and washed with sterile water 3 times to eliminate the toxic effects of Mercuric chloride.

### Mortality rate

Mortality rate, or death rate, is a measure of the number of deaths (in general, or due to a specific cause) in a particular population, scaled to the size of that population, per unit of time. The mortality rate was calculated by

$$\% \text{ Mortality} = (\text{Explants contaminated} / \text{Total no. of explants}) \times 100$$

### Multiplication of emerged shoots

The shoots that arose from the Nodal parts were further transferred to the multiplication stage for rapid propagation. The emerging shoots were properly trimmed and inoculated in the media consisting of basal

MS media + 3% Sucrose with the following.

M1: NAA – 0.1 mg/l + 6BAP – 1 mg/l

M2: NAA – 0.1 mg/l + 6BAP – 2 mg/l

M3: NAA – 0.1 mg/l + 6BAP – 3 mg/l

### Culture conditions

The trimmed explants were subjected to light intensity for 10-12 h in the growth room for rapid multiplication. Photoperiod is provided by cool white fluorescent lamps of 1500-3000 lux, a temperature of about  $25 \pm 2$  °C, and a humidity of 35 - 40%. The observation for the shoot formation was monitored often.

### Antimicrobial activity

#### Preparation of extract

The leaves of the plant were washed with distilled water, cut opened, and fresh pulp was collected. The gel was dried in an oven at 80°C for 48 hours and then powdered. An ethanol extract was obtained by dissolving 20 grams of the powder in 200 ml of ethanol. The contents were then filtered through Whatman filter paper no1, and the filtrate was evaporated for dryness. Anti-fungal activities were investigated against the fungal organisms and antibacterial activity using Bacterial species. The selected organisms were pre-cultured onto nutrient broth in a rotary shaker at 37 degrees C(Swamy, 2013).

#### Agar well diffusion method of *A. niger*

The antifungal activity of *A. niger* was screened by using the agar well diffusion method. The fungi were cultured for 4 days and grew on potato dextrose agar (PDA) and were used for inoculation of fungal strains on PDA plates. 100 µl of the aqueous and ethanol extracts were introduced into the wells, incubation period of 24-48 hours at 28°C was maintained for observation of the antifungal activity of plant extracts. The antifungal activity was evaluated by measuring zones of inhibition of the fungal growth surrounding the plant extracts. The complete antifungal analysis was carried out under strict aseptic conditions. The zones of inhibition were measured with antibiotic zone scale in mm and the experiment was carried out in triplicates. (Swamy, 2013).

### Agar well diffusion method of bacteria

Antibacterial activity of *B.subtilis* and *Staphylococcus* were screened by using the agar well diffusion method. The bacteria had been cultured for 2 days old grown on nutrient agar (NA) and were used for inoculation of bacterial strains on NA plates. 100 µl of the aqueous and ethanol extracts were introduced into the wells, incubation period of 24-48 hours at 28 degrees C was maintained for observation of the antibacterial activity of plant extracts. The antibacterial activity was evaluated by measuring zones of inhibition of the bacteria growth surrounding the plant extracts. The complete antibacterial analysis was carried out under strict aseptic conditions. The zones of inhibition were measured with antibiotic zone scale in mm and the experiment was carried out in triplicates (Swamy, 2013).

### Suspension culture media

The suspension culture media was used with the Plant hormones for the production of plant metabolite which was characterized by an HPTLC instrument where the identification of the metabolites was done. The suspension culture was prepared using MS media and 6BAP hormone of 2 mg/l was added for the production of the metabolites. Meanwhile, every 5 days, the release of phytochemicals was tested from the used suspension media.

### Results and discussion

#### Phytochemical analysis

The phytochemicals screening was done for the selected Aloe vera plant to ensure the presence of phytochemicals before using the plant for Initiation through Invitro propagation.

#### Collection of explants and sterilization

Healthy *Aloe vera* variety *barbadensis* was collected from the nursery and maintained in the Greenhouse. The shoots with young leaves were collected from the plant and treated as explants. The collected explants have proceeded with the surface sterilization procedure in order to standardize the survival of the explants.

When Mercuric chloride was used for the treatment exposed at various timings such as 3, 5, 7, and 9

minutes respectively, the mortality rate and the survival rate were analyzed, and concluded that 7 minutes of

exposure to mercuric chloride was found to survive with 88% and less mortality than the other time exposure.

**Table 2.** Preliminary phytochemical findings of *Aloe vera* extracts.

Phytochemicals	<i>Aloe vera</i> + water	<i>Aloe vera</i> + acetone	<i>Aloe vera</i> + iso-propanol
Alkaloids (Wagner's Test)	Yes	----	Yes
Saponins	----	----	Yes
Glycosides	----	----	----
Carbohydrates (Molish Test)	Yes	----	Yes
Carbohydrates (Benedict's)	----	Yes	----
Tannins	----	----	----
Flavonoids (H <sub>2</sub> SO <sub>4</sub> )	----	----	Yes
Fats and Oils	----	Yes	----
Mucilage and Gums	----	Yes	----
Phenolic Compounds (Lead Acetate Test)	Yes	Yes	Yes
Proteins	----	----	----

**Table 3.** Sterilization survival.

Surface sterilant used	Conc. (%)	exposed time (min)	Mortality (%)	Survival (%)
HgCl <sub>2</sub>	0.1%	3	100	0
		5	79	21
		7	12	88
		9	49	51
NaOCl <sub>2</sub>	20%	5	91	9
		8	80	20
		10	89	11
		12	94	6

When Sodium hypochlorite was used, it was found that the lowest mortality was found with 8 min exposure, and 20% survival was observed. On the whole, Mercuric chloride was found to be efficient in explant sterilization and showed the highest survival percentage when compared to the Sodium hypochlorite trials.

## Initiation

The survived explants after the sterilization were inoculated in the prepared media for the shoot formation. The observations were recorded.

**Table 4.** Effect of 6BAP & NAA concentration in MS medium in multiplication: after one week incubation:

Growth regulator concentration (mg/l)	Mean number of shoots	Mean shoot length (cm)
M1	4	6.2
M2	2	3.6
M3	1	2.4

From the table, it is evident that the aloe vera responded to the growth hormone and helped in the initiation of new multiple shoots which was then used for the

suspension culture media for the examination of the secondary metabolites.



## Quantitative analysis

### Determination of carbohydrates

The carbohydrates in the selected samples have been analysed for the presence of carbohydrates. In this study, the carbohydrates were found to be higher in the IP extract of *in vitro* followed by acetone and Water of *in vitro* and *ex-vivo* samples.

### Determination of proteins

The protein content in the samples was analyzed using the Lowry method and the results are tabulated.

S.no	Samples	Concentration (mg/ml)
<b><i>In vitro</i></b>		
1	Water	6.1
2	IP	6
3	Acetone	6.8
<b><i>Ex vivo</i></b>		
4	Water	5.4
5	IP	6
6	Acetone	6.4

In this test, the proteins were recorded with higher concentration in the Acetone extract of leaves of *in vitro* samples followed by the IP extracts of both *in vitro* and *ex-vivo* were found to be the same, and water extract of *in vitro* was higher than the *ex vivo*.

### Determination of total phenolics

The total phenolics were estimated in the chosen samples for the confirmation of the efficiency of the samples to confirm the antioxidants in the leaves, stem, and flower.

S.no	Samples	Concentration (mg/ml)
<b><i>In vitro</i></b>		
1	Water	49
2	ip	53
3	acetone	49.5
<b><i>Ex vivo</i></b>		
4	Water	46
5	Ip	51
6	Acetone	40

In this study, the IP extract was found to be high in the leaves of *in vitro* samples followed by Acetone and Water extract which showed almost the same amount as the *ex-vivo* samples of the phenolics content.

### Determination of total flavonoids

Flavonoids are one of the important antioxidants that are present in the samples and help in boosting the immune system. The flavonoids were analyzed in the selected samples and tabulated.

S.no	Samples	Concentration (mg/ml)
<b><i>In vitro</i></b>		
1	Water	26
2	ip	39.2
3	acetone	32
<b><i>Ex vivo</i></b>		
4	Water	21
5	Ip	22
6	Acetone	20

DPPH activity in the selected samples showed higher inhibition activity in Acetone extract followed by water extract and IP extract in *in vitro* samples than the solvents of *ex-vivo* samples proving the higher antioxidant content in *in vitro* than *ex-vivo*.

### Reducing power assay

The reducing power assay method is based on the principle that substances, that have reduction potential, react with potassium ferricyanide ( $\text{Fe}^{3+}$ ) to form potassium ferrocyanide ( $\text{Fe}^{2+}$ ), which then reacts with ferric chloride to form a ferric ferrous complex that has an absorption maximum at 700 nm.

S.no	Samples	Concentration (mg/ml)
<i>In vitro</i>		
1	Water	842
2	ip	1020
3	acetone	625
<i>Ex vivo</i>		
4	Water	820
5	Ip	955
6	Acetone	620

In this study, the reducing power content was found to be higher in the Acetone extract in the *in vitro* sample than the *ex-vivo* in all the solvents chosen.

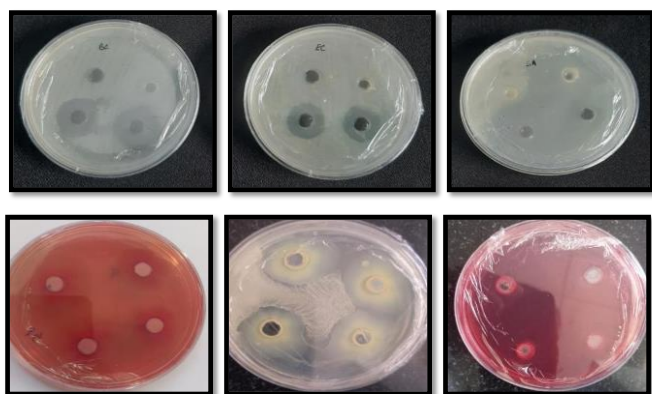
### Antimicrobial activity

*Ex vivo:*

Sample $\mu$ l)	<i>Pseudomonas</i>	<i>A.niger</i>	<i>Trichoderma</i>	<i>Bacillus</i>	<i>Staphylococcus</i>	<i>E.coli</i>
<b>Aloe vera + Water</b>	0.1 mm	0.1 mm	0.12 mm	-	0.13 mm	0.18 mm
<b>Aloe vera + Acetone</b>	0.13 mm	-	0.14 mm	-	-	0.22 mm
<b>Aloe vera + IP</b>	0.11 mm	-	-	-	-	-
<b>(Control-50 <math>\mu</math>l)</b>	0.22 mm	0.18 mm	0.24 mm	0.21 mm	0.33 mm	0.4 mm

*In vitro:*

Sample $\mu$ l)	<i>Pseudomonas</i>	<i>A.niger</i>	<i>Trichoderma</i>	<i>Bacillus</i>	<i>Staphylococcus</i>	<i>E.coli</i>
Aloe Vera+Water	0.43 mm	0.25 mm	0.2 mm	-	0.33 mm	0.34 mm
<b>Aloe Vera+Acetone</b>	0.22 mm	-	-	-	0.21 mm	0.3 mm
<b>Aloe Vera+IP</b>	-	-	-	-	0.15 mm	0.20 mm
<b>(Control-50 <math>\mu</math>l)</b>	0.22 mm	0.18 mm	0.24 mm	0.21 mm	0.33 mm	0.4 mm



It was found that the *Bacillus* species did not respond to the antibacterial activity using aloe vera extract while other bacterial and fungal organisms were found to exhibit antimicrobial activity with either of the chosen solvents resulting in the presence and efficiency of antimicrobial activity in the *in vitro* stage and the virulence activity in formation of zone was found to be higher in *in vitro* than *ex vivo*.

### Suspension culture

The grown Aloe vera culture was then suspended in the liquid media with the growth hormones in order to confirm the efficiency of the *in vitro* plants to produce secondary metabolites in the micropropagation stage itself. The secondary metabolites were tested every often and the results were tabulated.

### Estimation of secondary metabolites

Secondary metabolite	Day 1	Day 6	Day 11	Day 17
Carbohydrates ( $\mu$ g/ml)	56	100.3	156	188
Total flavonoids (mg/ml)	122.3	148.9	155	189
Total proteins (mg/ml)	119	156.3	204	287
Total phenolics ( $\mu$ g/ml)	95	110	133.5	156

It was found that the suspension culture produced secondary metabolites and was analyzed every 5 days

which showed the constant increase in the release of the metabolites that proved the efficiency of the Aloe vera micropropagation. With the suspension culture medium, the HPTLC analysis was done to check the metabolites.

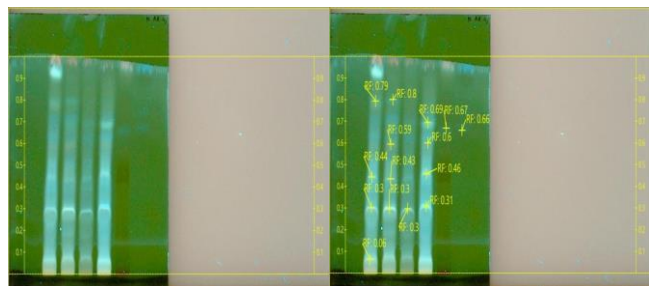


Fig. 1: TLC plates of *Aloe vera* plant.

Secondary metabolites are the major constituents in the *Aloe vera* plant. The solvent system containing Toluene: Ethyl acetate: Formic acid in the volume ratio of (6:4:1) (v/v/v) resulted in good separation (Fig. 1). TLC plate was observed under UV light and white light which were detected by prominent spots at UV 254 nm, dark grey spots appeared after derivatization at 540 nm. The Rf value of various metabolites was observed proving the presence of bioactive compounds that can be used for the therapeutics and also can be formulated in Ayurvedic medicine/ herbal formulation against the diseases.

## Test compounds

### Zebrafish line and maintenance

The strains of zebrafish (*Danio rerio*) used were wild-type animals adapted and bred at Crescent Innovation & Incubation Council. All fish were acclimated to constant laboratory conditions (14-h light: 10-h dark photoperiod, diet, water, 28°C) for at least one week in stock aquaria before all experiments were conducted. Adult fish were fed Tetra flakes (A complete pet food for Tropical fish from Tetra GmbH, Herrenteich) daily, until the beginning of experiments. All fish used in these experiments were random adults from different clutches. Groups of 40 adult fish were housed in transparent polycarbonate tanks with a light-dark cycle of 14/10hr with a water temperature of 28 +/- 1 °C and pH between 6.8-7.5. Good Animal Practice as per the Institutional Animal Ethics Committee in accordance with the Committee for the Purpose of Control and Supervision of Experiments (CPCSEA), India, was followed in adherence to established protocols. Housing

tanks were cleaned once in 4 days to keep the fish clean and free from infection.

## Group setting

Adult male fishes of similar body weight, totaling 0.8 grams per fish were selected for the study and housed as 8 per group in a polycarbonate tank at a stocking density of 2 liters of water per fish. Study fishes were grouped as per the table mentioned below.

Group study	Category
Group 1	Control
Group 2	Model
Group 3	Dilution 1
Group 4	Dilution 2
Group 5	Dilution 3
Group 6	Dilution 4

## Dosing

The stock compounds were prepared by dissolving the respective doses in the pellet feed. On the first day, the adult fish were screened and the best with a good growth phase was selected for dosing. The selected adult fishes were transferred to study cups containing housing water and were housed in a ratio of 1 per study cup for 5 groups. The groups were dosed every day. On days 14 and 21 the adult fish were screened for therapeutic activity.

## Dissection and smear pathology

Fish were euthanized and dissected as per ethical guidance. Fish were euthanized with 2-4 °C water. The fish was dissected through an incision in the viscera and organs of interest were isolated by cutting the localized tissue with a dissection knife. The caudal fin was cut by holding the fish with a lateral segment parallel to the dissection table. Cytology smears were made on glass slides and stained with Hematoxylin and eosin.

## H and E staining

Fixed glass slides were stained with Hematoxylin and eosin respectively for 2 minutes each, followed by PBS washes. Slides were viewed at 40X magnification.

## Imaging

Prepared slides were observed under a bright field microscope and images were captured by the Image

View image capture system.

## Mortality rate

Mortality was counted on an everyday basis to understand the mortality curve over therapeutic intervention.

## Assays

The study tanks were filled with system water and the fish were analyzed for any locomotor, neuromotor, and behavioral abnormality and they were categorized based on the abnormality score.

## Results and discussion

Preliminary tests such as Startle response, light & dark assay, memory maze assay, and locomotion abnormality assessment were conducted to determine the therapeutic efficacy of the compound at the given dilutions. Based on the activity of the compound the therapeutic dose was identified.

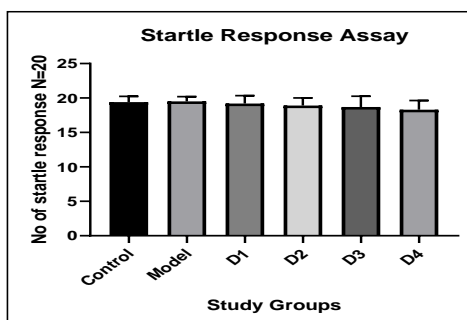
## Acute toxicity

Acute toxicity describes the adverse effects of a substance that result either from a single exposure or from multiple exposure in a short period of time.

## Acute toxicity-Day 7th:

### Startle response assay:

Study Groups	No of response N=20
Standard Control	19.4 ±1.238
Model	19.5 ±1.038
Dilution1	19.2 ±1.666
Dilution2	18.9 ±1.615
Dilution3	18.7 ±2.3
Dilution4	18.3 ±1.963

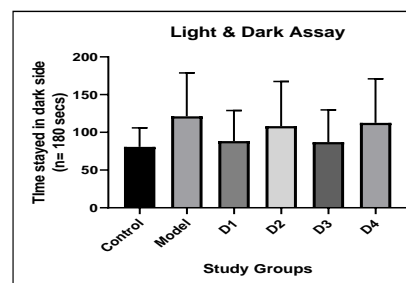


The startle response assay was conducted to identify the

neuromotor activity after dosing with the study compound. The model exhibits high startle response while the control spent the least. The study compound at dilution D1 shows similar activity to control, while dilution D2 and D3 exhibits low startle response than control which is observed to exhibits lesser therapeutic efficacy. The dilution D4 exhibits very low startle response than control and D1, D2&D3 which indicates very lesser therapeutic efficacy of the treated compound.

### Light and Dark assay:

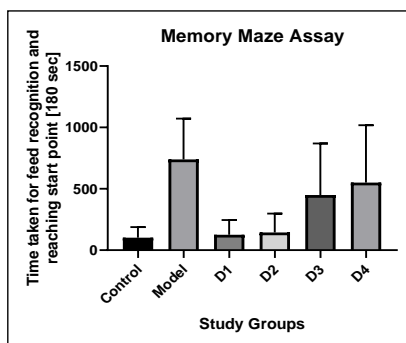
Light and dark assay	
Study Groups	Time taken on dark side (sec) N=180 secs
Standard Control	80.7 ± 36.954
Model	121.3 ± 84.194
Dilution1	88.4 ± 59.345
Dilution2	108.2 ± 87.008
Dilution3	87.1 ± 62.55
Dilution4	112.5 ± 85.802



The light and dark assay was conducted to identify the stress and anxiety level in adult fish which are dosed with the study compound at varying dilutions. The model has spent the highest time in the dark side while the control spent the least. The dilution D1 and D3 exhibits similar activity to control as it spends lesser time on darker side. The dilution D2 and D4 exhibits slightly higher time on darker side than control and D1 and D3 but lesser than model as a result of therapeutic efficacy of the treated compound.

### Memory maze assay:

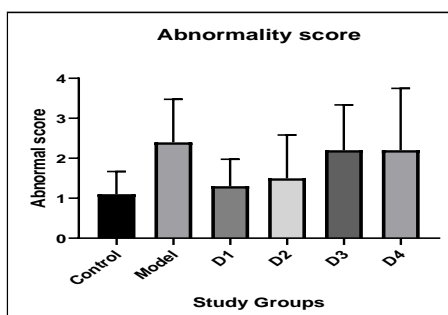
Memory maze test	
Study Groups	Time taken for feed and start checkpoint (sec) N= 180 secs
Standard Control	124 ± 146.856
Model	739.4 ± 487.953
Dilution1	126.2 ± 174.391
Dilution2	143.8 ±226.45
Dilution3	449.2 ±617.24
Dilution4	550.6 ±687.026



The memory maze assay of adult zebrafish is analysed at varying dilution of which at dilution D1 and D2 exhibits similar activity to control, while model has taken highest time to recognize the feed and reaching to start point. The dilution D1 and D2 has taken least time to recognize the feed and reaching to start point which indicates lesser therapeutic efficacy than control, while dilution D3 and D4 has taken more time to recognize the feed and reaching to start point which indicates lesser therapeutic efficacy than D1 and D2.

#### Abnormality score:

Abnormality score Assay	
Study Groups	Abnormal Score
Standard Control	1.1 ±0.833
Model	2.4 ±1.578
Dilution1	1.3 ±0.991
Dilution2	1.5 ±1.585
Dilution3	2.2 ±1.666
Dilution4	2.2 ±2.274



Burst and coast movement-1, movement towards edge of the tank-2, Circling-3, all of the above-4, absence-0;

The abnormality score of adult zebrafish is analyzed at varying dilution of which at dilution D1 exhibits similar activity to control, while model performed in contrast to control. The dilution D2 and D3 similarly exhibits lower activity as control. The dilution D3 and D4 exhibits high abnormality score than control but lesser than model which indicates high therapeutic efficacy.

#### Acute screening at 7th day post compound treatment

##### Gross anatomy of heart:

Control (a) possesses the opposite size and shape of the heart, with thin internal trabeculae and muscular walled atrium. The ventricle appears transparent with thick wall filled with blood. D1, D2 and D4 indicates apposite size and shape of the heart with bulbus arteriosus, ventricle and atrium. D3 indicates slightly swallow bulbus arteriosus, ventricle and atrium.

##### Smear pathology of heart

Control cytology of the heart shows well-defined cardiomyocytes with elongated nucleus that are found and distributed in the non-nucleated eosinophilic cytoplasm. Nucleated erythrocytes (E) are found distributed throughout abnormal count. D1 & D4 indicates cardiomyocytes with elongated nucleus that are scattered through the cytoplasm and has fewer necrotic and degenerative cells while D3 exhibits well defined cardiomyocytes scattered through cytoplasm and increase in degenerative and necrotic cell. Dilution D2 indicates the presence of well define cardiomyocytes with elongated nucleus with slightly higher necrotic and degenerative cells comparable to control.

##### Gross anatomy of brain

Control brain remains apposite size and shape which retains an opaque white colour. The brain is composed of soft parenchymal tissue layer with neuronal glial and endothelial cells. D1, D2, D3, and D4 indicates apposite size and shape of the brain with opaque white colour and composed of soft parenchymal tissue layer with neuronal glial and endothelial cells as of control which is an indication of normal anatomy similar to control.

##### Smear pathology of brain

Control cytology of the brain shows neuronal cells and schwann cells with eosinophilic cytoplasm which are uniformly distributed in the parenchyma. There is no observed necrosis or degenerative cells in D1 and D4 the Schwann cells appear normal with eosinophilic cytoplasm and are uniformly distributed in the parenchyma and few neuronal cells appear pale and swollen with less stain uptake characterizing degenerative. D2 and D3 is observed to indicate higher amount of degenerative and necrotic cell presence with

fewer neuronal and Schwann cells as a result of compound activity.

#### Gross anatomy of liver

Control liver lobe shows intact structure with portal vein (PV), Hepatic artery (HA) and the central vein (CV) scattered throughout the parenchyma. The Liver is of apposite size, shape and color. D1 and D2 indicate intact structure of the liver with central and portal vein scattered through the parenchyma while D4 indicates shrunken central vein with pale vein and hepatic artery scattered through packed parenchyma. D4 exhibits intact structure with shrunken central and portal vein scattered through the loosely packed parenchyma with higher amount of degenerative and necrotic cells.

#### Smear pathology of liver

Control cytology of the liver shows well defined hepatocytes, erythrocytes, eosinophilic cytoplasm and vacuoles and have very few degenerative cells. Treated compound D1, D2 and D3 shows well defined hepatocytes, erythrocytes, eosinophilic cytoplasm and vacuoles with presence of lesser necrotic and degenerative cells similar to control while D4 exhibits less defined liver cells, erythrocytes, eosinophilic cytoplasm and vacuoles and has fewer degenerative cells comparable to control

#### Gross anatomy of pancreas

Control shows tightly packed cells forming intact structure with pancreatic duct. The Pancreatic body appears opaque with densely packed endocrine cells, Morphologically the pancreas is of apposite size, shape and color. Study groups treated at D4 shows brightly coloured central vein and the pancreatic body appears opaque with densely packed endocrine cells as control. While at D1, D2 and D3 the pancreas remains intact with loosely packed cells and central vein is shrunken and mild discoloration in central and portal vein is observed.

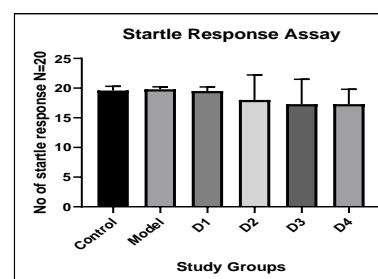
#### Smear pathology of pancreas

Control shows well-defined distribution of endocrine and exocrine cells and there is no degenerative and necrotic cells were observed. Study groups treated at D2 and D3 exhibit no degenerative cells and necrotic cells as control. Treated groups at D1 and D4 demonstrates

higher degenerative and necrotic cells with fewer healthy cells as a result of the compound activity cells containing nucleus is in fewer amount and is confirmed through nevsn stain of hematoxylin.

#### Subacute screening -Day 14th: Startle response assay:

Startle assay	
Study Groups	No of response N=20
Standard Control	19.6 ± 1.026
Model	19.8 ± 0.619
Dilution1	19.5 ± 1.038
Dilution2	18 ± 6.188
Dilution3	17.3 ± 6.151
Dilution4	17.3 ± 3.664



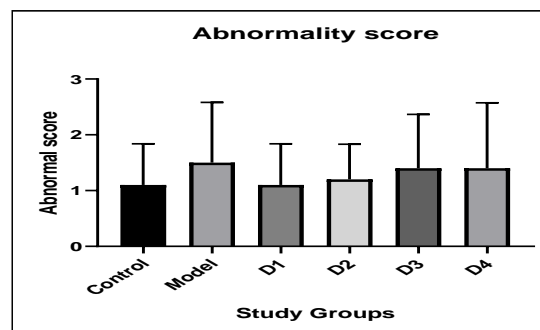
The startle response assay was conducted to identify the neuromotor activity after dosing with the study compound. The model exhibits high startle response, while the control shows least. The D1 shows similar activity and has comparable startle response to that of control, while the dilution D2 slightly exhibits low startle response. The dilution D3 and D4 exhibits very low startle response than control which indicates very lesser therapeutic efficacy.

Light and dark assay	
Study Groups	Time taken on dark side (sec) N=180 secs
Standard Control	99.4 ± 86.773
Model	158 ± 92.631
Dilution1	99.9 ± 71.542
Dilution2	122.5 ± 64.827
Dilution3	100 ± 85.684
Dilution4	122.8 ± 65.153

The light and dark assay was conducted to identify the stress and anxiety level in adult fish which are dosed with the study compound at varying dilutions. The model has spent the highest time in the darker side while the control spent the least. The dilution D1 and D3 shows similar activity to control as it spends lesser

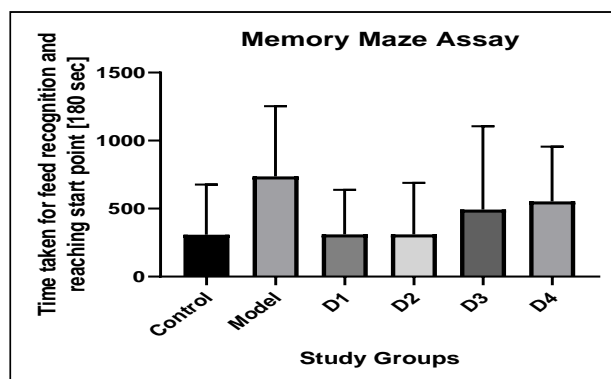
time on darker side, while the dilution D2 and D4 spends more time on the darker side than control and D1 & D3 but lesser than model as a result of therapeutic efficacy of the treated compound.

**Memory maze assay:**



Burst and coast movemnt-1, movement towards edge of the tank-2, Circling-3, all of the above-4, absence-0  
The abnormality score of adult zebrafish is analyzed at varying dilutions of which at dilution D1and D2 exhibits similar activity to control, while model performed in contrast to control. The dilution D3 and D4 high abnormality score than control and D1 and D2 but lesser than model which indicates high therapeutic efficacy.

Memory maze test	
Study Groups	Time taken for feed and start checkpoint (sec) N= 180 secs
Standard Control	307.8 ± 541.573
Model	736.9 ± 756.87
Dilution1	310.7 ± 481.385
Dilution2	310.7 ± 556.35
Dilution3	493.4 ± 898.16
Dilution4	553.3 ± 591.236

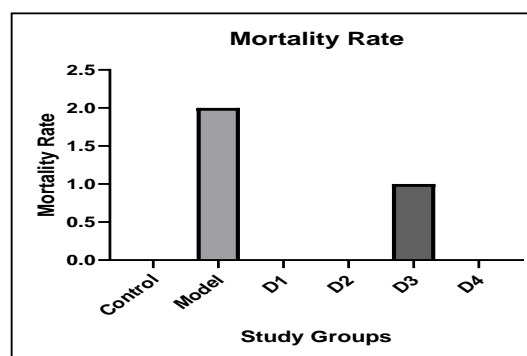


The memory maze assay of adult zebrafish is analysed at varying dilution of which at dilution D1and D2 exhibits similar activity as control, while model has taken least time to recognize the feed and reaching to start point. The dilution D3 and D4 has taken much more time to recognize the feed and reaching to start point which indicates lesser therapeutic efficacy than control and D1&D2 but lesser than model as result of therapeutic efficacy of the treated compound.

**Mortality rate**

Abnormality score Assay	
Study Groups	Abnormal Score
Standard Control	0
Model	2
Dilution1	0
Dilution2	0
Dilution3	1
Dilution4	0

**Abnormality score:**



Abnormality score Assay	
Study Groups	Abnormal Score
Standard Control	1.1 ± 1.083
Model	1.5 ± 1.585
Dilution1	1.1 ± 1.083
Dilution2	1.2 ± 0.928
Dilution3	1.4 ± 1.418
Dilution4	1.4 ± 1.723

The above table and graph are a representation of survival rate of the zebrafish throughout the study period. The survival rates are marked on the daily basis from start of the study. The treated compound at dilution D1, D2 and D4 shows 100% survival rate like control. The treated compound at dilution D3 marked the least survival rate than control towards the end of the study.

### Conflict of interest statement

Authors declare that they have no conflict of interest.

### Acknowledgement

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